

# The gut microbiome research: an epidemiologist's perspective

Mingyang Song, MBBS, ScD

# Outline

- Research question
- Study design
- Sample type
- Sample size
- Covariate assessment
- Microbiome among Nurses Study (MICRO-N)



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# Research question

- **Etiology**

- To identify a microbial exposure, represented by a specific microbiota composition or microbial activity, that is, **causally** related to a disease end point.

- **Prediction**

- To develop a microbiome-based biomarker for risk prediction and screening to facilitate interventions in **early** phases of disease.



# Causality criteria

## Koch's Postulates:

- The bacteria must be present in every case of the disease.
- The bacteria must be isolated from the host with the disease and grown in pure culture.
- The specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host.
- The bacteria must be recoverable from the experimentally infected host

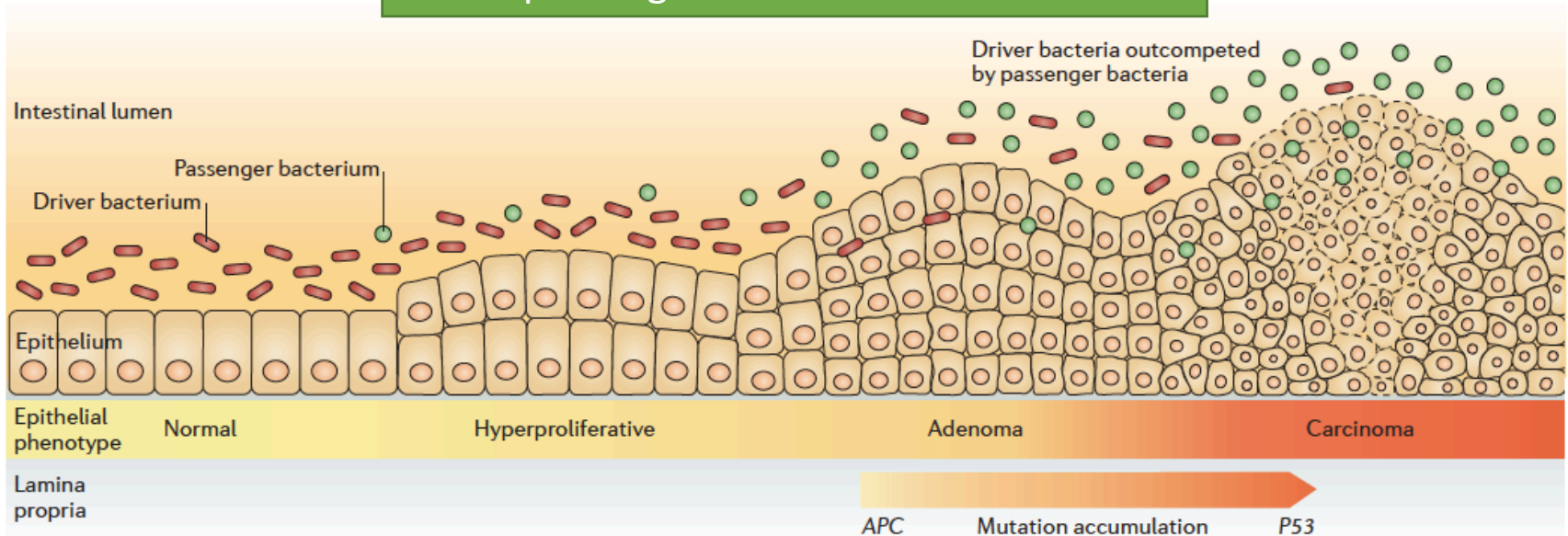
## Bradford Hill Criteria:

- Strength of the association
- Consistency
- Specificity
- Temporality
- Biological gradient
- Plausibility/Coherence
- Experiment
- Analogy

# Key considerations for different questions

- **Etiology**
  - Temporality

## Driver-passenger model in colorectal cancer



Driver bacteria ↑

Selective pressure due to  
carcinogenesis-induced  
microenvironmental changes

Driver bacteria ↓

Passenger bacteria ↑

# Key considerations for different questions

- **Etiology**

- Temporality
- Confounding
  - Microbial interactions
  - Environmental factors

Positive interaction	Negative interaction
Mutualism	Ammensalism (antagonism)
Proto-cooperation	Parasitism
Commensalism	Predation
	Competition

# Key considerations for different questions

- **Etiology**

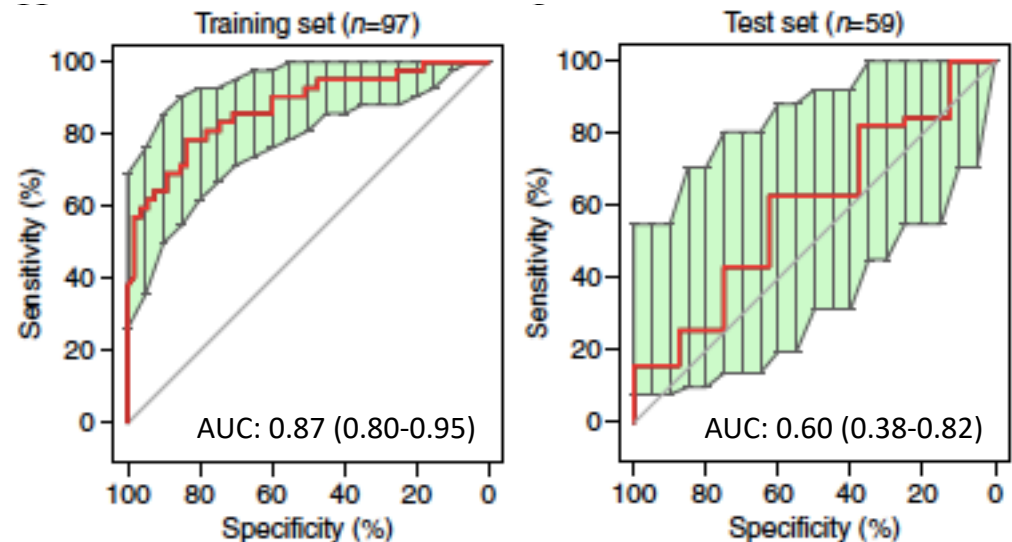
- Temporality
- Confounding
  - Microbial interactions
  - Environmental factors

- **Prediction**

- Discrimination → predictivity

Feng Q, et al. Nat Comm, 2015

ROC of 10 microbial features  
for colorectal neoplasia



# Key considerations for different questions

- **Etiology**

- Temporality
- Confounding
  - Microbial interactions
  - Environmental factors

- **Prediction**

- Discrimination → predictivity
- Reliability:
  - Context-dependent?
- Generalizability: universal vs. population-specific signatures?

# Microbiome as a screening tool for CRC

Study	microbes	Country	AUC for CRC	AUC for adenoma
Zeller, 2014	4 species (2 <i>Fusobacterium</i> species, <i>Porphyromonas asaccharolytica</i> , <i>Peptostreptococcus stomatis</i> )	France	0.84	
Zackular, 2014*	5 OTUs ( <i>Clostridiales</i> , <i>Clostridium</i> , <i>Lachnospiraceae</i> , <i>Bacteroides</i> )	USA	0.80 (0.69-0.91)	0.84 (0.74-0.94)
Feng, 2015	10 metagenomic groups ( <i>Bacteroides massiliensis</i> , <i>Bacteroides xylanisolvens</i> , <i>Bifidobacterium animalis</i> , <i>Paraprevotella clara</i> , <i>Streptococcus mutans</i> , 5 unclassified)	Austria	0.96 (0.88-1.00)	0.60 (0.38-0.82)
Baxter, 2016	34 OTUs (most belong to <i>Clostridiales</i> order and some to <i>Bacteroides</i> )	USA	0.85	0.67
Wong, 2017	1 species ( <i>F. nucleatum</i> )	China	0.89 (0.80-0.98)	0.58 (0.49-0.67)
Liang, 2017	4 species ( <i>F. nucleatum</i> , <i>Bacteroides clarus</i> , <i>Roseburia intestinalis</i> , <i>Clostridium hathewayi</i> , and one undefined)	China	0.76	
Thomas, 2019	16 species (e.g., <i>Peptostreptococcus stomatis</i> , <i>F. nucleatum</i> , <i>Parvimonas</i> spp., <i>Porphyromonas asaccharolytica</i> , <i>Gemella morbillorum</i> , <i>Clostridium symbiosum</i> and <i>Parvimonas micra</i> )	Multi	0.81	0.54

\*No validation was performed. The AUC was calculated in the training set.



# Outline

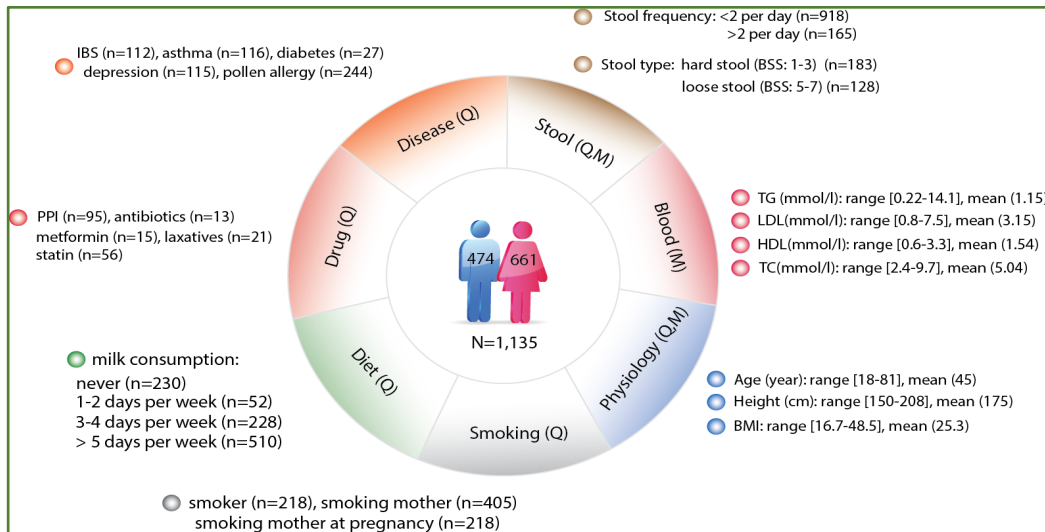
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- **Study design**
- Sample type
- Sample size
- Covariate assessment
- Microbiome among Nurses Study (MICRO-N)

# Study design

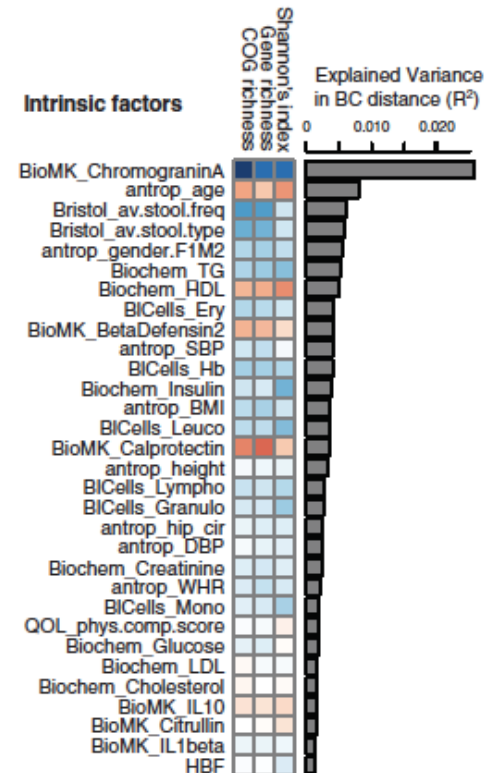
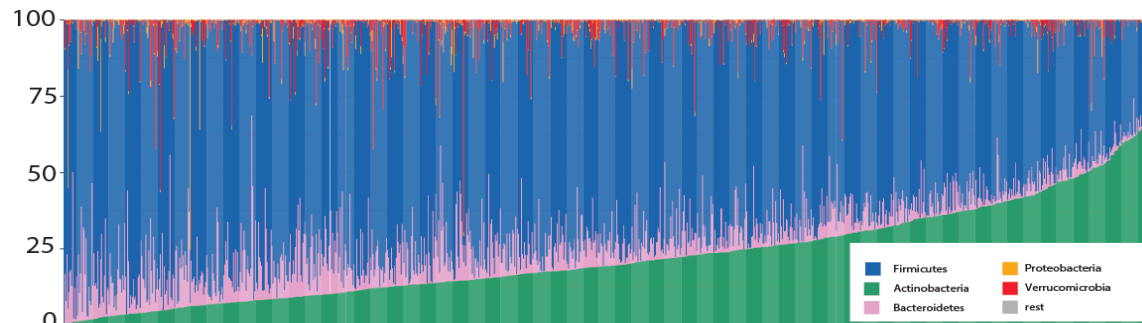
- Cross-sectional
- Case-control
- Prospective cohort (nested case-control)
- Meta-analysis
- Interventional

# Cross-sectional study

- Useful for describing the microbial pattern in a population
- Limited ability for causal inference



