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Liere, E.L.S.A. van; Ramsoekh, D.; Daulton, E.; Dakkak, M.; Lingen, J.M. van; Stewart, T.K.; ... ; Boer, N.K.H. de

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Faecal Volatile Organic Compounds to Detect Colorectal Neoplasia in Lynch Syndrome—A Prospective Longitudinal Multicentre Study

Elsa L. S. A. van Liere[1,2] | Dewkoemar Ramsoekh[1,2] | Emma Daulton[3] | Maya Dakkak[1,4] | Joris M. van Lingen[1,4] | Trenton K. Stewart[3] | Sofie Bosch[1,2] | Beatriz Carvalho[5] | Evelien Dekker[1,2] | Maarten A. J. M. Jacobs[1] | Jan Jacob Koornstra[6] | Johan P. Kuijvenhoven[7] | Monique E. van Leerdam[8,9] | Tim G. J. de Meij[2,10] | Gerrit A. Meijer[5] | Manon C. W. Spaander[11] | James A. Covington[3] | Nanne K. H. de Boer[1,2]

[1]Department of Gastroenterology and Hepatology, Amsterdam University Medical Center, Amsterdam, The Netherlands | [2]Amsterdam Gastroenterology Endocrinology Metabolism Research Institute, Amsterdam, The Netherlands | [3]School of Engineering, University of Warwick, Coventry, UK | [4]School of Medicine, Vrije Universiteit, Amsterdam, The Netherlands | [5]Department of Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands | [6]Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands | [7]Department of Gastroenterology and Hepatology, Spaarne Gasthuis, Hoofddorp, The Netherlands | [8]Department of Gastrointestinal Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands | [9]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands | [10]Department of Paediatric Gastroenterology, Amsterdam University Medical Center, Amsterdam, The Netherlands | [11]Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands

Correspondence: Elsa L. S. A. van Liere (elsa.vanliere@amsterdamumc.nl)

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ABSTRACT

Background: Non-invasive biomarkers may reduce post-colonoscopy colorectal cancer (CRC) rates and colonoscopy overuse in Lynch syndrome. Unlike faecal immunochemical test (FIT), faecal volatile organic compounds (VOCs) may accurately detect both advanced and non-advanced colorectal neoplasia.

Aim: The aim of this study was to evaluate the potential of faecal VOCs—separately and with FIT—to guide optimal colonoscopy intervals in Lynch syndrome.

Methods: Prospective longitudinal multicentre study in which individuals with Lynch syndrome collected faeces before and after high-quality surveillance colonoscopy. VOC-patterns were analysed using field asymmetric ion mobility spectrometry (FAIMS) and gas chromatography-ion mobility spectrometry (GC-IMS) followed by machine learning pipelines, and combined with FIT at 2.55 μg Hb/g faeces. Gas chromatography time-of-flight mass spectrometry analysed individual VOC abundance.

Results: Among 200 included individuals (57% female, median 51 years), 62 had relevant neoplasia at colonoscopy: 3 CRC, 6 advanced adenoma (AA), 3 advanced serrated lesion (ASL), and 50 non-advanced adenoma (NAA). Respective sensitivity and negative predictive value for CRC and AA (and also ASL in case of FAIMS) were 100% and 100% using FAIMS (54% specificity), and 89% and 99% using GC-IMS (58% specificity). Respective sensitivity and specificity for any relevant neoplasia were 88% and 44% (FAIMS) and 84% and 28% (GC-IMS); accuracy did not significantly improve upon VOC-FIT. VOC-patterns differed before and after polypectomy (AUC 0.70). NAA showed decreased faecal abundance of butanal, 2-oxohexane, dimethyldisulphide and dimethyltrisulphide.

Conclusions: In Lynch syndrome, faecal VOCs may be a promising strategy for postponing colonoscopy and for follow-up after polypectomy. Our results serve as a stepping stone for large validation studies.
Trial Registration: NL8749

1 | Introduction

Lynch syndrome is the most common hereditary colorectal cancer (CRC) predisposition syndrome, affecting an estimated 1 in 279 individuals [1]. Individuals with Lynch syndrome have an increased lifetime risk for CRC, varying from 15% to 70% between the different causal pathogenic germline variants [2]. CRC incidence and mortality have reduced considerably due to early detection of CRC and removal of premalignant adenomas, through biennial surveillance colonoscopies starting at the age of 25 years [2, 3].

However, regular and lifelong colonoscopy surveillance poses several challenges: the invasive procedures are not only costly and resource intensive but also experienced as time-consuming, burdensome, and detrimental to patients' quality of life [4–6]. As a result of these barriers, among others, as many as 28% of individuals demonstrate delayed surveillance, which undermines the effectiveness of the program [5, 7, 8]. Therefore, it would be valuable to lower the number of 'unnecessary' colonoscopies (colonoscopies without relevant neoplasia being present), which may be as many as 70% of currently performed colonoscopies for Lynch syndrome [9, 10]. On the other hand, despite strict colonoscopy surveillance, post-colonoscopy CRCs still occur [11]. Most post-colonoscopy CRCs likely result from accelerated transition of normal colonic mucosa to carcinoma within the surveillance interval, and from missed or incompletely resected premalignant lesions [12]. As such, some individuals may benefit from a shorter colonoscopy interval.

Post-colonoscopy CRC rates and colonoscopy overuse in Lynch syndrome may potentially reduce by using non-invasive biomarkers that guide optimal colonoscopy intervals [13]. Such biomarkers should have high sensitivity for CRC, advanced adenomas and ideally non-advanced adenomas, accepting suboptimal specificity. CRC and advanced adenomas, but not non-advanced adenomas, have been detected with high sensitivity in Lynch syndrome and other high-risk populations by the faecal immunochemical test (FIT) at thresholds $\leq 4\,\mu g$ Hb/g faeces [14–17]. In contrast, both advanced and non-advanced colorectal neoplasia have been accurately identified in average-risk populations using faecal volatile organic compounds (VOCs) [18–24]. Faecal VOCs also showed promising results for follow-up after polypectomy [19]. Nevertheless, the potential of faecal VOCs for surveillance in Lynch syndrome has not yet been studied.

Given this knowledge gap, we designed a prospective longitudinal multicentre study to evaluate the performance of faecal VOCs—as a single test and in combination with FIT—for detection of (non-)advanced colorectal neoplasia and for intra-individual follow-up after polypectomy in Lynch syndrome. A non-invasive biomarker panel of faecal VOCs might enable amore effective and personalised surveillance in Lynch syndrome.

2 | Methods

2.1 | Study Design

This prospective longitudinal multicentre study was performed from August 2021 to March 2023 at five hospitals across the Netherlands: three academic hospitals (Amsterdam University Medical Center, Erasmus University Medical Center, University Medical Center Groningen), one comprehensive cancer centre (Netherlands Cancer Institute) and one general hospital (Spaarne Gasthuis). The design and conduct of the study were endorsed by the Dutch Lynch Polyposis patient association. The study was approved by the Research Ethical Committee of Amsterdam UMC (2020.317) alongside the local ethical committees of the other participating centres, and was registered at the WHO International Clinical Trials Registry Platform (NL8749). All study participants provided written informed consent.

2.2 | Study Participants and Sample Collection

We invited consecutive individuals with a pathogenic germline mismatch repair gene variant and without a history of (sub-)total colectomy, who were scheduled for surveillance colonoscopy, to participate in our study. Included individuals were asked to collect faeces in the 3 months before surveillance colonoscopy and bowel preparation, as well as in the 3–6 months after the colonoscopy. At the time of sample collection, participants completed an online questionnaire concerning stool consistency, various lifestyle factors related to faecal VOC-composition [25–27], and patient acceptability regarding both faeces collection and surveillance colonoscopy on a scale ranging from extremely burdensome [0] to not burdensome [10].

