Protocollo studio

Combining faecal biomarkers to improve prediction of individual's risk ofpreinvasive and invasive colorectal lesions

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Background and rationale

Colorectal cancer (CRC) is the third most common cancer in men and the second most common in women, accounting for approximately 1.85 million new cases and 881,000 deaths in 2018 (1). The disease burden in 2016 was estimated as 17 million Disability-Adjusted Life Year (DALYs) (2). CRC is a highly preventable disease: a substantial proportion of CRC burden is attributable to modifiable lifestyle and environmental factors (2) and effective screening methods are available (3). Although in more recent years better understanding of the biology and of the natural history of the disease was associated with a trend toward more favorable outcomes, the stage at diagnosis remains the main determinant of survival and therefore screening can provide a crucial contribution to improve CRC outcomes. Population screening programs for CRC have been introduced in many countries (4): most of them are using faecal immunochemical tests (FIT), recommended as the faecal test of choice for population based screening (3), while some have implemented primary endoscopy screening (sigmoidoscopy – FS - or colonoscopy - TC). However, whilst almost certainly cost-effective, problems with CRC screening remain. FIT sensitivity, in particular for advanced adenomas, remains low on a single round, but lowering the cut-off might have a negative impact on the sustainability of the programs, given the generally limited TC capacity, stretched by an ageing population, too few trained staff and limited budgets. FIT remains also prone to false positive results, resulting in unnecessary TCs and many polypectomies are probably unnecessary (although which ones are unknown). Endoscopy based screening, although more accurate, as compared to FIT, and thus requiring less frequent screening, often shows a low acceptance and the harms benefit balance remains uncertain, at least for TC (5).
The implementation of more accurate non-invasive methods would then represent a priority for population based screening programs to face the budget limitations and the limited ability of endoscopy services to readily cope with increasing demand. This could take the form of a more intensive screening of those at higher risk and less intensive screening of those at lower risk using refined combinations of biomarkers.

One of the main advantages of FITs is that they allow for f-Hb quantification. This offers new options for screening, which have not been fully explored yet. In the context of population based programs, the test results are used in a dichotomized fashion, using quantitative information only to modulate the positivity cut-off, and to match the local endoscopy capacity. However, evidence from studies conducted among subjects with FIT results above or below the stipulated positivity cut-off suggested that combining f-Hb level and individuals characteristics may allow to define sub-groups showing a different risk of being detected with advanced neoplasia (CRC + advanced adenoma-AN). The positive predictive value (PPV) for AN was ranging between 24% among women aged 50 to 59 with 20-64 µg Hb /gr faeces and 76% among men aged 60 to 69 and f-Hb level > 175 µg /gr faeces in an average risk screening population in Spain (6). A positive correlation of f-Hb concentration at first screening (7), or over consecutive tests (8, 9), with the subsequent AN risk among subjects with FIT results below the positivity cut-off, has been documented. However, the small sample size of studies exploring the predictive role of cumulative f-Hb did not allow to assess its association with AN risk at subsequent screening rounds.

A limitation of current screening protocols is related to the lack of consideration of other potential predictors, apart from age, of individual's risk of CRC, or pre-invasive lesions (adenomas), while available evidence is suggesting that diet and other lifestyle related factors (10), microRNAs (miRNAs) and the gut microbiome

