Lo studio MITOS: il ruolo del microbioma e dei miRNA nello sviluppo delle neoplasie del colon-retto

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PROGRAMMA REGIONALE DI SCREENING COLORETTALE
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Colorectal cancer (CRC) and the need of biomarkers

CRC is the 3rd common malignancy and the 2nd leading cause of cancer-related deaths in Western countries.

Environmental and genetic/epigenetic factors in combination are relevant for CRC.

Before CRC onset there may be a long period in which «silent» precancer lesions and inflammatory processes can be observed.
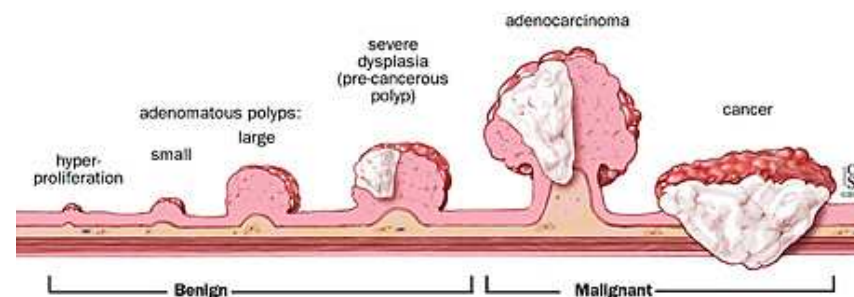

Fundamentally:

•Primary prevention and early detection are fundamental to reduce incidence and mortality for this cancer.

•New potential biomarkers are constantly proposed following the rapid development of molecular biology (such as DNA methylation, mutations, protein, metabolites).

•Markers based on RNA molecules are also of great interest, in particular microRNAs (miRNAs) and other sncRNAs.

•The gut microbiome composition is emerging as playing a fundamental role in CRC, and a potential source of biomarkers.
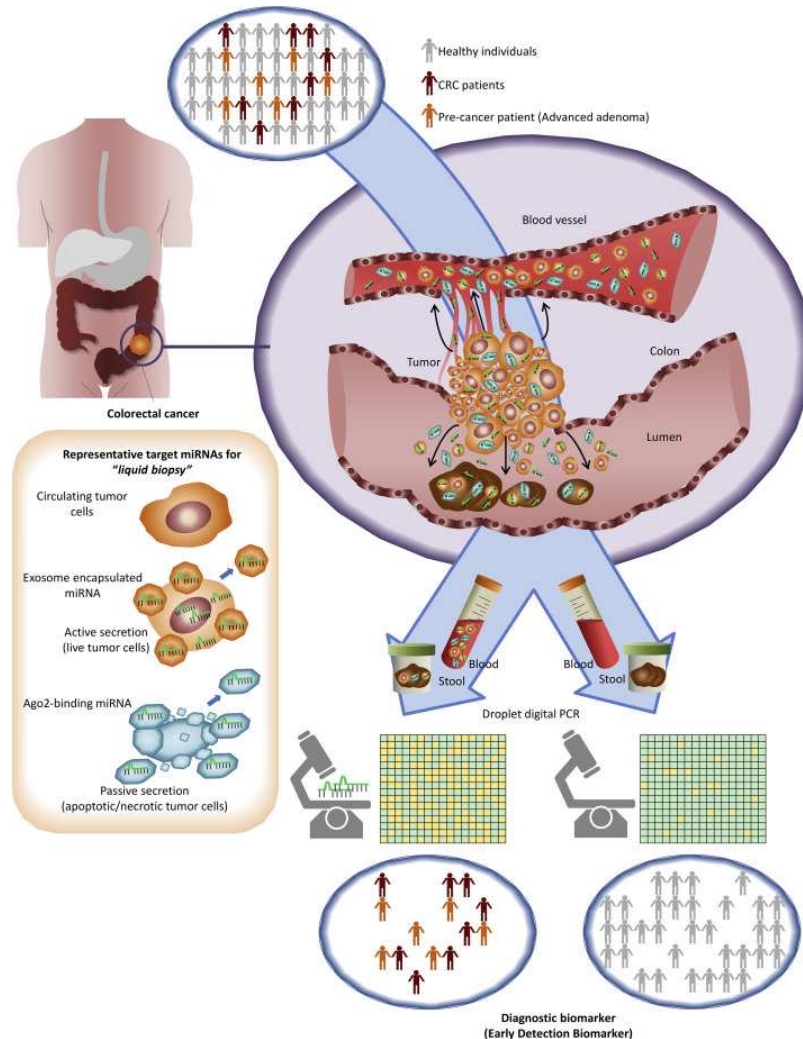
Small non-coding RNAs

- Small non-coding RNAs (sncRNAs) are a class of RNAs with important role in regulating gene expression;

- Altered levels of sncRNAs are found in patients with cancer and other diseases both in tissues and in body fluids, including serum, plasma, urine, and saliva;

- They have potential clinical applications as non invasive diagnostic and prognostic biomarkers.

NAME	DESCRIPTION
microRNAs (miRNAs)	are small, single-stranded, non-coding RNA (ncRNA) molecules with a characteristic length of approximately 21 ribonucleotides that are able to regulate gene expression.
P-element-induced wimpy testis (piwi)	interacting RNAs (piwiRNAs) are a class of small ncRNAs that interact with piwi-like proteins to form RNA–protein complexes. The size of piwiRNAs ranges from 26 to 31 nucleotides, and they have transposon-silencing capabilities.
5 tRNAs	tRNAs are fragments of 30–33 ribonucleotides in length that are generated through processing of tRNAs in response to cellular stressors (such as infection, heat shock, oxidative stress, or ultraviolet irradiation) and can regulate mRNA translation.
Small nuclear RNAs (snRNAs)	also known as u-RNAs or RNus) are RNA molecules comprising 100–300 ribonucleotides that form complex secondary structures through intrachain base pairing. snRNAs localize to the splicing speckles and Cajal bodies in the cell nucleus and, as key members of the major and minor spliceosomes, predominantly mediate mRNA processing and splicing.
Small nucleolar RNAs (snoRNAs)	are short sequences of 70 ribonucleotides that are located in nucleoli within the cell nucleus, where they regulate ribosomal RNA processing and modifications (methylation and pseudouridylation).
Small interfering RNA (siRNAs)	are short ncRNA molecules comprising 20–25 that are able to induce the degradation of perfectly complementary target RNAs.

Protein-coding region of target gene mRNA

3' UTR

miRNA

AAAAAA

miRNA

AAAAAA
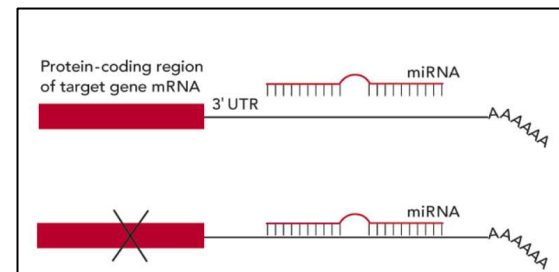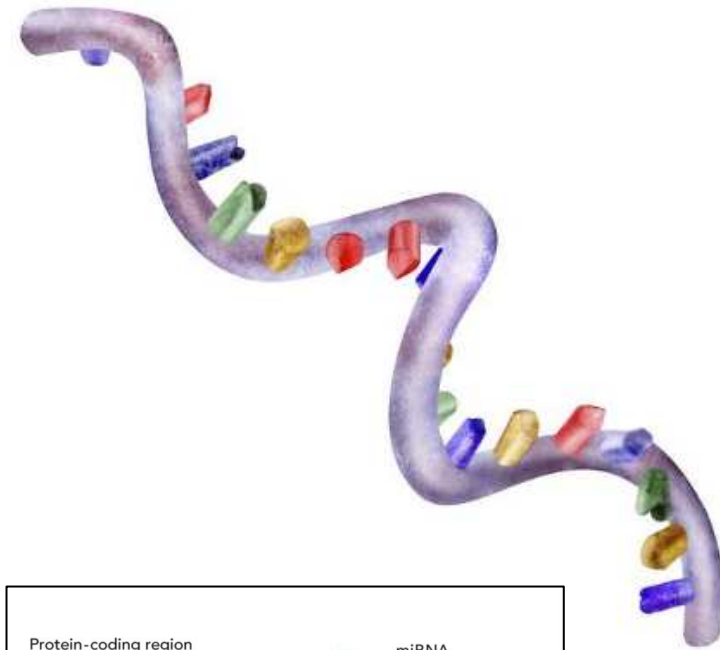
MicroRNAs as CRC biomarkers

Dysregulated miRNA expression has been identified in most malignancies, including CRC.

