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Concept Paper

Immunogenomic Biomarkers for Early Cancer Detection in Lynch Syndrome

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Abstract: Lynch syndrome (LS) is an inherited disorder in which affected individuals have a significantly higher-than-average risk of developing colorectal and non-colorectal cancers, often before the age of 50 years. In LS, mutations in DNA repair genes lead to a dysfunctional post-replication repair system. As a result, the unrepaired errors in coding regions of the genome produce novel proteins, called neoantigens. Neoantigens are recognised by the immune system as foreign and trigger an immune response. Due to the invasive nature of cancer screening tests, universal cancer screening guidelines unique for LS (including colonoscopy) are poorly adhered to by LS variant heterozygotes (LSVH). Currently, it is unclear whether immunogenomic components produced as a result of neoantigen formation can be used as novel biomarkers in LS. We hypothesize that: (i) LSVH produce measurable and dynamic immunogenomic components in blood, and (ii) these quantifiable immunogenomic components correlate with cancer onset and stage. Here, we discuss feasibility and propose the potential to: (a) identify personalised novel immunogenomic biomarkers, (b) reduce invasive cancer screening tests and thus increase non-invasive cancer surveillance, (c) improve compliance and adherence to recommended cancer screening guidelines in LSVH.

Keywords: Lynch syndrome variant heterozygotes; colorectal and non-colorectal cancers; frameshift mutations; neoantigens; immune responses; immunogenomic biomarkers

1. Introduction

Lynch syndrome (LS) is the most common inherited cancer predisposition syndrome caused by germline pathogenic variants (PV) in the DNA-mismatch repair (*path*_MMR) genes: *MLH1, MSH2, MSH6, PMS2* or by deletions in *EPCAM* [1, 2]. Approximately 10-15% of early-age-onset colorectal cancer (CRC) is attributable to LS, which has a prevalence of one in 280 people in Australia, Canada, and USA populations [3]. CRC, which is a traditional hallmark cancer of LS, accounts for up to 80% of primary tumour sites [4, 5]. CRC is commonly prevented and cured by screening, surveillance (mainly by colonoscopy), and modern surgical and medical treatments, with an average 10-year survival rate of 90% for stage I disease [6, 7]. About 5% to 10% of all CRC cases are caused by high penetrance familial cancer syndromes, including the LS [8, 9]. The majority of LS-variant heterozygotes (LSVH) develop colorectal, endometrial, ovarian, breast (in women) and prostate cancers, amongst others [10]. The incidence of multiple cancers in other body organs is also higher in LSVH than in the general population[11]. The higher mortality rate is of concern in developing countries notably due to poor surveillance and lack of

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early-stage screening methods, late diagnosis, and inadequate/inappropriate treatment [12].

Identification of individuals with hereditary cancers, and their surveillance based on empiric risks, leads to improved cancer outcomes [13, 14]. More specifically, ascertainment of families with LS (in which there are no premonitory lesions), the identification of PV and best-practise clinical surveillance, reduces morbidity and mortality dramatically [13-15]. The recommended frequency, invasiveness, and procedure-associated risks of colonoscopies (and other cancer-screening tests) are known to influence adherence and compliance to cancer-screening guidelines [16-19]. Existing screening and surveillance guidelines for LSVH are based on average age-specific cumulative cancer risk [20].

Furthermore, these guidelines fail to take into account the fact that there is substantial heterogeneity between LSVH with different PV, which presents challenges for diagnosis and management [21, 22]. Detection of tumour-derived cell-free nucleic acids in stool and blood has emerged as a promising biomarker in gastrointestinal cancers and various assays for their detection have been developed. These include the stool-based DNA multimarker (ColoGuard®) or the blood-based assay for methylated Septin 9 DNA [23, 24]. However, even with extremely sensitive techniques, most early-stage tumours and precancerous lesions do not release detectable amounts of circulating tumour DNA especially for non-colorectal cancers [25-27]. Due to a lack of prospective data, the current guidelines also rely on invasive cancer screening tests and retrospective data from patient cohorts whose molecular testing was initially biased and generalized using a "one-size-fits-all" approach for every LSVH [8, 28]. Therefore, there is a need to identify alternative personalised non-invasive means for detecting and monitoring for the development of both premalignant and malignant lesions with high sensitivity and specificity in LSVH [16, 17, 29].

