DEMOCRITUS UNIVERSITY OF THRACE
SCHOOL OF HEALTH SCIENCES
DEPARTMENT OF MEDICINE

POSTGRADUATE COURSE
"Food, Nutrition and Microbiome"

MASTER DISSERTATION

Microbiome Analysis in Patients with Colonic Neoplastic and Metastatic Disease

Clinical Study

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Alexandroupolis, June 2025

ΔΗΜΟΚΡΙΤΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΡΑΚΗΣ
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ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ ΣΠΟΥΔΩΝ
«ΤΡΟΦΙΜΑ, ΔΙΑΤΡΟΦΗ ΚΑΙ ΜΙΚΡΟΒΙΩΜΑ»

ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ ΕΙΔΙΚΕΥΣΗΣ

Ανάλυση μικροβιώματος σε ασθενείς με νεοπλασματική νόσο του παχέος εντέρου και με μεταστατική νόσο

Χαραλαμπία- Χρυσούλα Καρούσου-Τσελέντη, ΑΕΜ:

Η παρούσα Μεταπτυχιακή Εργασία Ειδίκευσης υποβλήθηκε στο Τμήμα Ιατρικής του Δημοκρίτειου Πανεπιστημίου Θράκης για την απόκτηση του τίτλου μεταπτυχιακών σπουδών ειδίκευσης στις Επιστήμες της Υγείας

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Αλεξανδρούπολη, Ιούνιος 2025

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Charalambia Karousou Tselenti, Student ID Number:

A thesis submitted in partial fulfilment of the requirements for the degree of Master in health Sciences, Department of Medicine,Democritus University of Thrace

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Alexandroupolis, June 2025

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I extend my heartfelt thanks to all those who stood by me throughout the journey of this thesis.
To Dr. Alexandros Ardavanis, Dr. Gerasimos Ardavanis, and Dr. Maria Giannakou from the General Oncology Hospital of Athens "Agios Savvas", whose guidance and trust made this study possible.
To Dr. Kyriakos Pispirigos and Dr. G. Pantazidis, for their generous support and collaboration at the microbiology laboratory.
Above all, I am deeply grateful to my professors, whose knowledge and encouragement shaped every step of this work, and to my parents, whose love, patience, and unwavering faith carried me through to the very end.

Abstract

The synergy between the gut microbiome and host cells plays a crucial role in maintaining homeostasis. The dysbiotic profile observed in patients with colorectal cancer (CRC) is linked both to the hypothesis of disease pathogenesis and to disease progression and therapeutic response. This highlights the microbiome as a subject of growing research interest.

Understanding the microbial fingerprint in CRC is of critical importance to public health. In this context, the present work was designed as a clinical study involving patients with CRC, in which their gut microbiota was analyzed using high-throughput 16S rRNA sequencing technology. Preliminary results are presented herein.

The differences in dominant genera between patients and healthy controls reveal a clear pattern of dysbiosis: an increased presence of pathogenic species (e.g., *Escherichia coli*, *Parvimonas micra*, *Bacteroides fragilis*) is observed, alongside a reduction in beneficial, anti-inflammatory bacteria such as *Faecalibacterium prausnitzii*, *Anaerobutyricum hallii*, and *Roseburia inulinivorans*.

This observed microbial imbalance, combined with existing literature, supports the hypothesis that microbiome composition may offer valuable insight into carcinogenic risk factors, disease prognosis and progression, and could be further utilized for early screening and as part of combined or adjunctive therapeutic approaches.

Keywords: Colorectal Cancer (CRC); Gut Microbiome; Dysbiosis; Short-Chain Fatty Acids (SCFAs); 16S rRNA Sequencing; Microbial Biomarkers; Microbiota-Host Interactions

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Human microbiome

General information

The field of study of the human microbiome has existed since 1900, and has been a subject of interest to scientists ever since. (1). However, today the term has been refined with multiple clarifications. This is due to the fact that the human microbiome operates within a cooperative and multidimensional framework. It is dynamic in nature—cooperative, because it engages in direct and essential interactions with human cells that are critical for physiological function, and multidimensional, because its activity can influence homeostasis, disease development, and therapeutic outcomes.

The term "human microbiome" is used to denote the set of microorganisms and their genomes that have been identified within the human body (2). In essence, these are the microbial communities that inhabit the cavities of the body, both externally and internally. These communities possess distinctive characteristics that define their ecological niche (3). Additionally, when referring to the human microbiome, we also refer to the functional scope of these microorganisms—namely, their metabolic activity and their interactions with human cells (4).The total number of microorganisms, which can include bacteria, archaea, archaebacteria, viruses (bacteriophages), and fungi, is estimated to be approximately 100 trillion (3). It has been established that the number of genes in question is approximately 200 times greater than the number of human genes, which is estimated to be approximately 22,000 to 23,000 (5). Approximately 3.3 million genes and over 500,000 gene products have been identified in the gut microbiome. According to the study by Qin J. et al., around 40% of these genes appear to be shared among half of the individuals studied. (6). Bacteria constitute the predominant microbial community within the human microbiome (7).

The human microbiome develops a relationship with host cells. Its interaction with the human body—that is, with human cells—can be saprophytic, symbiotic, or dysbiotic in nature. (8).

Researchers began studying the human microbiome as early as the 1900s (1).. Medical microbiologists gradually began to conceptualize this, until then inconceivable, term in the context of their research. As early as 1885, Louis Pasteur observed in experimental models that germ-free mice exhibited significant pathological alterations in immune system development and lacked essential vitamins such as vitamin K and vitamin B12 (9), (10) In the early 1990s, Joshua Lederberg helped systematize the term and referred specifically to the human microbiome (11). He described it as an environment within the human body composed of microorganisms, their genes, and the specific conditions that define this ecological niche (11). Moreover, researchers observed that the disruption of the balance between microbes and host cells could lead to pathological conditions,

primarily infectious diseases, which further intensified scientific interest in the field (12).

Nowadays, microbiome research extends to chronic illnesses, neoplastic diseases, psychiatric disorders, and even genetic conditions.

Findings also suggest that immunotherapy increases the metastatic tumor microbiome and reduces bacterial diversity — a phenomenon observed more prominently in patients who respond positively to treatment (13). Recent studies have shown that certain bacteria are capable of invading cancer cells, and that their peptides can be presented on the HLA molecules of tumor cells.

This suggests that these bacteria may serve as a potential source of immunogenic peptides, thereby acting as substrates for cytotoxic immune responses (14), (15).

Microbial communities in the human body and their living conditions

The human microbiome is present in various anatomical niches, including both external and internal body cavities (12). Externally, the microbiome colonizes sites such as the skin and nasal cavities, while internally it is found in organs including the lungs, small and large intestines, stomach, urinary bladder, and reproductive system (2).

In each bodily cavity, distinct types of microorganisms reside, exhibiting specific characteristics that allow them to survive and co-exist in these environments—such as pH regulation, in which they also actively participate.

Each cavity harbors a unique microbial composition. The number and type of microorganisms present are determined by the local environmental conditions, which promote the growth of certain strains while inhibiting others. One of the primary factors influencing microbial composition is pH. For instance, *Staphylococcus epidermidis* thrives on the skin in pH levels ranging from 5 to 14 (14), B*ifidobacterium* species are typically found in the intestine and the oral cavity, where they thrive within a pH range of 4.5–8.5. *Lactobacilli* are also present in both the gut and the oral cavity, showing optimal growth at a pH range of 5.8–6.0. *Clostridia*, on the other hand, are predominantly located in the intestine and can tolerate a pH range of 7.0–11.0.