	Number of studies	miRNAs deregulated in colorectal cancer
Up-regulated	15	miR-21
	11	miR-31
	9	miR-135b
	8	miR-183, miR-20a
	7	miR-19a, miR-203, miR-96
	5	miR-18a, miR-92, miR-181b
	4	miR-15b, miR-17, miR-17-5p, miR-19b, miR-20, miR-25, miR-93, miR-106a, miR-182, miR-200c, miR-224
	3	miR-15a, miR-29a, miR-95, miR-103, miR-106b, miR-130b, miR-142-3p, miR-148a, miR-221, miR-191
	2	let-7f, let-7 g, miR-10a, miR-17-3p, miR-27a, miR-29b, miR-32, miR-34a, miR-92a, miR-98, miR-105, miR-107, miR-133b, miR-135a, miR-182*, miR-188, miR-200a*, miR-210, miR-213, miR-223, miR-301b, miR-320, miR-324-5p, miR-424, miR-493, miR-513a-5p, miR-552, miR-584
Down-regulated	15	miR-145
	9	miR-143
	7	miR-1, miR-195, miR-378
	5	miR-133a, miR-133b, miR-139-5p, miR-192, miR-215
	4	miR-30a-3p, miR-375, miR-422a
	3	miR-10b, miR-26b, miR-30b, miR-30c, miR-138, miR-139, miR-194, miR-363, miR-378*, miR-490-3p, miR-497, miR-551b
	2	miR-9, miR-9*, miR-16, miR-28-3p, miR-30a*, miR-30a-5p, miR-30e, miR-101, miR-125b, miR-137, miR-149, miR-150, miR-192*, miR-204, miR-320a, miR-328, miR-365, miR-486-5p, miR-598, miR-642

*Up- or down-regulated miRNAs in CRC tissue* (Slaby and Calin, Non-coding RNAs in colorectal cancer 2016)

Alterations in miRNA expression may be detected in **surrogate tissues, such as stool/plasma**

Toiyama Y, et al. Biochim Biophys Acta Rev Cancer. 2018

Systematic Review of the studies on miRNAs and CRC analysed in stool

Reference	Year	Country	Cases	Controls	Technique	n. miRNA	Up-regulated	Down-regulated
Wu C.W., et al., Dig Dis Sci	2017	USA	75 CRC tissue 20 CRC stool (Exploratory set) 29 CRC stool, 31 advanced adenoma stool (Feasibility set)	25 whole blood 20 tissue 40 stool (Exploratory set) 115 stool (Feasibility set)	small RNAseq (Discovery phase) blood/tissue RT-qPCR (Validation phase) in stool	7 (Discovery phase) 3 (Validation phase)	miR-144-5p and 451a	
Bastaminejad S. et al., Iranian Biomedical Journal	2017	Iran	40 CRC stool and plasma	40 CRC stool and plasma	RT-qPCR	1	miR-21	
Chang P., et al. Oncotarget	2016	Taiwan	62 stool/plasma/tissue (Discovery phase), 70(stool), 158 (plasma) no tissue (Validation phase)	62 stool/plasma/tissue (Discovery phase), 217 stool, 121 plasma (Validation phase)	multiplex RT-qPCR	40 miRNA selected from literature	miR-92a miR-223	
Yau T.O, et al. Oncotarget	2015	China	40 Tumour/Healthy tissues (Discovery phase) 198 CRC, 159 adenoma stool (Validation phase)	198 Healthy stool (Validation phase)	Microarray, RT-qPCR for Validation	miRNA-20a (selected from a previous array study on CRC tissue. Wu CW, et al)	miR-20a	
Ghanbari R., et al. Linbertas Academica	2015	Iran	8 CRC stool and plasma (Discovery phase) 51 CRC stool/plasma (Validation phase)	4 Healthy stool/plasma (Discovery phase) 26 Healthy stool/plasma (Validation phase)	Microarray, RT-qPCR for Validation	2,006 Discovery 2 validated		let-7a-5p let-7f-5p
Rotelli M. T., et al. Int J Colorectal Dis	2015	Italy	20 CRC stool and Tumor tissue	20 Healthy stool and Healthy tissue from negative biopsy	RT-qPCR	13	miR-17-3p, miR-18a-5p, miR-19b-3p, miR-19b-3p, miR-20a-5, miR-21-3p miR-92a-3p, miR-106a, miR-135e-3p, miR-135b-5p miR-141	
Ghanbari R. et al., Cancer Biomarkers	2015	Iran	40 CRC stool	16 stool	Microarray (Discovery phase) RT-qPCR (Validation phase)	2006 (Discovery phase) 2 (Validation phase)		miR-4478 miR-1295b-3p in CRC early stage
Zhao H., et al., Theranostics	2014	China	20 CRA stool, 23 CRC stool, 40 CRC tissues, 40 Adenoma	20 stool 10 tissue	RT-qPCR	1		miR-194
Yau T.O, et al., British Journal of Cancer	2014	China	40 tumor/Healthy tissue 199 CRC stool, 198 Adenoma	198 stool	Array RT-qPCR	667 Discovery 2 Validation	miR-221 miR-18a	
Wu C.W., et al., Clin canc Res	2014	China	5 CRC tissue, 2 adenoma tissue (Discovery phase) 40 Tumor/Healthy tissues, 16 adenoma/Healthy tissues (Validation phase) 104 CRCs, 169 adenomas, 42 IBDs stool (Validation phase)	169 healthy controls (Validation)	Microarray, RT-qPCR	667 Discovery 2 Validation	miR-135b	
Phua L. C., et al., Oncology Reports	2014	Malesia	8 CRC stool 8 tumor/Healthy tissue (Discovery phase) 17 CRC stool	8 Healthy stool 18 Healthy stool	Microarray RT-qPCR	1,347 Discovery 3 Validation	miR-451 miR-223	
Koga Y. et al., Cancer Epidemiol Biomarkers Prev	2013	Japan	117 CRC stool	101 stool	RT-qPCR	14	miR-106a	
Ahmed F.E., et al., Cancer Genomics & Proteomics	2010	USA	12 CRC stool (Discovery phase) 40 CRC stool (Validation phase) 12 CRC tissue	3 stool (Discovery phase) 20 stool (Validation phase) 3 tissue	Microarray (Discovery phase) RT-qPCR (Validation phase)	1,733 (Discovery phase) 20 (Validation phase)	miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR214	miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938
Yamazaki N., et al. Jpn J Clin Oncol	2013	Japan	5 CRC stool	5 stool	RT-qPCR	3	miR-106a	
Kalimutho M, J., et al.Gastroenterol	2011	Italy	15 CRC tissues (Discovery phase), 35 CRC stool (Validation phase)	15 tissue (Discovery phase), 40 stool (Validation phase)	RT-qPCR	645 (Discovery phase) 2 (Validation)	miR 144, miR 532-3p,	
Wu C.W., et al., Gut	2011	China	88 CRC stool 57 Polyp stool 40 tumor/Healthy tissue	101 stool	RT-qPCR	2	miR-92a miR 21	
Link A., et al., Cancer Epidemiol Biomarkers Prev	2010	Japan	9 Adenoma 10 CRC from FOBT leftover	18 stool 10 from FOBT leftover	Microarray RT-qPCR	1145 first investigation 6	miR-21 miR 106a	
Koga Y, Cancer Cancer, et al., Prevention research	2010	China	206 CRC stool 197 CRC tissue	134 stool 110 tissue	RT-qPCR	9	miR-17-92 cluster miR-135	
Ahmed F.E., et al. Cancer Genomics & Proteomics	2009	USA	5 polyp, 15 CRC, 5 UC 5 CD stool and tissue	5 stool and tissue	RT-qPCR	16	miR-21, miR-106a, miR-96, miR-203, miR-20a, miR-326, and miR-92	miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16 and miR-125b

Aims of our study

• Next generation Sequencing (small RNA-seq) in **stool and plasma** for the **identification** of **miRNA signatures** for the identification of CRC/precancerous lesions.

• Investigation on other **small noncoding RNAs**, (such as piRNAs and tRNAs) in relation to health status.

• (small) RNA-seq **in colorectal tissues:** do differentially expressed small noncoding RNAs in plasma and stool reflect primary tissues?

• The role of **diet and lifestyle habits** on miRNA profiles.