Phyla across individuals



# Case-control study

- Useful to identify potential signals for future studies

Author, Year	Sample Size	Main Findings Comparing Cases to Controls
Scanlan, 2008	20 cancers / 20 polyps / 20 controls	↑ <i>Clostridium leptum</i> and <i>C. coccoides</i>
Sobhani, 2011	60 cancers / 119 controls	↑ <i>Bacteroides/Prevotella</i>
Wang, 2012	46 cancers / 56 controls	↑ <i>Bacteroides fragilis</i> and opportunistic pathogens; ↓ butyrate-producing bacteria
Chen, 2012	21 cancers / 22 controls	↑ <i>Lactobacillales</i> ; ↓ <i>Faecalibacterium</i>
Ahn, 2013	47 cancers / 94 controls	↓ <i>Clostridia</i> ; ↑ <i>Fusobacterium</i> , <i>Porphyromonas</i> ;
Zackular, 2014	30 cancers / 30 adenomas / 30 controls	↑ <i>Bacteroides fragilis</i> , <i>Fusobacterium</i> , <i>Porphyromonas</i> ; ↓ butyrate-producing bacteria
Zeller, 2014	91 cancers / 42 adenomas / 358 controls	Metabolic shift from fiber degradation to carb and amino acid utilization; ↑ LPS
Yu, 2015	74 cancers / 54 controls	↑ <i>Peptostreptococcus</i> ; <i>F. nucleatum</i>
Feng, 2015	41 cancers / 42 adenomas / 55 controls	↑ <i>B. dorei</i> , <i>B. vulgatus</i> , <i>E. coli</i> , <i>Fusobacterium</i> ; ↓ <i>Lactobacillus</i> and <i>Bifidobacterium</i>
Nakatsu, 2015	52 cancers / 47 adenomas / 61 controls	↑ <i>E. coli</i> , <i>Bacteroides fragilis</i> , <i>Gemella</i> , <i>Peptostreptococcus</i> , <i>Parvimonas</i>
Liang, 2016	203 cancers / 236 controls	↑ <i>F. nucleatum</i> , <i>Clostridium hathewayi</i> ; ↓ <i>B. clarus</i>
Flemer, 2016	59 cancers / 21 polyps / 56 controls	↑ <i>Fusobacterium</i> , <i>Porphyromonas</i> , <i>Anaerococcus</i> , <i>Parvimonas</i> , <i>Granulicatella</i> , <i>Prevotella</i>
Vogtmann, 2016	52 cancers / 52 controls	↑ <i>Fusobacterium</i> , <i>Porphyromonas</i>

# Case-control study

- Useful to identify potential signals for future studies
- Cons:
  - Reverse causality
  - Selection bias
  - Confounding

# Prospective cohort/case-control study

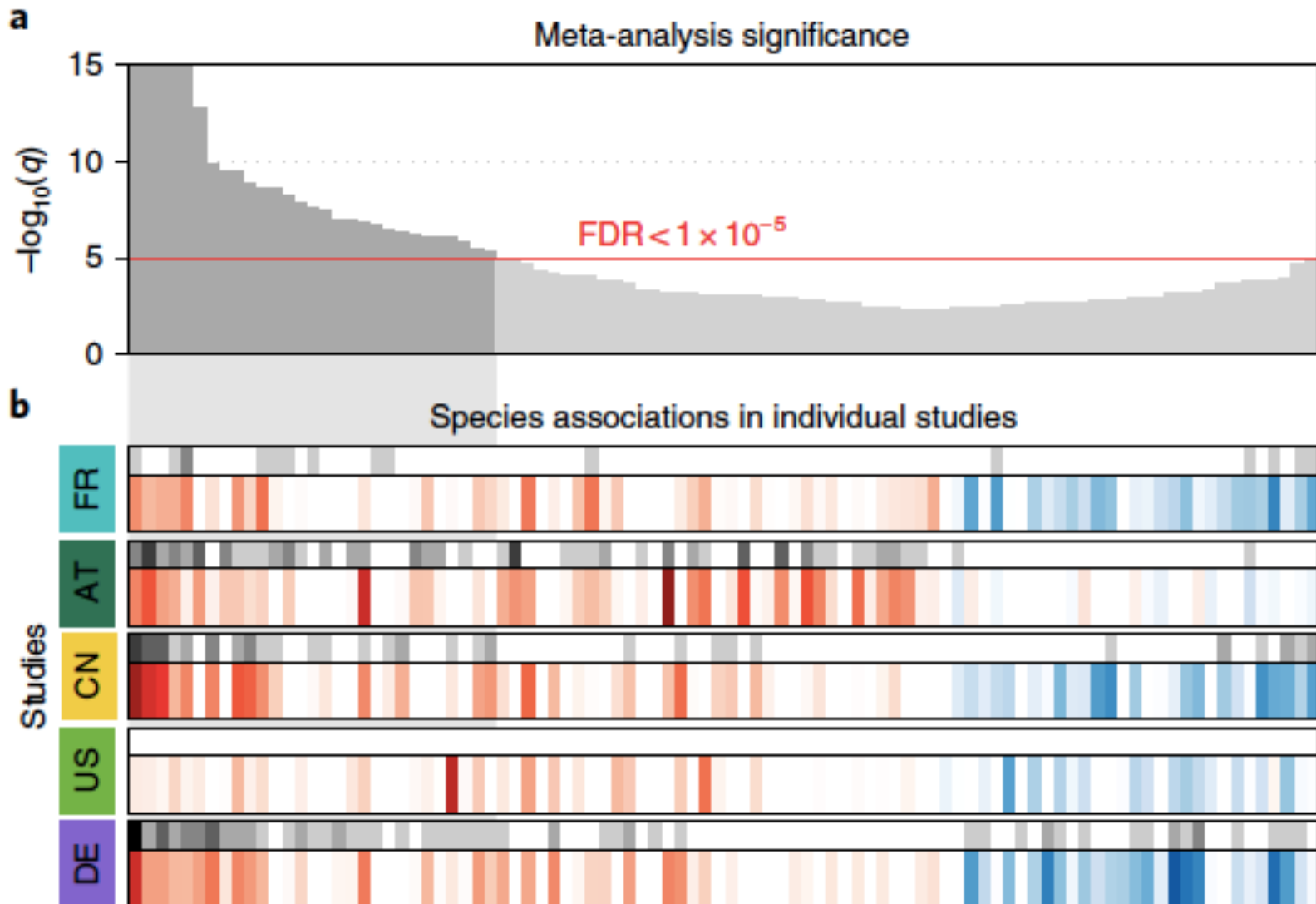
- Pros:
  - Established temporality
- Cons:
  - Difficult to enroll and follow up participants
  - Confounding



# Meta-analysis

- Pros:
  - Increased sample size
  - Comparison across different populations
- Cons:
  - Heterogeneity across studies
    - Sample collection, processing, sequencing, annotation, etc
  - Dependence on the quality of the original studies

# Meta-analysis



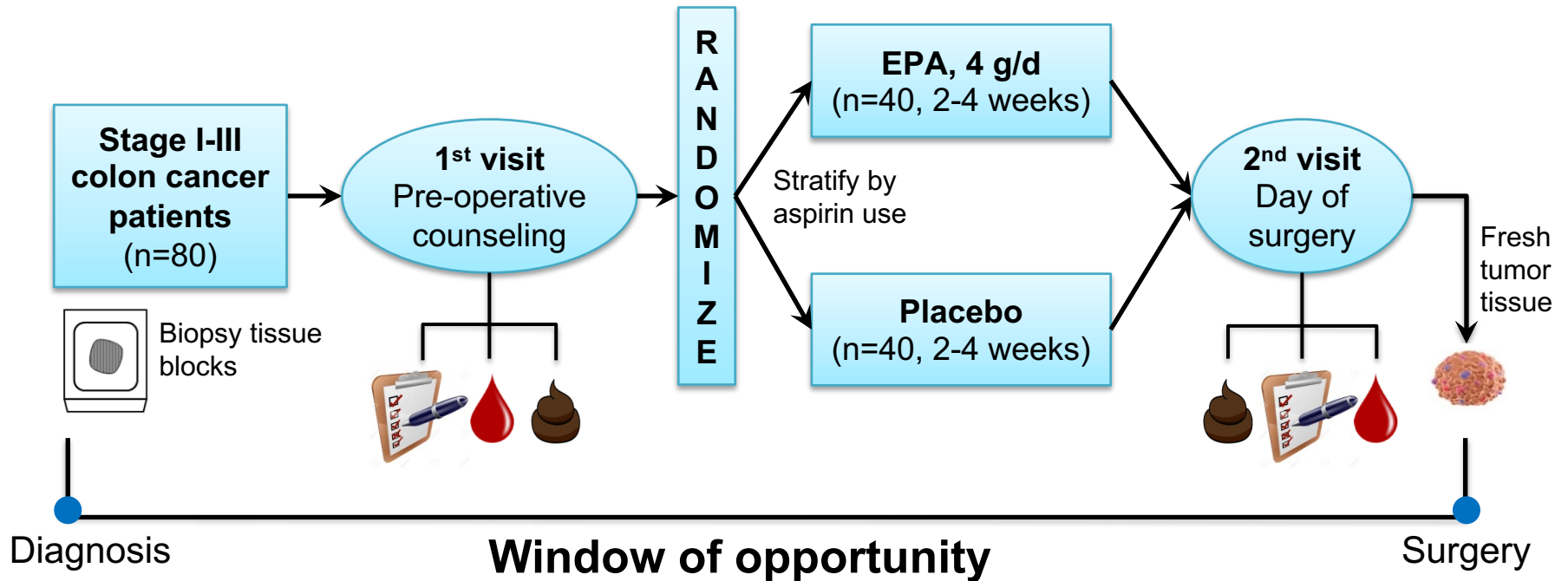


# Interventional study

- Pros:
  - Reduced confounding
  - Well-specified intervention
- Cons:
  - Limited sample size
  - Compliance: can be difficult
  - Reductionistic vs. holistic

# Interventional study

**OMICC: OM**ega-3 fatty acid for the **I**mmune modulation of **C**olon **C**ancer



# Outline

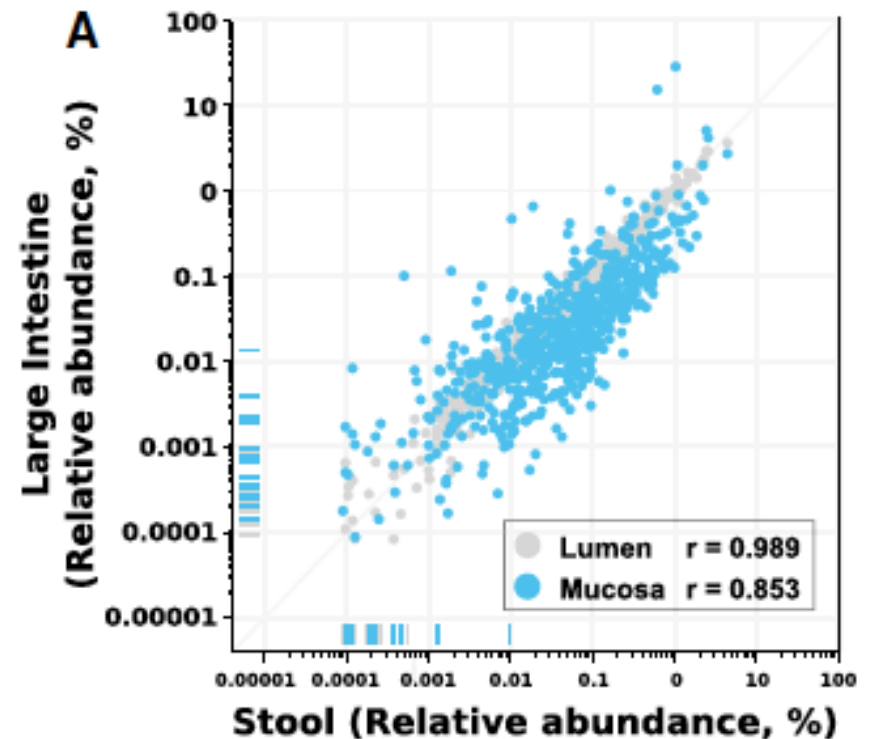
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# Sample type

- Stool
- Tissue
- Blood
- Urine

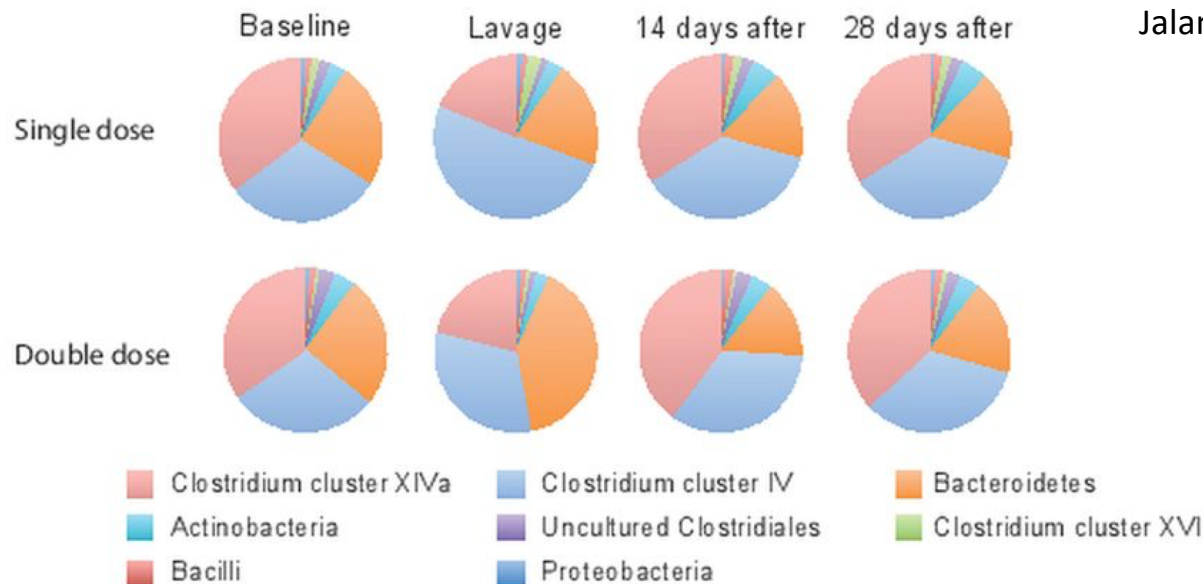
# Sample type

- Stool
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  - Cons: Representativeness of the gut microbial community; inability to study the biogeography of the gut microbiota



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- Tissue (from colonoscopy or surgery)
  - Pros: Mucosal microbiome
  - Cons: Difficult to collect; Influence of bowel prep