alterations are also involved in the cascade of events leading to carcinogenesis, contributing to disease development.
miRNAs are small noncoding RNAs (about 18-25 nucleotide long), that regulate gene expression inside cells by degrading mRNA or inhibiting protein synthesis, which may be released outside cells. Their stability in the faeces (and in body fluids) has opened new opportunities for anticipating CRC diagnosis (11.12). The potential use of such molecules for diagnostic/prognostic purposes regarding CRC has been extensively evaluated and there is growing evidence that altered stool miRNA signatures may reflect precancerous lesions and early stages of CRC, providing noninvasive and sensitive biomarkers for CRC early detection especially for subjects at higher risk (13). Also, a growing body of evidence is implicating the gut microbiome in CRC development. Cross-sectional studies demonstrated differences in the relative distribution of bacterial taxa between CRC cases and controls with enrichment of BacteroidetesFusobacterium, Atopobium and Porphyromonas and depletion of Firmicutes (14). Fusobacterium is prevalent in colon tissue and is maintained in distal metastases and is thought to be pro-inflammatory (15). These studies are consistent with dysbiosis, or microbiotic imbalance, leading to a pro-inflammatory microenvironment that is conducive to colorectal tumorigenesis. Recent discoveries point to a role of faecal miRNAs on shaping the human microbiota (16) introduced by the diet, but the link between diet, miRNAs and microbial diversity and CRC status require replication in independent population studies. Available studies, though showing promising results, still have limitations. Many of them had small sample sizes, they adopted a cross-sectional design and they were often including both symptomatic and asymptomatic subjects. Also, studies linking variation in these biomarkers with CRC in humans are limited (even more for their association with adenomas), and caution is required in the interpretation of case-control and cross-sectional studies due to the potential of reverse causality (17). To gain further insight into the role of these biomarkers in the natural history of CRC it would be important to compare the faecal microbiome and miRNome profile in healthy individuals and in patients with early to advanced adenomas, or CRCs. Moreover, to assess their potential role as screening tools, they should be compared to established screening test, such as faecal tests.

Preliminary data

Predictive value of f-HB level: We conducted a retrospective study (18) in the context of 4 population based programs in Northern-Central Italy, all adopting the same FIT (OC Sensor, Eiken Japan), performed every 2 years, on a single sample, with the same positivity cut-off (20 µg Hb /gr faeces). Among subjects aged 50 to 69, we found a strong positive correlation of the cumulative fHb concentration over the first two consecutive tests with the positivity rate (PR) and the AN PPV among subjects performing their third FIT: the PR ranged between 2.6% among subjects in the lowest and 25.9% among in the highest cumulative f-Hb category; the PPV for AN, ranging between 16.3% and 50.6%, as well as the DR for CRC, ranging from 0.04% to 1.86% and for AA, ranging from 0.3% to 9.8%, showed a parallel increasing trend. The cumulative probability of having a positive FIT result over the subsequent two rounds ranged between 7.8% (95%CI:7.5-8.2) for subjects who had no f-Hb detected at the initial 2 tests and 48.4% (95%CI:44.0-53.0) among those who had a cumulative f-HB concentration ≥ 20 µg Hb /gr faeces. The corresponding figures for cumulative DR were: 1.4% (95%CI:1.3-1.6) and 25.5% (95%CI: 21.4-30.2) for AN; 0.17% (95%CI:0.12-0.23) and 4.5% (95%CI: 2.8-7.1) for CRC.
In order to increase faecalhaemoglobin stability in collected faecal samples, a new sampling buffer has been introduced by the company providing the kit and the analytic method in the years following the completion of the analysis reported above. As a result the distribution of f-Hb levels in the screening samples was changed (19), with an increase in the proportion of cases with f-Hb level in the range 1-4 µg Hb /gr faeces and a parallel reduction of the proportion of samples with undetectable f-Hb. According to a previous health technology assessment report (20), 4 µg Hb/gr. faeces may represent the detectability threshold of the method and the modest increase in the probability of being detected with an AN at subsequent rounds in the sub-group of subjects with cumulative f-Hb in the range 0-3.9 µg Hb /gr faeces observed in our study was not associated with an increase of the rate of IC. It seems therefore reasonable to assume that subjects now