• The **relationship between miRNA profiles and gut microbiome composition.**
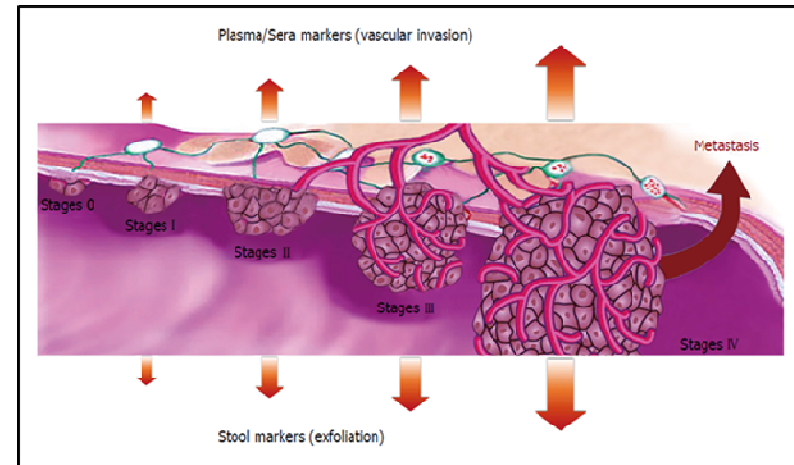
(Aghagolzadeh and Radpour, World Journal of Gastroenterology 2016)
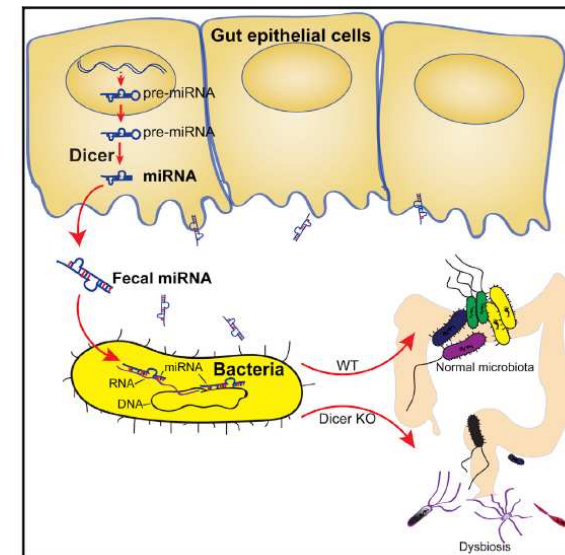
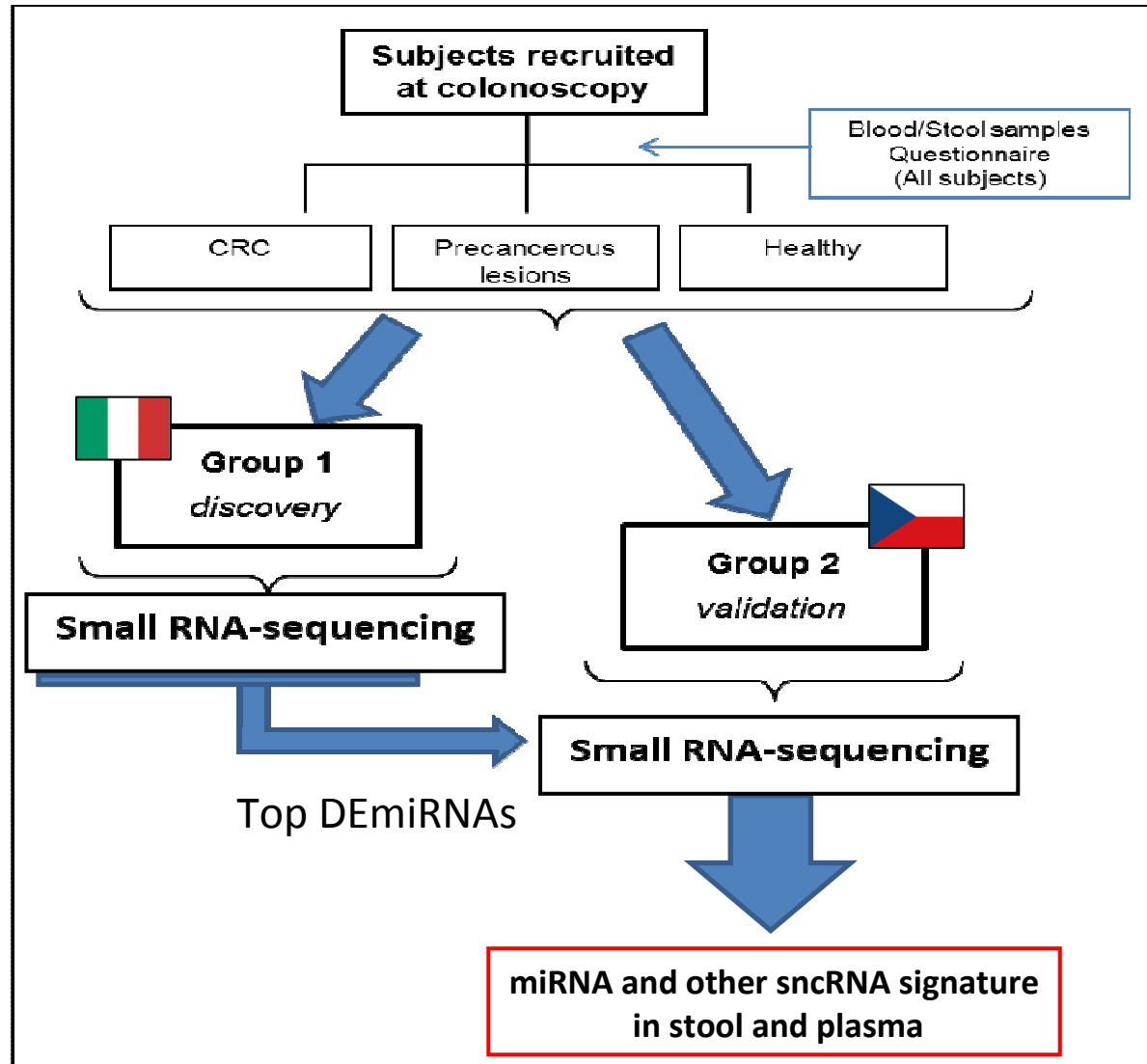*(Liu et al, Cell Host & Microb 2016)*

Organization of the study

- In collaboration with Clinica S.Rita (Vercelli) we have set up a **cross-sectional study** to evaluate miRNA expression levels in relation to CRC and precancerous lesions in plasma/stool samples.
- At present, we have recruited more than 400 subjects.

Molecular endpoints

miRNAs   microbiome   miRNAs *in exosomes*   Coding/noncoding RNAs   DNA

Biospecimen collection

Stool   *Blood/plasma*   *Tumor/healthy tissue (in RNA later)*

Recruitment → **Colonoscopy** → **Diagnosis** → **Surgical resection**

Questionnaire administration
Dietary & lifestlye information
Additional info
Demographic data collection

Clinical data recording:
1) *Healthy controls and hernia*
2) *Hemorroids*
3) *Inflammatory disease*
4) *Polyps*
5) *CRC*