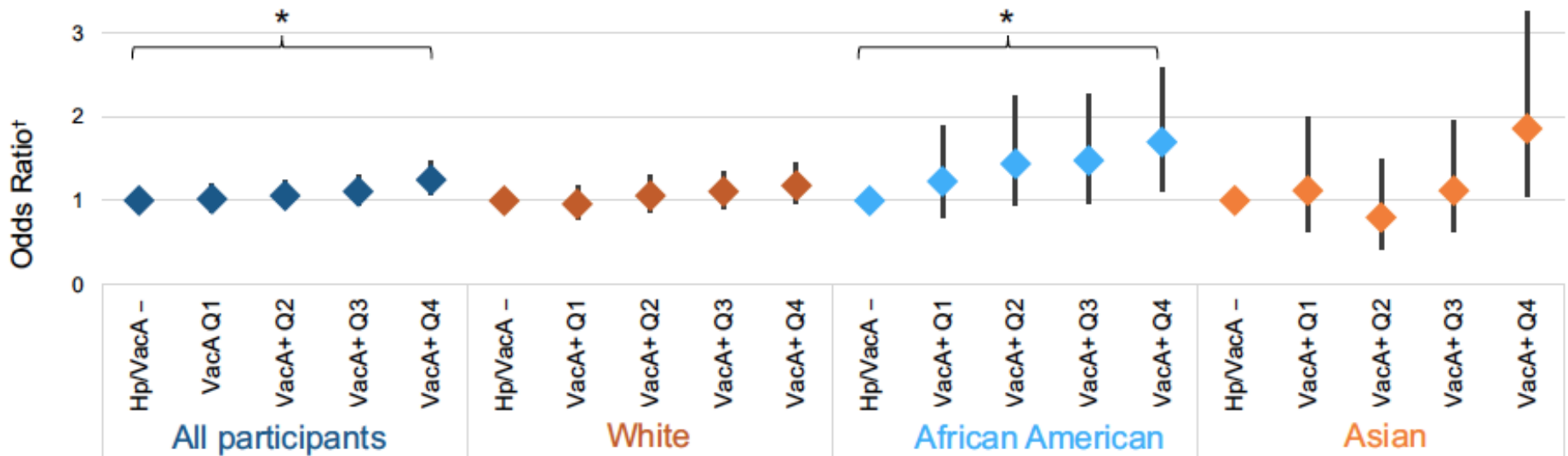


Jalanka J, et al. Gut. 2015

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- Blood
  - Pros: Able to study antigens/antibodies and microbial metabolites; Use of archived blood samples
  - Cons: Inability to distinguish active vs. past infection; Systemic response/level vs. local colonization/level;

## Odds of colorectal cancer incidence by strength of antibody response to *H. pylori* VacA, among participants in 10 US cohorts

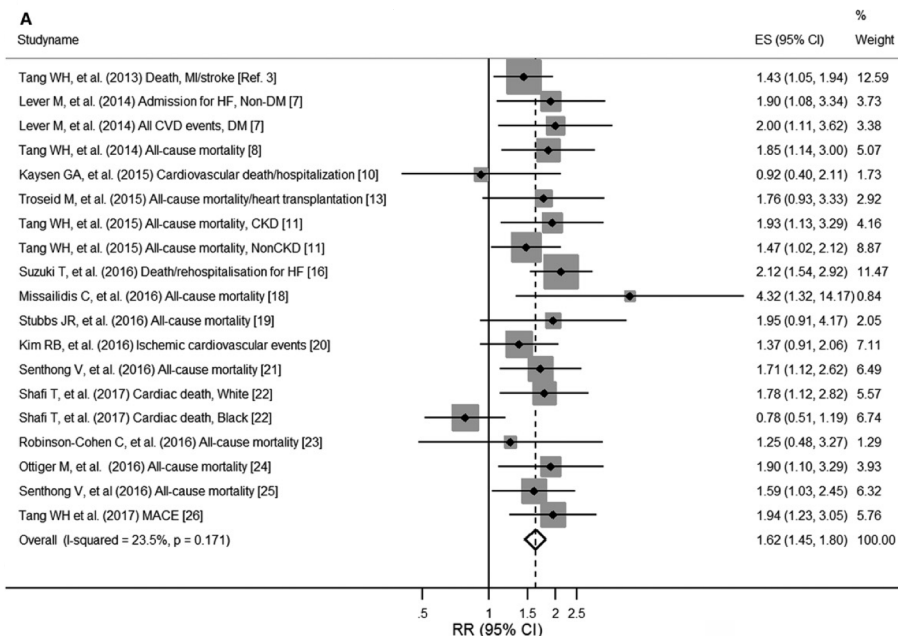


*H. pylori* multiplex serologic assays: ICC: 0.92 to 1.0 for reproducibility

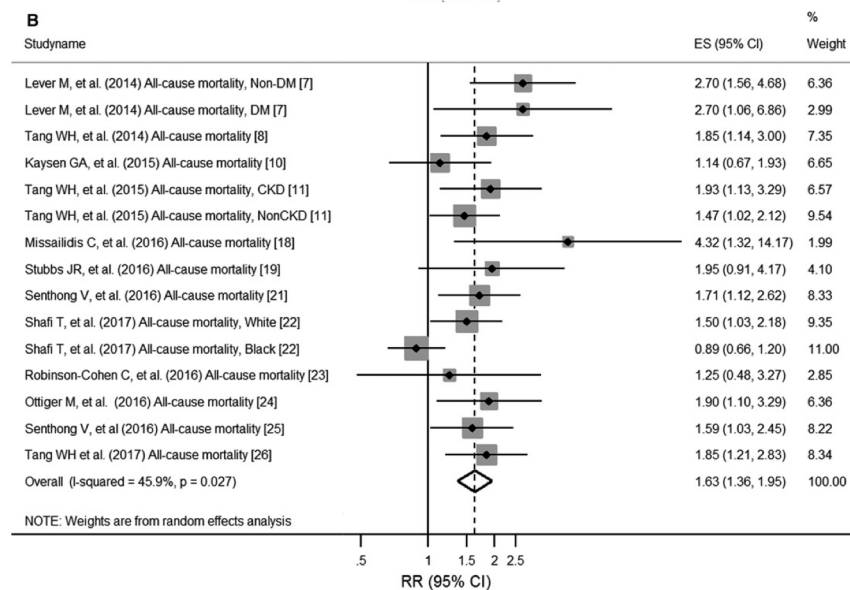


# Circulating levels of Trimethylamine-N-oxide (TMAO)

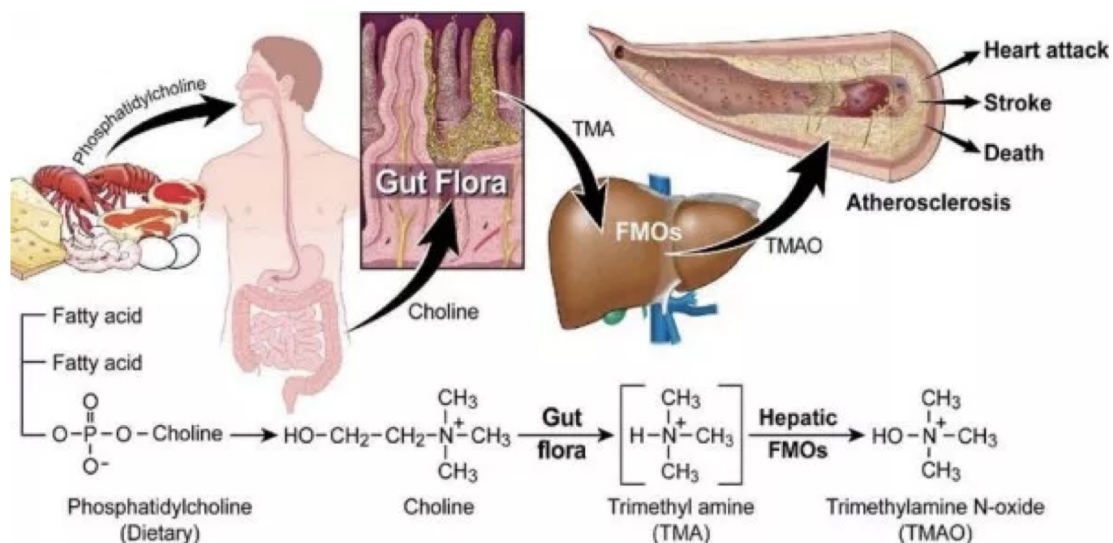
## Major adverse cardiovascular events



## All-cause mortality



Heianza Y, et al. J Am Heart Assoc. 2017



# Sample type

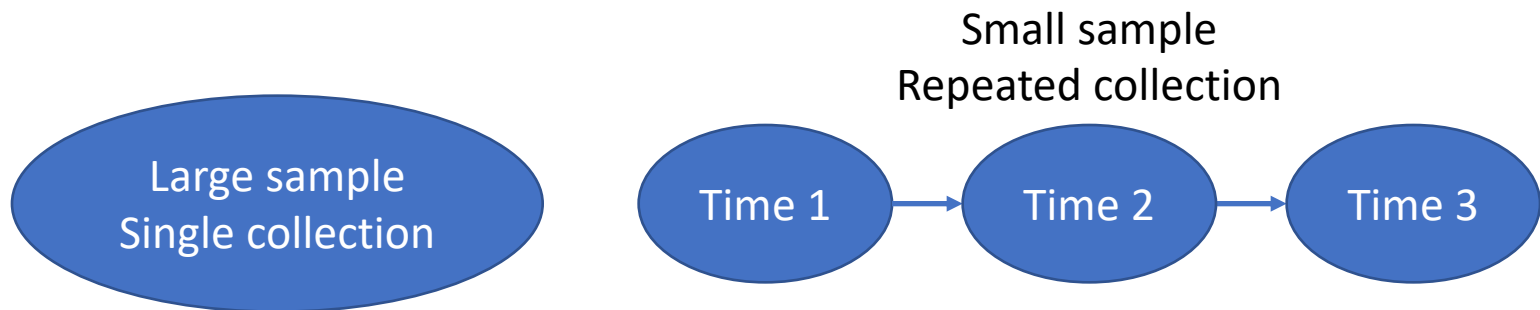
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- Tissue (from colonoscopy or surgery)
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- Blood
  - Pros: Able to study antigens/antibodies and microbial metabolites (TMAO and CVD); Use of archived blood samples
  - Cons: Inability to distinguish active vs. past infection; Systemic response/level vs. local colonization/level;
- Urine
  - Microbial metabolites

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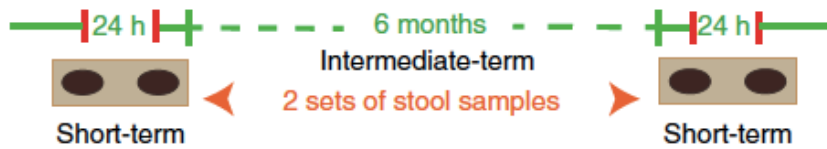
# Sample size

- Statistical power
  - Discovery-based study
    - Overall diversity
    - Abundance of individual microbes
  - Hypothesis-driven study
- Longitudinal study:
  - Budget = # of collections per participant \* # of participants



## Key question:

- Does a single assessment provide sufficient information for long-term exposure?

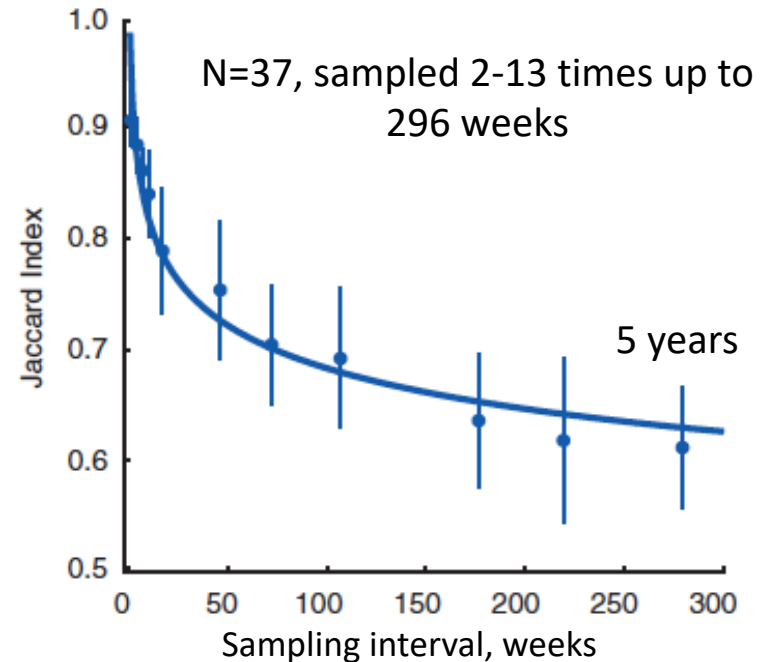
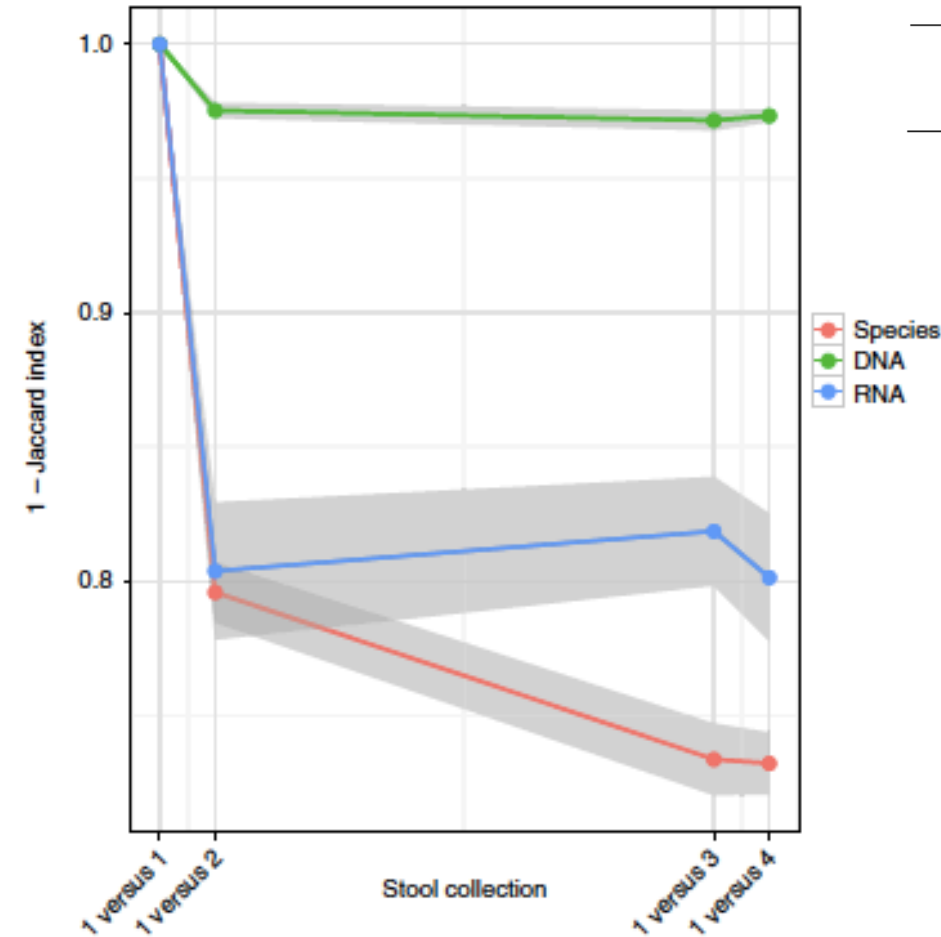