Through genetic mutations or epigenetic silencing, MMR-deficiency (dMMR) significantly increases the genomic mutation rate and predisposes LSVH to a remarkably higher-than-average risk and an excess incidence for all types of cancers [30]. Despite the good recovery rate in first cancers, LSVH often develop more lethal cancers at a relatively young age [31]. This highlights the need for better molecular evaluation and identification of patients who require more intensive molecular and clinical surveillance. Personalized medicine plays an important role in managing patients, and particularly patients with PV in genes that predispose them to cancer [32].

LS cancers have a high mutational burden that results in a defined set of frameshift peptide neoantigens [33]. Based on the increasing knowledge of the mutational landscapes of cancers with dMMR, it can be predicted that mutant neoantigens trigger strong immune responses by CD8+ cytotoxic T cells functioning as major mediators of anti-cancer immunity [29, 34]. Insertion and deletion mutations in microsatellites occur during DNA replication, and dMMR phenotype failing to repair the mutations, that contributes to tumorigenesis [35]. The induced shift in the protein reading frame generates neoantigens that are recognized as foreign by the immune system [34]. T-cell immune responses specific to the frameshift peptides (FSPs) have previously been observed in the peripheral blood of both LSVH with CRC and in LSVH who had never developed cancer or adenomas [36-38]. Immune responses such as these suggest that the immune system has been pre-symptomatically exposed to FSPs generated by dMMR cells during life [29, 39]. Furthermore, there is a high prevalence of non-neoplastic or early dysplastic dMMR cells in the intestine and other organs of LSVH, as well as mutations causing FSPs in the colonic crypts [33]. In addition, LS has been associated with marked local immune responses, including LS-related CRCs, adenomas, dMMR crypts, and even completely normal-appearing colonic mucosa [29, 33, 40]. All of these observations suggest that the adaptive immune system plays a critical role in suppressing and controlling the growth of dMMR cancers in the host [29, 41].

A LS cohort provides an ideal population for assessing biomarkers due to a well-predicted cancer risk of patients who are under frequent surveillance [29, 42]. We hypothesize that the presence of PV and expression of neoantigens in LS generate measurable

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and dynamic immune components that may correlate with cancer initiation and/or progression [29]. If effectively characterised, this phenomenon could be used as an alternative to the regular invasive cancer screening tests and as novel immunogenomic biomarkers for early cancer detection, progression or control in LSVH.

2. Frameshift neopeptides, neoantigens, and the immune responses in LS

When cancers with a deficiency of DNA mismatch repair (dMMR) develop, they accumulate a large number of mutations [43, 44]. Physiologically, the mismatch repair system detects and corrects base mismatches caused by polymerase slippage during DNA replication. Microsatellites are repeated sequence segments that are frequently affected by these mismatches. Uncorrected mismatches lead to the accumulation of insertion/deletion mutations (indels) in dMMR cells [33, 44, 45] (**Figure 1A**). Indels of specific coding microsatellites (MS) located within tumour suppressor genes, particularly *TGFBR2* and *ACVR2*, are major causes of malignant transformation and cancer progression of dMMR cells [33, 44]. MS indels are functionally significant, and their distribution in manifest dMMR cancers is not random but follows Darwinian principles of selection [33]. Recurrent MS indels however, are well known and have been documented in many independent studies for different types of dMMR tumours [45]. Mutations such as these not only inactivate tumour-suppressive signalling pathways, but also cause a shift in the translational reading frame, resulting in novel FSPs as neoantigens [45] (**Figure 1B**). As opposed to point mutations, which lead to the alteration of single amino acids, indel-mediated frameshifts give rise to long segments of amino acid sequences that are completely foreign to the host immune system [33, 44, 45]. As a result, the immunogenicity of dMMR cancers is not only due to the sheer number of somatic mutations, but also to the number of potential epitopes in FSPs caused by indel mutations [44].