Table 1: Tolerable pH ranges for selected microbial taxa

Scientific name	pH	Body part	References
Staphylococcus epidermis	5–14	Skin	Pandey et al. (2015)
Cladosporium spp.	3.5–6.7	Oral Cavity	Gross and Robbins (2000)
Streptococcus pyogenes	6.5	Respiratory Tract	Savic and McShan (2012)
Helicobacter pylori	2.7–7.4	Stomach	Sidebotham et al. (2003)
Lactobacillus bulgaricus	5.8–6	bowel; Stomach	Rault et al. (2009)
Lactococcus lactis subspp. cremoris	6.3–6.9	bowel; Stomach	Rault et al. (2009)
Peptoniphilus stercorisuis spp.	6–9 (7.75)	Urinary Tract	Johnson et al. (2014)

Porphyromonas gingivalis	6.5–7	Uterus	Takahashi and Schachtele (1990)
Bacteroide intermedius	5–7	Uterus; bowel	Takahashi and Schachtele (1990)
Pseudomonas spp.	7	Respiratory Tract	Mishra et al. (2008)
Neisseria gonorrhoeae	6.75	Uterus	Brookes and Sikyta (1967)
Bifidobacterium	4.5–8.5	Έντερο, Στοματική Κοιλότητα	Biavatiet al. (2000)
Streptococcus mutans	6.5–7	Στοματική Κοιλότητα	Handelman and Kreinces (1973)
Clostridium spp.	7–11	Έντερο	Li et al. (1993)
Candida albicans	2–10	Αναπνευστικό, Ουροποιητικό	Sherrington et al. (2017)

Table 1: Tolerable pH range of various microorganisms found in the natural environment and their corresponding anatomical location in the human body

The quantitative and qualitative composition of the human microbiome varies across different anatomical sites of the body. In the gastrointestinal tract, approximately 29% of the total microbiome is located, followed by 26% in the oral cavity, 21% on the skin, 14% in the respiratory system, and 9% in the urogenital tract (2). More specifically, microbial concentration differs across anatomical sites. The highest microbial densities are observed in the ileum, the colon, and the upper respiratory tract

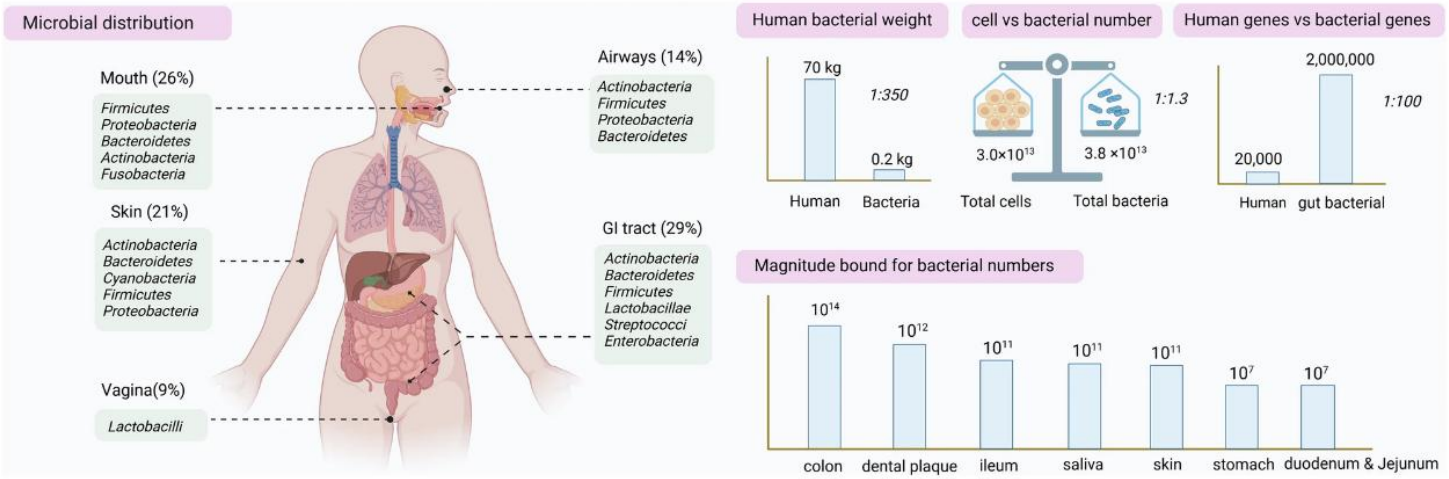

Figure 1: ***Percentages of microbial communities and dominant representation across anatomical sites.***
Source: Ma, Z., Zuo, T., Frey, N. et al. A systematic framework for understanding the microbiome in human health and disease: from basic principles to clinical translation. Signal Transduction and Targeted Therapy 9, 237 (2024). https://doi.org/10.1038/s41392-024-01946-6

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In addition to the well-characterized anatomical sites, microbiota have now been identified in regions such as blood vessels, where Proteobacteria, Firmicutes, and Actinobacteria have been detected as the dominant phyla (16).The eyes also harbor a distinct microbiome. Intraocular samples commonly reveal genera such as *Pseudomonas*, *Bradyrhizobium*, *Propionibacterium*, *Acinetobacter*, and *Corynebacterium*. On the conjunctival surface, predominant genera include *Staphylococci*, *Aquabacterium*, *Sphingomonas*, *Streptococcus*, and *Methylobacterium*. (17), (18), brain (19), gallbladder (20), and the breast (mostly Proteobacteria) (21).

Microbial composition in each body site may be influenced by modifiable factors (22). or example, the composition of the oral microbiome is particularly susceptible to dietary changes, smoking habits, and oral hygiene practices. This vulnerability stems primarily from its direct exposure to food intake and its role as a gateway for microorganisms and metabolites that can influence the broader human microbiome(22).

The following table presents a list of the micro-organisms found in each cavity:.

Human Microbiome by Organ/System\

Organ / Site	Dominant Microbes	Microbial Role / Notes
Blood vessels	Proteobacteria, Firmicutes, Actinobacteria	Dominant phyla in vascular microbiome studies

Eyes	Pseudomonas, Bradyrhizobium, Propionibacterium, Acinetobacter, Corynebacterium	Detected on ocular surface in healthy individuals
Conjunctiva	Staphylococcus, Aquabacterium, Sphingomonas, Streptococcus, Methylobacterium	Common in conjunctival microbiome
Brain	Not well-characterized; evidence of sterile to low-biomass microbiome	Emerging evidence; traditionally considered sterile
Gallbladder	Low biomass; some evidence of microbial DNA, unclear viability	Emerging research; possible microbial DNA presence
Breast	Mainly Proteobacteria	Colonized by skin and environmental microbes
Oral cavity	Firmicutes, Fusobacteria, Proteobacteria, Tenericutes; Streptococcus, Gemella, Lactobacillus, Fusobacterium, Haemophilus, Rothia; with teeth: Fusobacteriota, TM7, SR1, Neisseria	Highly diverse; influenced by hygiene, diet, and tooth eruption
Stomach	Helicobacter, Lactobacillus	Acid-tolerant genera; gastric colonizers
Esophagus	Helicobacter	Transitional microbiota; few species adapted to acidic conditions
Duodenum	Prevotella (carbohydrate breakdown and amino acid synthesis)	Involved in digestion and nutrient processing
Ileum	Enterococcus, Bacteroides (amino acid synthesis, bile acid metabolism)	Associated with bile acid metabolism and immune interaction
Right colon	Klebsiella, Enterococcus, Lactobacillus (SCFA production)	Fermentation and SCFA production
Left colon	Parabacteroides, Bifidobacterium, Dorea	Anaerobic fermentation; fiber metabolism
Urethra / Bladder	Lactobacillus, Staphylococcus, Gardnerella, Corynebacterium, Streptococcus	Involved in urinary tract health and dysbiosis
Skin	Staphylococcus epidermidis, Corynebacterium, Pseudomonas, Enterobacter,	Metabolically active; lipid and energy metabolism