Mehta RS, et al, Nat Microbiol. 2018

	Percent of features with an ICC > 0.40	
	Short-term	Long-Term
Species (n=146)	96.8%	86.8%
DNA (n=1951)	99.9%	92.8%
RNA (n=3566)	1.3%	0.79%

## The Long-Term Stability of the Human Gut Microbiota

Jeremiah J. Faith, Janaki L. Guruge, Mark Charbonneau, Sathish Subramanian, Henning Seedorf, Andrew L. Goodman, Jose C. Clemente, Rob Knight, Andrew C. Heath, Rudolph L. Leibel, Michael Rosenbaum, Jeffrey I. Gordon\*



Metagenomic potential >  
Functional profile >  
Taxonomic profile abundance

Faith JJ et al, Science. 2013








# Outline

- Research question
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- **Covariate assessment**
- Microbiome among Nurses Study (MICRO-N)

# Covariate assessment

- Major determinants of the gut microbiome
  - Bristol Stool Scale
  - Medication use: e.g., antibiotics
  - Diet/lifestyle: short- and long-term
- Information about the collection
  - Collection & arrival time:
    - Shipping delay
  - Pattern of bowel movement
  - Contamination

## Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on its surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges (passed easily)
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. <b>Entirely Liquid</b>

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- **Microbiome among Nurses Study (MICRO-N)**



# Microbiome among Nurses Study (MICRO-N)

<https://www.nurseshealthstudy.org/participants/micro-n>



Nurses'  
Health Study

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**Micro-N**

Microbiome Among Nurses

# Importance of prospective studies

- To disentangle cause vs. effect → key to establish **causality**
  - “While the microbiota plays a key pathogenic role in IBD, chronic inflammation, in turn, promotes dysbiosis by altering the oxidative and metabolic environment of the gut.”  
--Nat Rev Gastroenterol Hepatol, 2017
- To identify early microbial changes → improve **prediction**

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## *Research Article*

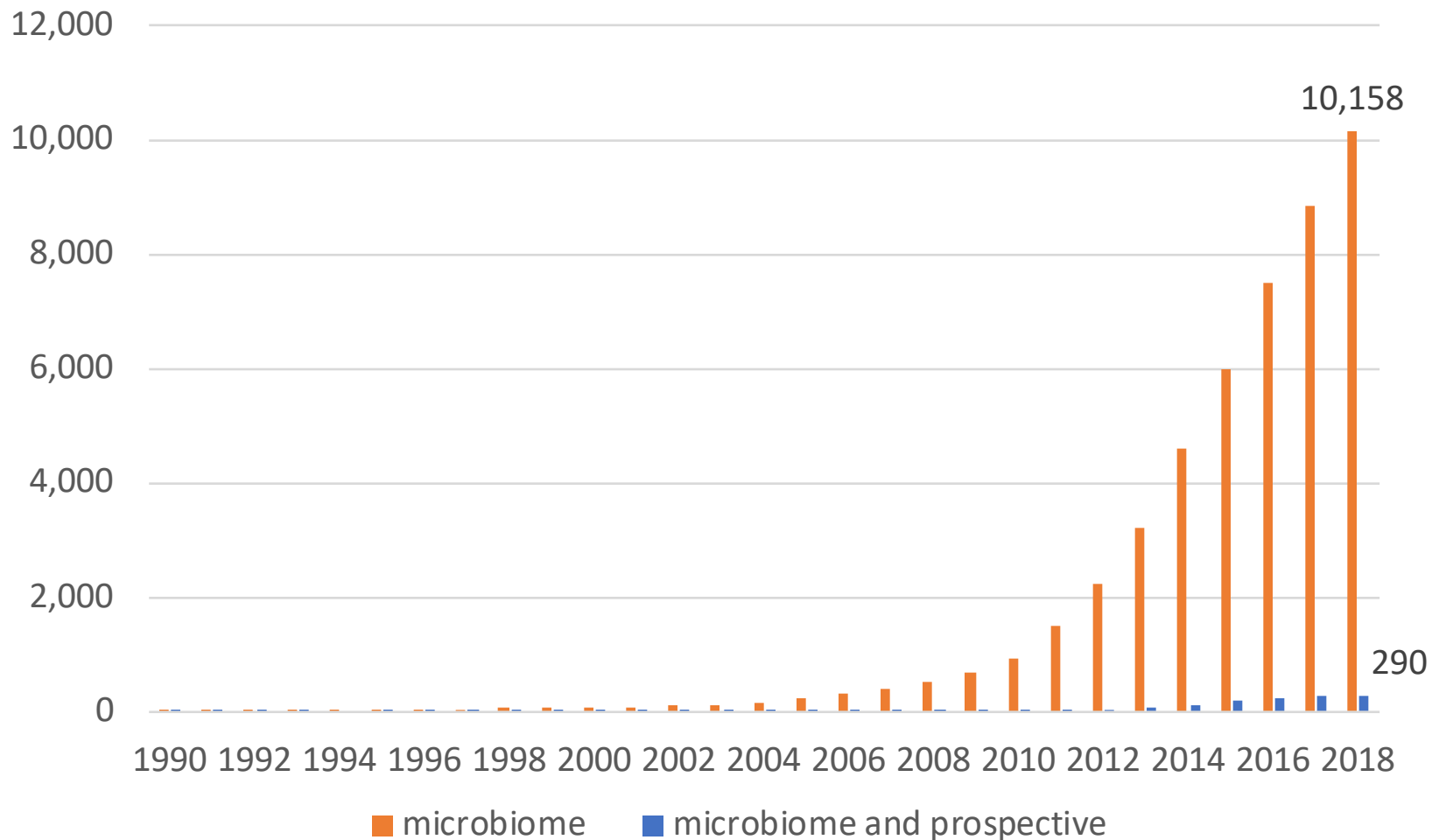
*See related article by Narayanan et al., p. 1108*

## **The Human Gut Microbiome as a Screening Tool for Colorectal Cancer**

Joseph P. Zackular<sup>1</sup>, Mary A.M. Rogers<sup>2</sup>, Mack T. Ruffin IV<sup>3</sup>, and Patrick D. Schloss<sup>1</sup>

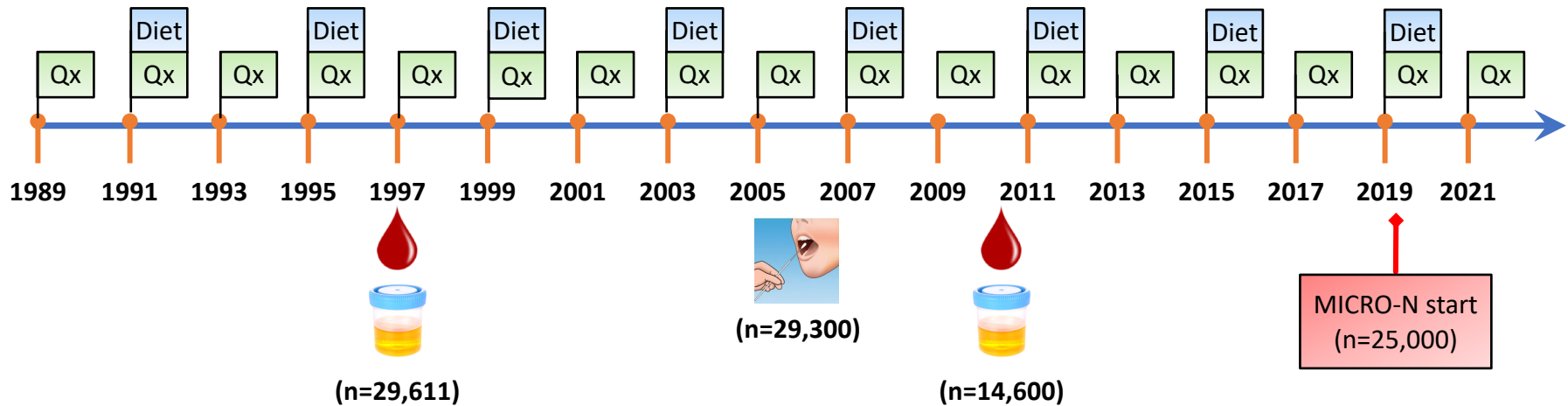
**Cancer  
Prevention  
Research**

# PubMed search



# Nurses' Health Study II

- 116,430 female registered nurses across the U.S. enrolled in 1989, aged 25-42 years



- Microbiome among Nurses Study (MICRO-N)
  - Primarily funded by the Massachusetts Life Sciences Center
  - Goal: to build an integrated microbiome research platform allowing collection, use, and analysis of microbiome-targeted biospecimens.

# Outcomes assessed in the NHS II

Group	Health conditions
Cardiovascular disease	Coronary heart disease, high blood pressure, cardiac arrest, congestive heart failure, arrhythmia, stroke, elevated cholesterol,
Cancer	Breast, colon or rectum, endometrium, ovary, melanoma, basal cell skin cancer, squamous cell skin cancer, other cancer
GI disease	Colon or rectal polyp, ulcerative colitis/Crohn's, gastric or duodenal ulcer, Barrett's esophagus, gallstones, cholecystectomy
Respiratory disease	Emphysema/Chronic Bronchitis
Metabolic diseases	Diabetes, obesity
Mental and neurological disorders	Multiple Sclerosis, Parkinsons' disease, depression
Diseases of the genitourinary system	Fibrocystic/other benign breast disease, endometriosis, kidney stones
Immune diseases	Asthma, Graves' Disease/Hyperthyroidism, hypothyroidism, hyperparathyroidism, gout, SLE (systemic lupus), rheumatoid arthritis

# Basic characteristics of the NHS II participants in 2015

Variable	Mean (SD) or %
Age, year	60.7 (0.2)
Menopause, %	93
Age at menopause, year	48.6 (6.4)
Current use of postmenopausal hormone, %	16
Multivitamin use, %	51
High blood pressure, %	27
Elevated cholesterol, %	29
Diabetes, %	7
Current smoking, %	5
Body mass index, kg/m <sup>2</sup>	27.8 (6.4)
Physical activity, MET-hours/week	28.4 (32.1)
Alcohol consumption, g/d	7.2 (11.1)
Total calorie intake, kcal/d	1,778 (570)
Red meat intake, serving/week	2.9 (2.9)
Processed meat intake, serving/week	1.4 (2.0)
Total fiber intake, g/d	23.9 (6.8)

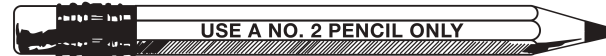
# Workflow

## Interest survey (2017 questionnaire)

### HARVARD UNIVERSITY

### NURSES' HEALTH STUDY II

#### INSTRUCTIONS



##### INTERNET:

Go to our website at [www.NHS2.org](http://www.NHS2.org) and use your ID number (see front of this page) and your birth date to log in and complete the survey online.

##### PAPER FORM:

Please use an ordinary No. 2 pencil to answer all questions. Fill response circles completely. If you have comments, please write them on a separate piece of paper.

**Please remove the cover letter (to preserve confidentiality) and return the questionnaire in the enclosed postage-paid envelope.**

#### Exciting New Research: How the Microbiome Affects Health

In Question 16 of the attached survey we ask you to participate in a ground-breaking new sub-study to examine the role that gut bacteria (aka the microbiome) play in affecting human health. To make this important research possible, we are asking everyone to help us by providing a sample of their saliva and of their stool. As always, we will ensure the privacy of all your results.

Scientists are just beginning to examine how the human microbiome works. Humans and microbes depend on one another – our bodies provide microbes with resources, and the microbes provide functions necessary for our health. It is crucial to learn what types of microbes live in a healthy human, what they are doing, and how they can influence the development of disease. By collecting saliva and stool samples from tens of thousands of women like you, we can begin to describe what makes up a healthy microbiome and also start to define when it may be unhealthy.