In addition to an already elevated immune-system in LSVH (as a result of new coding mutations arising every cycle of cell division), dMMR further generates frameshift mutations that lead to highly immunogenic neoantigens that trigger an immune response in the body (**Figure 1B**) [29, 34, 36]. The association between strong immunogenicity and dMMR is generally explained by the accumulation of frameshift mutations within runs of coding mononucleotide sequences, and the synthesis of neoantigens [36, 46, 47]. Neoantigens can trigger the immune system to launch an attack against the cells producing these proteins (**Figure 1B**) [34, 36, 48]. Neoantigens are also capable of eliciting a CD4+ T cell-specific response in addition to CD8+ T cells, while T lymphocytes play a critical role in controlling cancer progression (**Figure 1B**) [29, 34, 49, 50]. In our recent work [51], we observed that cancers attributable to HIV/HPV infection were the least reported histologically confirmed cancers in a large European cohort of LSVH [52]. We hypothesized that LSVH may control a range of acute and chronic infections, including HIV/HPV, and perhaps also cancers attributable to these infections. We originally speculated that this may be due to the continuous hyperinflammatory status of the immune system in LSVH caused by the uninterrupted production of neoantigens [29, 33, 40].

Increased density of tumour-infiltrating lymphocytes and heightened T-cell responses are a cardinal feature of LS [53]. Moreover, mutated cancer proteins are known to elicit strong antitumour-T-cell responses that correlate with clinical findings [34, 36, 54]. Neoantigen degradation releases immunogenic neo-peptides on the surface of tumour cells presented by human leukocyte antigen class I (HLA-I) molecules, against which a specific $CD8^+$ T-cell immune response is directed (**Figure 1B**) [34, 47]. In addition, both cell-mediated and humoral responses of the immune system are also central to inflammation influencing tumorigenesis [55, 56]. The observation of an elevated immune system in LSVH, as a response to neoantigens generated by cells acquiring secondary (unrepaired) mutations during the replication process and inflammation during carcinogenesis, warrants attention as a potential means for monitoring cancer development in pre-symptomatic LSVH at the molecular level (using immunogenomic biomarkers) [36, 44, 57, 58].

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In a previous study [36], including both healthy LS patients without a cancer history and LSVH with CRC, FSP-specific effector T cells were detected in peripheral blood. Analysis of the immune responses in these individuals revealed that the observed T-cell responses were directed toward 14 different FSP antigens predicted from human genome databases. These antigens exhibited different mutation frequencies. A number of neoantigens derived from genes with high mutation frequencies that exhibited immunogenic properties *in vitro* were also found in LS [59].

The activation of immune responses against neopeptides in healthy LS mutation carriers without a history of tumour development can be explained by the generation of frameshift peptides already in haploinsufficiency when a dMMR gene becomes relevant. In LSVH, the type and intensity of the infiltrating immune cells may reflect pathological tumour stage [60]. It is also possible that CD8+ T-cell immune responses predict outcome in early-stage tumours, as the immunogenomic load correlates with cancer outcomes in LSVH (**Figure 1C**) [60]. This implies that immune responses may not only be a predictor but also a means to intervene in cancer development as immunogenomic biomarker profiles correlate with clinical parameters and pathology results for cancer diagnosis and prognosis assessments (**Figure 1C**) [61].