Exclusion of subjects:
No colonoscopy,
Familiar cases
No colorectal cancers
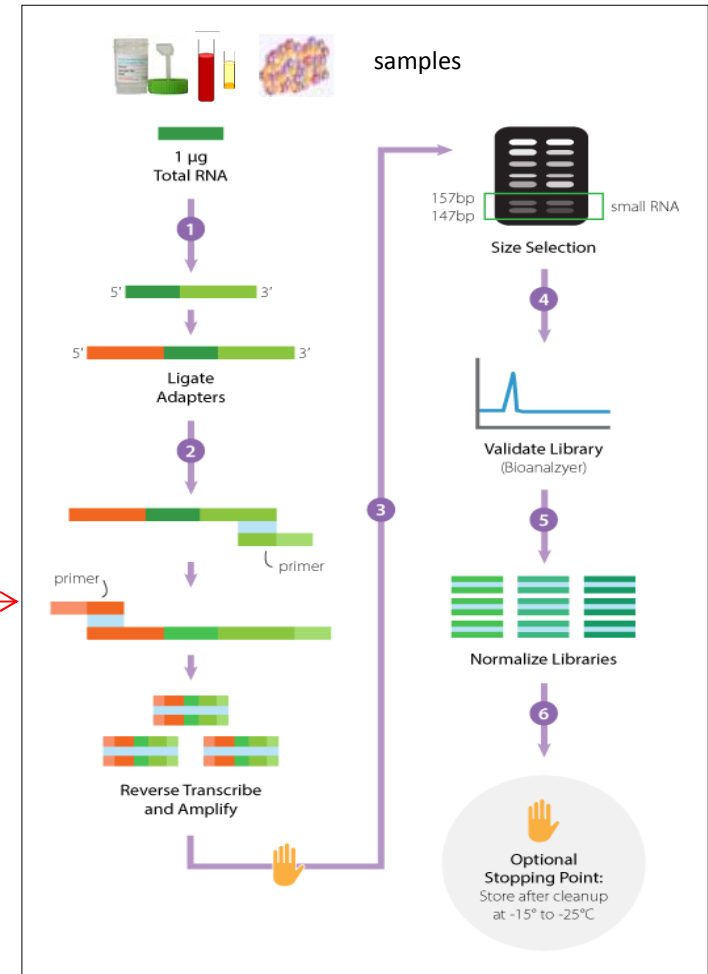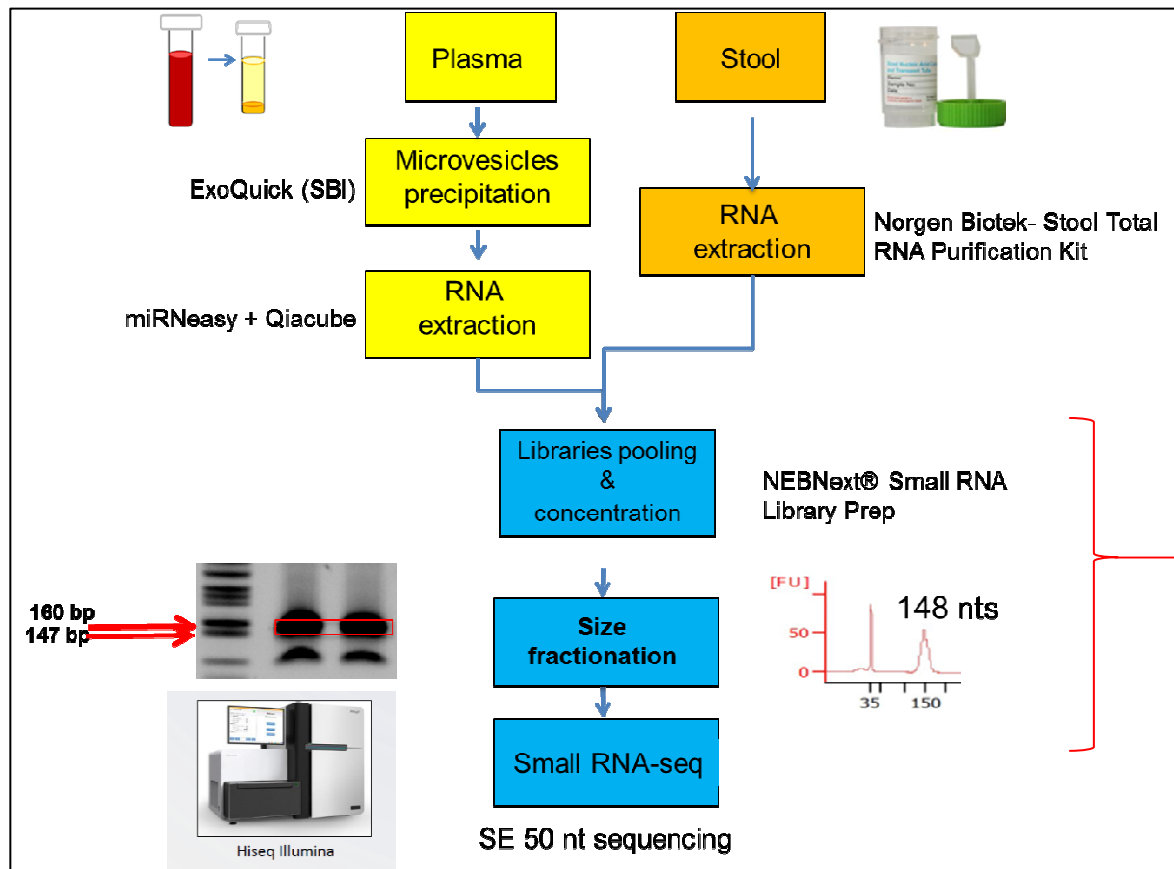No biospecimen collection

Study design

Library preparation and NGS

- RNA extraction for stool library preparation do not require mechanical homogenization of the samples.
- Analyses on plasma exosomes require a first phase of precipitation of the microvesicles

Ferrero et al., Oncotarget 2017

DISCOVERY PHASE: DESeq2 analysis – stool DEmiRNAs in CRC, Inflammation, Adenoma vs. Healthy

DE analysis:

Filtered data: Excluding healthy female with < 40 years

Category (Stool): 80 Healthy, 41 Inflammation, 43 Adenoma, 57 CRC

Adjusted p-value < 0.05
Median reads > 20 in at least one group

A miRNA signature accurately discriminates CRC (preliminary data)
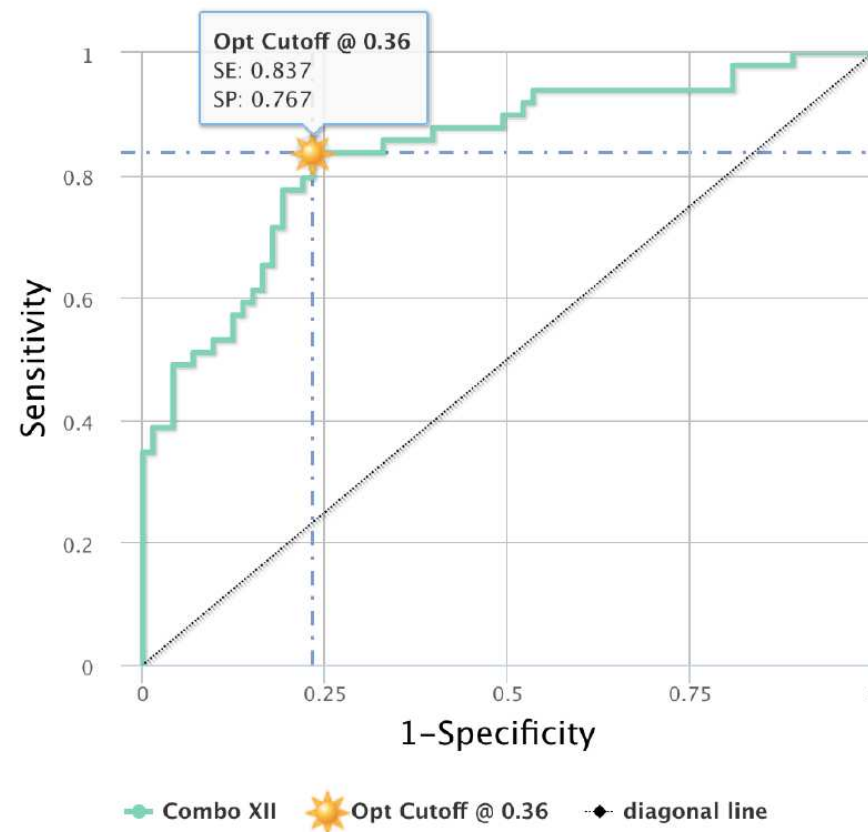
hsa-miR-1246-3p
hsa-miR-21-5p
hsa-miR-1290-5p
hsa-let-7g-5p
hsa-miR-200c-3p
hsa-miR-320a-3p
hsa-miR-378a-3p

Some of the DEmiRNAs were previously reported in literature (Francavilla et al., 2019; Slaby, 2016) - both in stool or in primary tissue, others were newly identified.
From the best performing DEmiRNAs retrieved, a 7-miRNA candidate signature has been identified that accurately classifies CRC from healthy subjects (AUC 0.84)

Czech Validation: Preliminary results

In collaboration with the Inst. of Exp. Medicine Prague (CZ) we have recruited 170 subjects at colonoscopy (CRC, polyps, inflammations and healthy). The design of the study and collection of samples has been organized as similar as possible to that of the Italian cohort.
So far, we have performed **small RNA-seq** of 96 stool samples

Characteristics		Healthy (n=22)	Adenoma (n=21)	Inflammation (n=19)	CRC (n=34)
Age (years)	Mean (range)	57.6 (40-76)	62.7 (51-76)	60.1 (52-75)	65.0 (40-88)
Sex	Male	11	12	11	20
	Female	11	9	8	14