	Enterococcus, Proteus, Klebsiella, Propionibacterium acnes, Malassezia globosa, M. restricta	
Vagina	Lactobacillus (L. crispatus, L. iners, L. jensenii, L. gasseri), Gardnerella, Prevotella, Atopobium, Sneathia, Megasphaera, Peptoniphilus	Dominant in reproductive age; varies with hormones and life stages
Nasal cavity / Nasopharynx	Moraxella, Staphylococcus, Corynebacterium, Haemophilus, Streptococcus, Propionibacteria, Gammaproteobacteria	Important in respiratory defense; dynamic with environment
Bronchi	Prevotella, Veillonella, Streptococcus, Fusobacterium, Dolosigranulum, Haemophilus	Present in healthy lower airways; anaerobic and facultative taxa
Oropharynx	Prevotella, Veillonella, Streptococcus, Leptotrichia, Rothia, Neisseria, Haemophilus	Reflects both oral and airway microbiota

Figure 2: he microorganisms in the internal and external cavities of the body

Microbiome and Cells: A Unified Human Entity Rather Than Independent Systems

The totality of human cells and the microbiome-associated microorganisms living within the human body form a collaborative supersystem known as a 'superorganism' or 'meta-organism (6). This new conceptualization of the human entity also encompasses genes; therefore, the collective genome is referred to as the 'hologenome (22). Every component of this 'meta-organism' is essential for sustaining physiological homeostasis. It is particularly noteworthy that microbial cells significantly outnumber human cells within this integrated biological system (21).

Developmental Programming of the Human Microbiome and Epigenetics

The understanding of the human microbiome and its functional implications has been significantly enhanced through the development of molecular techniques capable of investigating its role in both homeostasis and disease.
Next-Generation Sequencing (NGS), multi-omics approaches, and artificial intelligence have emerged as powerful and transformative tools in this domain. Major international initiatives have been established to characterize the microbiome's genomic landscape, such as the **Human Microbiome Project (HMP)** (23) and the **Integrative Human Microbiome Project (iHMP)** (24), the European MetaHIT project (Metagenomics of the Human Intestinal Tract) (25), the American Gut Project

(AGP) (26),the Dutch Microbiome Project (27)(DMP) These tools can provide significant associations and new perspectives for the modulation and clinical exploitation of the human microbiome.

The Human Microbiome Project (HMP) established three foundational principles that researchers in the field now consider: (1) microbial communities vary across different body sites within the same individual and also between individuals; (2) the human microbiome undergoes dynamic changes over time; and (3) alterations or imbalances in the microbiome have been associated with various diseases. (28). Moreover, it is of particular significance that the human genome and the microbiome genome can interact at the epigenetic level. The epigenetic influence of the microbiome has already demonstrated implications in resistance to immunotherapy, as well as in the development of neoplastic disease. When such interactions occur early in life, they may have profound effects on homeostasis and disease susceptibility. Studies have identified pre-treatment bacterial populations associated with a lack of response to immune checkpoint blockade (ICB), likely due to the ability of certain microbes to shape an immunosuppressive microenvironment (29). The mechanisms of action at the epigenetic level include histone modifications (such as methylation and acetylation), non-coding RNAs (ncRNAs)—including microRNAs (miRNAs), circular RNAs (circRNAs), and long non-coding RNAs (lncRNAs)—as well as chromatin remodeling.

Bacterial-derived metabolites can act as substrates or cofactors for DNA methylation, which typically occurs at CpG islands, thereby preventing RNA polymerase complexes from initiating transcription. Moreover, butyric acid can inhibit histone deacetylases (HDACs), allowing for the acetylation of histone H3. Other microbial products can influence additional histone marks, such as H3K4me1 and H3K27ac (30). Histone modifications resulting from dysbiosis have been implicated in the pathogenesis of disease, such as inflammatory bowel diseases (IBD). (2).

Non-coding RNAs exert regulatory effects at the post-transcriptional level, thereby modulating the production of gene products indirectly (2). The microbiome can influence the activity of miR-181 (a microRNA), leading to increased insulin sensitivity, and may also affect the expression of lncRNAs that broadly participate in gene regulation (31).

Gut microbiome

Among all microbial communities comprising the human microbiome, the gut microbiota is considered the largest and most functionally important (12), (32). It comprises over 3 million genes—compared to approximately 23,000 human genes—5,000 different species, and a total of 100 trillion microbes (33), (28).

It is referred to as the gastrointestinal microbiome (gut microbiome or GM). Below are the main species and genera identified in the gut.

Table 3: The data have been obtained from the literature and the Human Microbiome Project (HMP) (34), (35), (36), (37)

Phylum	Representative Genera/Species
Firmicutes	Lactobacillus, Ruminococcus, Clostridium, Eubacterium, Faecalibacterium, Roseburia, Streptococcus
Bacteroidetes	Bacteroides, Prevotella, Xylanibacter
Proteobacteria	Escherichia, Desulfovibrio
Actinobacteria	Collinsella, Bifidobacterium
Euryarchaeota	Methanobrevibacter

Verrucomicrobia	Akkermansia

also identified

Species / Genus	Higher Taxonomy	Notes
Methanobrevibacter smithii	Archaea > Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae	
Clostridium	Bacteria > Firmicutes > Clostridia > Clostridiales > Clostridiaceae	Some species reclassified (e.g., Clostridioides)
Ruminococcus	Bacteria > Firmicutes > Clostridia > Oscillospirales > Ruminococcaceae	
Ruminococcus gnavus (Mediterraneibacter gnavus)	Bacteria > Firmicutes > Clostridia > Lachnospirales > Lachnospiraceae	Reclassified as Mediterraneibacter gnavus
Haemophilus	Bacteria > Proteobacteria > Gammaproteobacteria > Pasteurellales > Pasteurellaceae	
Haemophilus parainfluenzae	Bacteria > Proteobacteria > Gammaproteobacteria > Pasteurellales > Pasteurellaceae	
Coprococcus	Bacteria > Firmicutes > Clostridia > Lachnospirales > Lachnospiraceae	
Dialister	Bacteria > Firmicutes > Negativicutes > Selenomonadales > Veillonellaceae	
Veillonella	Bacteria > Firmicutes > Negativicutes > Selenomonadales > Veillonellaceae	
Collinsella aerofaciens	Bacteria > Actinobacteria > Coriobacteriia > Coriobacteriales > Coriobacteriaceae	
Eubacterium hallii (Anaerobutyricum hallii)	Bacteria > Firmicutes > Clostridia > Lachnospirales > Lachnospiraceae	Reclassified as Anaerobutyricum hallii
Enterobacteriaceae	Bacteria > Proteobacteria > Gammaproteobacteria > Enterobacterales	Family level
Oxalobacter formigenes	Bacteria > Proteobacteria > Betaproteobacteria > Burkholderiales > Oxalobacteraceae	

Streptococcus mitis	Bacteria > Firmicutes > Bacilli > Lactobacillales > Streptococcaceae	
Faecalibacterium cf	Bacteria > Firmicutes > Clostridia > Oscillospirales > Ruminococcaceae	Likely Faecalibacterium prausnitzii
Faecalibacterium prausnitzii	Bacteria > Firmicutes > Clostridia > Oscillospirales > Ruminococcaceae	
Lachnobacterium	Bacteria > Firmicutes > Clostridia > Lachnospirales > Lachnospiraceae	
Parabacteroides merdae	Bacteria > Bacteroidota > Bacteroidia > Bacteroidales > Tannerellaceae	
Desulfovibrionaceae	Bacteria > Proteobacteria > Deltaproteobacteria > Desulfovibrionales	Family level
Escherichia coli	Bacteria > Proteobacteria > Gammaproteobacteria > Enterobacterales > Enterobacteriaceae	