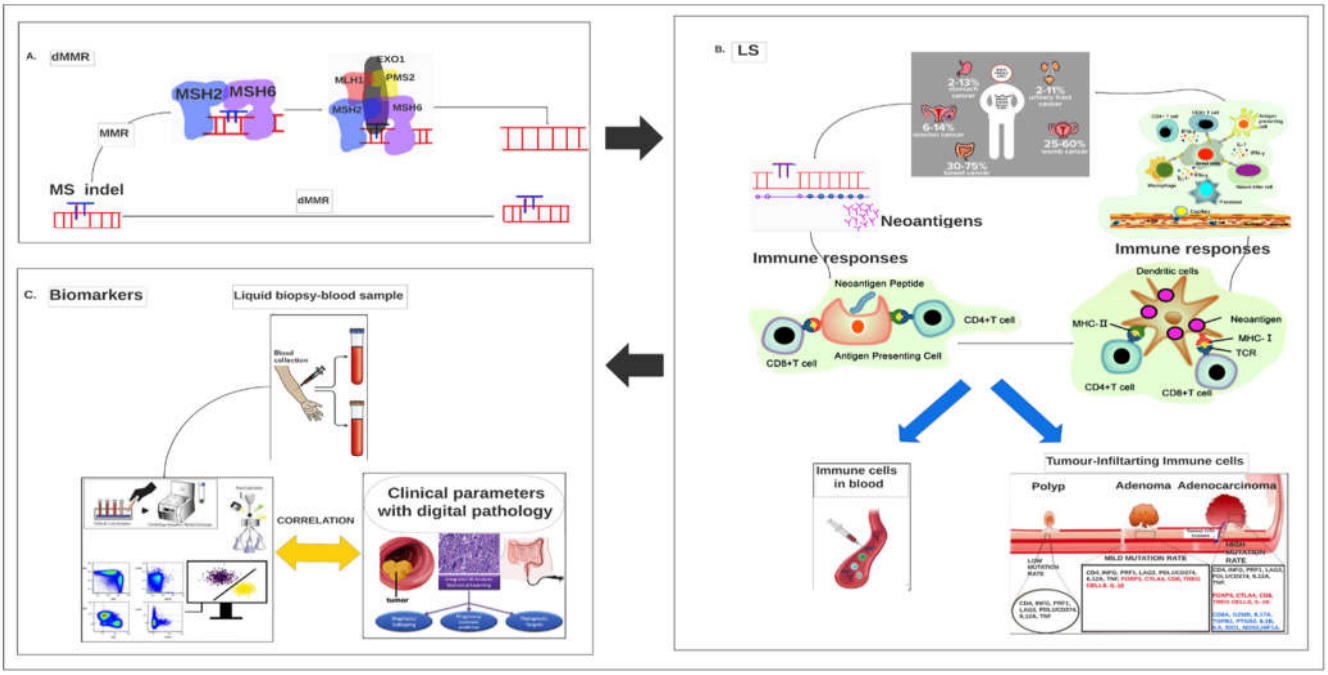

Figure 1. Schematic illustration of pathogenesis and characterization of immunogenomic biomarkers in blood. (**A**) Deficiency in mismatch repair (dMMR) occurs when the microsatellite (MS) indel is not repaired by MMR genes. (**B**) LS, which occurs due to dMMR. When MS indels are not repaired in LSVH, frameshift neopeptides are formed and accumulate in the bloodstream as neoantigens. Neoantigens in the blood trigger immune responses that accumulate in the circulation and infiltrate the tumour during early carcinogenesis. (**C**) Biomarker analysis by serial centrifugation of blood collected in either K2EDTA tubes or cell stabilising tubes. After immunological library preparation, multicolor flow cytometry would be performed to detect cellular and acellular immune components in blood [29]. Computer screens are used to display multicolor immunogenomic biomarker profiles. Biomarker profiles correlate with clinical parameters and digital pathology results for cancer diagnosis and prognosis assessments.

3. Research vision and study concept

a) Research vision

To analyse immunogenomic components in peripheral blood as biomarkers for colorectal and non-colorectal cancers in LSVH [29].

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Rationale

We hypothesize that neo-epitopic provocation of the immune system generates measurable and dynamic immunogenomic components that, if effectively characterized, could be used as diagnostic and prognostic biomarkers for cancer initiation or progression in LSVH [4, 29, 34, 36, 44, 51].