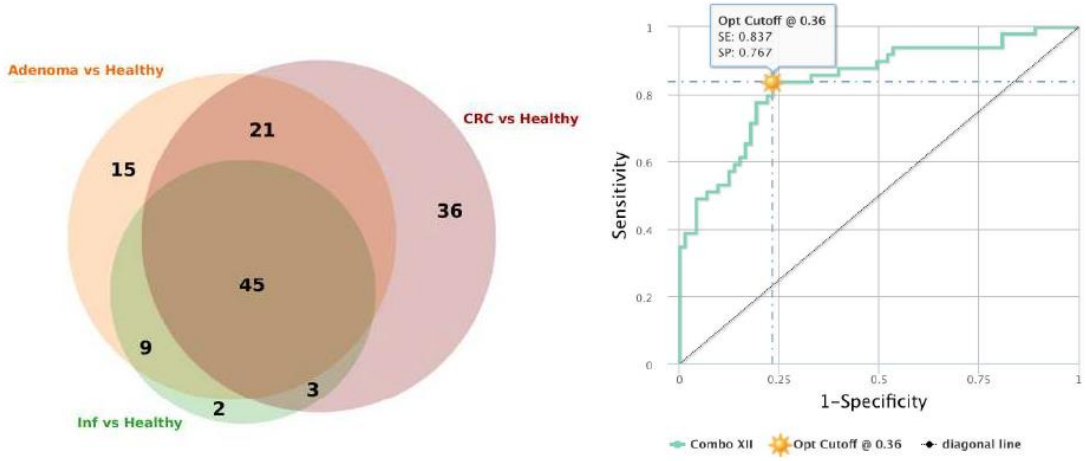
presenting with f-Hb level in the range 0-3.9 µg Hb /gr faeces would be comparable to those previously classified as having undetectable f-Hb. We will therefore include in the low-risk group in our project all subjects with cumulative f-Hb<4 µg Hb /gr faeces.

Predictive value of biomarkers: A miRNA signature by arrays discriminating CRC and precancerous lesions has been described in plasma samples by collaborators of the IIGM/UniTO group (21). Other studies have been performed in groups of subjects with different dietary habits (22). A set of stool samples (N=250) has been sequenced for small RNAs as part of a cross-sectional study currently ongoing where profiles miRNA and other small noncoding RNAs of similar size in subjects newly diagnosed with CRC, or with precancerous lesions are analysed. The average number of unique miRNAs identified in stool (>1000) is comparable with that of other specimens. Several differentially expressed miRNAs (DEmiRNAs) have been detected among the categories analysed (Figure 1), especially when comparing CRC vs. healthy subjects (N=105). Some of the DEmiRNAs were observed in the literature (23) - both in stool or in primary tissue- others were newly identified. From the best performing DEmiRNAs retrieved, a 7-miRNA candidate signature (hsa-let-7g-5p, hsa-miR-1246-3p, hsa-miR-1290-5p, hsa-miR-200c-3p, hsa-miR-21-5p, hsa-miR-320a-3p, hsa-miR-378a-3p) has been identified that accurately classifies CRC from healthy subjects (AUC 0.84, Fig 1). Similarly, a signature discriminating adenoma patients from healthy controls has been detected. Additional stool samples from all categories are currently being sequenced to improve classification according to cancer/pre-cancer subtypes.

The IIGM/UNITO group performed, in collaboration with CIBIO, a meta-analysis of five publicly available datasets and two new cohorts (one is the same as for miRNAs previously described) and validated the findings on two additional cohorts, considering in total 969 faecal metagenomes. The gut microbiome in CRC showed reproducibly higher richness than controls ($P < 0.01$), partially due to expansions of species typically from the oral cavity. Predictive microbiome signatures for CRC trained on multiple datasets showed consistently high accuracy in datasets not considered for model training and independent validation cohorts (average AUC 0.84). The combined analysis of heterogeneous CRC cohorts thus identified reproducible microbiome biomarkers and accurate disease-predictive models, that can form the basis for clinical prognostic tests and hypothesis-driven mechanistic studies (24).