Overlap DE miRNA CRC vs. Healthy
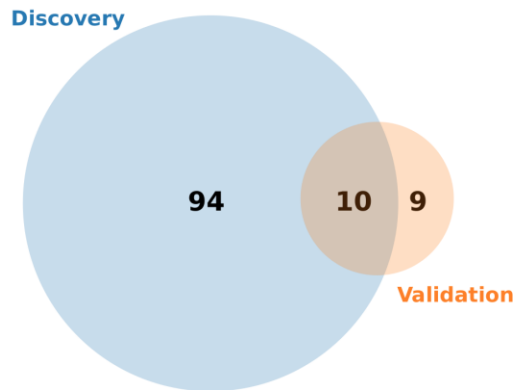
Examples of miRNAs validated in Czech cohort

miR-4488-3p

miR-1290-5p
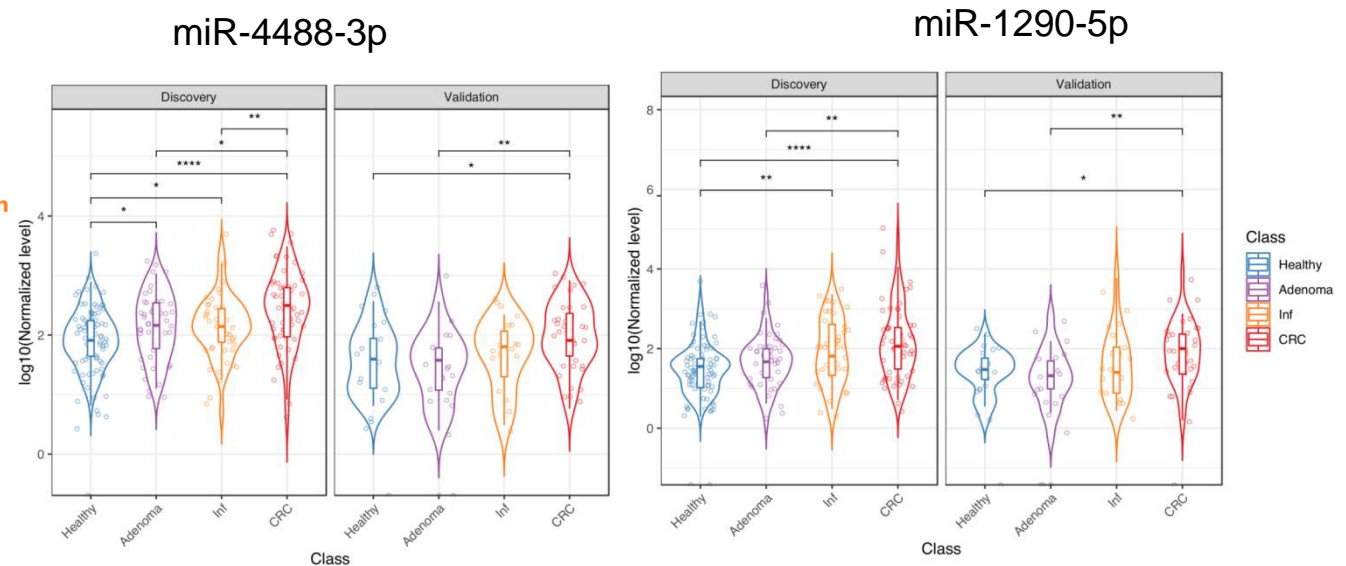
Involvement of microbiota in Cancer
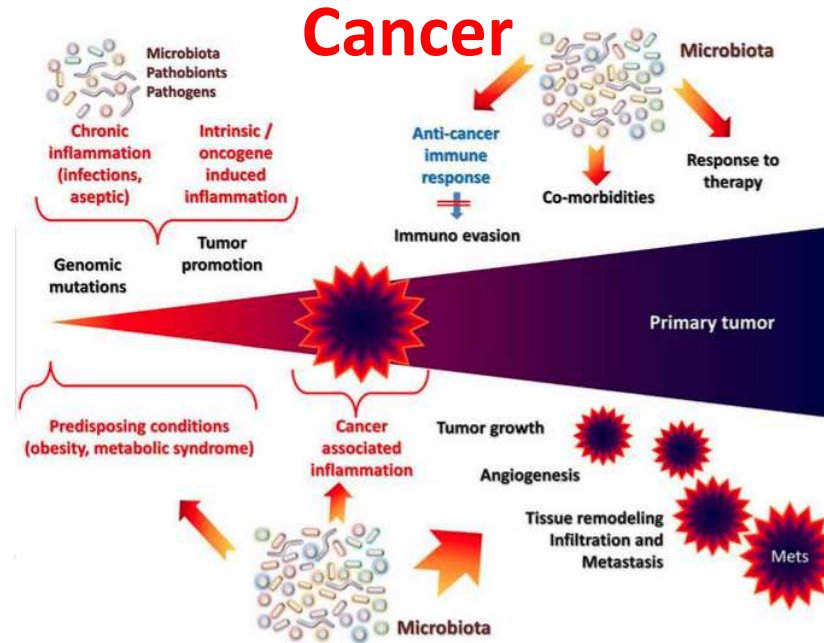
Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation

Andrew Maltez Thomas [1,2,3,32], Paolo Manghi[1,32], Francesco Asnicar [1], Edoardo Pasolli[1], Federica Armanini[1], Moreno Zolfo [1], Francesco Beghini [1], Serena Manara[1], Nicolai Karcher[1], Chiara Pozzi[4], Sara Gandini [4], Davide Serrano[4], Sonia Tarallo [5], Antonio Francavilla[5], Gaetano Gallo [6,7], Mario Trompetto[7], Giulio Ferrero [8], Sayaka Mizutani[9,10], Hirotsugu Shiroma[9], Satoshi Shiba[11], Tatsuhiro Shibata [11,12], Shinichi Yachida[11,13], Takuji Yamada[9,14], Jakob Wirbel [15], Petra Schrotz-King [16], Cornelia M. Ulrich[17], Hermann Brenner[16,18,19], Manimozhiyan Arumugam [20,21], Peer Bork [15,22,23,24], Georg Zeller [15], Francesca Cordero[8], Emmanuel Dias-Neto [3,25], João Carlos Setubal[2,26], Adrian Tett[1], Barbara Pardini [5,27], Maria Rescigno[28], Levi Waldron [29,30,33], Alessio Naccarati [5,31,33] and Nicola Segata [1,33]*

Metagenomic analyses performed on stool samples of two Italian Cohort, 5 publicly available datasets and two additional cohort, considering in total 969 fecal metagenomes revealed:

- higher species richness in CRC-associated samples but not diversity.
- a panel of microbial biomarkers for CRC is reproducible across cohorts.
- choline trimethylaminelyase gene overabundant in CRC ($P = 0.001$), identifying a relationship between microbiome choline metabolism and CRC.

ONCOBIOME

Microbiota against cancer
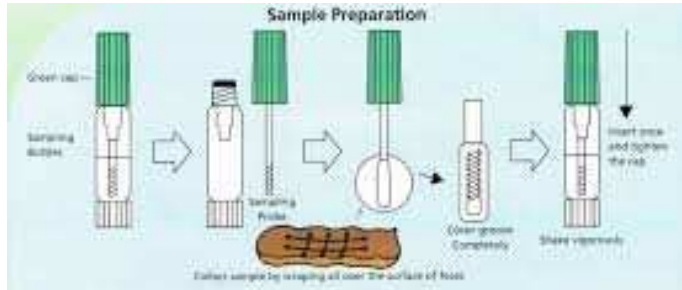International research program

Reciprocal regulation of miRNAs and gut microbiota in colorectal cancer

miRNAs have an important link in host microbiota interactions to regulate gut health and CRC tumorigenesis

Dong J et al 2019
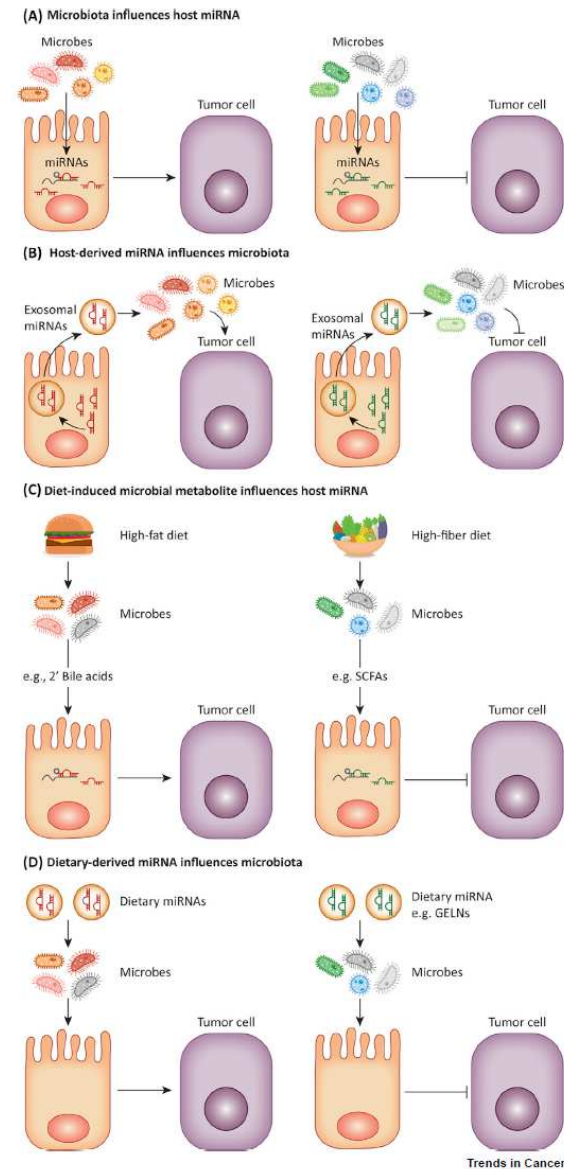
MITOS project and CRC screening in Piedmont