b) Study concept

In our laboratory, we are currently studying the immunogenomic biomarker profiles of LS patients carrying the same germline pathogenic variant *hMLH1* c.1528C>T. Cases (N=500), the majority of whom will be pre-symptomatic and without any suspicious features during colonoscopy; however, of whom it is expected that at least 10% will have lesions ranging from early polyps to frank cancers, will be compared to their previously genetically-tested age- and sex-matched (confirmed) mutation-negative family members (Controls, N=400). Cases will be followed up in a five-year prospective study [29]. Peripheral whole blood samples will be collected during the first and fourth years of follow-up from both cases and controls by venepuncture in K2 ethylenediaminetetraacetic acid (EDTA), Acid Citrate Dextrose (ACD), and red-top tubes (5 ml each). Plasma and cells will be separated by centrifugation. The supernatant (plasma) will be carefully removed from the cell pellet using a Pasteur pipette. Multicolour flow cytometry will be used to analyse CD8+ cytotoxic T cells, CD4+ helper T cells, Treg cells, and B cells. Inflammatory cytokines (IFN-γ, TNF- α, and IL -6) will be quantified from plasma (separated from EDTA-collected fresh whole blood) using Becton Dickinson Cytometric Bead Array Flex Set kits (BD Cytometric Bead Array, USA) in the Immunology Laboratory, Institute for Infectious Diseases and Molecular Medicine, in the Faculty of Health Sciences at UCT [62-64].

c) Research questions in our study concept for immunogenomics of LS

i. How can the measurable immunogenomic components in LSVH that occur as a result of mutations in the mismatch-repair genes be characterised?

Rationale:

LS cancers are characterized by a higher-than-average burden of mutational frameshift neoantigens, which trigger a large pool of immune response components in the bloodstream [29, 33]. To date, there have been no previous approaches that followed this logic, either by studying recurrent FSPs derived from functionally relevant driver mutations or by evaluating dynamic immune components responsive to frameshift neoantigens in blood as immunogenomic biomarkers for high-sensitivity cancer detection in LS [29, 61].

ii. Could immunogenomic biomarker profiles serve endophenotypically as potential biomarkers to reflect neoplastic changes (from early-stage to invasive and metastatic cancer) in LSVH?

Rationale:

In a previous study, healthy LSVH and LS patients with CRC were shown to have a FSPs-specific effector T cell population in peripheral blood [36]. Several neoantigens from genes with high mutation frequency that showed immunogenic properties *in vitro* were also found in healthy LSVH. The immune responses may suggest a pathological tumour stage in LSVH [60]. In addition, the burden of blood immune responses may correlate with cancer initiation in the earliest stages for clinical applications of immunogenomic biomarkers [29] (**Figure 2**). Since immunogenomic biomarker profiles may correlate with clinical parameters and pathology outcomes for cancer diagnosis and prognosis, this implies that immune responses may serve not only as a predictor but also as a means to intervene in cancer development [29, 61].

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iii. Can immunogenomic biomarker profiles serve to prognosticate, i.e., predict disease-free survival and overall survival for LSVH carrying the same or different novel PV?

Rationale:

Generally, certain aspects of the immune profile can predict a patient's cancer prognosis [29, 61]. However, it has proven difficult to establish a standard prognostic criterion for LSVH. It is possible to predict cancer prognosis, survival and treatment response in LSVH using immunogenomic biomarkers alone or in combination with other factors [29] (**Table 1**). In addition, immunogenomic biomarker profiling can be used to build a prognostic model to provide clinicians with simple tools to accurately predict prognosis and treatment outcomes of cancer in LSVH [29, 65], or simple evaluation of systemic immune-inflammation index as biomarkers in LSVH, an example from urinary system cancers [66].