A collection of leftover from FIT samples for microbiome analyses and the recruitment of FIT + subjects for the stool/plasma samples collection and diet and lifestyle questionnaires is currently ongoing in the context of a collaborative study (MITOS) involving the applicant, the IIGM, the US National Cancer Institute (resp. Dr. Rashmi Sinha) and the International Agency for Research on Cancer (resp. Dr Marc Gunter).As of March 2019, over 3,200 samples have been collected and are available for microbiome analyses. A dataset for the collection of anamnestic and clinical data has been set up. From FIT+ subjects, a subset of 270 provided stool and blood for miRNA and microbiome analyses.

Figure 1. Left: DEmiRNAs between different categories of patients and healthy subjects. Right: ROC curves for a 7-miRNA signature in stool classifying CRC patients vs. controls.



General aims

This project aims to assess the potential impact of a screening approach using the quantitative information available from the determination of the faecalhaemoglobin (f-Hb) level at each FIT exam, combined with the information about diet and lifestyle related risk factors and/or with novel biomarkers of increased CRC risk, to allow CRC screening to be focused on those most likely to benefit and/or more rapid and tailored investigation of those found by screening potentially to harbour CRC or polyps.
Specific objectives include:
The identification of CRC risk predictors to stratify populations into different levels of CRC risk; the identification of a set of strategies, encompassing modulation of FIT positivity thresholds, to differences in screening methods, age and frequency, which could be proposed to target CRC screening more effectively; the development of pipelines for molecular testing and patient data accrual that allow rapid incorporation of risk scores into CRC screening programmes. In particular, the combination of miRNome-wide and metagenomic approaches to study faecal markers, adopted in this study, has not yet used in a screening setting.

Expected impact

In the short term, this risk based approach could allow current resources to be used more effectively. In the future, any increase in resources may allow screening to be modulated so as to maximize cost-effectiveness. As the proposed study will be performed in the context of ongoing population based programs, its results will be directly relevant to the definition of policy recommendations for the implementation of a risk based tailored screening approach on a larger population basis.

A strength of the study is represented by the prospective uniform nature of specimen collection for all subjects from a single cohort. This eliminates systematic biases by ensuring that samples from case patients and control subjects are collected in the same way, within the screening setting for which biomarker is intended. Moreover, as all subjects recruited in the study are included in an ongoing screening program and they will then be invited again for screening every two years, future analyses of specimens collected and stored before detecting neoplasms at subsequent screening examination, or in the interval between screening rounds, may provide information on the time dimension, that is, the timing of the deviation of biomarker levels among cases as compared to controls with respect to the time of diagnosis. Sample storing will allow as well future discovery of novel blood or faecal markers, if more standardized and reliable techniques will become available.

The project has been designed as a **randomized controlled trial with a prospective case control study nested in the trial cohort**

RCT

Hypothesis and aims

FaecalHb concentration could be used to categorize different risk groups for AN. Subgroups at different risk would then be offered tailored protocols, adopting risk based screening intervals and TC referral criteria. Anticipating TC in the high-risk group might result in down-staging, or preventing, those CRCs now detected at subsequent rounds, improving FIT performance, while reducing the burden and costs of unnecessary TCs in the low-risk group.

The primary aim is to assess the potential impact of a screening protocol tailored to the AN risk by class of cumulative f-Hb level, in a large cohort of screenees.

The study is designed to achieve the following secondary aims:

1.1 To measure risk-specific (by gender, age, f-Hb level and Life Style Questionnaire – LSQ- score) screening performance indicators (i.e. positivity rate - PR, PPV, detection rate- DR).

1.2 To assess the feasibility of a risk based tailored intervention within an ongoing screening program and to quantify its costs and its potential impact on TC and organisational workload.

Experimental Design

We plan to conduct two parallel randomised trials among subjects undergoing screening in our population based screening program.