Table 3 : Bacteria in the Colon :Acidaminococcus, Faecalibacterium, Veillonella, Pseudomonas, Bacteroides, Bifidobacterium, Coprococcus, Staphylococcus, Enterobacter, Escherichia, Eubacterium, Fusobacterium, Klebsiella, Lactobacillus, Megamonas, Salmonella, Megasphaera, Peptostreptococcus, Enterococcus, Peptococcus, Proteus, Ruminococcus, Clostridium.Bacteria in the Intestinal Lumen: Bacteroides, Bifidobacterium, Streptococcus, Enterococcus, Clostridium, Lactobacillus, Ruminococcus. Mucosal Layer of the Small Intestine: Enterococcus, Clostridium, Lactobacillus. General Bacteria in the Digestive System : Veillonella, Escherichia, Bacteroides, Clostridium, Lactobacillus, Streptococcus. Dominant and Specific Microbial Species: Escherichia coli, Bacteroidetes, and Firmicutes usually dominate in the digestive system

Synergy Between the Human Genome and the Human Microbiome: Evolution and Homeostatic Reprogramming

The synergy between microbes and human cells is crucial for the proper functioning of the human body. This is not merely a balance of functions within a shared environment, but rather a dynamic interaction and harmony between their respective activities—many of which are interdependent.

In 1992, Bocci referred to the microbiome as an **organ**, highlighting its essential and integrated role within the host organism (38). This theory opens new horizons in both research and clinical practice—especially as we recognize that the microbiome lacks classical organ characteristics, yet there is an urgent need to understand how bacteria residing within the host, as well as within tumors, can reshape tumor biology, immune system dynamics, metabolism, and therapeutic response.

Humans are not composed solely of, nor do they rely exclusively on, human cells. Instead, their function is fundamentally influenced by the activity of the microbiome. The synergy between microbial elements and human cells, which emerges from cohabitation, predates our current biology—evident in mitochondria and chloroplasts, once independent organisms, now organelles essential for cellular survival.

By deduction, the human microbiome plays a pivotal role in maintaining homeostasis

and, consequently, in sustaining life. The entirety of human cells represents only the apex of a biological framework that supports both human and microbial existence (39).

Nevertheless, the interdependence that has been identified—likely influenced by environmental conditions, particularly given the microbiome's capacity for dynamic adaptation—has introduced new terms and prerequisites for life and evolution.

The environment itself hosts organisms with their own microbiomes, co-existing in a shared biological space. The spatial-temporal selection of specific microbes and host cells constitutes a fundamental step in natural selection—one that first facilitates adaptation and subsequently enables synergy. This step concerns both microbes and host cells alike.

Synergy in life gives rise to a harmonious coexistence between human cells and the microbiome. Their trajectory follows a proportional continuum.

The diversity and functional influence of the microbiome on homeostasis evolves over time. This influence is strongest during early life and adulthood, whereas it diminishes with aging—a process in which environmental factors play a pivotal role. Thus, despite differing origins, the microbiome is affected by and behaves in a manner analogous to that of human cells. ***Figure 2:****The concept of homeostatic reprogramming, as supported by evidence from various studies, also pertains to the microbiome.* (40), (41).

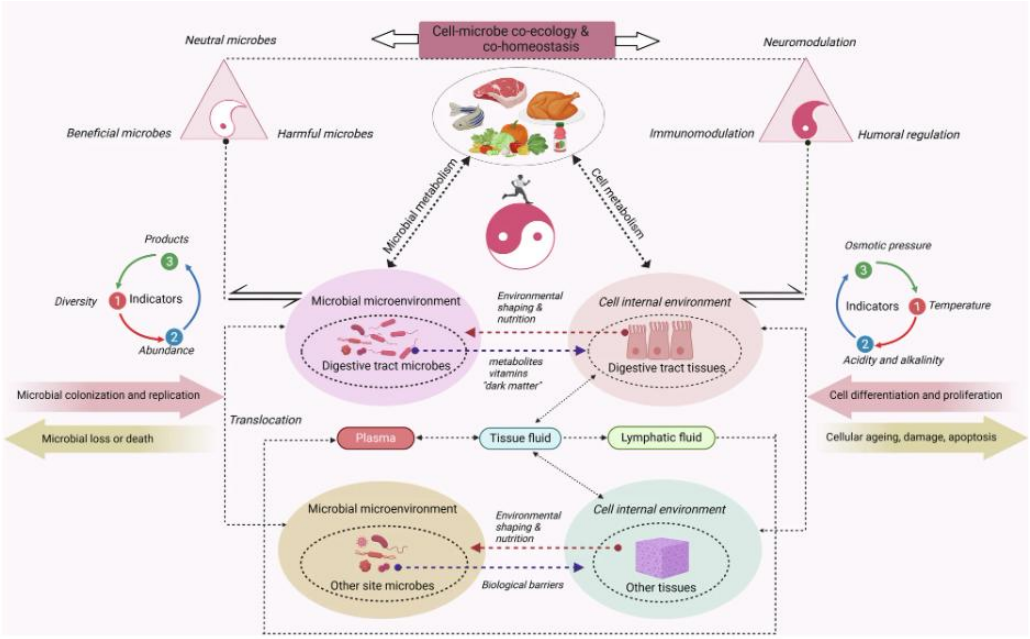

Figure 3 A comprehensive overview of the presence and role of the human microbiome in maintaining homeostasis and supporting physiological functions of the human body

The interaction between the genomes of human cells and microbes, under the influence of environmental factors, shapes the framework through which homeostatic regulation and reprogramming occur. Within the human body, the organ-specific synergy between microbiota and host cells is orchestrated to maintain physicochemical properties and compositional stability—such as pH, osmotic pressure, metabolic and signaling pathways, metabolism, immune function, brain activity, and inflammatory responses.

"Diversity, relative abundance, and the functional products of beneficial, harmful, and neutral microorganisms (i.e., the microbiome composition) serve as critical indicators for assessing environmental balance. Microorganisms also contribute to the formation of the microenvironment by creating a barrier that modulates responses to

external environmental changes. This overall modulation governs the sensitivity of the internal milieu to external disturbances, acting as an additional regulatory force for maintaining homeostasis. Conversely, internal imbalance directly disrupts the microenvironment they construct."- Ma, Z., Zuo, T., Frey, N. et al. A systematic framework for understanding the microbiome in human health and disease: from basic principles to clinical translation. Sig Transduct Target Ther 9, 237 (2024)-. Essentially, the microbiome and the host cell genome coexist, offering the human organism an evolutionary advantage in the context of natural selection.
Loss of interaction between these components reflects the onset of pathological conditions stemming from disrupted homeostasis and may represent a "second wave" of disease manifestation in genetically predisposed individuals.Studying the intrinsic relationship between the microbial genome and human cells may provide both mechanistic insights and practical tools for understanding diseases and protecting human health. This protection applies both individually and collectively—whether in the context of chronic diseases such as cancer, infectious epidemics, or anthropogenic challenges such as antimicrobial resistance (42).

Development, Early Establishment of the Human Microbiome, and Aging

The presence of the human microbiome is critical even during fetal life, influencing the regulation of endocrine, neural, gonadal, and immune development of the embryo (2). The massive transfer of the microbiome occurs during the passage of the fetus through the vaginal canal in a normal delivery. Additional exposure to the skin, breast milk, and maternal feces further reinforces the initial microbial transfer that takes place at birth (43). Moreover, although the mode of delivery may vary, there is an independent pattern of colonization by certain microbes—such as *Bifidobacterium*—which are critical for both the development of the host organism and the maturation of the microbiome (44).
The microbiome can be detected as early as the fourth day of life, with species such as *Staphylococcus* and γ-Proteobacteria already identified (45), while there is evidence of microbiome transfer from mother to newborn (12). Studies in mice have shown that short-chain fatty acids (SCFAs) can be transferred from the mother to the fetus and contribute to the development of the metabolic program, providing an advantage and enhancing resilience to metabolic syndromes in adulthoodα (46). Studies in children have identified a correlation between the maternal microbiome and the child's neurodevelopment during the first year after birth (47).