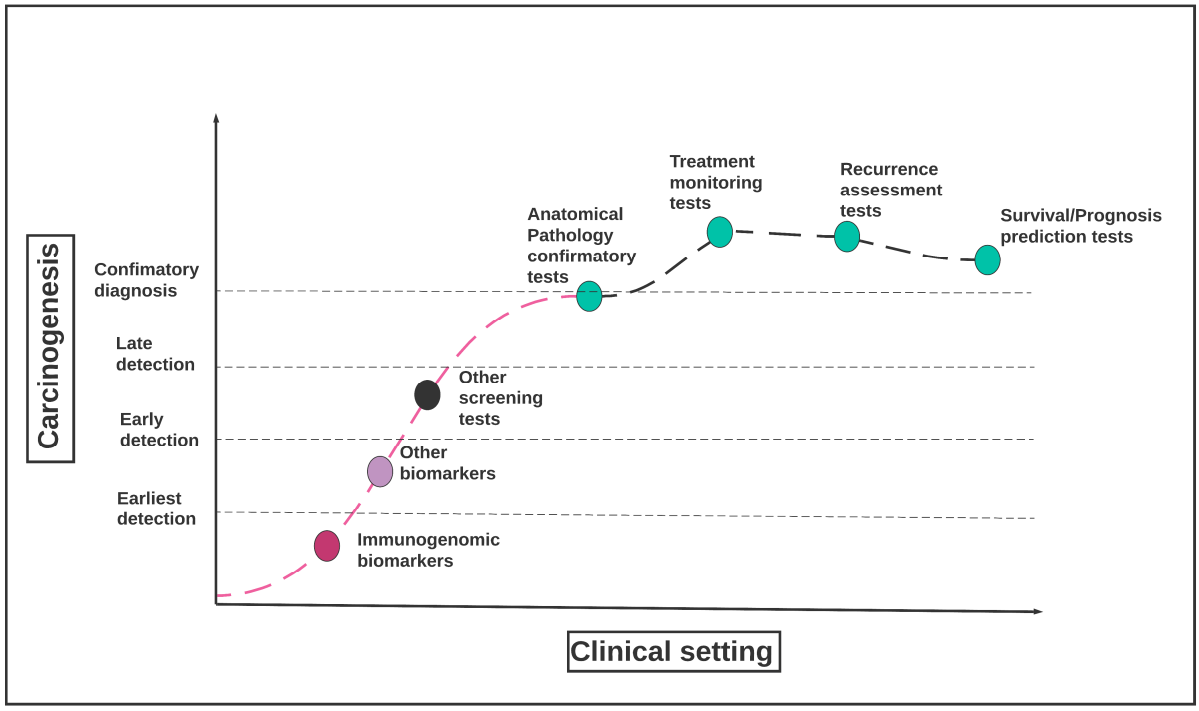

Figure 2. Clinical applications of immunogenomic biomarkers. In LS, immunogenomic biomarkers could be more sensitive for detecting malignancies than conventional imaging or other approaches. This sensitivity can be exploited in several ways, such as detecting cancers in LSVH before symptoms or radiological manifestations appear and detecting minimal residual disease. As an alternative to invasive surveillance and cancer screening, immunogenomic biomarkers can be used to screen for cancer even in the absence of other clinical evidence. They can also be used to assess cancer prognosis in patients with LS who have completed all potentially curative therapies. In patients with radiographically detectable disease, immunogenomic biomarkers may also be more sensitive for tailored monitoring of tumour response.

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Table 1. Suggested cancer screening, diagnosis, treatment, recurrence and prognosis tests in LS.