Collection of stool for FIT

Analyses of FIT tubes

In collaboration with the **Cancer Prevention Center of Piedmont Region** (Dr. Carlo Senore), the **National Cancer Institute** (Prof Rashmi Sinha), **IARC** (Dr Marc Gunter), we have started a collection of samples from the Torino CRC screening:

Aim 1: In subjects positive to fecal immunochemical test (FIT) who undergo colonoscopy, we will evaluate the relationship between miRNAs (selected from our cross-sectional study), microbiome (16S rRNA) in stool samples and life style related risk factors.

Aim 2: We are collect FIT negative subjects to set up a cohort study within the Screening Program in order to prospectively study miRNA expression levels and microbiome composition.

miToS

microRNA&**mi**crobioma
Torino **S**creening

CPO
Centro di Riferimento per l'Epidemiologia
e la Prevenzione Oncologica in Piemonte

NIH
NATIONAL CANCER INSTITUTE
Division of Cancer Epidemiology & Genetics

International Agency Research on Cancer
World Health Organization
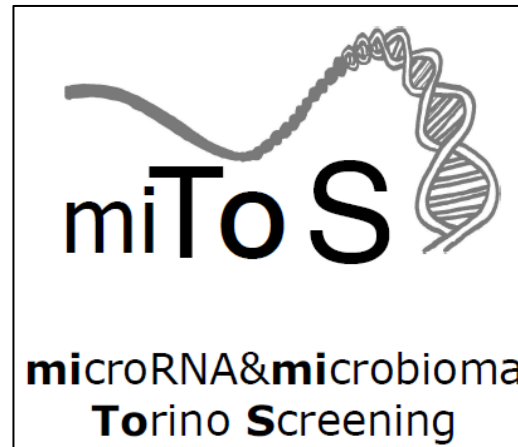
miToS - Workflow FIT tube leftover collection for microbiome analysis

List of subjects recruited

Periodo 18/09/2018--- 18/09/2018

CAMPIONI VALIDATI

N° Re	Codice	Cognome	Nome	Cognome Coniuge	Data di Nascita	Recapiti Telefonici	Data Validazione	Operatore di Validazione	Codice Provetta
1	6550482412	▮▮	▮▮		04/10/1959	3470068969	18/09/2018	5656	ZGK4J4
2	6501215319	▮▮	▮▮		12/04/1959	3490957756	18/09/2018	5656	ZGTY8P

Results from Lab

Tumori 2 CPO SGAS
Feci
Feci Sg Occulto

GIUSEPPE
19/09/2018

Feci Sg Occulto

M 96093476 MOL Epidemiologia

| 0 |

Tumori 2 CPO SGAS
Feci
Feci Sg Occulto

19/09/2018

Feci Sg Occulto

F 96093345 MOL Epidemiologia

| 51 |

Selection and matching of samples

TUR 0001 1002

FIT aliquot 2

250ul   250ul   ~500ul

Aliquote FIT1   Aliquote FIT2   Aliquote FIT stock

Storage immediately at -80°C
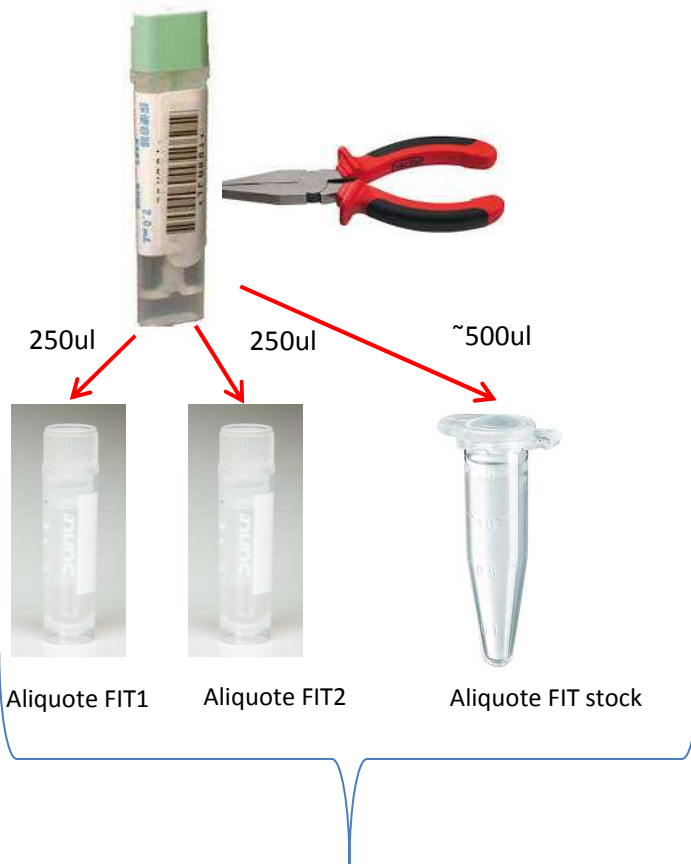
4398 FIT LEFTOVER collected (November 2019):

2925 FIT-
1472 FIT+

miToS study – Recruitment of FIT + subjects

Fit + subjects
HB ≥20µg/gr

→

Called for appointment for colonoscopy and invited to pick up the bowel preparation

→

FIT+ SUBJECTS COME TO THE UNIT TO PICK UP THE BOWEL PREPARATION, on this occasion the **screening staff**:

provides detailed information and asks for informed consent to participate in the miTos study

provides stool sample tubes and questionnaires

stool samples

blood samples

LSQ questionnaires

260 subjects

DURING THE APPOINTMENT FOR BLOOD COLLECTION

- **Subjects** return: with **signed consent form, tube for faecal collection** and the **questionnaire** (completely filled)

- **Laboratory staff** :
  - **Measures** height, body weight, and waist circumference
  - **Venepuncture** for blood collection
  - **Apply univocal barcode (see Rashmi's labels)**

Codice identificativo paziente

Studio

miToS

microRNA&**mi**crobioma
Torino **S**creening

QUESTIONARIO SUGLI STILI DI VITA

Outcomes of FIT positive subjects
(data are considering the tests performed until September 2019)

		TC performed		Completed		Histology	
		YES n. %	NO n.	YES n. %	NO n.	Advanced adenoma n. %	CRC n. %
Study participation *	YES (n=320)	289	31	265	24	71	7
		90.3%		91.7%		24.6%	2.4%
	NO (n=648)	550	98	511	39	139	26
		84.9%		98.3%		25.3%	4.7%
Not contacted/ proposed	(n=347)	181	166	167	14	38	6
		52.2%				21.0%	3.3%
Total	N=1315	1020	295	943	77	248	39
		77.6%		92.4%		24.3%	3.8%

* Consent to be recruited in the miRNA study (blood sampling)

Fit + Subjects:

Response Rate To The Questionnaire

|		YES*	NO	In progress	Not called yet	Total
Study participants	YES	297	25			322
		92.2%	7.8%			
	NO	384	190	54	26	654
		58.7%	29.1%	12.9%	8.3%	
Not contacted/ proposed		107	109	16	158	390
		27.4%	27.9%	4.1%	4.5%	
Total		788	324	70	184	1366
		57.7%	23.7%	5.1%	13.5%	