The transfer of microorganisms from the mother begins already during pregnancy, as indicated by the presence of microbes in the placenta, umbilical cord, and meconium (48), (49).
Indeed, the microbiome, even from fetal life, can modulate fetal T-cell inflammatory ability (50).
The development and activity of microbes are crucial for the maturation of the immune system and the proper immune response. Moreover, dysbiosis may be responsible for immune-related disorders, such as autoimmunity. Imbalances and alterations in the gut microbiota as early as 120 days after birth can serve as biomarkers for the prevention of pediatric diseases such as childhood asthma and airway inflammation. For example, one study showed that elevated levels of 12,13-DiHOME, produced by bacterial hydrolases during dysbiosis, can exacerbate airway allergic responses (51), (50).

The hypothesis of microbiome inheritance from mother to fetus is not universally accepted. The transmission of the maternal microbiome occurs primarily

through contact during and after birth, as well as via microbial-derived substances that reach the fetus; however, these do not constitute true genetic inheritance. Importantly, it should be considered that the maternal microbiota undergoes significant changes, particularly during the third trimester of pregnancy (52).

Developmental Timeline of Key Microbial Genera in the Human Neonatal and Infant Microbiome

Timepoint / Factor	Dominant Microbial Genera	Body Site(s)	Notes / Transitions
At birth	Bifidobacterium, Granulicatella, Veillonella, Lactobacillus	Gut / Skin	Initial colonization; strongly influenced by delivery mode
Day 4 of life	Staphylococcus, γ-Proteobacteria (Escherichia, Klebsiella, Enterobacter)	Skin / Gut	Early environmental colonizers (hospital/skin flora)
1–6 weeks	Bifidobacterium, Staphylococcus, Clostridium, Bacteroides	Gut	Transition to a mixed community; promoted by breastfeeding
By end of 1st year	Staphylococcus, Streptococcus, Prevotella, Porphyromonas, Veillonella, Ureaplasma	Skin / Respiratory tract / Nasal cavity	Increasing diversity; emergence of anaerobic taxa
After 1st year	↑ Firmicutes (Clostridia, Faecalibacterium, Ruminococcus, Veillonella); ↓ Bifidobacterium	Gut	Maturation and expansion of obligate anaerobes
Around age 3	Firmicutes, Bacteroidetes (dominant)	Gut	Microbiota reaches adult-like stability
Vaginal delivery	Lactobacillus, Prevotella, Sneathia	General	Reflects maternal vaginal microbiota
Cesarean section	Staphylococcus, Corynebacterium, Propionibacterium	General	Reflects maternal skin and hospital flora

Table 4: Table illustrating the microbial genera that constitute the human microbiome during the early stages of life. Although components of the maternal microbiome may influence the fetus in utero, the major microbial transfer begins at birth. The mode of delivery plays a crucial role in initial colonization: vaginal delivery is associated with genera such as Lactobacillus, Prevotella, and Sneathia, while cesarean section favors colonization by Staphylococcus, Corynebacterium, and Propionibacterium. Within the first days of life, γ-proteobacteria and Staphylococcus predominate, followed by the gradual emergence of a more complex and anaerobic community including Bifidobacterium, Clostridium, Bacteroides, and Veillonella. By the age of three, the gut microbiota reaches relative stability, dominated by the phyla Firmicutes and Bacteroidetes, resembling the composition of the adult microbiome. (2), (53), (54)

Moreover, the human microbiome changes throughout the lifespan. During puberty, significant shifts occur, while in older age, both the quantitative and qualitative composition of the microbiota is reduced. This decline has been associated with life expectancy. Specifically, the Firmicutes/Bacteroidetes ratio is lower in the elderly compared to younger adults (55). Quantitative and qualitative changes in the human microbiome due to aging alter its capacity to regulate homeostasis. As time progresses, microbial diversity shifts toward dysbiosis—for example, a decrease in *Faecalibacterium prausnitzii* and an increase in *Eubacterium limosum*. Additionally, the aging microbiome becomes more susceptible to colonization (56).

The rate at which this occurs depends on the extent to which microbiome-modifying factors have affected the organism (e.g., extensive exposure to antibiotics). This weakened influence of the microbiome leads to immunosenescence (a decline in immune competence) and increased inflammation—factors that can accelerate aging-related events on predisposed ground, such as neurodegenerative diseases. Aging of the gut–brain, gut–muscle, and gut–immune axes may act as a “trigger point” for the onset of these diseases. Therefore, managing and “reprogramming” the microbiome are considered scientifically achievable goals and important interventions for the geriatric population.

Factors Influencing the Human Microbiome

The human microbiome can serve both as a biomarker and as a pathological factor in disease development. A notable example is the observation that microbiota transplantation from a diseased human donor into mice can induce the pathological phenotype, as seen in cases of obesity. (57). Furthermore, it has been observed that germ-free mice develop pathological conditions that are attributed to the absence of a functional microbiome (2).

The essential synergy between the microbiome and human cells is crucial for maintaining homeostasis, yet it is also highly sensitive and individualized. It is individualized in that it is entirely unique for each meta-organism, and sensitive because it is easily influenced by variable conditions—although it maintains a degree of stability and resilience. The most sensitive component is the gut microbiota.

In every meta-organism, the microbiome maintains a relatively stable dynamic equilibrium, and certain compositional ratios are consistently observed among individuals. Nevertheless, the quantitative and qualitative composition of the microbiome can be influenced by both exogenous and endogenous factors throughout life (from the fetal stage to old age—as discussed in the section ‘Development, Early Development of the Human Microbiome, and Aging’).

These include modifiable and non-modifiable factors. Non-modifiable factors comprise sex, age, behavior, disease status (particularly in chronic conditions), and ancestry (including genetic mutations). Modifiable factors include diet; antibiotics (as well as other medications, such as antidepressants, which alter microbiome composition and promote the presence of resistant pathogens); lifestyle habits affecting microbial niches (such as the use of toothpaste or deodorant); and environmental exposure (58), (59).

The environment includes factors such as geographic isolation and urbanization, both of which influence the genetic diversity and composition of the microbiome. Diet pertains to the dietary patterns of the community to which an individual belongs, shaped by cultural norms and personal preferences, as well as by the use of dietary supplements and the intake of food preservatives. Furthermore, dietary influence

involves the quantity and type of glucose, fats, dietary fiber, carbohydrates, polyphenols, and meat consumed. (2). On the other hand, microbiome-derived metabolites—primarily those produced by the gut microbiota—shape the function of human cells both locally and at distant sites (60), (12).
The human microbiome exerts both localized effects within the host organism and collective influences at the community level. The microbial composition of a population can promote local immune tolerance and modulate immune responses based on population-specific conditions. For example, variations may arise due to living environments, dietary patterns, and the presence of nomadic populations. (12).
The scientific observation regarding the manifestation of autoimmune diseases within a population is of great importance: individuals who migrate from countries with low prevalence rates to countries with high prevalence rates of autoimmune diseases—such as allergic asthma and type 2 diabetes mellitus—tend to exhibit a corresponding increase in disease prevalence, provided they migrate before a certain age.- (2). For example, in the case of asthma, this critical age is up to 5 years.
Any condition that can disrupt the balance among microbial communities—or their interactions—may serve as an indicator or even a cause of disease.