Samples were collected in plastic containers with a spoon embedded into the cap (Sarstedt, Germany), using the 'FecesCatcher' (TAG Hemi, The Netherlands) to ease sample collection and prevent contamination from urine and toilet water. Samples were stored in participant's own freezer within 1 h after collection, and transported to the hospital either by the participant using icepacks (De Ridder Packaging, The Netherlands) or by an investigator using dry ice. Upon arrival at the hospital, the samples were directly stored at −20°C until further analysis.

Individuals were excluded if during surveillance colonoscopy the cecum was not intubated, the Boston Bowel Preparation Score was < 2 in one or more segments, the endoscopist observed signs of infection or inflammation, or the histopathology report was not available after polypectomy. Individuals were also excluded in case of insufficient faecal sample for analysis or bowel preparation within 3 months before sample collection.

2.3 | Colonoscopy and Histopathology

Colonoscopies were either performed or supervised by a consultant gastroenterologist who had performed over 2000 colonoscopies. In line with the European Society of Gastrointestinal Endoscopy guideline for colonoscopies in Lynch syndrome, high-definition white light endoscopy was used with additional use of advanced imaging techniques at the discretion of the endoscopist [2]. Following the European Society of Gastrointestinal Endoscopy quality measures, withdrawal time was at least 6 min [28]. Except for obvious hyperplastic polyps ≤ 5 mm in the rectosigmoid [29], all polyps were resected using standard polypectomy techniques. Histopathology was assessed by experienced gastrointestinal pathologists and was described according to the Vienna classification of gastrointestinal neoplasia [30]. Neoplasia size, morphology and location were obtained from the endoscopy report. Neoplasia located in the splenic flexure, descending colon, sigmoid and rectum were classified as distal, whereas neoplasia in the cecum, ascending colon and transverse colon were classified as proximal.

2.4 | Sample and Data Analysis

Samples were analysed after a median storage period of 1 year, as VOCs are considered stable for this period when stored frozen [31]. Using a calibrated scale and electric drill (Dremel 4250, United States), sub-samples of 0.50 g faeces (range 0.45–0.55 g) were obtained and transferred to glass vials. Researchers blinded to colonoscopy results analysed headspace VOCs of faecal samples with three advanced systems, all analysing different chemical windows and having different advantages and disadvantages: gas chromatography—ion mobility spectrometry (GC-IMS), field asymmetric ion mobility spectrometry (FAIMS) and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Analytical methodology of each system is detailed in the supporting infomation.

For data analysis, the GC-IMS and FAIMS output underwent four pre-processing steps: data alignment and data scaling to correct for environmental and instrumental disturbances, manual cropping to reduce data dimensionality, and threshold application to remove background noise. Next, faecal VOC patterns (i.e., the 'VOC-fingerprint') were compared between the groups detailed below, using a custom data analysis platform in R version 2022.07.1–554 [19]. This platform performed binary class prediction using 10-fold cross validation, following which discriminatory data point features were implemented into five separate machine learning algorithms (random forest, support vector machine, XGBoost, Gaussian process and sparse logistic regression). For each algorithm, performance of VOC patterns was calculated in terms of sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and the area under the curve (AUC). The processing of the GC-TOF-MS output involved dynamic background compensation as well as integration and deconvolution of peaks. Upon forward and reverse searching set to 450, peaks were matched to individual VOCs registered in the NIST 2020 library.

Differences in patient characteristics between groups were assessed using the Mann–Whitney *U* test for continuous variables, and the Chi-squared or Fisher's exact test (in case ≥ 20% of cells had expected counts < 5) for categorical variables. Patient acceptability of faeces collection and colonoscopy were examined with the Mann–Whitney *U* test, followed by ordinal logistic regression to explore correlations with age and gender.

2.5 | Outcome Parameters

Individuals were classified by their most relevant neoplasia at colonoscopy. Relevant neoplasia included CRC, advanced adenoma (adenomas ≥ 10 mm, with villous component or with high-grade dysplasia [32]), advanced serrated lesion (serrated lesions ≥ 10 mm or with dysplasia [33]) and non-advanced adenoma. Serrated lesions involved traditional serrated adenoma, sessile serrated lesion (SSL), and hyperplastic polyp [34]. Individuals with non-advanced serrated lesions only, along with individuals having no colorectal lesions, were deemed as controls, since they are considered to have a negligible risk for CRC [35].

Figure 1 presents an overview of the outcome parameters. The samples collected before colonoscopy were used to evaluate the diagnostic performance of faecal VOC patterns (i.e., the 'VOC-fingerprint' analysed using FAIMS and GC-IMS) for the following target groups: (i) any relevant neoplasia (CRC, advanced serrated lesion, any adenoma), (ii) advanced neoplasia (CRC, advanced adenoma, advanced serrated lesion) and (iii) CRC plus advanced adenoma. To evaluate the performance of faecal VOC patterns for intra-individual follow-up after polypectomy, we compared GC-IMS outcomes of samples collected before and after colonoscopy of both controls and patients with successful polypectomy (insufficient faecal sample hindered these analyses for FAIMS). Next, we investigated whether neoplasia detection and follow-up was affected by external factors related to VOC composition, by performing sensitivity analyses in individuals with and without neoplasia that were matched 1:1 on gender, age (± 10 years), body mass index (18.5–25, 25–30, or ≥ 30 kg/m^2), smoking habits (yes/no active smoker), and dietary habits (yes/no vegetarian) [25–27]. In another sub-analysis, we investigated the diagnostic performance of combined sequential FIT-VOC for any relevant neoplasia in Lynch syndrome (the vast majority of CRCs and advanced adenomas are already detected by FIT [17]). This sub-analysis involved faecal VOC pattern analysis in individuals with Lynch syndrome having a negative FIT at the lower limit of quantitation of 2.55 μg Hb/g faeces (SENTiFIT—FOB gold test collected in our previous study [17], within 30 days of the VOC sample), for which we used Fagan's nomogram in RStudio version 4.2.1 with the probability of any relevant neoplasia in FIT-negatives being the pre-test probability for subsequent VOC-analysis.

Due to financial constraints, GC-TOF-MS analyses were performed in a subset of samples, consisting of randomly selected controls and individuals with non-advanced adenomas (advanced neoplasia were not included to promote homogeneity).