Trial A) Screenees who performed their second negative FIT, detected with cumulative f-Hb concentration over the two tests >=20 µg Hb /gr faeces and providing their consent to the study, will be enrolled and randomized (ratio 1:1:1) into 3 arms: 1) immediate TC referral 2) FIT screening at 1 year interval; 3) standard 2-year FIT screening. Subjects randomized to arms 2 and 3 will be referred for a TC following a positive FIT result.

Trial B) Subjects attending the screening invitation during year 1 and 2, with undetectable f-Hb at both tests (present and previous round), who will provide their consent to the study, will be enrolled and randomized (ratio 2:1) into 2 arms: 1) FIT screening at 3 year interval; 2) standard 2-year FIT screening.

Subjects with a negative FIT and cumulative Hb in the range 4-19.9 µg Hb/gr will not be targeted for the trial and they will be managed according to the standard screening protocol (i.ereinvited at the standard two year interval).

Study population: All residents, aged 59 to 69 in the Piedmont Region screening program are invited to undergo single sample biennial FIT with a personal invitation letter, signed by their GP. All programs use the same test. Subjects with a FIT+ result (positivity cut-off: 20 µg/gr. faeces) are contacted by phone to fix an appointment for the assessment TC. Information about the f-Hb level measured at each exam is available in the screening database and the IT system governing the screenees flow will be modified to calculate the cumulative f-Hb over the previous rounds.
During the study period, all eligible subjects (considering the screening activity in the Turin and Biella programs, we can estimate that they will be about 28,000 /year in year 1 and 2, and 23,000 / year in year 3 and 4) will be targeted for recruitment in the trials, if younger than 67 at the time of the index exam, to

ensure that all subjects enrolled will be eligible for receiving the invitation in the program over the following 2-year period.

Trial A) Starting in year 1 and ending in year 4, all subjects attending the screening invitation with a cumulative f-Hb level ≥ 20 μg/gr. faeces will targeted for recruitment. During the initial 3 years, subjects will be randomized in the 3 arms, while, during year 4 only arm 1 and arm 2 will be active, using a 1:1 ratio (to ensure completion of the recruitment procedures and of the first test at 1 year interval before the end of the 5-year period covered by the study).
Subjects randomized to arm 1 will be contacted by the screening staff and they will be asked for their consent to undergo a TC and to provide a blood and stool sample.
Subjects randomized to arm 2 will receive a response letter indicating that they have been enrolled in a study aimed to assess the potential benefit of using a shorter screening interval for subjects with high levels of f-HB at repeated exams. The letter will also include the indication for withdrawing the consent to the participation in the study FIT, requesting to be referred to the standard interval.
Trial B) In year 1 and 2 all subjects attending the screening invitation with undetectable f-Hb at both tests (present and previous round) will be asked for their consent to be randomized in the study.
Subjects randomized to arm 1 will receive a response letter indicating that they have been enrolled in a study aimed to assess the potential benefit of using a longer screening interval for subjects with very low levels of f-HB at repeated exams. The letter will also include the indication for withdrawing the consent to the participation in the study FIT, requesting to be referred to the standard interval.

Sample size, power calculations and statistical analysis
Trial A) Assuming a 1% prevalence of subjects with a cumulative f-Hb concentration above the positivity threshold at any round following the initial one, about 1,100 subjects should be eligible for enrolment over a 4-year period. Accounting for the expected reduction in screening uptake at subsequent rounds and of the expected refusal rate for TC referral, the planned arm size (320:320:200), would allow detecting as statistically significant a 9% absolute difference in the AN yield when comparing arms 1 with arm 2 or arm 3 (expected AN yield: arm 1: 25%; arm 2; 15%; arm 3:10%).

Primary outcome: AN detection rate (DR) and CRC distribution by colonic site and stage at diagnosis of screen-detected CRCs, measured at the index TC in group 1, over the two FIT examinations performed at 1-year interval in group 2 (cumulative AN yield over 2 tests) and at the second round in group 3. In the main analysis we will compare these outcomes in the 3 arms (comparisons of proportions) using the relative risk as a measure of association. Multivariable models will be used to get estimates adjusted for gender age and screening history (i.e. number of previous tests).