Homeostasis and Functions of the Human Microbiome

The synergy between human cells and the microbiome involves interactions mediated by microbiota-derived metabolites, proteins, and bioactive compounds such as vitamins. The microbiome produces proteins, enzymes, neurotransmitters, and other essential molecules—many of which cannot be synthesized by human cells.
Examples include compounds that reduce nitrates to nitrites (through nitric oxide metabolism by bacterial strains such as *Veillonella* and *Actinomyces*) and vitamin biosynthesis. Certain bacterial species synthesize vitamin K and B-complex vitamins—molecules involved in critical processes such as blood coagulation (e.g., prothrombin production). Other microbiota-derived substances include short-chain fatty acids (SCFAs), branched-chain amino acids (BCAAs), secondary bile acids (BAs), polyamines, lipids, and a still poorly understood group of compounds referred to as "microbial dark matter." These metabolites are not only important for digestion but also essential for tissue integrity, immune system function, and nervous system activity. Moreover, they participate in key cellular signaling pathways.

The importance of the human microbiome for host cells extends to maintaining homeostasis, supporting normal physiological development from the embryonic stage, and enabling the organism to adapt to environmental conditions. The microbiome contributes to both fundamental biological functions and complex inter-system cooperation. For instance, thermoregulation is influenced by the microbiota; alterations in microbial composition—such as those induced by antibiotic treatment—can impair the body’s thermogenic capacity and specifically affect gut temperature, particularly under conditions like sepsis (61).
The gut microbiome may contribute, in coordination with the liver, to the regulation of circadian rhythms—a phenomenon linked to its metabolic and physiological functions (62). The gut microbiome displays circadian rhythms in its microbial composition—characterized by minor diurnal shifts in dominant bacterial species—as well as in its metabolic activity, both of which are influenced by feeding time (63). Additionally, genetic mutations in circadian rhythm regulatory pathways may result in shifts in the bacterial diversity of the gut microbiome (63). Accordingly, the interplay between gut

microbiome metabolites—particularly short-chain fatty acids (SCFAs)—and circadian rhythm regulation should be carefully considered.

Metabolic and Regulatory Substances

Short-chain fatty acids (SCFAs): actions on the digestive and immune systems

Short-chain fatty acids (SCFAs)—namely acetate, propionate, and butyrate—are produced by specific gut microbiota and act on various cell types, regulating biological processes including host metabolism, intestinal function, and immune system activity (64).

Acetate contributes to cholesterol metabolism and fatty acid synthesis, whereas propionate counteracts cholesterol processing, supports gluconeogenesis, and plays a role in the regulation of satiety (65), (66), (67). Butyrate plays a role in modulating satiety, appetite, and the secretion of gut hormones, and additionally functions as a primary energy substrate for the colon (2).

SCFAs, are produced through fermentation by anaerobic bacteria and are capable of diffusing into all cell types, including across the blood–brain barrier (68). SCFAs influence both innate and adaptive immunity, and there is a strong correlation with autoimmunity.

In autoimmune conditions, homeostatic reprogramming and microbiome imbalance are often observed. Autoimmune diseases are characterized by the recognition of specific antigens and are, in part, triggered by insufficient immunological tolerance mechanisms that fail to eliminate self-reactive B and T cell receptors (such as TCRs and BCRs).

SCFAs further interact with the immune system by competing with pathogenic microorganisms in the gut, including *Escherichia coli*, *Clostridium difficile*, and *Salmonella* (69), (70), (71). SCFAs also contribute to immune regulation through the differentiation and activation of T cells, the maturation of macrophages (with butyrate guiding their differentiation by inhibiting histone deacetylase 3 [HDAC3]), and the activation of monocytes (72), (73), (74), (140), as well as the activation of basophils, through the induction of CD69 expression on the cell surface by interleukin-3 (IL-3) (75) (76) (77), and degranulation. SCFAs also recruit neutrophils to sites of inflammation by increasing L-selectin expression on the neutrophil surface, which promotes their chemotactic recruitment to the inflamed tissue (78). Moreover, SCFAs enhance the cytotoxic activity of natural killer (NK) cells, contributing to innate immune defense mechanisms (79), they regulate B cells and contribute to the generation of regulatory B cells. (80) and they regulate B cells, contribute to the generation of regulatory B cells, and promote B cell differentiation and antibody production through histone deacetylase (HDAC) inhibition and G-protein-coupled receptor (GPR) signaling (81).

In addition, SCFAs are involved in signaling pathways that indirectly influence the function of immune-related molecules and cells, at times exerting pro-inflammatory effects depending on the context (68). SCFAs are thus involved in apoptotic pathways in neutrophils, limit eosinophilic infiltration (e.g., in the lungs), can promote the production of pro-inflammatory mediators, and inhibit cytokine release.

Their immunomodulatory activity includes the modulation of receptor signaling, such as the suppression of Th2 cytokine production. SCFAs may act through G-protein-coupled receptors (GPCRs), such as GPR41 and GPR43, or via intracellular pathways including mTOR signaling, particularly through mTORC1, (82), by activating pathways such as the mitogen-activated protein kinase (MAPK) cascade,

through the stimulation of the GPR43 receptor (83) , leading to the production of TNF-α, TNF-β, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-12, IL-17A, IL-18, as well as the chemokines CXCL1 and CXCL2 (108), (84) and IL-10 by macrophages—processes that can attenuate the inflammatory response and suppress autoimmunity (85). ecent evidence has shown that a soluble microbiome-derived metabolite, trimethylamine N-oxide (TMAO), can drive macrophage polarization in mice in an NLRP3 inflammasome–dependent manner (86).

Digestive System, Metabolism, and Secondary Bile Acids

The human microbiome, primarily through the gut microbiome, contributes to and regulates the metabolism of nutrients as well as the normal function of the digestive system.

The cellular architecture of the gastrointestinal tract displays a distinct structural organization that supports microbial metabolism, colonization, and function.

The apical and basal surfaces of intestinal epithelial cells, through the diffusion barrier, enable the transport of soluble substances absorbed from the lumen into the underlying tissues and systemic circulation (92) . The intestinal epithelium is renewed approximately every 4 to 7 days (87). The intestinal epithelium originates from stem cells located in the crypt region. The newly generated cells undergo differentiation as they migrate from the crypts toward the tips of the adjacent villi, where they ultimately shed into the intestinal lumen (87).

The architecture of the gastrointestinal tract, both morphologically and functionally, is shaped by the presence and activity of the gut microbiota (2). In cases of quantitative and qualitative alterations, intestinal villi may exhibit structural abnormalities and functional impairments, the regenerative rate of crypt-derived cells is reduced, and cecal enlargement is observed (as seen in germ-free mice) (88), (2). Moreover, the intestine itself supports bacterial survival through its contractile and propulsive movements, while intestinal atrophy may lead to pathological alterations of the tissue (77). Additionally, further interactions may exert synergistic or antagonistic effects with pharmaceutical compounds, due to the influence of the gut microbiota on drug metabolism (47).

The metabolism of food by the gut microbiome constitutes a dynamic triangle: the microbiota present in food—particularly in fermented products—interacts with the gut microbiome (both in the small and large intestine), while the human microbiome contributes to overall metabolism and nutrient absorption.

There is, therefore, a bidirectional relationship between the food microbiome and the host, as well as between the microbiome and host cells. What varies is the role each component plays.

The microbiota and nutrients found in fermented foods can induce shifts in the composition of the gut microbiome (through colonization and population shifts caused by both microorganisms and food substrates, such as dairy products—with kefir being prominent—sauerkraut, and Cha-Koji tea), while specific dietary compounds can promote microbiome growth—for example, polyphenols and flavonoids (89). These properties of certain foods grant them a potential “Trojan horse” role, allowing them to serve as effective vehicles for the safe delivery of probiotic strains to the intestine (89).