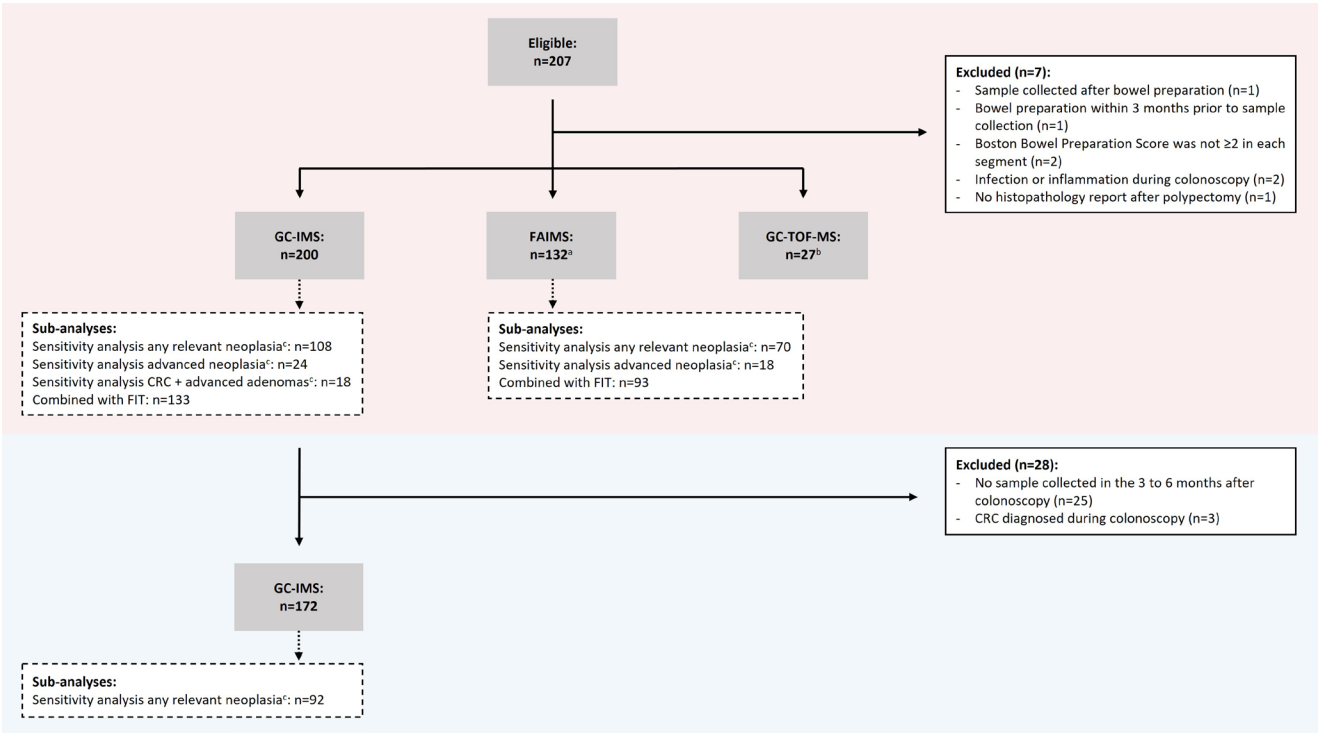

FIGURE 1 | Flow diagram showing the different analyses conducted, including numbers of patients analysed, to evaluate the performance of faecal volatile organic compounds for detection of colorectal neoplasia (upper red box) and for intra-individual follow-up after polypectomy (lower blue box). Abbreviations: CRC = colorectal cancer; FAIMS = field asymmetric ion mobility spectrometry; FIT = faecal immunochemical test; GC-IMS = gas chromatography—ion mobility spectrometry; GC-TOF-MS = gas chromatography time-of-flight mass spectrometry. [a]Due to insufficient faecal sample, FAIMS could not be evaluated for neoplasia detection in 68 individuals nor for neoplasia follow-up in any individual. [b]Due to financial constraints, GC-TOF-MS was performed in a random selection of 13 non-advanced adenomas and 14 controls. [c]For sensitivity analysis, individuals with and without colorectal neoplasia were matched 1:1 on possible confounders: Gender, age, body mass index, smoking habits and dietary habits. Only $n = 8$ (GC-IMS) and $n = 6$ (FAIMS) non-advanced adenomas could not be matched and therefore were excluded. Moreover, the analysis 'CRC + advanced adenomas' could not be performed for FAIMS due to insufficient number of CRC and advanced adenomas in this group.

Between groups, the Mann–Whitney *U* test compared the abundance (i.e., peak area as analysed by GC-TOF-MS) of those individual faecal VOCs present in 40% of samples; this criterion avoided possible over-or underestimation of the results. The names of discriminatory VOCs were standardised against the Human Metabolome Database and Pubchem.

2.6 | Sample Size Calculation

In PASS 2022 version 22.0.3, the required sample size was calculated by a non-inferiority test for paired dichotomous data with 80% power and a one-sided significance level of 5%. The non-inferiority margin for the absolute difference in relevant neoplasia detection between colonoscopy and faecal VOCs was set at 10%. We assumed that the nuisance parameter would be ≤10% [19]. Under the alternative hypothesis of equal positive tests by the two methods (i.e., the actual absolute difference being 0%), 81 individuals were required to prove non-inferiority. As the current study was part of a larger study in which individuals were asked to collect multiple samples over a 2-year period, dropout or lost to follow-up rates were expected to be significant over time, therefore, the sample size was set at 200 individuals.

3 | Results

3.1 | Patient Characteristics

In total, 200 individuals with Lynch syndrome were included in the study (Figure 1; Table 1). Among inclusions, 87 (44%) were male, median age was 51 years (IQR 42–62) and 39 (20%) had a personal history of CRC. The most common mutated genes were *MSH6* and *PMS2*, followed by *MSH2* and *MLH1*, and then *EPCAM*. The vast majority of individuals (83%) had undergone two or more colonoscopies before inclusion, with a median time since last colonoscopy of 24 months (IQR 20–25). Patient characteristics were largely similar in the cohorts analysed with FAIMS and GC-TOF-MS, except that in the latter most individuals had no history of CRC and had pathogenic variants in *PMS2*, or in *MSH6* and *MLH1* (Tables S2 and S4).

3.2 | Neoplasia Characteristics

Among 200 included individuals, 62 (31%) had relevant neoplasia diagnosed at colonoscopy. The most relevant neoplasia was CRC in 3/200 (1.5%; all adenocarcinomas), advanced adenoma in 6/200 (3.0%; all ≥ 10 mm with low-grade dysplasia), advanced

TABLE 1 | Characteristics of patients analysed with gas chromatography—ion mobility spectrometry, *n* (%) or median (IQR).

	To evaluate neoplasia detection			To evaluate follow-up after polypectomy		
	Relevant neoplasia (*n* = 62)	Controls (*n* = 138)	*p*	Relevant neoplasia (*n* = 52)	Controls (*n* = 120)	*p*
Male	34 (55)	53 (38)	0.030	28 (54)	46 (38)	0.059
Age	59 years (48–68)	50 years (40–60)	0.003	58 years (48–68)	51 years (41–61)	0.025
Pathogenic variant						
MLH1	9 (15)	28 (20)	0.617	7 (13)	23 (19)	0.823
MSH2	16 (26)	29 (22)		13 (25)	26 (22)	
MSH6	16 (26)	44 (32)		14 (27)	37 (31)	
PMS2	20 (32)	35 (25)		17 (33)	32 (27)	
EPCAM	1 (1)	2 (1)		1 (2)	2 (1)	
History of colorectal cancer	14 (23)	25 (18)	0.461	10 (19)	22 (18)	0.890
History of bowel resection						
No	48 (77)	113 (82)	0.536	42 (81)	98 (82)	0.366
Left hemicolectomy	3 (5)	2 (1)		3 (5)	2 (1)	
Right hemicolectomy	8 (13)	13 (9)		6 (12)	11 (9)	
Proctectomy or sigmoidectomy	3 (5)	9 (7)		1 (2)	8 (7)	
Ileocecal resection	—	1 (1)		—	1 (1)	
Number of previous colonoscopies						
0	9 (15)	5 (3)	0.018	6 (12)	5 (4)	0.217
1	5 (8)	16 (12)		4 (8)	12 (10)	
2+	48 (77)	117 (85)		42 (80)	103 (86)	
Surveillance interval	24 months (15–26)	24 months (22–25)	0.790	24 months (15–25)	24 months (22–25)	0.592
Comorbidity						
Diabetes mellitus type I or II	4 (6)	4 (3)	0.257	4 (8)	4 (3)	0.246
Hypertension	8 (13)	8 (6)	0.097	7 (13)	8 (7)	0.154
Medication use in the 3 months before sample collection						
Oral antibiotics	—	4 (3)	0.313	—	7 (6)	0.103
Proton pump inhibitors	12 (19)	14 (10)	0.073	12 (23)	11 (9)	0.014
Laxatives	5 (8)	13 (10)	0.757	4 (8)	8 (7)	0.756
Probiotics	6 (10)	8 (6)	0.372	6 (12)	4 (3)	0.068
Vitamin supplements	32 (52)	79 (57)	0.458	25 (48)	66 (55)	0.403
Body mass index[a]						
18.5–25 kg/m^2	24 (39)	65 (47)	0.540	22 (42)	55 (46)	0.917
25–30 kg/m^2	29 (46)	55 (40)		23 (44)	50 (41)	
≥ 30 kg/m^2	9 (15)	18 (13)		6 (12)	15 (13)	