Trial B) Assuming a 40% prevalence of subjects with undetectable (or below the analytic detectability level of 4 μg/gr. faeces) f-Hb concentration at 2 consecutive FITs, about 15,000 will be randomized to 3-year screening and 8,000 to the standard 2 year interval. Accounting for the expected reduction in screening uptake at the subsequent round the planned arm size would allow detecting as statistically significant a 0.3%, 0.8% and 1.7% absolute increase in the AN yield, PR and AN PPV respectively when comparing arms 1 with arm 2 (expected AN yield: 0.3%; PR: 2.5;% AN PPV: 16%).

Primary outcome: PR, PPV and AN DR, measured at the subsequent screening round in each group, and CRC distribution by colonic site and stage at diagnosis of screen-detected CRCs.
In the main analysis we will compare these outcomes in the 2 arms (comparisons of proportions) using the relative risk as a measure of association. Multivariable models will be used to get estimates adjusted for gender age and screening history (i.e. number of previous tests).

Secondary outcomes for both trials: Interval cancer (IC) rate and IC; PPV for advanced adenomas and for CRC of TC referral.

Nested case-control study
Hypothesis and aims
Risk-based screening programmes according to different CRC risk criteria (i.e. f-Hb concentration, life-styles) may maximize the impact of screening in groups of subjects characterized by different risks. Validated highly accurate faecal biomarkers associated with CRC risk may represent a complementary tool to FIT in the primary screening setting.

The primary aim is then to investigate whether altered expression of selected stool miRNA signatures, or gut microbiome profiles, previously found associated with CRC risk are significantly more frequent in the faeces of patients with CRC, or advanced adenomas, compared to matched healthy controls and if they satisfy pre-specified true- and false-positive rates that are considered minimally acceptable in the screening setting.

The study is designed for achieving the following secondary aims:

2.1 To assess the association between factors such as age, gender, as well as diet and life-style and miRNA expression levels, or microbiome composition, in the cancer-free population. If such factors affect the biomarkers, the threshold for screening positivity may need to be defined separately for different subpopulations, in order to keep the false-positive rate at a low level for each.

2.2 To assess factors associated with miRNA levels/microbiome composition in cancer cases – in particular, disease characteristics such as stage, grade, and available prognostic factors. Understanding the nature of pre-invasive and invasive lesions detected with a biomarker is a key issue. A biomarker that detects cancer at an early stage or pre-invasive lesions is more valuable for preventive purposes than one that detects only late-stage cancers.

Recruitment Each year, during the initial 4 years of the study, all screenees with a positive FIT result and those referred for immediate TC in trial A, arm 1, will be administered a validated, detailed, questionnaire collecting information about dietary habits, physical activity, smoking, recent drug consumption (NSAIDs and antibiotics in particular) and other potential risk factors (LSQ) and they will also be asked to provide a blood sample and a second faecal sample which will be stored, together with the aliquots of the left-over fecal material from their FIT sample after f-Hb determination. A matched (by gender and age) sample of subjects enrolled in trial B (N=1,000) will be asked for their consent to answer the LSQ and to provide the same biological samples as subjects with positive FIT. Life-style related CRC risk score will be derived based on the results of previous research (25). An external control group (N=500 per year) will also be sampled among subjects eligible for regular 2-year interval screening (cumulative f-Hb in the range 4-19.9 µg Hb /gr faeces).
Overall, we can, therefore, estimate to store biological samples from 4,000 FIT positive subjects, 2,000 from subjects with intermediate f-Hb levels and 4,000 from subjects with very low, or undetectable, f-Hb. These samples will be available for the activities related to aim 2, as well as for future analyses during the follow-up of the study cohort within the screening program.
Cases. To assess the predictive role of the selected biomarkers for CRC, as well as for pre-invasive lesions, we will consider two sets of **cases**: a) all subjects detected with a CRC; b) a random sample of subjects detected with advanced adenomas.
Matched controls will be selected among subjects with undetectable f-Hb enrolled in trial B (see above - hypothesis 1). Although the expected prevalence of AN (1.4%), and in particular of CRC (0.2%), in this sub-