* Any questionnaire or interview

Study participants (FIT +): available information
Questionnaires: 299
Fecal samples: 294
Blood samples: 296
Questionnaire+faeces+blood: 268
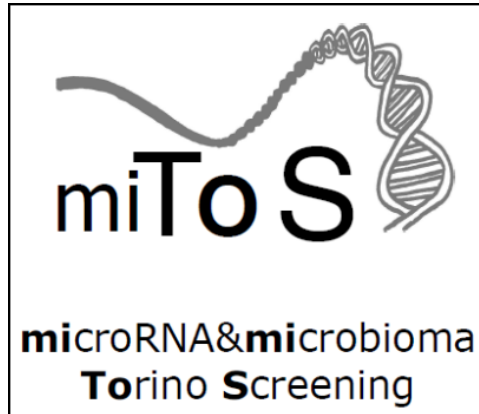
Mitos prospective study

"Combining faecal biomarkers to improve prediction of individual's risk of pre-invasive and invasive colorectal lesions"
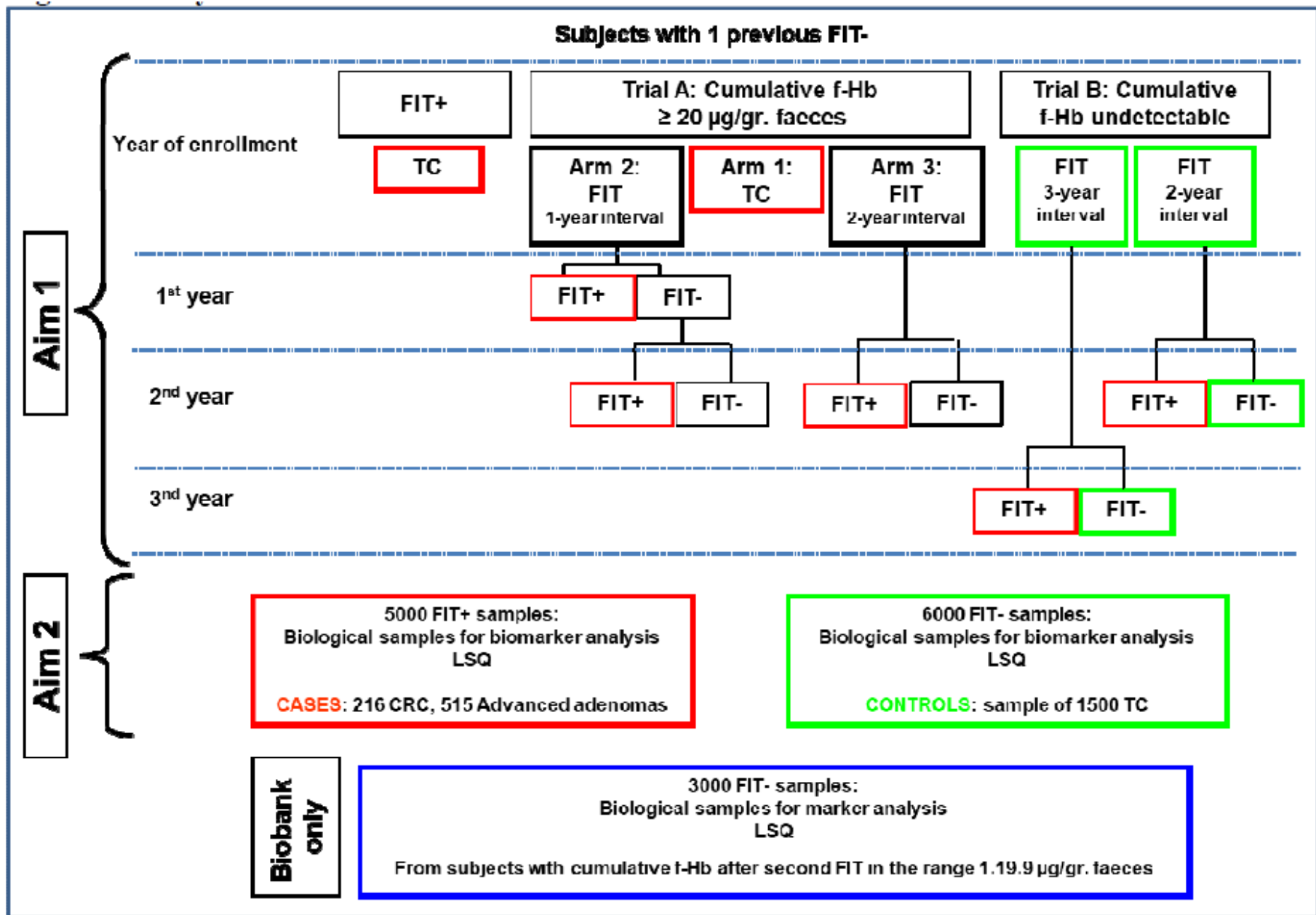**PI Dr Carlo Senore**
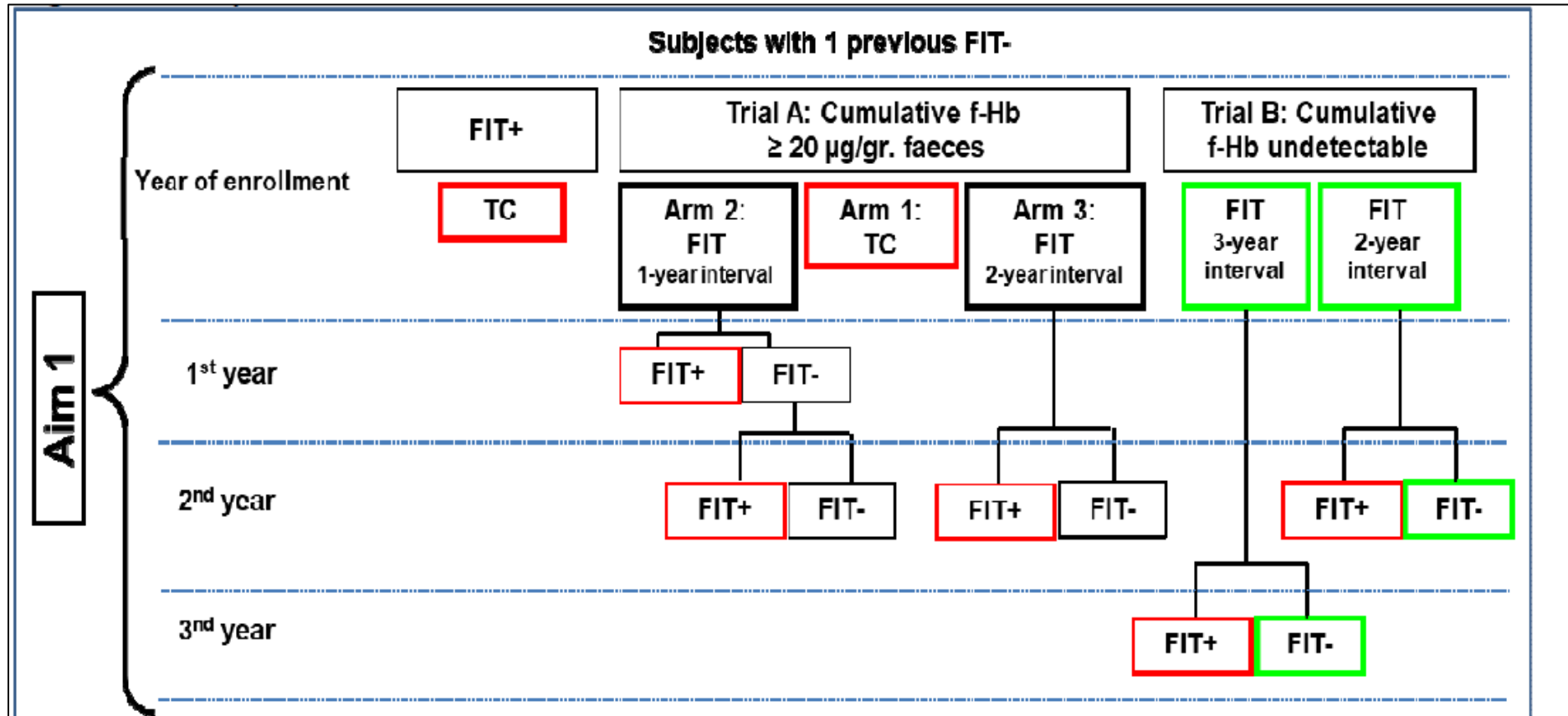**IIGM Partner**

AIRC IG 2019

Fit – Subjects:

Response Rate To The Phone Interview

Response rate to the questionnaire	YES	NO	In progress	Not called yet	Total
Total	968	505	344	699	2516 (1817 contacted)
% overall % contacted	38.5% (53.3%)	20.1%	13.7% (18.9%)	27.8%	