1. IMMUNOGENOMIC BIOMARKERS	2. OTHER BIOMARKERS	3. BASIC CLINICAL SCREENING TESTS	4. PATHOLOGY AND ADVANCED IMAGING TESTS	5. TREATMENT MONITORING TESTS	6. RECURRENCE TESTS	7. SURVIVAL/PROGNOSIS PREDICTION TESTS
Panel 1 CD4,INFG,PRF1, LAG3,PDL1,/CD274, IL12A, TNF [34] **Panel 2** FOXP3,CTLA4,CD8, TREG CELLS, IL-10 [34] **Panel 3** CD8A, GZMB, IL17A, TGFB1, PTGS2, IL1B, IL6, IDO1, NOS2, HIF1A [34]	**A. Specific for colorectal cancer** i) Faecal occult blood testing ii) Stool DNA, miRNA [24, 67] iii) Faecal immunological test (FIT) [68] iv) Faecal bacteria v) Gut microbiota signatures [69] **B. Colorectal and non-colorectal cancers** i) DNA, RNA, cfDNA, ctDNA, cfRNA, mRNA, microRNA, IncRNA ii) Circulating tumour cells iii) CA 125 Blood test [70] iv) Methylation tests [23] iv) Growth factors tests v) Tissue tests vi) Proteins and Glycoproteins tests [71] v) Tissue tests vi) Volatile organic compounds (VOC) [72] vii) Immune-Inflammation index [66] viii) Prostate cancer antigen 3 test (PCA3) [73] ix) Genomic Prostate Score [74]	**A. Gastric cancer** i) Upper endoscopy ii) Barium meal [75] **B. Breast cancer** i) Mammography and/or automated breast ultrasound (ABUS) [76] **C. Ovarian and womb** (Endometrium) cancers i) Transvaginal ultrasound **D. Colorectal cancer** i) Colonoscopy ii) Barium enema [75] **E. Urinary system cancers** i) DRE with prostatic massage [73] ii) PSA isoforms and prostate health index [73] iii) Urinary cytology iv) Cystoscopy	i) Biopsy ii) 2D or 3D Ultrasound [77] iii) CT scan iv) MRI v) PET scan [78] vi) Bone scintigraphy [79]	i) Biopsy ii) Other biomarkers [67] iii) X-ray iv) Ultrasound v) CT scan vi) MRI vii) PET scan [78] viii) Bone scintigraphy [79]	i) Biopsy ii) Blood [67] iii) X-ray iv) Ultrasound v) CT scan vi) MRI vii) PET scan [78] viii) Bone scintigraphy [79]	i) Immunogenomic biomarkers ii) Other biomarkers [67] iii) Circulating nucleic acids iv) Serum vitamin D levels [80] v) Tumour size vi) Radiomic signature PET/CT [81] vii) Diffusion weighed imaging/apparent diffusion coefficient (ADC) [82] viii) MRI ix) Bone scintigraphy [79]

4. Potential outcomes and future perspective

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a. Direct potential outcomes

i. Re-engagement, improved adherence, and motivation to participate in cancer screening in a substantial number of LSVH who have been lost-to-follow-up [83].
ii. Discovery of a novel, reliable, non-invasive, cost-effective, and accessible immunogenomic biomarker profiles applicable to LSVH.
iii. Potential research to develop cancer immunotherapy strategies based on *in vitro* stimulation of circulating immune cells in blood against tumour-specific immunogenic peptides derived from frameshift mutations found in LSVH and to develop individualised cancer vaccines using neoantigens.
iv. Establishment of a unique resource of phenotypic and clinical follow-up data to analyse cancer heterogeneity and cancer expression as a function of immunogenic, genetic, and environmental factors in LSVH carrying the same or different novel PV [84-86].
v. Improved quality of life, with reduced morbidity and mortality, and lower cancer-related costs for LSVH.

b. Indirect outcomes

i. The creation of a distinct research niche focusing on immunology, genomics and cancer.
ii. Enhancing scientific knowledge, technical ability, and/or clinical practice in a transdisciplinary fashion and advancing the uptake basic biomedical concepts in the clinical realm.
iii. Growing the accessibility of genomics and molecular biology to a wider application in developing world settings. Bringing together laboratory science, immunology, genetics, and oncology in research especially in the underserved and underrepresented populations of familial cancers in Africa, and translating this into a more effective relationship with the community and society.

5. Conclusions

Developments in cancer immunogenomics could lead to a substantial reduction in morbidity and mortality of familial cancers such as LS. In LS, frameshift germline mutations in DNA mismatch repair genes lead to post-replication errors and neoantigen production. This can cause pre-symptomatic chronic immune responses in LSVH. It is possible to identify measurable and dynamic immune components that, if effectively characterised, can be used as novel diagnostic and prognostic biomarkers to monitor cancer progression in both healthy and cancer diagnosed LSVH as an alternative to invasive cancer screening tests including colonoscopies. The use of immunogenomics in LSVH may provide (i) novel and personalised immunogenomic biomarkers for non-invasive cancer screening and surveillance, (ii) fewer invasive colonoscopies for CRC screening and diagnosis, and (iii) novel insights into cancer immunotherapies and vaccine development. Ongoing and future vaccine immunoprevention studies in LS will guide the development of technologies that can then be applied more broadly to help these individuals with a higher-than-average risk for cancer.

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