(Continues)

TABLE 1 | (Continued)

	To evaluate neoplasia detection			To evaluate follow-up after polypectomy		
	Relevant neoplasia ($n=62$)	**Controls ($n=138$)**	***p***	**Relevant neoplasia ($n=52$)**	**Controls ($n=120$)**	***p***
Smoking status						
Smoker	9 (15)	8 (6)	0.074	7 (13)	7 (6)	0.227
Ex-smoker (not smoked for > 6 months)	26 (42)	53 (38)		19 (36)	44 (36)	
Never smoked	27 (43)	77 (56)		26 (50)	69 (58)	
Diet[a,b]						
Regular diet	59 (95)	119 (86)	0.123	47 (90)	106 (88)	0.182
Vegetarian	3 (5)	13 (10)		4 (8)	7 (6)	
Other	—	6 (4)		—	7 (6)	
Stool consistency[a,c]						
BSC 1	—	2 (1)	0.506	1 (2)	4 (3)	0.459
BSC 2	4 (6)	9 (14)		4 (8)	18 (15)	
BSC 3	16 (26)	32 (23)		14 (27)	25 (21)	
BSC 4	24 (39)	55 (40)		18 (35)	49 (40)	
BSC 5	3 (5)	6 (4)		2 (4)	8 (7)	
BSC 6	11 (18)	20 (14)		9 (17)	15 (13)	
BSC 7	4 (6)	3 (2)		2 (4)	1 (1)	
Stool collection season						
Winter	15 (25)	39 (28)	0.332	13 (25)	34 (28)	0.529
Spring	18 (29)	40 (29)		16 (31)	35 (30)	
Summer	4 (6)	18 (13)		4 (8)	17 (14)	
Autumn	25 (40)	41 (30)		19 (36)	34 (28)	
Number of relevant neoplasia at study colonoscopy per patient						
1	36 (58)	n.a.	n.a.	31 (59)	n.a.	n.a.
2	20 (33)			17 (33)		
3	4 (6)			3 (6)		
4+	2 (3)			1 (2)		
Most relevant neoplasia at study colon						
Colorectal cancer	3	n.a.	n.a.	n.a.	n.a.	n.a.
Advanced adenoma	6			5		
Advanced serrated lesion	3			3		
Non-advanced adenoma	50			44		

[a]Cumulative percentage is not 100% due to some missing values.
[b]Other dietary habits included vegan, gluten-free and/or lactose-free.
[c]BSC: Bristol Stool Chart, which classifies stool into seven categories, with 1 being severe constipation and 7 severe diarrhoea.

serrated lesion in 3/200 (1.5%; one SSL < 10 mm with dysplasia and two SSLs ≥ 10 mm without dysplasia), and non-advanced adenoma in 50/200 (25.0%). Of the three CRCs, one was stage III (proximal cT2N1) and two were stage I (one proximal and one distal pT1N0). The adenomas and advanced serrated lesions were proximally located in 54% and had sessile morphology in 54% (Table S1). Neoplasia characteristics were similar in the cohorts analysed with FAIMS and GC-TOF-MS (Tables S3 and S4).

Compared to controls, individuals with relevant neoplasia differed in terms of age, gender, number of previous colonoscopies, hypertension and proton pump inhibitor usage (Tables 1, S2 and S4). Patients with CRC or advanced adenomas were more likely to be male, carry *MSH2* or *MLH1* variants, to have a personal history of CRC and/or to have never had a colonoscopy.

3.3 | Diagnostic Accuracy—Any Relevant Neoplasia

Tables 2 and S5 and Figures 2 and S1 show the accuracy and receiver operating characteristic curves to detect each colorectal neoplasia group by faecal VOC patterns as analysed with FAIMS and GC-IMS. To detect any relevant colorectal neoplasia, respective sensitivity and NPV were 84% and 80% for GC-IMS, whereas 88% and 89% for FAIMS. However, specificity was poor; 28% for GC-IMS and 44% for FAIMS. On sensitivity analysis, sensitivity lowered to 74% (GC-IMS) or remained 88% (FAIMS), and specificity remained poor.

3.4 | Diagnostic Accuracy—Advanced Neoplasia

Assessing advanced neoplasia as the target lesions, respective sensitivity and NPV were 67% and 95% for GC-IMS, whereas 100% and 100% for FAIMS—at moderate specificity of 61% (GC-IMS) and 54% (FAIMS). On sensitivity analysis, sensitivity improved to 92% (GC-IMS) or remained 100% (FAIMS), whilst specificity improved to 67% (GC-IMS) and 89% (FAIMS).

3.5 | Diagnostic Accuracy—CRC and Advanced Adenomas

The FAIMS group included only 6 CRCs and advanced adenomas, hence, detection of this target group could only be assessed in the GC-IMS group (9 lesions), which demonstrated 89% sensitivity, 99% NPV and 58% specificity. On sensitivity analysis, sensitivity lowered to 78% at equal specificity.

3.6 | Follow-Up After Polypectomy

The performance of VOC patterns for intra-individual follow-up after polypectomy was assessed in 120 controls and 52 individuals with complete removal of advanced adenomas ($n = 5$), advanced serrated lesions ($n = 3$) or non-advanced adenomas ($n = 44$, Figure 3). We observed that VOC patterns measured with GC-IMS seem to be similar before and after normal colonoscopy ('controls', AUC 0.56), but different before and after successful polypectomy (AUC 0.70). Following successful polypectomy, VOC patterns seem to be similar to those of controls (AUC 0.50)—although this was not confirmed when we corrected for external confounders on sensitivity analysis (AUC 0.67).

3.7 | Combined FIT-VOC

The probabilities of any relevant neoplasia and specifically non-advanced adenomas were 32% and 26% prior to FIT, respectively, whereas 28% and 26% after negative FIT (Figure 4). When subsequent VOC test was also negative, the probability of any relevant neoplasia reduced to 19% (FAIMS) or 16% (GC-IMS), indicating that sensitivity of FIT-VOC to detect non-advanced adenomas was at least 27% (FIT with FAIMS) and 39% (FIT with GC-IMS). When individuals had a positive VOC test after a negative FIT, the probability of any relevant neoplasia was 39% (GC-IMS) and 37% (FAIMS), representing 56% specificity.

3.8 | Adenoma-Associated VOCs

Among the 27 samples analysed, GC-TOF-MS identified 737 different individual faecal VOCs, of which 605 were excluded from further statistical analysis because they were present in less than 40% of samples. Upon statistical analysis of the remaining 132 VOCs, we found that the presence of non-advanced adenomas was associated with decreased faecal abundance of the following endogenous VOCs: butanal (aldehyde), 2-oxohexane (ketone), dimethyldisulphide and dimethyltrisulphide (both sulphides, Table S6). As mentioned in the method section, advanced neoplasia were not assessed by GC-TOF-MS.