Subjects with 1 previous FIT-

TC: total colonoscopy; HB: haemoglobin; LSQ: Life-styles questionnaire
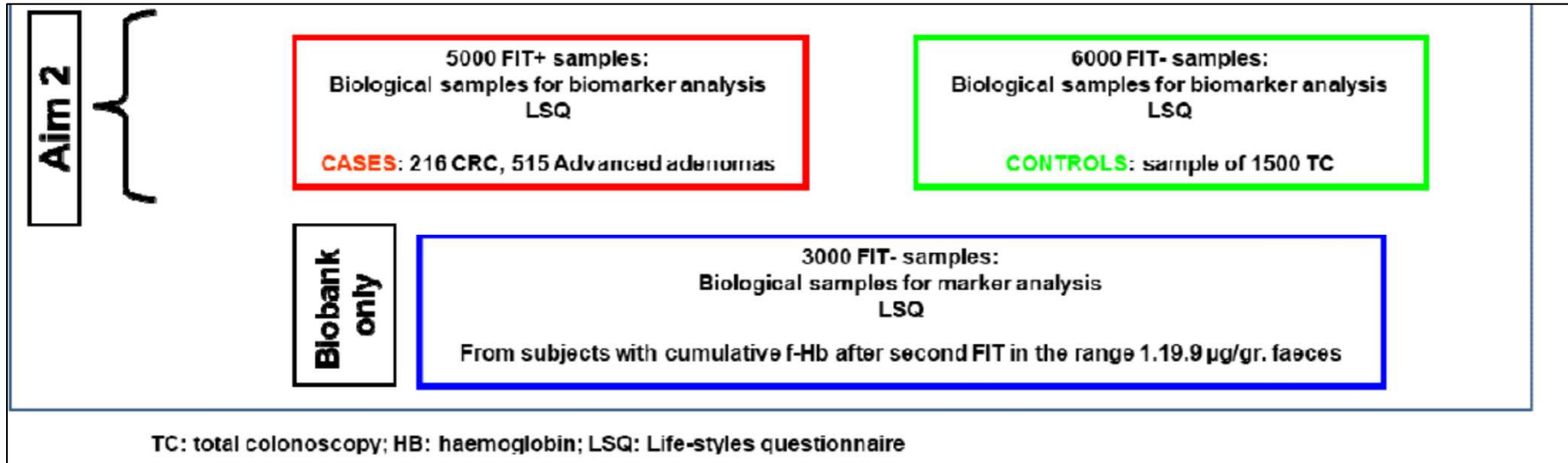
MITOS-AIRC: study design 1

AIM:

To assess the potential impact of screening protocol tailored to the subsequent AN risk by class of cumulative f-HB level in a large cohort of screenees.

MITOS-AIRC: study design 2

Aim 2

5000 FIT+ samples:
Biological samples for biomarker analysis
LSQ

CASES: 216 CRC, 515 Advanced adenomas

6000 FIT- samples:
Biological samples for biomarker analysis
LSQ

CONTROLS: sample of 1500 TC

Biobank only

3000 FIT- samples:
Biological samples for marker analysis
LSQ

From subjects with cumulative f-Hb after second FIT in the range 1.19.9 µg/gr. faeces

TC: total colonoscopy; HB: haemoglobin; LSQ: Life-styles questionnaire

AIM:

- To investigate whether altered expression of selected stool miRNA signature or gut microbiome profiles previously found associated with CRC risk are significantly more frequent in the faeces of patients with CRC or advanced adenoma, compared to mathched healthy controls and if they satisfay pre-specified true- and false positive rates that are considered minimally acceptable in the screening setting.

Conclusions

- ✓ Stool miRNA profiles analysed by NGS by us seem to provide reliable and comparable results to other specimens.

- ✓ Several miRNAs are dysregulated in stool of CRC patients according to grade and tumor location (colon or rectum), reflecting results in tissues.

- ✓ Gut microbiome composition in different study populations shows strong and reproducible results in relation to CRC

Future Perspectives

- ➢ To implement data analyses in stool and plasma and complete a validation of the main results also for other sncRNAs.

- ➢ To relate information on diet and lifestyle habits and microbiome with sncRNA profiles.

- ➢ To define a broadly informative miRNA / other sncRNAs marker panel in the surrogate specimens to test the in the FIT screenes population.

- ➢ To set up an international collaboration within similar studies (Microbiome studies within colorectal cancer screening Programme) coordinated by IARC

Collaborations

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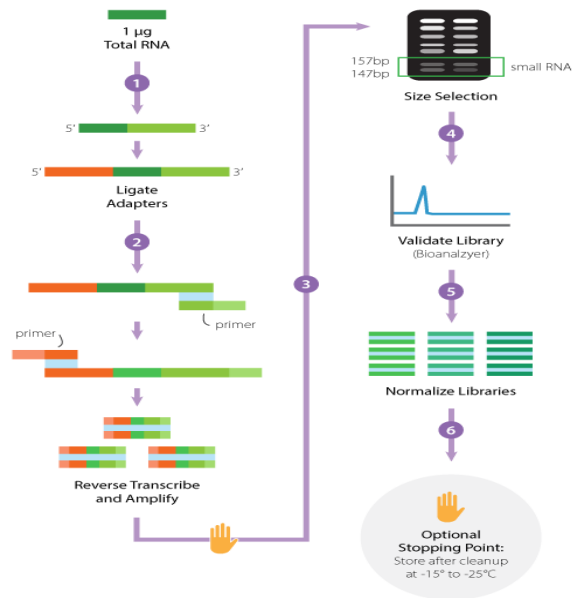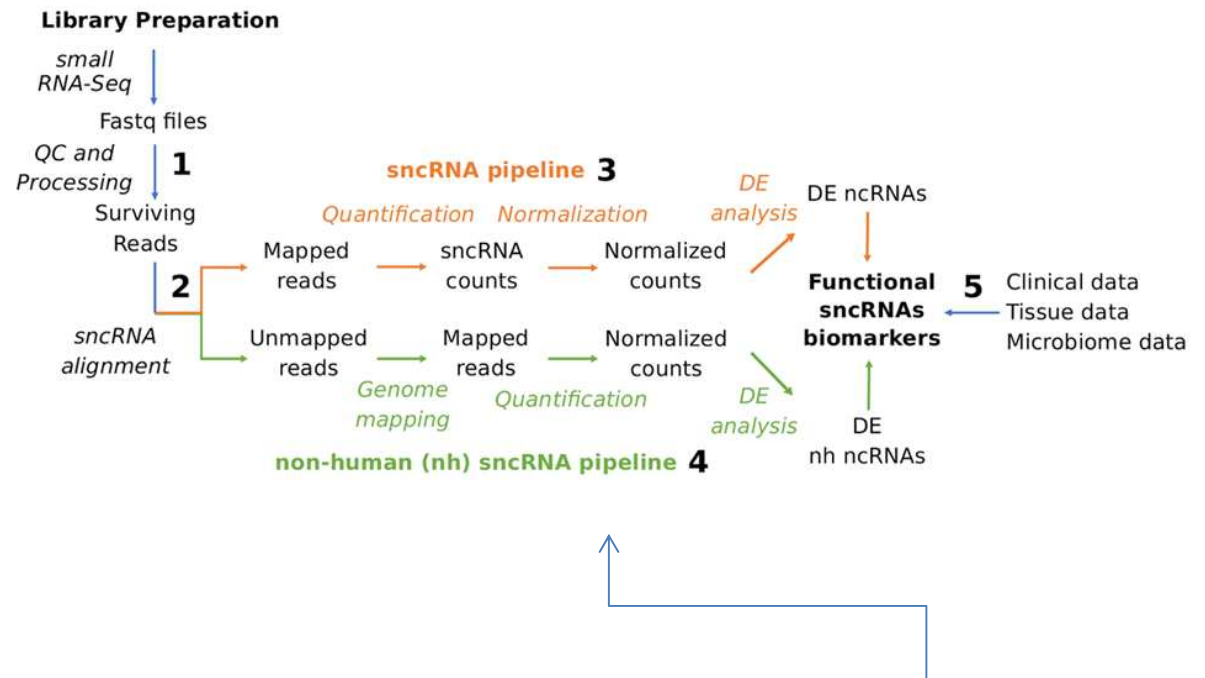Gaetano Gallo
Alberto Realis
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Experimental approach

The library preparation for smallRNA-seq by Next-generation sequencing and the pipeline for computational analyses have been previously implemented by our group (*Ferrero et al., 2017*)

Library Preparation

Computational Analysis

We have recently included an analysis of non-human small RNAs in stool

(Ferrero et al., 2017; Tarallo et al., 2019)