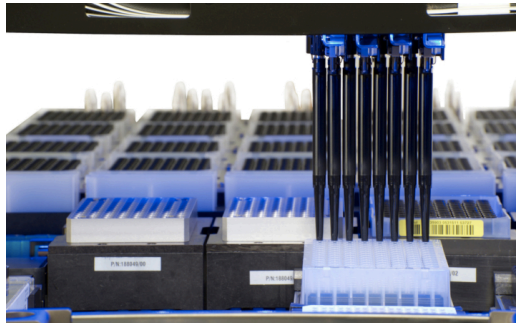
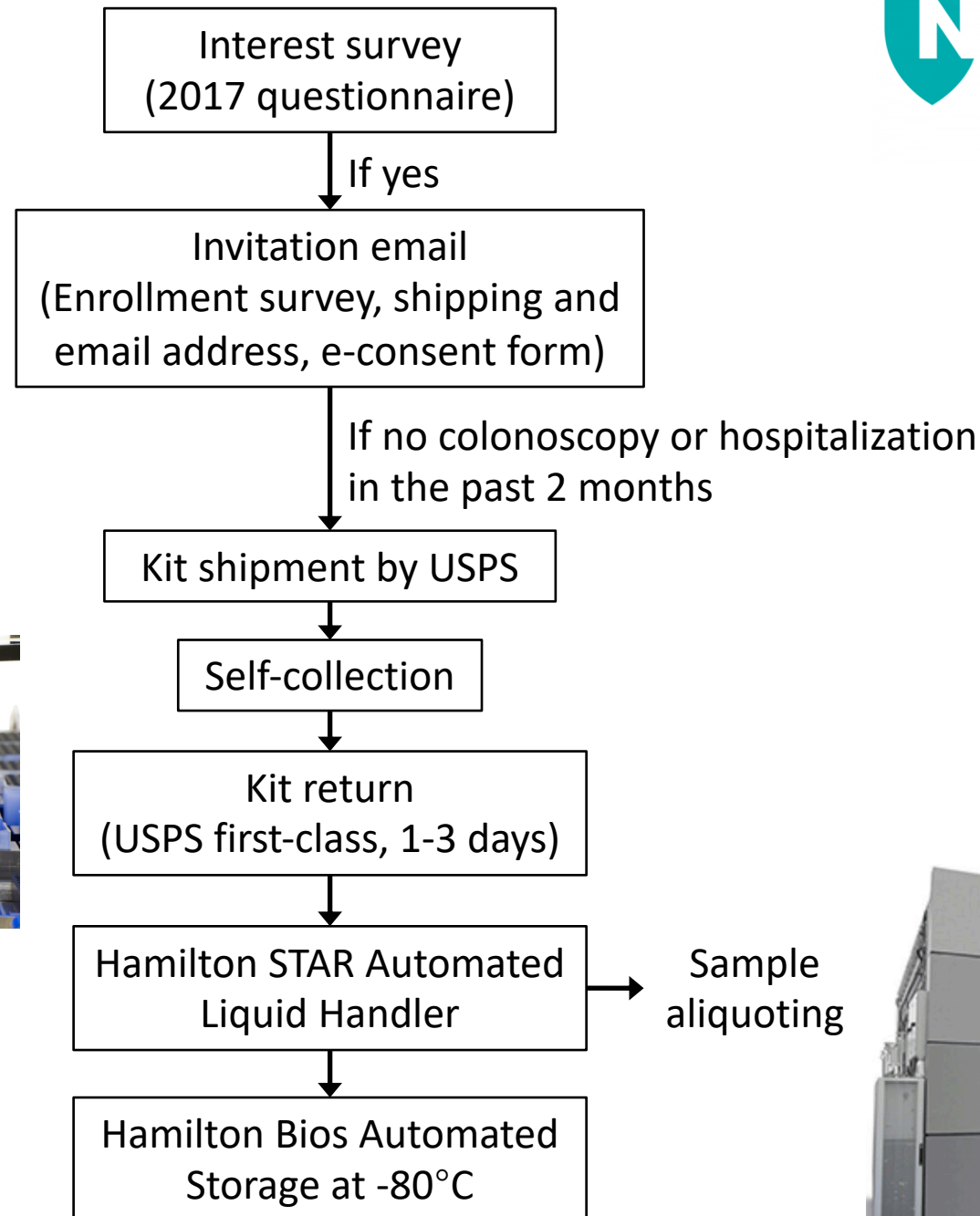
**The collection process is surprisingly easy, hygienic (and not particularly gross). You will be able to provide a sample at a time that is convenient for you. If you agree, we will send you a consent form and detailed instructions with all the supplies you will need, including a postage-paid shipping box that can be dropped into any mailbox; no special handling required. For more information, visit our microbiome info page at [nhs2.org/Micro-N](http://nhs2.org/Micro-N)**

Response	N (%)
Yes, definitely	42,093 (50)
Yes, possibly	13,122 (16)
No	28,480 (34)
Total	83,695

**16. The Nurses' Health Study is in a position to be a leader in the study of how gut bacteria (aka the microbiome) influence human disease. If we send you a convenient, hygienic, pre-paid collection kit, would you be willing to provide a sample of your saliva and of your stool? (The back of the cover letter has more detailed information.)**

☐ Yes, definitely    ☐ Yes, possibly    ☐ No

# Workflow





# Collection kits

- Stool microbiome collection
  - 95% Ethanol Kit
    - Pros: Cheap, can be used for stool metagenomics, metatranscriptomics and metabolomics.
    - Cons: volatile, flammable and considered hazardous.
  - OMNIgene<sup>®</sup>·Gut Kit
    - Pros: Good stabilization property for both DNA and RNA.
    - Cons: Costly; utility for metabolomics remains to be established.
  - Anaerobic Stool Collection Kit: Specialized kit for future culture studies
- Oral microbiome collection
  - OMNIgene<sup>®</sup>·ORAL Tongue Swab Kit

# Validation studies of stool collection methods

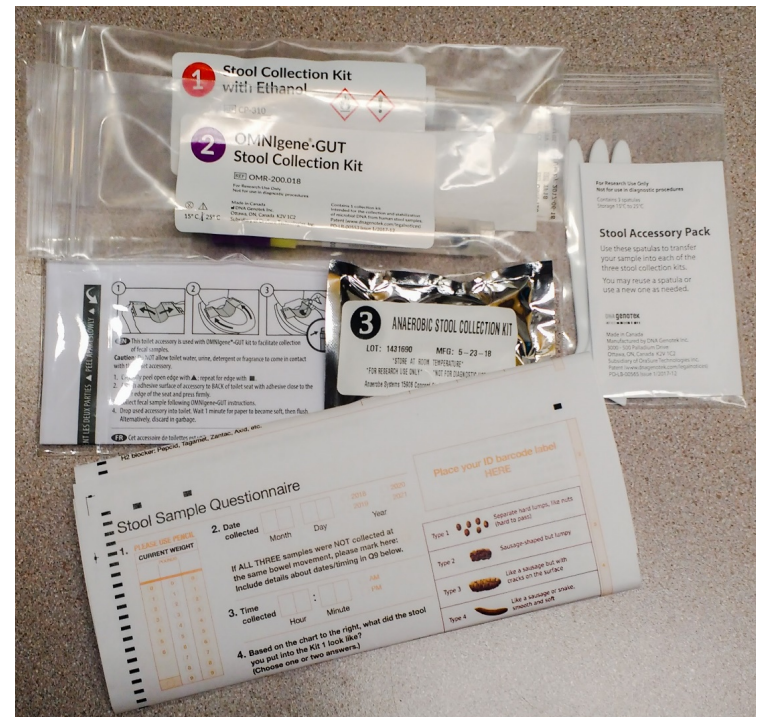
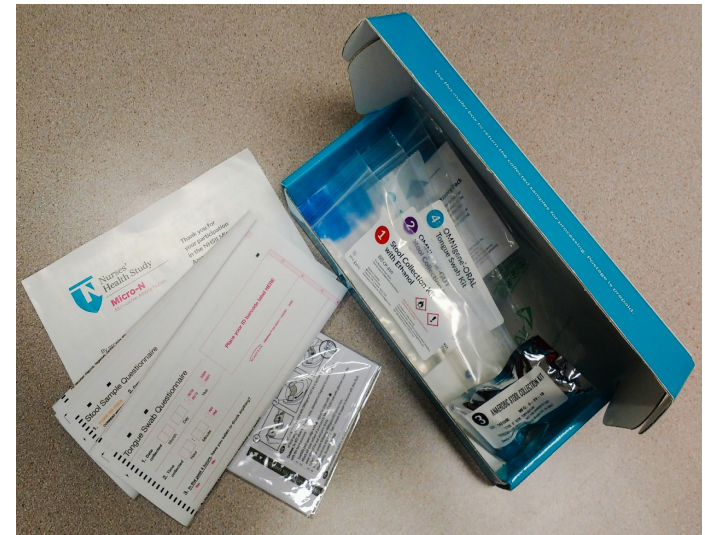
Year	Study (First/last authors)	Tested collection methods	Microbiome analysis	Storage time	Temperature	Repeated sampling	Sample size	Invalid method
2010	Lauber/Fierer	No buffer	16s rRNA	3-14d	-20, -80, +4, +20	-	2	NO
2012	Carroll/Ringel	All fresh	16s rRNA	24h/6mo	RT/-80°C	-	4	NO
2014	Dominianni/Ahn	RNAlater, FOBT card, Eppendorf tube	16s rRNA	3d	RT	-	3	NO
2014	Franzosa/Huttenhower	RNAlater, 95% ethanol	Metagenomics, Metatranscriptomics	48h	RT	-	8	NO
2015	Sinha/Knight	RNAlater, FOBT card, 70% ethanol, EDTA, dry swab	16s rRNA	4d	RT	-	20	NO
2015	Voigt/Bork	RNAlater	Metagenomics	7d	-20, +4-10, RT	+	3-7	NO
2015	Choo/Rogers	RNAlater, OMNIgene GUT, Tris-EDTA	16s rRNA	3d	4°C, RT	-	1	Tris-EDTA
2015	Flores/Sinha	RNAlater, No buffer	16s rRNA	3-7d	25°C	-	10	NO
2015	Reck/COMBACTE Consortium	RNAlater, RNA protect, All protect, DNA stabiliser	Metagenomics, Metatranscriptomics	15d	4°C, RT		1	RNAlater valid for 6d at RT; RNA Protect invalid
2015	Gorzelak/Gibson	RNAlater	16s rRNA	15min at RT, up to 30d at domestic freezer		-	4	RNAlater for the studied conditions
2016	Anderson/Jones	OMNIgene GUT	Metagenomics	28d	RT	-	16	NO
2016	Song/Knight	RNAlater, 95% ethanol, OMNIgene GUT, FTA card, 70% ethanol	16s rRNA	8wk	4-40°C	-	10 human+5 dogs	70% ethanol
2016	Hill/O'Toole	OMNIgene GUT	16s rRNA	1-2wk	RT	-	22 infants, 20 adults	for infant samples
2017	Vogtmann/Sinha	RNAlater, 95% ethanol, FOBT card, FIT tube	16s rRNA	4d	RT	-	52	NO
2017	Vogtmann/Sinha	RNAlater, 95% ethanol, FOBT card, FIT tube	16s rRNA	4d	RT	-	20	FIT tube

# Comparison of stool collection methods

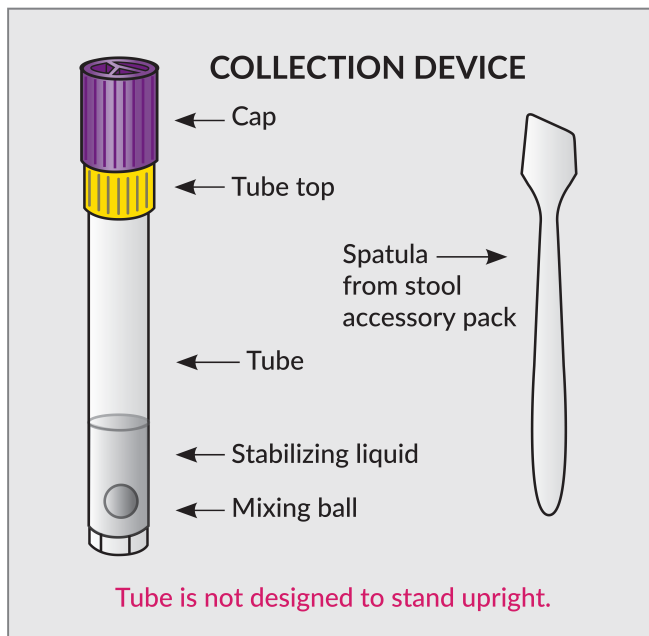
Method	16s	Metagenomics	Metatranscriptomics	Metabolomics	Stabilization ability at RT	Selected applications in cohort studies
RNA <i>later</i>	✓	✓	✓	✗	<2 weeks	Multiethnic Cohort HCHS/SOL Study CARDIA study
95% ethanol	✓	✓	✓	✓	Up to 8 weeks	Human Microbiome Project 2 Shanghai cohorts
OMNIgene GUT	✓	✓	?	?	Up to 8 weeks	Personalized Nutrition Study (Israel)
FOBT/FIT card	✓	?	✗	✓	Up to 4 days	-
Whatman FTA card	✓	?	✗	?	Up to 8 weeks	American Gut Project



- User instruction trifold brochure
- Barcode labels (6)
- Bio-specimen bags with absorbent pads (4)
- Air cushion
- Stool collection kits (3)
- Toilet accessory (2)
- Stool accessory pack
- Stool sample questionnaire
- Tongue swab kit
- Tongue swab questionnaire



# Day 1: Stool collection kits 1-2

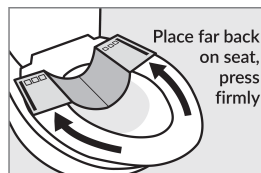


- 2.5 mL ethanol + 0.5 mL stool
- 2.0 mL OMNIgene.GUT fixative + 0.5 mL stool

Collect both specimens from the same stool sample.

## PROCEDURE

1



## IMPORTANT PREPARATIONS:

- Empty your bladder before beginning the collection.
- Follow the instructions on the toilet accessory to **affix to the back of the toilet seat. Press firmly.**
- If stool sample is liquid or donor has diarrhea, wait until the next bowel movement to collect the sample. A second toilet accessory has been provided if needed.
- Collect stool sample free of urine or toilet water. If sample becomes contaminated with urine or falls into the toilet, do NOT collect. Use the extra toilet accessory to provide a new stool sample when possible.