3.9 | Patient Acceptability

Ranging from extremely burdensome [0] to not burdensome [10], median patient acceptability was 7 (IQR 6–9) for faeces collection and 6 (IQR 4–8) for surveillance colonoscopy ($p < 0.001$, Figure 5). Faeces collection was more often experienced as not burdensome by patients under 39 years than those over 60 years, irrespective of gender (OR 0.484, p 0.045).

4 | Discussion

This prospective longitudinal multicentre study is the first study evaluating the performance of faecal VOCs, separately and in combination with FIT, as non-invasive biomarker for colorectal neoplasia in Lynch syndrome. We analysed VOC patterns by different advanced systems and found that both GC-IMS and FAIMS detect CRC and advanced adenomas (and also advanced serrated lesions in case of FAIMS) with high sensitivity and NPV, which were, respectively, 89% and 99% for GC-IMS, whereas 100% and 100% for FAIMS—although specificity was moderate (GC-IMS: 58%, FAIMS: 54%). Our study also showed that successful polypectomy may lead to normalisation of VOC patterns, and that patients better tolerate faeces collection than colonoscopy.

Although analysing VOC patterns (or the 'VOC-fingerprint') would be suitable for clinical practice because it is rapid, simple and low-cost, only few studies have explored its potential to diagnose colorectal neoplasia. Nevertheless, diagnostic accuracy for CRC has consistently been high, with AUCs ranging between 0.84–0.96, independent of analytical system or biological specimen (faeces, urine or exhaled breath) [19, 36–39]. When VOCs were combined with FIT in a symptomatic population, the 0.5% probability of CRC after negative FIT at threshold 10 μg Hb/g faeces decreased to 0.1% after negative FIT-VOC [20]. Faecal and exhaled breath VOC patterns have also shown high

TABLE 2 | The accuracy of faecal volatile organic compounds, as analysed with different advanced systems, to detect colorectal neoplasia in Lynch syndrome.

	Machine learning algorithm[a]	Sensitivity (95% CI)	Specificity (95% CI)	Negative predictive value (95% CI)	Positive predictive value (95% CI)	AUC (95% CI)
Any relevant neoplasia[b]						
GC-IMS	SupportVectorMachine	84% (72–92)	28% (21–37)	80% (65–89)	34% (27–43)	0.51 (0.42–0.59)
GC-IMS—sensitivity analysis	SparseLogisticRegression	74% (60–85)	41% (28–55)	61% (44–76)	56% (43–67)	0.56 (0.45–0.67)
FAIMS	SupportVectorMachine	88% (74–96)	44% (34–55)	89% (75–96)	41% (31–52)	0.70 (0.60–0.79)
FAIMS—sensitivity analysis	XgBoost	88% (73–97)	40% (24–58)	78% (52–93)	59% (45–73)	0.60 (0.46–0.73)
Advanced neoplasia[b]						
GC-IMS	XGBoost	67% (35–90)	61% (52–69)	95% (88–99)	13% (6–24)	0.60 (0.39–0.81)
GC-IMS—sensitivity analysis	GaussianProcess	92% (62–100)	67% (35–90)	89% (51–99)	73% (45–91)	0.78 (0.58–0.98)
FAIMS	RandomForest	100% (66–100)	54% (43–64)	100% (91–100)	18% (9–31)	0.74 (0.61–0.86)
FAIMS—sensitivity analysis	SparseLogisticRegression	100% (66–100)	89% (52–100)	100% (60–100)	90% (54–99)	0.99 (0.98–1.00)
CRC + advanced adenomas[b]						
GC-IMS	GaussianProcess	89% (52–100)	58% (49–66)	99% (92–100)	12% (6–23)	0.71 (0.53–0.89)
GC-IMS—sensitivity analysis	XGBoost	78% (40–97)	56% (21–86)	71% (30–95)	64% (32–88)	0.62 (0.34–0.90)

Abbreviations: AUC = area under the curve; CI = confidence interval; FAIMS = field asymmetric ion mobility spectrometry; GC-IMS = gas chromatography—ion mobility spectrometry.
[a]Results are shown for the best performing machine learning algorithm, out of the five algorithms tested (random forest, support vector machine, XGBoost, Gaussian process and sparse logistic regression).
[b]Due to the smaller sample size, sensitivity analysis resulted in wider confidence intervals. In the sensitivity analyses, the neoplasia prevalence did not reflect reality, so the negative and positive predictive values are meaningless.

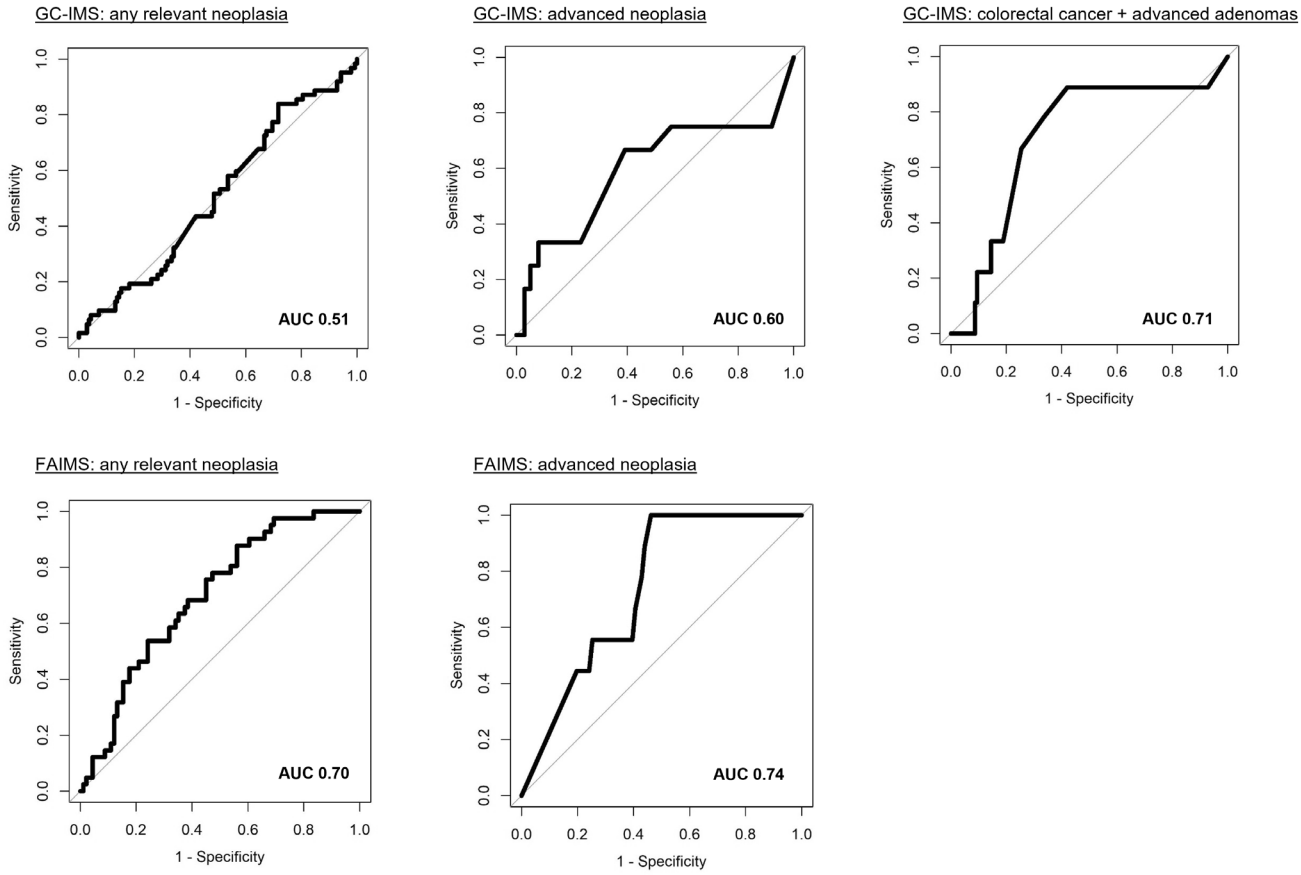

FIGURE 2 | Receiver operating characteristic curves to detect colorectal neoplasia in Lynch syndrome by faecal volatile organic compounds, as measured with gas chromatography—ion mobility spectrometry (GC-IMS) and field asymmetric ion mobility spectrometry (FAIMS).