The gut microbiome also plays a significant role in nutrient absorption and metabolism. Through saccharolytic bacterial fermentation, gut microbes can produce various compounds—such as short-chain fatty acids (SCFAs) and gases—

metabolize intermediate fermentation products (e.g., polyphenols converted into aglycones), exhibit proteolytic activity, and degrade polymers.
In the case of polyphenols, microbial metabolism may involve dehydroxylation, decarboxylation, and ring cleavage, ultimately generating simpler phenolic compounds, such as hydroxyphenylacetic and hydroxyphenylpropionic acids.
In addition, the gut microbiota synthesizes vitamins and other bioactive molecules, and plays a central role in bile acid metabolism.
Gases are also produced as both primary products and byproducts—for example, hydrogen by *Bacteroides* and *Clostridium*, methane via the methanogenesis reaction ($4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$), and carbon dioxide as a metabolic end product (90), (91), (92). Proteolytic activity—primarily attributed to *Bacteroides* and *Propionibacterium* as the dominant proteolytic species—can lead to the production of peptides, short-chain fatty acids (SCFAs), and amino acids (93), (92).

The vitamins synthesized by the gut microbiome include B-complex vitamins and vitamin K. More specifically, the microbiota contributes to the production of:vitamin B1 (thiamine),vitamin B2 (riboflavin),vitamin B3 (niacin),vitamin B5 (pantothenic acid),
vitamin B6 (pyridoxine),vitamin B7 (biotin),vitamin B9 (folic acid), and
vitamin B12 (cobalamin) (39). The dual action of vitamins benefits both microorganisms and the human host.
Metabolites produced by the gut microbiota participate in metabolic processes and in the complex biosynthesis of bile acids in the liver. These microbial metabolites can regulate the activity of key enzymes such as CYP7A1, CYP7B1, and CYP27A1 (94), They can participate in the conversion of primary to secondary bile acids, glucose homeostasis (including absorption and gluconeogenesis), insulin secretion, and inflammation—primarily through the regulation of FXR and TGR5 receptors.
Quantitative and qualitative imbalances in bacterial populations have been associated with irritable bowel syndrome (IBS), colorectal neoplastic disease, neuroinflammation, and non-alcoholic fatty liver disease (NAFLD) (2).
Secondary bile acids (DCA, LCA, UDCA), which are microbiota-derived metabolites, have been associated with the development of colorectal neoplastic disease through multiple mechanisms:
induction of oxidative DNA damage (notably by DCA), inhibition of apoptotic programs, and activation of signaling pathways such as EGFR, ERK, and COX-2, which promote cancer cell proliferation and survival.
Additionally, these bile acids may suppress $CD8^+$ T cell function, thereby reducing immune surveillance against tumors (95), (96). More specifically, deoxycholic acid (DCA) can alter gene expression by interfering with regulatory proteins—for example, through the activation of the Nur77 protein, which is positively associated with colorectal cancer (CRC) when overexpressed (97), through the downregulation of miR-199a-5p, which normally degrades CAC1—a tumor suppressor gene implicated in colorectal cancer (CRC) (98). Both lithocholic acid (LCA) and deoxycholic acid (DCA) have been shown to enhance cancer stemness (99). Furthermore, both lithocholic acid (LCA) and deoxycholic acid (DCA) activate the EGFR signaling pathway, inducing DNA damage and triggering oxidative stress, apoptosis, mutations, and activation of the protein kinase C (PKC) pathway (99).
Ursodeoxycholic acid (UDCA) may also exert a protective effect (100).

Bile Acid	Action	Effect on Intestinal Epithelium
DCA (Deoxycholic acid)	Induces oxidative stress; activates oncogenic pathways	Increases cancer risk
LCA (Lithocholic acid)	Promotes inflammation and cytotoxicity	Causes epithelial damage
UDCA (Ursodeoxycholic acid)	Exhibits anti-inflammatory and protective effects	Reduces cancer risk

Table 5: Effects of Secondary Bile Acids on the Intestinal Epithelium

Metabolites derived from the gut microbiome can be absorbed through the intestinal epithelium and enter both endogenous and exogenous pathways of the host, thereby influencing the host's overall metabolic phenotype.

The Axes of the Human Microbiome

Gut- Brain Axis

The idea that the microbiome could influence the brain was almost unthinkable just a few years ago. It wasn't until 2006 that neuroscientist Jane Foster made a discovery that, in her view, pointed toward the emergence of an entirely new field of research.

Although her initial findings were rejected twice, her study was eventually published three years later, marking the beginning of a shift in our understanding (101).

Another neuroscientist, John Cryan from University College Cork in Ireland, entered this field of research around the same time as Foster (102).

Nowadays, the gut–brain axis is considered a fundamental component of neuroscience.The links formed between the microbiome and host cells constitute the chain upon which this bidirectional interaction is built. Each link defines the function of a specific axis—such as the microbiome–immune axis, the metabolic axis, or the gut–liver axis.

Perhaps the most important of these is the gut–brain axis, as microbial activity can influence brain function through the production of neurotransmitters.

Conversely, the central nervous system can affect gut physiology via cortisol and the hypothalamic–pituitary–adrenal (HPA) axis (103).

The interaction within this axis spans the entire human lifespan—from the embryonic stage to adulthood.

The gut–brain axis is a complex, bidirectional communication system linking the brain and the gut via neurotransmitters such as serotonin, norepinephrine, dopamine, melatonin, histamine, gamma-aminobutyric acid (GABA), acetylcholine, BDNF, and GDNF.

These molecules are utilized by both the enteric nervous system and the central nervous system.Certain gut bacteria are capable of producing neurotransmitters and have notable effects on the dopaminergic system and tryptophan metabolism (103).

For example, gamma-aminobutyric acid (GABA) is produced by *Lactobacillus* spp. and *Bifidobacterium* spp.; acetylcholine is produced by *Lactobacillus* spp.; and dopamine is synthesized by *Bacillus* spp. (103).

The microbiome can modulate the brain through three principal routes—the immune pathway, the neuronal pathway, and the endocrine/systemic pathway—while extensive bidirectional crosstalk exists among all thre (104), (42).

Numerous publications support the notion that the trillions of bacteria residing in the gut could have profound effects on brain disorders, including autism.
A disrupted microbiome negatively affects the host's microbial biodiversity, transmitting deficient proteins and pathogenic agents along the vagus nerve. However, the underlying cause of microbiome disruption prior to the onset of neurological conditions remains unclear, as dysbiosis and gastrointestinal disturbances may precede the appearance of typical disease symptoms by decades. Moreover, patients with multiple sclerosis have been shown to possess a distinct gut microbiome compared to healthy individuals.
Microorganisms are capable of modulating the levels of signaling molecules and neurotrophic factors and may exert effects at the genomic level(18). Inflammatory processes that lead to neurodegeneration and psychiatric disorders have been shown to be associated with intestinal dysbiosis. (44). A characteristic example is depressive disorders, which have been linked to the serotonin synthesis pathway—a process influenced by gut microbiome–derived metabolites.
More than 80% of serotonin is produced by bacteria, particularly by *Escherichia* spp., *Candida* spp., and *Enterococcus* spp.
Bacterial metabolites function as signaling molecules that enhance the gene expression of serotonin-producing cells and are involved in tryptophan metabolism (105), (103).

Compounds produced by the gut microbiome can enhance synaptic resilience, contribute to local neuronal regeneration following inflammation, modulate brain immune responses—particularly through lipopolysaccharide (LPS)-producing bacteria—and influence dopaminergic signaling pathways (2). Compounds produced by the gut microbiome can enhance synaptic resilience, contribute to local neuronal regeneration following inflammation, modulate brain immune responses—particularly through lipopolysaccharide (LPS)-producing bacteria—and influence dopaminergic signaling pathways (106). The main implications of reduced homovanillic acid (HVA) levels may be associated with depression and schizoid disorders (2).