2



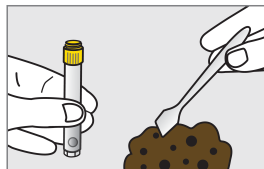
**Handle Kit ① with extra care as it contains Ethanol.**

While holding the yellow tube top, unscrew **ONLY** the purple cap from the tube top and set aside for later use.

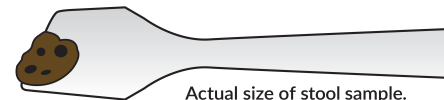
**IMPORTANT:** Do NOT remove the yellow tube top. Do NOT spill the stabilizing liquid in the tube.



3



Use the spatula to collect a small amount of stool sample.



4



Transfer the stool sample into the yellow tube top. Repeat until the sample fills the upper part as shown here in the fill close up.

**IMPORTANT:** Do NOT push sample into the tube.

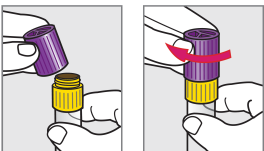


5



Scrape horizontally across the tube top to level the sample and remove any excess.

6

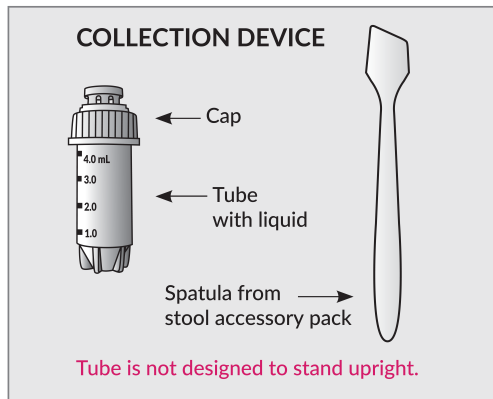


Pick up the purple cap with the solid end facing down and screw onto the yellow tube top until tightly closed. If stool overflows, wipe exterior with toilet paper or tissue.

**Repeat step 2 to 6 using Kit ②.**



# Day 1: Stool collection kit 3



Mix vigorously,  
then barcode the  
three stool samples.

Complete the  
stool questionnaire  
and barcode it.

Collect the stool sample for Kit 3 on the same day and from the same sample already deposited on the toilet accessory. Extra spatulas have been provided.

## PROCEDURE



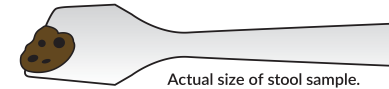
Locate Anaerobic Stool Collection Kit 3. Open the packaging by tearing the perforated edge.

Remove tube and unscrew cap. Set cap aside on a clean surface for later use. Tube is not designed to stand upright.

**IMPORTANT:** Do NOT spill the liquid inside the tube.



Use a spatula to collect a small scoop of stool sample.

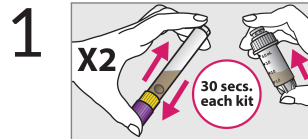


Transfer the sample into the anaerobic tube. Scrape horizontally across the tube top to remove any excess. Repeat with a second small scoop of stool, additional scoops are NOT required.

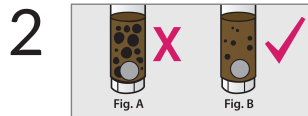
Wipe exterior of tube with toilet paper or tissue as needed.



Tightly screw the cap back onto the tube.

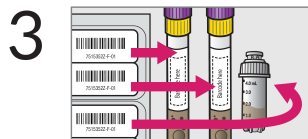


Shake the three tightly sealed tubes as hard and fast as possible in a back and forth motion for a minimum of 30 seconds each.



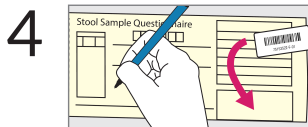
Each stool sample will be mixed with the stabilizing liquid in the tube; not all particles will dissolve.

**IMPORTANT:** Continue shaking if large particles remain as shown in Figure A.



**IMPORTANT:** Locate a barcode label and affix one to each tube. For the small anaerobic kit, wrap the barcode label around the tube.

Discard all stool collector packaging and spatula(s) in the garbage.



**IMPORTANT:** Once Kit 1, Kit 2 and Kit 3 collections are completed, fill in the stool sample section on the questionnaire and affix a barcode in the space provided.

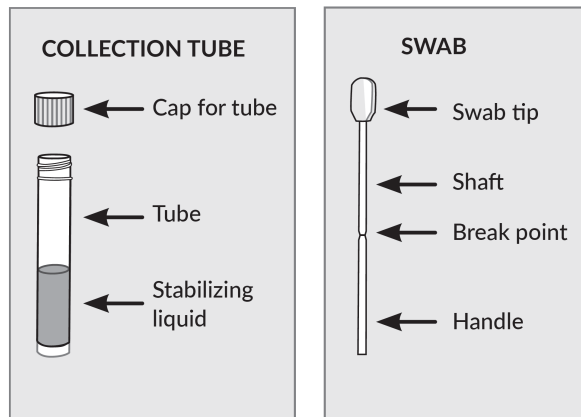


# Day 2: Tongue swab sample collection

**IMPORTANT:** You will need to use Kit ④ immediately upon waking on the day following your stool collections.

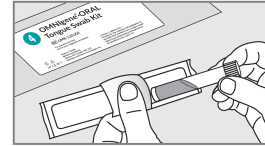
Do NOT eat, drink, smoke, use mouth wash or brush your teeth before providing a tongue swab sample.

Read all instructions prior to collection.



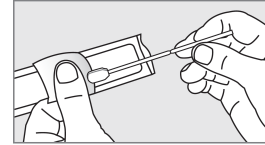
## PROCEDURE

1



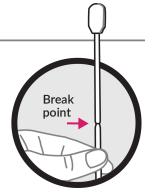
Locate Kit ④ and remove the collection tube containing stabilizing liquid from the packaging. Set the tube aside on a clean surface for later use.

2

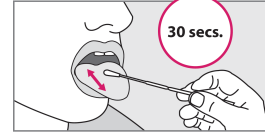


Remove the Kit ④ swab from its packaging using the handle.

**IMPORTANT:** Hold the swab only by the handle. Do NOT touch swab above the break point.



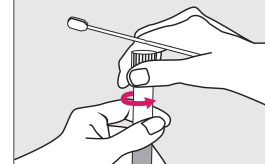
3



Gently rub the tongue for a minimum of 30 seconds. Avoid rubbing the teeth.

**IMPORTANT:** Do NOT bend swab during collection.

4

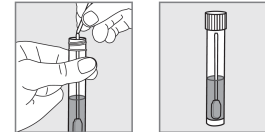


While still holding the swab in one hand, carefully unscrew the cap from the collection tube with stabilizing liquid.

**IMPORTANT:** Do NOT spill the stabilizing liquid in the tube. Do NOT touch the swab tip to any other surface.



5



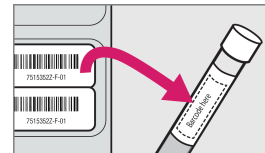
Immediately insert the swab into the bottom of the tube and snap the shaft off at the break point. The swab tip remains in the tube of liquid.

6



Tightly screw the cap back onto the tube.

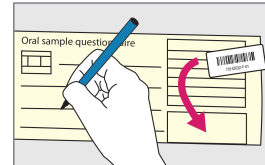
7



**IMPORTANT:** Locate a barcode label and affix it to the tube.

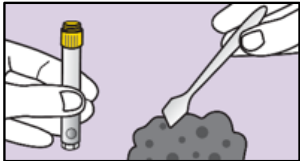


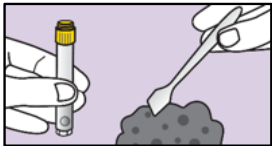


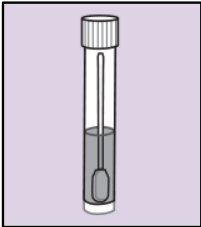


Discard the OMNIgene•ORAL packaging and swab handle in the garbage.

8



**IMPORTANT:** Once you have finished collecting with Kit ④, complete the oral sample section on the questionnaire and affix the barcode in the space provided.

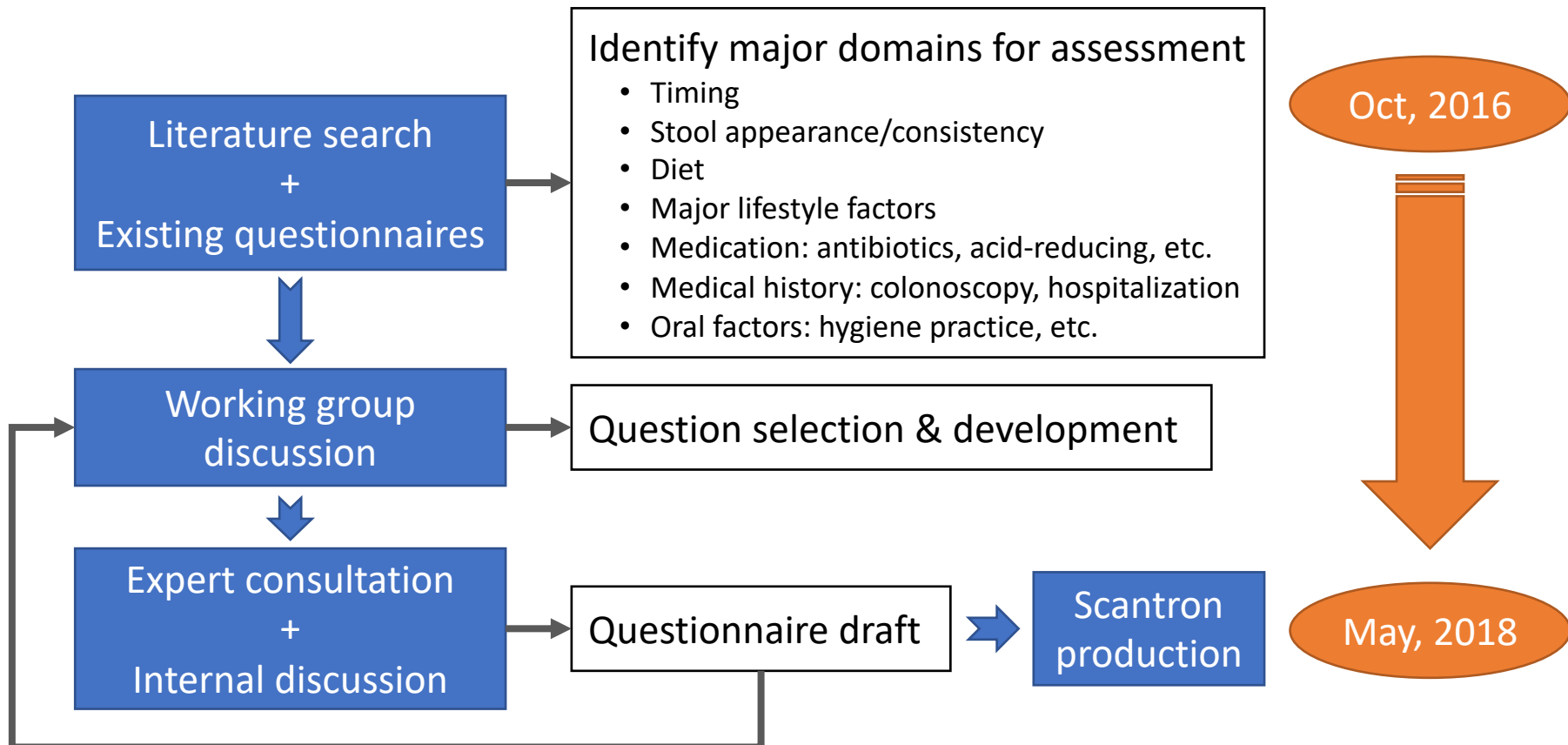
# Aliquoting after receiving

<p><b>OMNIgene.GUT Stool Collector (OMR-200.018)</b></p>  <p>2.0 mL OMNIgene.GUT fixative + 0.5 mL stool</p>	<p>4.5 mL liquefaction reagent</p> 	 <p>4 x 500 µL 1 x 400 µL</p>
<p><b>Stool Collector With Ethanol (CP-310)</b></p>  <p>2.5 mL ethanol + 0.5 mL stool</p>		 <p>4 x 500 µL 1 x 400 µL</p>
<p><b>OMNIgene.ORAL Tongue Swab (OMR-120.002)</b></p>  <p>tongue swab + 1mL OMNIgene.ORAL fixative</p>		 <p>2 x 450 µL</p>
<p><b>Anaerobic Stool Collector (AS-690)</b> 2.5 mL liquid dental transport medium</p>		Remains in tube



# Questionnaire development

- Aims:
  - Assess major determinants of the microbiome for future analysis.
  - Document potential problems that may have occurred during collection.