accuracy for detection of advanced adenomas, with AUCs ranging between 0.73–0.96 [19, 36–38] (for urine data were scarce and more conflicting [40, 41]). The higher AUCs for CRC and advanced adenomas observed in other studies compared to ours might be attributed to a type II error stemming from the small number of advanced neoplasia assessed in our study, as our study population was already under strict colonoscopy surveillance. Additionally, the lower AUCs in our study were the result of moderate specificity, which may be acceptable in Lynch syndrome because high test-sensitivity is prioritised over specificity. One should also appreciate that a test with a moderate specificity of ~55% would still avoid unnecessary, burdensome and invasive colonoscopies in over half of individuals without advanced neoplasia, thereby potentially improving patients' quality of life and reducing costs and burden to health systems. Yet, it would be key to adequately inform patients about the possibility of a false-positive faeces test.

Next to CRC and advanced adenomas, we evaluated diagnostic performance of VOC patterns for advanced serrated lesions and non-advanced adenomas. Though the short-term malignant potential of advanced serrated lesions seems to be low in Lynch syndrome (granting a window for their detection) [35, 42–45], our results may suggest that faecal VOC patterns detect these lesions with high sensitivity when analysed by FAIMS but not by GC-IMS. Although these results should be interpreted with caution due to the small number of lesions, explanations for the difference in sensitivity may be that either technique analyses a different chemical window or that GC-IMS analyses involved an additional freeze–thaw cycle [46]. For non-advanced adenomas, identification is relevant in Lynch syndrome given the accelerated adenoma-carcinoma sequence, however, these lesions (the majority being ≤ 5 mm in our study) were poorly detected by both FAIMS and GC-IMS. Detection did not significantly improve when VOCs were combined with FIT at the lowest threshold of 2.55 μg Hb/g faeces: sensitivity was 27% (FIT with FAIMS) and 39% (FIT with GC-IMS) at 56% specificity. However, these numbers must be viewed alongside the 28% miss rate of adenomas ≤ 5 mm at (high-quality) colonoscopy [47]. Moreover, in a recent study including twice as many non-advanced adenomas as the current study, faecal VOC patterns analysed by GC-IMS did accurately detect non-advanced adenomas in average-risk individuals [19]. Similar to our study, VOC patterns differed before and after adenoma removal while after adenoma removal they were similar to those of controls [19], providing further evidence that the presence of adenomas may result in a distinct VOC profile.

The normalisation of VOC patterns after successful polypectomy also suggests that these biomarkers may be useful for intra-individual follow-up after polypectomy, in an effort to early detect missed or incompletely resected premalignant lesions and thereby potentially decrease post-colonoscopy CRC in Lynch syndrome. Yet, the question remains whether such tailored monitoring of VOC patterns would be cost-effective; perhaps it would only be feasible for individuals at highest risk of post-colonoscopy CRC (e.g., *MLH1* and *MSH2* carriers

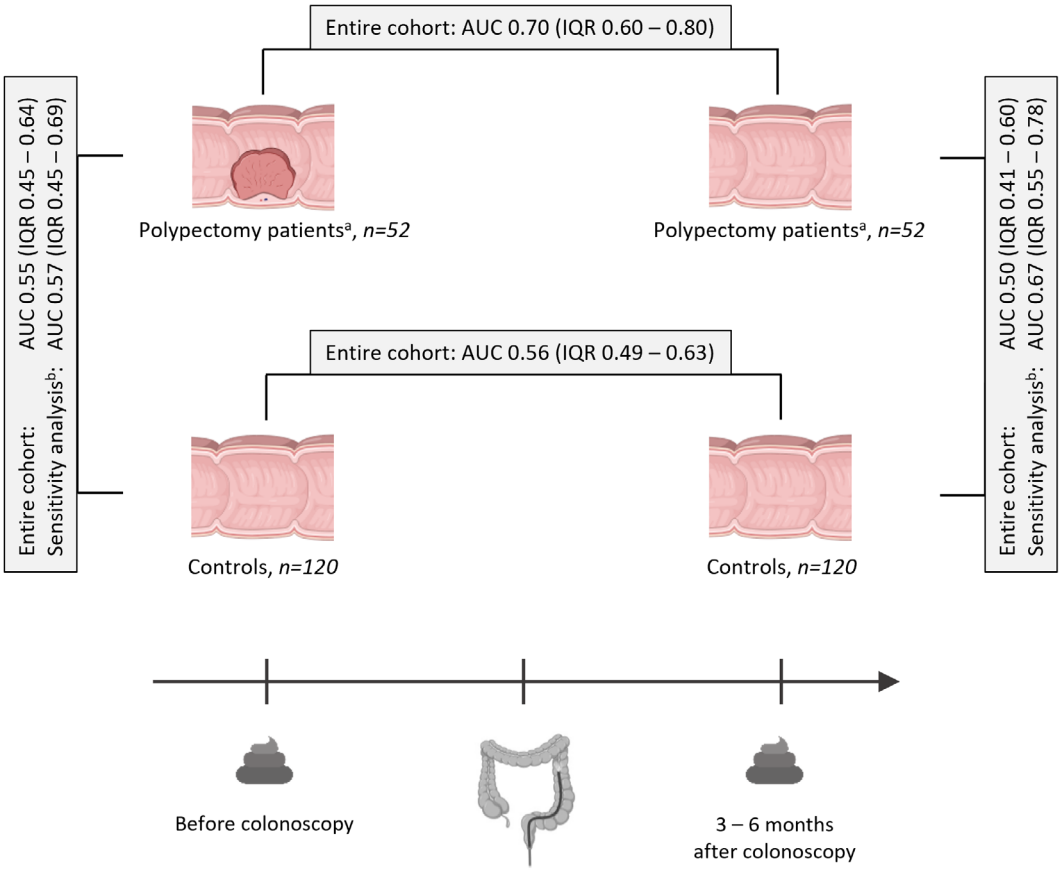

FIGURE 3 | The performance of faecal volatile organic compounds, as analysed with gas chromatography—ion mobility spectrometry, for intra-individual follow-up after polypectomy in Lynch syndrome. Samples collected before and after normal colonoscopy were analysed ('controls') as well as samples collected before and after complete removal of adenomas and advanced serrated lesions ('polypectomy patients'). [a]Of the 52 polypectomy patients, 5 (10%) had advanced adenomas, 3 (6%) advanced serrated lesions and 44 (85%) non-advanced adenomas. All neoplasia were resected en-bloc, except from one lesion which was resected piecemeal. [b]Sensitivity analysis included 46 controls and 46 polypectomy patients, of which 4/46 (9%) had advanced adenomas, 2/46 (4%) advanced serrated lesions and 40/46 (87%) non-advanced adenomas.

whom also have other risk factors for CRC such as personal history of CRC, smoking and overweight). Moreover, one should consider the dilemma of a negative colonoscopy after a positive faecal test, which may relate to a false-positive faecal test (further supporting its use in high-risk carriers only) but also to missed neoplasia at colonoscopy. Hence, to prevent (late-stage) CRC, close monitoring of such individuals would be essential, alongside continuous improvement of colonoscopy quality standards.