group is very low, all controls will be asked to undergo TC to exclude false negative cases (TC negative control cohort).
The same subjects free of CRC or advanced adenoma will be used as controls for assessing the biomarker performance for detecting CRC or advanced adenomas. Therefore the sample size of the control group will be based on the required number of advanced adenoma cases (see below - sample size). For each advanced adenoma case, 2 control subjects will be selected among those included in the sample of 4,000 subjects with very low, or undetectable, f-Hb, who will be asked to provide biological samples and to answer the LSQ, based on the following matching criteria: gender, age at enrolment (within 5 years), availability of blood sample, similar date of blood draw. They will be asked for their consent to undergo a TC to exclude CRC, or advanced adenoma (i.e. 1,000 subjects with undetectable f-Hb will be asked to undergo a TC)

The *choice of the biomarkers signatures* tested in this analysis will be mainly based on the findings of preliminary studies (see below). In addition, we will also perform an updated systematic review of available evidence to identify additional markers of interest. Considering that markers potentially associated with early detection of more aggressive lesions represent the main target of interest in a screening setting, the review will also be focused on the characterization of novel and already tested markers for their diagnostic and/or prognostic role.

Sample collection.Faecal samples will be obtained from all patients previously instructed to evacuate the bowel at home. Stool for microbiome analyses will be collected from leftover from FIT tubes used for automated tests (OC-sensor®, Eiken Chemical Co.) with a quantitative outcome. Stool for miRNA analyses will be collected in Stool Nucleic Acid Collection and Transport Tubes with RNA stabilisingsolution . All samples will be stored at -80°C until RNA/DNA extraction.

Laboratory procedures
RNA extraction and sequencing. RNA from stool will be extracted using the Stool Total RNA Purification Kit (NorgenBiotek Corp) and measured by Qubit® microRNA Assay Kit (Invitrogen) RNA quality will be verified (MIQE guidelines). Small RNA transcripts are converted into barcoded cDNA libraries. Library preparation is performed with the NEBNext Multiplex Small RNA Library Prep Set for Illumina (Protocol E7330, New England BioLabs Inc., USA) as described in Ferrero et al (21). The obtained libraries are subjected to the Illumina sequencing pipeline, passing through clonal cluster generation on a single-read flow cell (Illumina Inc., USA) by bridge amplification on the cBot (TruSeq SR Cluster Kit v3-cBOT-HS, Illumina Inc., USA) and 50 cycles sequencing-by-synthesis on the HiSeq 2500 (Illumina Inc., USA) .
DNA extraction and sequencing. Approximately 10 µg of microbial DNA is extracted from the FIT residual . Sequencing libraries were prepared using the NexteraXT DNA Library Preparation Kit (Illumina Inc., USA), following the manufacturer's guidelines. In all sequencing runs, we will include quality control samples. Sequencing will be performed on the NovaSeq (Illumina Inc., USA) with the expertise for the analyses of the outcome from the Centre for Integrative Biology, Trento, Italy.
Small-RNA sequencing analyses. The pre-processing step is based on the removal of the adaptor sequences and low-quality reads, then the survival reads will be aligned to a reference sequence. The reads will be first mapped to the reference miRNA precursor sequences available from mirBase (www.mirbase.org) using SHRiMPalgorithm which guarantees precise and accurate count detection. A high percentage of reads is related to other families of small noncoding molecules other than miRNAs (e.g. tRNAs, piRNAs, snoRNAs) that will also be explored. The set of candidate miRNAs will be defined considering: 1) the selection of differentially expressed miRNAs (DEmiRNAs) (using DESeq2 package in the Bioconductor suite for R); 2) the identification of good predictor, based on the computation of a regression model in which single miRNA is used to predict the class label of each subject; 3) the selection of the specific good predictors. Finally, a semisupervised classifier based method will be applied to predict relations between the candidate biomarkers and covariates. miRNA signatures tested in the study populations will be also integrated with data from FIT, microbiome and questionnaires about dietary and lifestyle habits. Metagenomic analyses Faecalmetagenomic