Google Drive ▶ 1 Research-related ▶ Gut microbiota ▶ MICRO-N ▶ Questionnaires ▶

Share with ▼ Burn New folder

Name	Date modified	Type	Size
From Gary	7/13/2018 9:08 PM	File folder	
HMP2	7/13/2018 9:17 PM	File folder	
oral_Joshipura	7/13/2018 8:37 PM	File folder	
Other studies	7/13/2018 5:36 PM	File folder	
scantron	7/13/2018 3:03 PM	File folder	
Combined Questionnaire_V1_20170614	6/14/2017 1:14 PM	Microsoft Word D...	926 KB
Combined Questionnaire_V1_20170614.docx.wv	6/15/2017 3:07 PM	Microsoft Word D...	927 KB
Combined Questionnaire_V2_20170629	6/29/2017 7:52 AM	Microsoft Word D...	994 KB
microbiome q 5-5 with antibiotics	5/18/2017 10:36 AM	Microsoft Word D...	929 KB
microbiome q 5-5	5/10/2017 8:52 PM	Microsoft Word D...	921 KB
NHS2_presentation_Stool Collection Questionnaire_20170308	3/8/2017 3:45 PM	Microsoft Word D...	456 KB
Questionnaire_scantron_microbiome q 3-8	3/8/2017 3:28 PM	Microsoft Word D...	654 KB
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Short-term_diet_20170604	6/4/2017 9:28 PM	Microsoft Word D...	2,527 KB
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Stool Collection Questionnaire_V4_20170204			
Stool Collection Questionnaire_V4_20170204-gec			
Stool Collection Questionnaire_V5_20170227			
Stool Collection Questionnaire_V5_20170227_QS			
Stool Collection Questionnaire_V6_20170304			
Stool Collection Questionnaire_V6_20170304_SST			
Stool Collection Questionnaire_V6_20170304_SST_ss			
Stool Collection Questionnaire_V7_20170308			
Stool Collection Questionnaire_V8_20170309			
Stool Collection Questionnaire_V8_20170309_clean			
Stool Collection Questionnaire_V8_20170604			

Google Drive ▶ 1 Research-related ▶ Gut microbiota ▶ MICRO-N ▶ Questionnaires ▶ scantron ▶

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Quote	7/13/2018 5:34 PM	File folder	
V1	7/13/2018 8:53 PM	File folder	
V2	7/13/2018 8:27 PM	File folder	
V3	7/13/2018 8:23 PM	File folder	
V4	7/13/2018 6:03 PM	File folder	
V5	7/13/2018 5:42 PM	File folder	

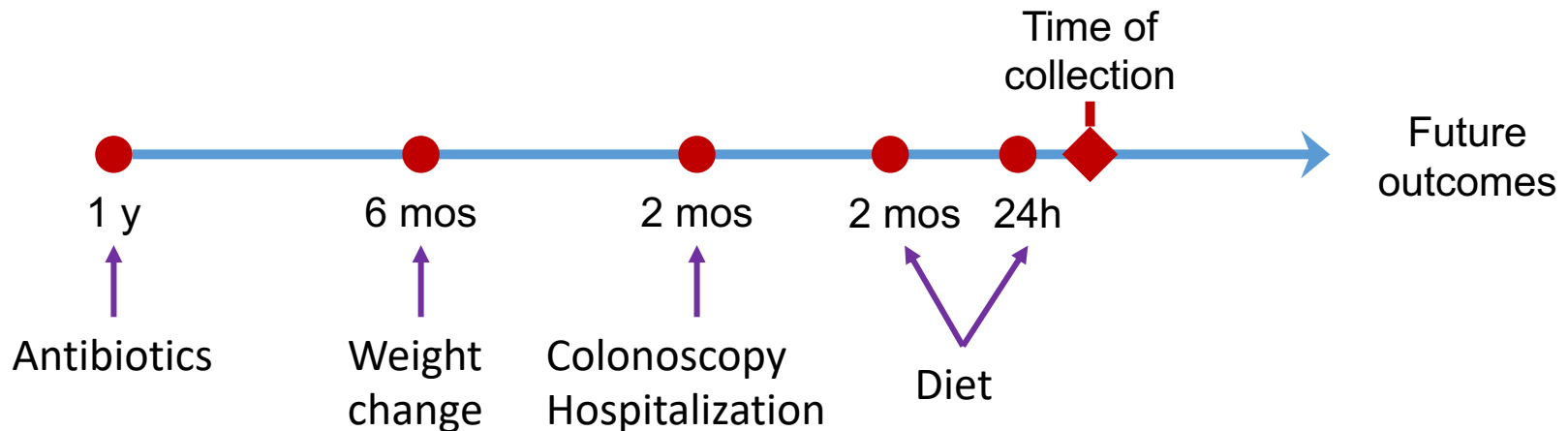
# Microbiome Working Group

- Goal: to develop a standard pipeline for microbiome sample collection and analysis → facilitate future pooling analysis across cohorts
- 27 participants from 10 institutions, 3 conference calls:
  - Nov 28, 2016: collection methods; questionnaire design; protocol elements
  - Jan 04, 2017: ethanol shipping; questionnaire items
  - Feb 03, 2017: questionnaire items
- NCI meeting, March 2017

Institution	Cohort
HSPH/HMS	NHS, HPFS
BWH/HMS	COSMOS / WHS, PHS, VITAL
Einstein	SOL, HIV+ cohort studies
Fred Hutch Cancer Research Center	MEC
NCI	PMI
NYU	NYU family study
U Hawaii	MEC
U Hawaii	MEC
UNC	CARDIA, ARIC, SEARCH
Vanderbilt	SCCS, Shanghai Men/Women's Cohort

# Key issues for questionnaire development

- Time frame for assessment



- Prioritization of the questions
  - Space vs. information
  - Balance with the main questionnaire
- Others: wording, response options, list examples

Stool Sample Questionnaire

1. PLEASE USE PENCIL

CURRENT WEIGHT

POUNDS

0

1

2

3

4

5

6

7

8

9

0

1

2

3

4

5

6

7

8

9

0

1

2

3

4

5

6

7

8

9

2. Date collected

MonthDayYear

2018

2019

2020

2021

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: Include details about dates/timing in Q9 below.

3. Time collected

HourMinute

AM

PM

4. Based on the chart to the right, what did the stool you put into the Kit 1 look like? (Choose one or two answers.)

Type 1

Type 2

Type 3

Type 4

Type 5

Type 6

Type 7

5. Prior to this collection, when was your last bowel movement?

Earlier today, in the last 6 hours

Earlier today, more than 6 hours ago

Yesterday

Two days ago

More than two days ago

6. In the past 2 months, please mark how often you have had the following types of bowel movements:

	More than twice per day	Twice per day	Once per day	Every other day	Every 3-6 days	Once a week or less	Never
Hard / lumpy	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>
Soft / smooth	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>
Watery liquid	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>
ANY bowel movement	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>

7. In the past year, have you used any of the following medications?

	Not used	Past one month	1 to 6 months	6+ months
Oral antibiotics	<div></div>	<div></div>	<div></div>	<div></div>
Injected antibiotics	<div></div>	<div></div>	<div></div>	<div></div>
Proton Pump Inhibitors: Prilosec, Nexium, Prevacid, Protonix, Aciphex, etc.	<div></div>	<div></div>	<div></div>	<div></div>
H2 blocker: Pepcid, Tagamet, Zantac, Axid, etc.	<div></div>	<div></div>	<div></div>	<div></div>

8. Compared to 6 months ago, how would you characterize your weight?

Lost >5 lbs.	Lost ≤5 lbs.	No change	Gained ≤5 lbs.	Gained >5 lbs.	Not sure
<div></div>	<div></div>	<div></div>	<div></div>	<div></div>	<div></div>

9. Did you have any problems or concerns with the stool sample collection, for example the solution spilled out of the tube or you had problems with catching stool in the toilet accessory? (Please describe)

Place your ID barcode label HERE

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. Entirely Liquid

10. In the past 2 months, have you undergone a colonoscopy or other procedure requiring bowel preparation?

No

Yes

11. In the past 2 months, have you been hospitalized for any reason?

No

Yes

12. For each food/supplement listed, please indicate whether you consumed it in the past 24 hours AND how often, on average, you consumed in the last week:

	DID YOU EAT THIS ITEM IN THE LAST 24 HOURS? (Mark if Yes)	HOW OFTEN DID YOU EAT OR DRINK THE FOLLOWING PRODUCTS IN THE LAST WEEK?							
		Not in the last week	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Prebiotic supplements (insulin, FOS, GOS, etc.)	<div></div>								
Probiotic supplements (Lactobacillus, Bifidobacterium, etc.)	<div></div>								
Fiber supplement (Metamucil, Konsyl or Citracel, etc.)	<div></div>								
Laxatives (Ex-lax, Dulcolax, MiraLax, Senna, enema, etc.)	<div></div>								
Stool softener (Colace, etc.)	<div></div>								
Soda, energy drinks, or fruit drinks with sugar (one can or bottle)	<div></div>								
Low-calorie beverage (one can or bottle) (Diet Coke, Diet 7Up, etc.)	<div></div>								
Alcoholic beverage (beer, brandy, spirits, hard liquor, wine, aperitif, etc.) (1 drink)	<div></div>								
Milk (Whole, skim, 1 or 2% milk) (1 cup)	<div></div>								
Soy milk (1 cup)	<div></div>								
Almond milk (1 cup)	<div></div>								
Cheese (cottage, ricotta, cream cheese, etc.) (1 oz.)	<div></div>								
Yogurt or kefir (1 cup)	<div></div>								
Fruits (no juice) (apples, raisins, bananas, oranges, strawberries, blueberries, etc.)	<div></div>								
Vegetables (salad, tomatoes, onions, greens, carrots, peppers, etc.)	<div></div>								
Tofu, soy burger, soybeans	<div></div>								
Fermented soy products (miso, etc.)	<div></div>								
Other fermented foods (kombucha, sauerkraut, etc.)	<div></div>								
Beans or lentils (baked, dried, or soup)	<div></div>								
Whole grain cold breakfast cereal	<div></div>								
Cooked oatmeal/cooked oat bran (including instant)	<div></div>								
Whole grain bread (1 slice)	<div></div>								
Other whole grains (brown rice, wheat pasta, etc.)	<div></div>								
Potatoes (baked, boiled or mashed)	<div></div>								
Refined grains (white bread, white rice, white pasta)	<div></div>								
Eggs (1)	<div></div>								
Red meat (beef, hamburger, pork, lamb)	<div></div>								
Poultry (chicken, turkey, etc.)	<div></div>								
Processed meat (lunch meat, sandwich meat, ham, salami, bologna, sausage, kielbasa, hot dog, bacon, etc.)	<div></div>								
Fish (fish nuggets, breaded fish, fish cakes, salmon, tuna, etc.)	<div></div>								
Sweets (pies, jam, chocolate, cake, cookies, etc.)	<div></div>								
Nuts (peanuts, walnuts, almonds, etc.)	<div></div>								
Flax seeds (1 tbs)	<div></div>								

SCANTRON EliteView™ EM-300913-1:554321 ED06

PLEASE CONTINUE ON THE REVERSE SIDE

## Timing of collection

## Stool Sample Questionnaire

2. Date collected

Month

Day

☐ 2018

☐ 2020

☐ 2019

☐ 2021

Year

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: ☐  
Include details about dates/timing in Q9 below.

3. Time collected

Hour

Minute

☐ AM

☐ PM

Place your ID barcode label  
HERE

Time of collection



Time of receipt



Sitting and shipping time

# Stool Sample Questionnaire

Timing of collection

Body weight

1. PLEASE USE PENCIL

CURRENT WEIGHT

POUNDS		
0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
	7	7
	8	8
	9	9

2. Date collected

Month

Day

☐ 2018

☐ 2020

☐ 2019

☐ 2021

Year

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: ☐  
Include details about dates/timing in Q9 below.

3. Time collected

Hour

Minute

☐ AM

☐ PM

Place your ID barcode label  
HERE

Energy harvest, gut barrier function,  
inflammation, host metabolism



Inflammation, gut barrier function

8. Compared to 6 months ago, how would you characterize your weight?

Lost >5 lbs.

☐

Lost ≤5 lbs.

☐

No change

☐

Gained ≤5 lbs.

☐

Gained >5 lbs.

☐

Not sure

☐

# Stool Sample Questionnaire

Timing of collection

Body weight

Stool consistency

1. PLEASE USE PENCIL

CURRENT WEIGHT

POUNDS

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
	7	7
	8	8
	9	9

2. Date collected

Month

Day

Year

☐ 2018 ☐ 2020  
☐ 2019 ☐ 2021

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: ☐  
Include details about dates/timing in Q9 below.

3. Time collected

Hour

Minute

☐ AM  
☐ PM

4. Based on the chart to the right, what did the stool you put into the Kit 1 look like? (Choose one or two answers.)

☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4  
☐ Type 5 ☐ Type 6 ☐ Type 7

Place your ID barcode label  
HERE

Type 1



Separate hard lumps, like nuts (hard to pass)

Type 2



Sausage-shaped but lumpy

Type 3



Like a sausage but with cracks on the surface

Type 4



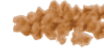
Like a sausage or snake, smooth and soft

Type 5



Soft blobs with clear-cut edges

Type 6



Fluffy pieces with ragged edges, a mushy stool

Type 7



Watery, no solid pieces. Entirely liquid

8. Compared to 6 months ago, how would you characterize your weight?

Lost >5 lbs.

☐

Lost ≤5 lbs.

☐

No change

☐

Gained ≤5 lbs.

☐

Gained >5 lbs.

☐

Not sure

☐



# Stool Sample Questionnaire

Timing of collection

Body weight

Stool consistency

Pattern & type of bowel movement

1. PLEASE USE PENCIL

CURRENT WEIGHT

POUNDS		
0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9

2. Date collected       ☐ 2018 ☐ 2020  
☐ 2019 ☐ 2021  
Month Day Year

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: ☐  
Include details about dates/timing in Q9 below.