Next to VOC patterns, we analysed individual faecal VOCs by GC-TOF-MS, which is a complex, time-consuming, and expensive technique that provides detailed results on molecular level. We found that the presence of non-advanced adenomas was associated with decreased abundance of butanal, 2-oxohexane, dimethyldisulphide and dimethyltrisulphide. Although these VOCs need to be validated in other Lynch syndrome populations and for advanced neoplasia detection, previous studies have yet shown that these VOCs significantly differed in faeces, urine and breath of individuals with and without (colorectal) cancer [21, 39, 48–51]. Multiple other VOCs have been reported as CRC-associated yet none have shown consistent results across studies, which likely reflect geographical and particularly methodological variability [18, 23, 39, 50]. To ascertain the origin of the VOCs postulated as potential biomarkers for (non-advanced) adenomas in our study, these VOCs have to be linked to metabolic pathways. In this regard, we found that the decreased levels of butanal may have resulted from increased oxidisation of butanal into butyric acid to support tumorigenesis [52], whereas the decreased levels of dimethyldisulphide and -trisulphide from disrupted composition of (anaerobic) sulphate-reducing bacteria during tumour progression [24]. The alterations in 2-oxohexane and other ketones may be linked to the peroxidation of fatty acids and other lipids, which is a hallmark of tumour growth [53, 54].

An acknowledged limitation of our study is that it was not powered for detection of advanced neoplasia (only for detection of any relevant neoplasia) which resulted in a relatively small number of advanced neoplasia assessed, nevertheless, our promising results were in line with previous studies including large numbers of advanced neoplasia (as described above). The small number of advanced neoplasia also hampered external validation, although we did perform internal validation. Another limitation is that storage period varied by several months between samples to analyse all samples in one batch, however, previous research showed that accuracy of faecal VOCs was not influenced by storage duration

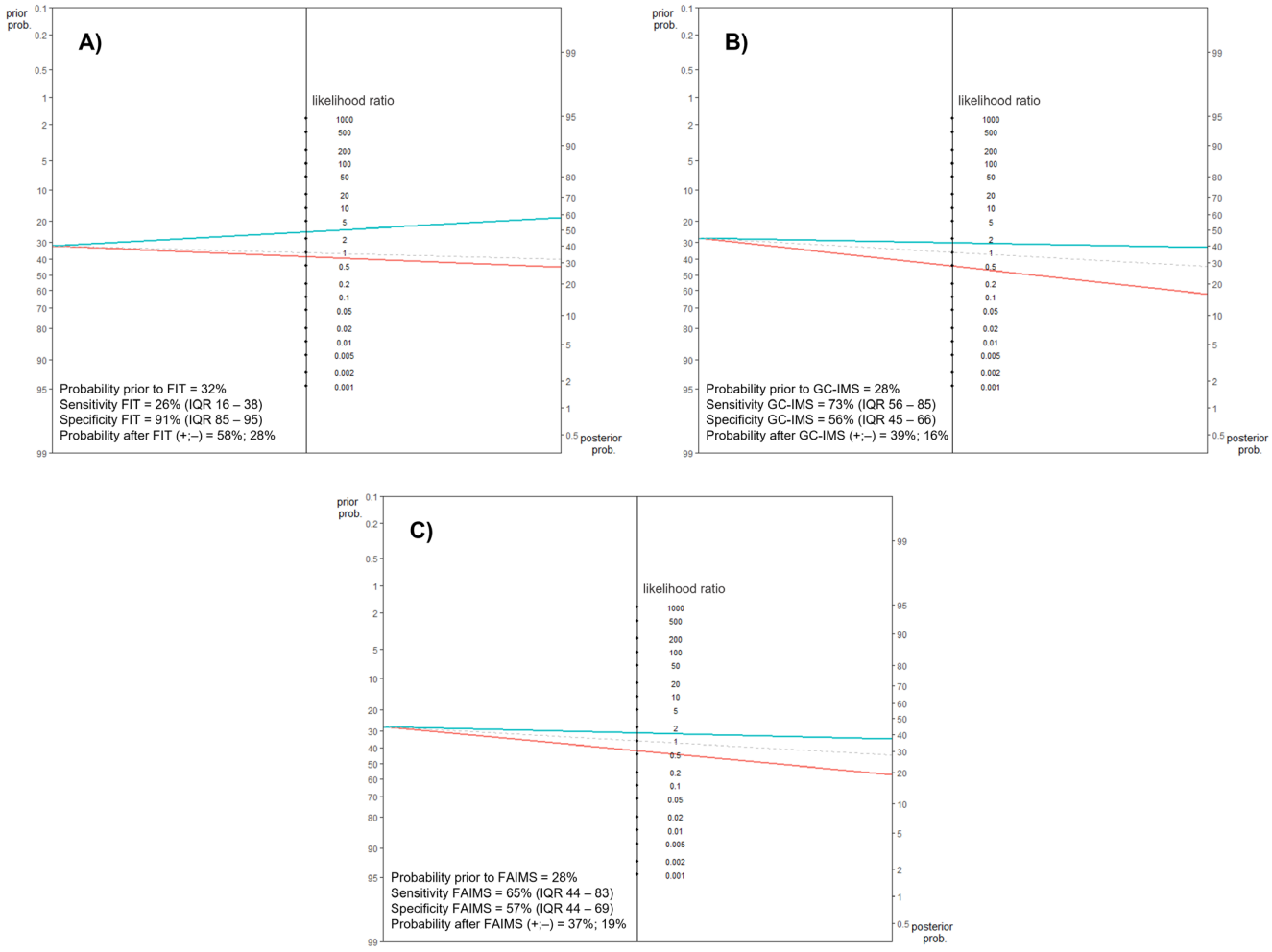

FIGURE 4 | Fagan's nomograms illustrating the probability of relevant neoplasia (colorectal cancer, advanced serrated lesions and all adenomas), following positive (blue line) or negative (red line) results of (A) FIT at threshold 2.55 μg Hb/g faeces[a], (B) GC-IMS analysis in FIT-negatives and (C) FAIMS analysis in FIT-negatives. The grey line represents the situation when the post-test probability remains unchanged from the pre-test probability, which represents the relevant neoplasia prevalence. Abbreviations: FAIMS = field asymmetric ion mobility spectrometry; FIT = faecal immunochemical test; GC-IMS = gas chromatography—ion mobility spectrometry; IQR = interquartile range. [a]Figure 4A Is adapted from our previous study [17]. This cohort consisted of 217 individuals with Lynch syndrome, in which FIT detected 4/4 colorectal cancers, 4/5 advanced adenomas, 1/4 advanced serrated lesions and 9/57 non-advanced adenomas. As such, relevant neoplasia were present in 28% of FIT-negatives further tested for VOC patterns: 0.5% had advanced adenomas, 1.5% advanced serrated lesions, and 26% non-advanced adenomas.

differences of 20 months [46]. A strength of this study is its prospective multicentre design, wherein participants collected samples both before and after high-quality colonoscopy in a standardised matter which was consistent with previously published key considerations for VOC-analysis [31, 46, 55, 56]. Moreover, we assessed the performance of various advanced systems for VOC analysis and the robustness of our outcomes through sensitivity analyses.