shotgun sequences are subjected to a pre-processing pipeline whereby sequences were quality filtered using trim_galore discarding all reads with quality less than 20 and shorter than 75 nucleotides. Filtered reads are then aligned to the human genome (hg19) and the PhiX genome for human and contaminant DNA removal using bowtie2 61. We use MetaPhlAn2 62 for quantitative profiling of the taxonomic composition of the microbial communities of all metagenomic samples, whereas HUMANn2 63 is used to profile pathway and gene family abundances.

Sample size, power calculations and statistical analysis

To calculate the sample size necessary for the evaluation of the biomarkers (expressed on a continuous scale), minimally acceptable and desirable levels of the true-positive rate (TPR) and the false-positive rate (FPR) have been defined (26). For general population screening, FPR must be quite low to avoid huge numbers of people undergoing unnecessary costly medical procedures. Thus, we hypothesized a maximally acceptable FPR0 of 5% (the minimally acceptable specificity is therefore 95%). The minimally accepted sensitivity was set at 60%, or at 72%, for CRC and at 30% for advanced adenomas (TPR0), based on the reported estimates of among subjects undergoing TC. The null hypothesis H0 to be rejected is then the following: FPR0≥0.05, both for advanced adenoma and CRC; TPR0a ≤ 0.60, or TPR0b ≤ 0.72 for CRC and TPR0=0.30 for advanced adenoma. The sample size required with an 80% power (alpha=5%) and assuming desirable FPR1=0.06 and true-positive rates TPR1a=0.72 or TPR1b=0.82 for CRC and TPR1=0.40 for advanced adenoma, was of 206 CRC cases, 513 advanced adenoma cases and 1,539 controls. Sample size computation was based on theory on the ROC curve.

Assuming a 6% FIT positivity rate and a 3% and 18% PPV for CRC and advanced adenoma respectively, a total of 216 CRC patients and 1,311 subjects with advanced adenoma will be diagnosed in the first four years of the study, thus exceeding the required numbers. These estimates are based on the screening performance indicators in the previous years in the same centres participating in this study. Thus, the molecular analyses will be performed on 216 CRC cases, 515 advanced adenoma cases and 1,540 matched controls.

To assess the predictive ability of the criteria considered (life-style risk score, f-Hb, molecular biomarkers) to identify groups of screens with different risk levels, the positive- and negative predictive values and ratios of predictive values will be calculated for each criteria.
The discrimination ability of single biomarkers will be evaluated by means of the Receiver Operating Characteristic (ROC) curves, whereas to test if biomarker levels are significantly higher, or lower, in cases and controls, logistic regression models will be used. To evaluate the association between screens' characteristics or tumour features and biomarker level, a linear regression model or the most appropriate model will be used.

As long as all controls will be referred for TC, it will be possible to assess, among subjects with very low/undetectable f-Hb, the AN PPV of the biomarkers of interest in an average risk population setting. Assuming a 20% prevalence of biomarkers of interest (corresponding for example to the observed prevalence of altered miRNA signature in the preliminary studies mentioned above), the planned size would allow detecting as a statistical significance an 8% absolute increase, or a 6% absolute decrease in the AN PPV of the marker, as compared to the expected AN PPV (16%) of FIT in this sub-group.

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