3. Time collected   :   ☐ AM ☐ PM  
Hour Minute

4. Based on the chart to the right, what did the stool you put into the Kit 1 look like? (Choose one or two answers.)

☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4  
☐ Type 5 ☐ Type 6 ☐ Type 7

5. Prior to this collection, when was your last bowel movement?

Earlier today, in the last 6 hours ☐ Earlier today, more than 6 hours ago ☐ Yesterday ☐ Two days ago ☐ More than two days ago ☐

6. In the past 2 months, please mark how often you have had the following types of bowel movements:

	More than twice per day	Twice per day	Once per day	Every other day	Every 3-6 days	Once a week or less	Never
Hard / lumpy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Soft / smooth	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Watery liquid	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
ANY bowel movement	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

8. Compared to 6 months ago, how would you characterize your weight?

Lost >5 lbs. ☐ Lost ≤5 lbs. ☐ No change ☐ Gained ≤5 lbs. ☐ Gained >5 lbs. ☐ Not sure ☐

Place your ID barcode label HERE

Type 1  Separate hard lumps, like nuts (hard to pass)

Type 2  Sausage-shaped but lumpy

Type 3  Like a sausage but with cracks on the surface

Type 4  Like a sausage or snake, smooth and soft

Type 5  Soft blobs with clear-cut edges

Type 6  Fluffy pieces with ragged edges, a mushy stool

Type 7  Watery, no solid pieces. Entirely Liquid

# Stool Sample Questionnaire

Timing of collection

1. PLEASE USE PENCIL

CURRENT WEIGHT

POUNDS

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9

2. Date collected





Month

Day

Year

If ALL THREE samples were NOT collected at the same bowel movement, please mark here: ☐ Include details about dates/timing in Q9 below.

3. Time collected




Hour

Minute

4. Based on the chart to the right, what did the stool you put into the Kit 1 look like? (Choose one or two answers.)

- ☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4  
☐ Type 5 ☐ Type 6 ☐ Type 7

5. Prior to this collection, when was your last bowel movement?

Earlier today, in the last 6 hours

Earlier today, more than 6 hours ago

Yesterday

Two days ago

More than two days ago

☐
☐
☐
☐
☐

6. In the past 2 months, please mark how often you have had the following types of bowel movements:

	More than twice per day	Twice per day	Once per day	Every other day	Every 3-6 days	Once a week or less	Never
Hard / lumpy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Soft / smooth	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Watery liquid	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
ANY bowel movement	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

7. In the past year, have you used any of the following medications?

	Not used	Past one month	1 to 6 months	6+ months
Oral antibiotics	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Injected antibiotics	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Proton Pump Inhibitors: Prilosec, Nexium, Prevacid, Protonix, Aciphex, etc.	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
H2 blocker: Pepcid, Tagamet, Zantac, Axid, etc.	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

8. Compared to 6 months ago, how would you characterize your weight?

Lost >5 lbs.

Lost ≤5 lbs.

No change

Gained ≤5 lbs.

Gained >5 lbs.

Not sure

☐
☐
☐
☐
☐
☐

Place your ID barcode label HERE

Type 1



Separate hard lumps, like nuts (hard to pass)

Type 2



Sausage-shaped but lumpy

Type 3



Like a sausage but with cracks on the surface

Type 4



Like a sausage or snake, smooth and soft

Type 5



Soft blobs with clear-cut edges

Type 6



Fluffy pieces with ragged edges, a mushy stool

Type 7



Watery, no solid pieces. Entirely liquid

Body weight

Stool consistency

Pattern & type of bowel movement

Medication use

Colonoscopy



10. In the past 2 months, have you undergone a colonoscopy or other procedure requiring bowel preparation?

☐ No

☐ Yes

Hospitalization



11. In the past 2 months, have you been hospitalized for any reason?

☐ No

☐ Yes



Useful for future flagging

Collection  
problems

9. Did you have any problems or concerns with the stool sample collection, for example the solution spilled out of the tube or you had problems with catching stool in the toilet accessory? (Please describe)

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Colonoscopy

10. In the past 2 months, have you undergone a colonoscopy or other procedure requiring bowel preparation?

☐ No

☐ Yes

Hospitalization

11. In the past 2 months, have you been hospitalized for any reason?

☐ No

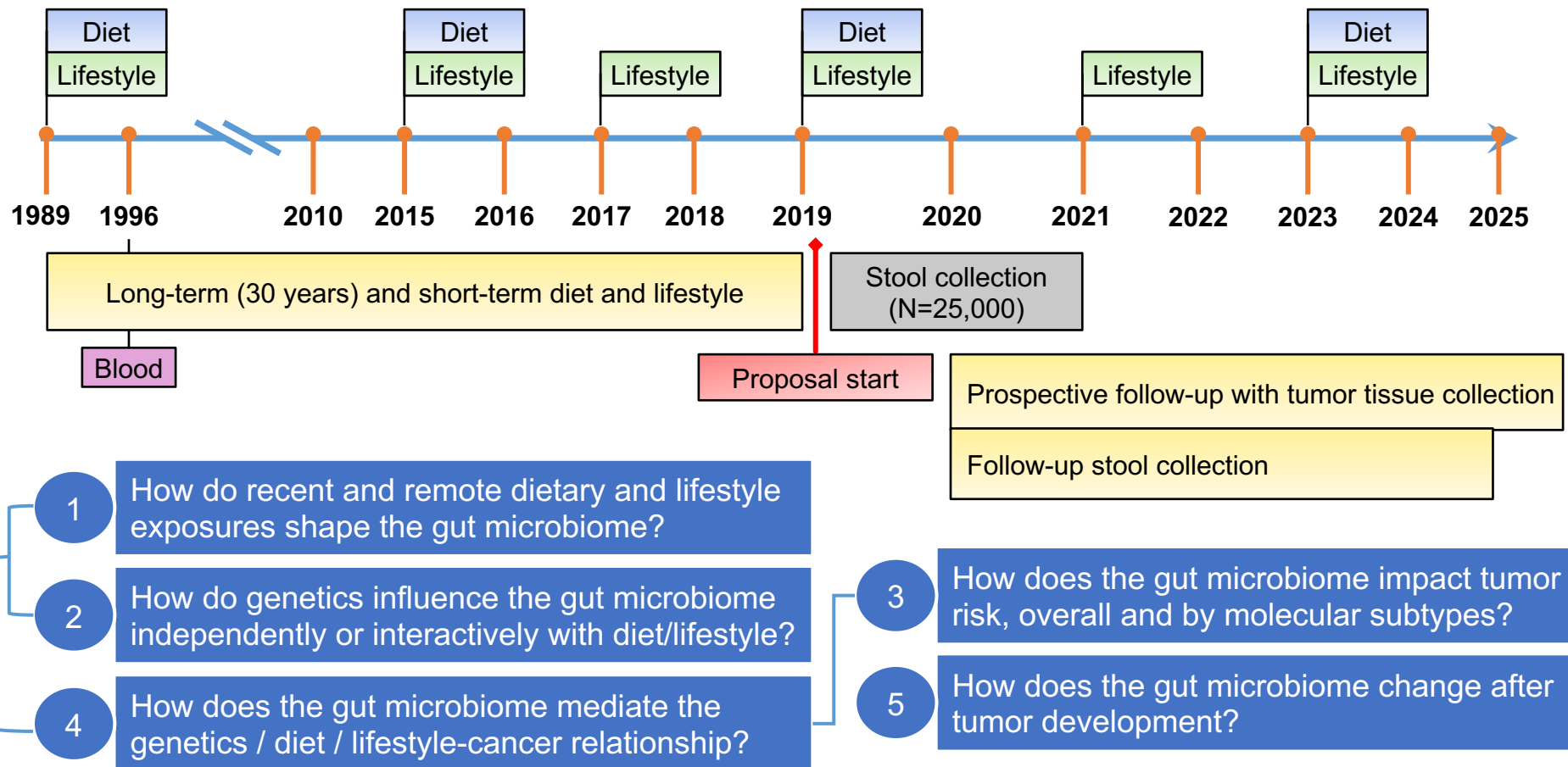
☐ Yes

Pre-/probiotic supplements

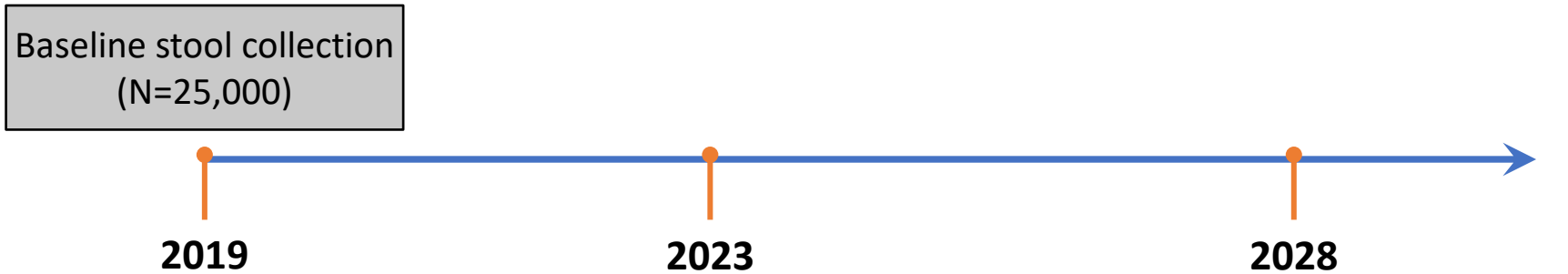
Major food items

[illegible]

# Study questions we can address: an example project proposal



# Projection of # cancer outcomes

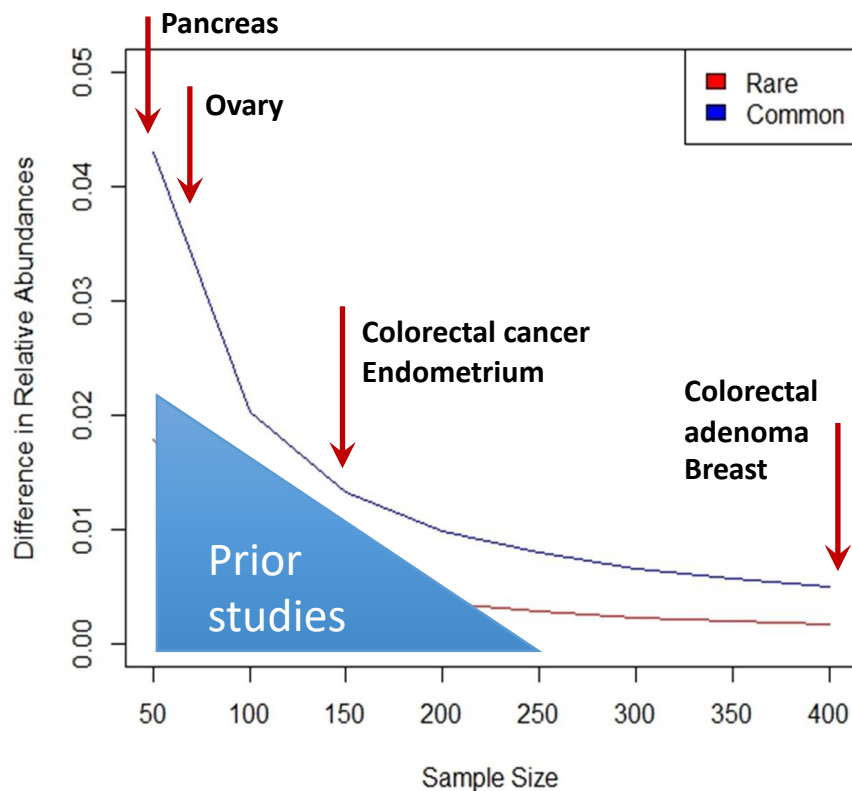


Outcomes	Projected no. of incident cases over 5 years	Projected no. of incident cases over 10 years
Colorectal adenoma*	735	945
Breast cancer	611	1,210
Colorectal cancer	144	308
Endometrial cancer	130	253
Ovarian cancer	61	132
Pancreatic cancer	30	77

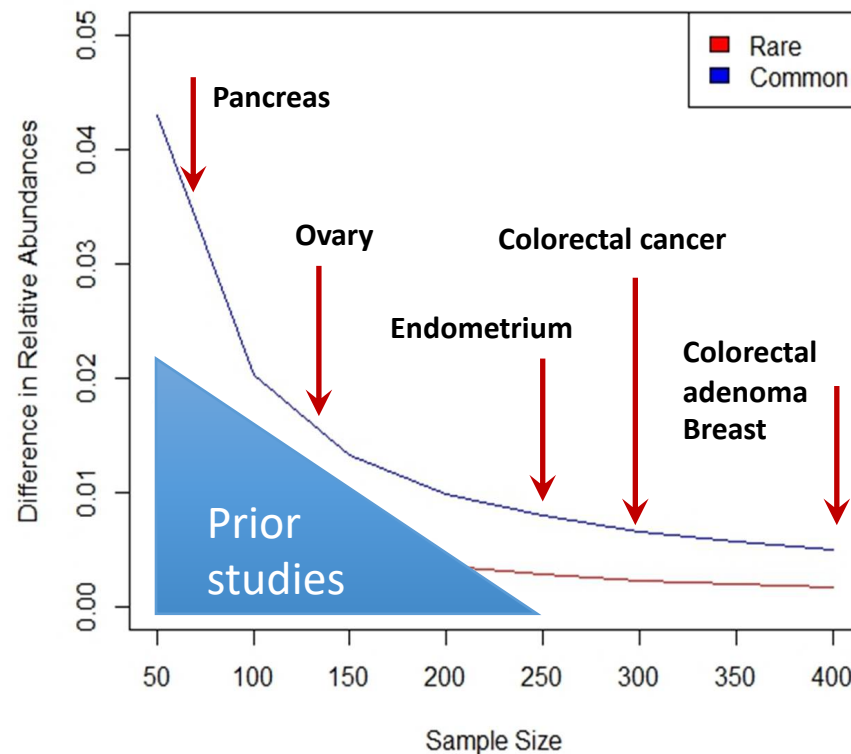
\*Restricted to cases that have a “negative” endoscopy free of adenoma before stool collection.

# Statistical power

After 5 years of follow-up



After 10 years of follow-up





# Significance and impact

- Provide the much-needed **prospective** data on the intricate relationship between the gut microbiome, lifestyle factors, and disease outcomes.
- Provide the scientific evidence and resources for development of gut microbiota-based diagnostics and therapeutics.



**Clinical translation**

# Summary

- Think carefully about the research questions to be addressed
- Select the most suitable & feasible design and biospecimen type
  - Lack of prospective data has been the major barrier to establishing causality.
- Do power calculation
- Collect the essential covariates

Questions?

**Thank you!**