To advance faecal VOCs towards clinical application, several issues must be addressed. First, validation of our findings in studies that include larger numbers of both advanced and non-advanced neoplasia (our data can be used to calculate the required sample size). Second, methods to enhance acceptability of faeces collection among individuals over 60 years (whom in our study showed lower acceptability than their younger peers), after multiple surveillance rounds, and in populations outside The Netherlands considering that in some countries sampling faeces is taboo [57, 58]. Third, potential strategies to improve the observed moderate specificity of VOC patterns for colorectal neoplasia, such as adding other faecal biomarkers (e.g., FIT or DNA [59]), adding risk factors for CRC (e.g., *MLH1* or *MSH2* gene variants, personal history of CRC, smoking, overweight), or correcting for external confounders (e.g., gender, age, body mass index, smoking, diet), although studies must first determine which confounders primarily impact accuracy of VOCs and how these confounders can be corrected for. Lastly, the influence of various instrumental and sampling variables, including storage duration and storage at −20°C or −80°C (it is yet known that room temperature significantly affects VOC composition [46]). Next, an appropriate, evidence-based protocol for standardised VOC-analysis should be developed by various international experts, for example using the Delphi Method.

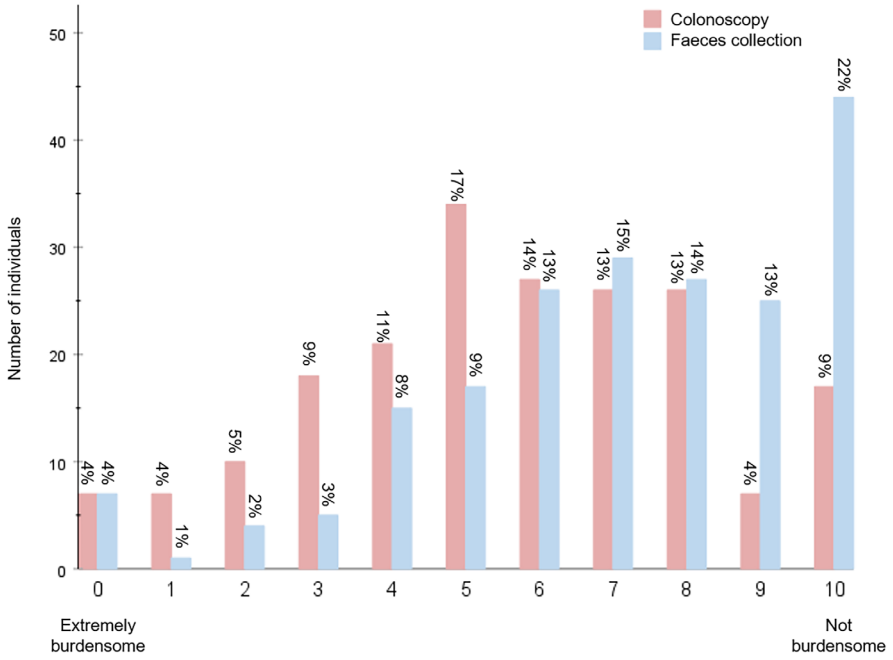

FIGURE 5 | Acceptability of faeces collection and surveillance colonoscopy by 200 individuals with Lynch syndrome on a scale from extremely burdensome [0] to not burdensome [10]. Median patient acceptability was 7 (IQR 6–9) for faeces collection and 6 (IQR 4–8) for colonoscopy, *p*-value < 0.001.

To conclude, faecal VOCs may be a promising strategy for postponing colonoscopy and for intra-individual follow-up after polypectomy in Lynch syndrome, aiming to reduce colonoscopy burden and post-colonoscopy CRC rates. Our results serve as a stepping stone for large validation studies that also consider external confounders and patient acceptability. In addition to their diagnostic potential, we showed that VOC identification on molecular level provides insight into the—not yet unravelled—pathophysiology of adenomas in Lynch syndrome.

Author Contributions

Elsa L. S. A. van Liere: conceptualization, writing – original draft, methodology, project administration, formal analysis. **Dewkoemar Ramsoekh:** conceptualization, writing – review and editing, funding acquisition, methodology, supervision. **Emma Daulton:** writing – review and editing, methodology, formal analysis. **Maya Dakkak:** writing – review and editing, project administration, formal analysis. **Joris M. van Lingen:** writing – review and editing, project administration, formal analysis. **Trenton K. Stewart:** writing – review and editing, methodology, formal analysis. **Sofie Bosch:** conceptualization, writing – review and editing, methodology. **Beatriz Carvalho:** conceptualization, writing – review and editing, methodology. **Evelien Dekker:** conceptualization, writing – review and editing, methodology, supervision. **Maarten A. J. M. Jacobs:** conceptualization, writing – review and editing, methodology, supervision. **Jan Jacob Koornstra:** conceptualization, writing – review and editing, methodology, supervision. **Johan P. Kuijvenhoven:** conceptualization, writing – review and editing, methodology, supervision. **Monique E. van Leerdam:** conceptualization, writing – review and editing, methodology, supervision. **Tim G. J. de Meij:** conceptualization, writing – review and editing, methodology. **Gerrit A. Meijer:** writing – review and editing, methodology. **Manon C. W. Spaander:** conceptualization, writing – review and editing, methodology, supervision. **James A. Covington:** conceptualization, writing – review and editing, methodology, formal analysis, supervision. **Nanne K. H. de Boer:** conceptualization, writing – review and editing, funding acquisition, methodology, supervision.

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Ethics Statement

The study was approved by the Research Ethical Committee of Amsterdam UMC (2020.317) alongside the local ethical committees of the other participating centres. All study participants provided written informed consent.

Conflicts of Interest

E.L.S.A.L., Emma D., M.D., J.M.L., T.K.S., S.B., M.A.J.M.J., J.J.K., J.P.K., M.E.L. and J.A.C. declare no competing interests. D.R. has received a research grant (unrestricted) from AbbVie. He has served as a member of the data safety monitoring board of the VIVIAD trial. B.C. has several patents pending and/or issued. Evelien D. has endoscopic equipment on a loan from FujiFilm and has received a research grant from FujiFilm. She has received an honorarium for a consultancy from FujiFilm, Olympus, InterVenn and Ambu, and speakers' fees from Olympus, GI Supply, Norgine, IPSEN, PAION and FujiFilm. T.G.J.M. has served as a speaker for Nutricia, Mead Johnson and Winclove. He has served as an advisory board member for Nutricia. G.A.M. is co-founder and board member (CSO) of CRCbioscreen BV. He has a research collaboration with CZ Health Insurances (cash matching to ZonMW grant) and with Exact Sciences, Sysmex, Sentinel Ch. SpA, Personal Genome Diagnostics (PGDX), DELFi and Hartwig Medical Foundation; these companies provide materials, equipment, and/or sample/genomic analyses. G.A.M. is an Advisory Board member of 'Missie Tumor Onbekend'. M.C.W.S. has received research support from Sysmex, Sentinel, Medtronic and Norgine. N.K.H.B. has served as

a speaker for AbbVie and MSD and has served as a consultant and principal investigator for TEVA Pharma BV and Takeda. He has received a research grant (unrestricted) from Dr. Falk, TEVA Pharma BV, Dutch Digestive Foundation (MLDS) and Takeda.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.