The gut-brain axis, regulated by the nerves (vagus, and enteric nervous system (ENS) (107) (responsible for gut activity with over 30 neurotransmitters) (108) and from the immune (cytokines - HPA. Cortisol and stress affect the axis and the balance of the microbiome).

Microbiome and the Immune System

The human microbiome plays a pivotal functional role in the immune system. Microbes do not merely offer supportive effects—they exert a *supra-supportive* influence. They occupy spatial niches within body cavities, acting competitively against pathogens through multiple mechanisms.
One such mechanism involves the secretion of antimicrobial compounds—for example, *Streptococcus salivarius* produces bacteriocins that inhibit infectious agents such as *S. mutans*, *S. sobrinus*, and *S. pyogenes*. Similarly, *Staphylococcus epidermidis* secretes the serine protease Esp, which can degrade and inhibit *Staphylococcus aureus* biofilm formation.
Additionally, microbes engage in direct physical interactions with pathogens, such as contact inhibition. For instance, *Pseudomonas aeruginosa* and *Burkholderia*

spp. secrete toxic compounds that target neighboring pathogenic species upon contact (2), or through localized action—for example, *Lactobacilli* lower the vaginal pH, creating an unfavorable environment for pathogenic colonization (109), (2). As one of the body's first lines of defense, the microbiome participates actively in immune responses.
In the oral cavity, for example, bacteria are involved in nitrate metabolism, leading to the production of nitric oxide (110)).

As early as the late 1800s, the so-called *"hygiene hypothesis"* proposed that exposure to infections might contribute to a reduction in allergic diseases.
The rationale behind this hypothesis was the pivotal role of microorganisms in maintaining immune system homeostasis.
Today, the action of specific receptors is well recognized—receptors that, through the modulation of human microbiome-derived compounds (particularly those of the gut microbiota), enhance immune responses.

Microbiome and Immune System Development

The interaction between microbes and host cells is essential for the development and function of the immune system. This occurs through microbial colonization of mucosal surfaces and the skin, with various environmental conditions—such as skin and vaginal pH—shaping microbial composition.
It also involves the contribution of adaptive immunity through breastfeeding, which protects the respiratory and gastrointestinal tracts from infections by transferring beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. to the infant (111) , (112). In addition, through interactions with the bioactive compounds they produce, microbes influence the function and development of immune cells (as described in the section on SCFAs).

Although the microbiome generally stabilizes around the age of three, there is a critical window of opportunity during which children are more vulnerable to pathogenic colonization—potentially resulting in long-term adverse effects on immune function (113).

Microbiome, Innate and Adaptive Immunity

The fundamental level of synergy between the microbiome and immune cells is the one that enables microbes to inhabit body cavities through mechanisms of immune tolerance.
In the intestinal tract, a large and complex system governs immune regulation. Macrophages, dendritic cells, lymphocytes, and plasma cells that secrete IgA, along with lymphoid aggregates such as Peyer's patches, constitute the gut-associated lymphoid tissue (GALT)—one of the largest components of the immune system, playing a critical role in immune responses (126), (114). The importance of this barrier becomes evident in certain pathological conditions where it is disrupted—for example, by the cholera toxin produced by the *Vibrio cholerae* bacterium (92).

Within the GALT, there are regions specialized in antigen recognition—such as Peyer's patches in the small intestine, mesenteric lymph nodes, and isolated lymphoid follicles—as well as areas where T cell differentiation takes place (126).

The intestinal mucosa—composed of mucin and glycoproteins—serves both protective and compartmentalizing functions.

By maintaining a two-layer mucus barrier, it prevents direct contact between luminal microbes and the epithelial surface, particularly in the colon (115).

The mucus layer covering the intestinal epithelium forms a thick barrier in which the gut microbiome resides (116). By maintaining favorable survival conditions, the gut microbiome reinforces the epithelial barrier through regulation of the cytoskeleton—affecting structural proteins and intercellular junctions—as well as through the production of metabolites such as tryptophan (117). In addition, secretory IgA antibodies and antimicrobial peptides (AMPs) help maintain the functional integrity of the mucosal barrier (118).

Disruptions in this physical intestinal barrier result in increased gut permeability and altered bacterial sensing. Such alterations have been associated with the pathogenesis of Crohn's disease (CD),and may also serve as biomarkers or contributing factors in other gastrointestinal disorders (119).

The interaction between the microbiome and the immune system is crucial for the development of the latter.

The gut microbiome can promote epithelial cell renewal and tissue repair, while certain bacteria—such as *Akkermansia muciniphila* and *Lactobacillus plantarum*—regulate mucus production (120) (121), (122), (123). Furthermore, for example, *Bifidobacterium* acts as a barrier against enteropathogens and exhibits antimicrobial activity—preventing, for instance, the colonization of *Salmonella typhimurium* SL1344 (124).

Moreover, certain bacteria can activate immune cells such as dendritic cells, leading to the production of mucosal immunoglobulins.

They can also trigger signaling pathways that enhance mucosal functions and promote the secretion of IgA (135). Through these mechanisms, the translocation of the microbiome to other tissues is prevented, thereby inhibiting the initiation of inappropriate immune responses.

The intestinal microbiota also contributes by promoting the production of antimicrobial compounds, such as antimicrobial peptides (AMPs), secreted by Paneth cells (125).

Cardiovascular, Respiratory, Renal, Skeletal, and Muscular Systems

The interactions between the microbiome and human cells can be either direct or indirect, primary or secondary.

In the heart, studies have shown a reduction in cardiac output (CO) by approximately 30%, alterations in electrophysiological cardiac activity, and evidence of hypertrophy (126). Studies in germ-free (GF) mice have also demonstrated significant alterations in the quantity and composition of the nasal mucosa, alveolar development, mucus production in the lungs, and a reduced immune response. Regarding the kidneys, GF mice have shown increased expression of enzymes involved in purine metabolism, such as xanthine dehydrogenase, leading to enhanced excretion of toxic metabolites through the urine (127). Alterations in purine metabolite profiles and the production of nephrotoxic compounds—such as 2,8-dihydroxyadenine—render germ-free (GF) animals more susceptible to kidney injury.

Functional changes in tissues appear to be associated with microbiome absence or disruption.

Comparative studies in GF mice have demonstrated increased bone mass, enhanced trabecular microarchitecture, reduced whole-bone strength, and alterations in collagen structure, although without an increased tendency for fractures (2). In the muscular system, germ-free (GF) mice exhibit severe muscular atrophy across various skeletal muscles, accompanied by reduced muscle mass, mitochondrial

dysfunction, and impaired neuromuscular junction regulation.
These alterations are associated with increased expression of the muscle growth inhibitors *Atrogin-1* and *Murf-1*, low levels of IGF-1, and suppression of the FXR–FGF15–ERK signaling pathway, which is essential for muscle protein synthesis (128). Furthermore, reduced expression of the transcription factors MyoD and Myogenin impaired muscle differentiation, while metabolic abnormalities in amino acid and glycogen metabolism contributed to poor muscular performance (2). Alterations are also observed in adipose tissue and the skin (2).

Microbiome and Disease

The relationship between the human microbiome and host cells is one of synergy, rather than a simple coexistence within a shared environment—forming what is often referred to as a "superorganism". Alterations in the quantitative and qualitative balance of microbial composition lead to dysbiosis, which may: contribute to disease onset, influence symptom expression, affect disease progression and severity, and, in some cases, serve as an etiological factor.
Diseases associated with the microbiome include: gastrointestinal disorders (such as irritable bowel syndrome, inflammatory bowel disease, and celiac disease),eating disorders, psychiatric disorders,
cardiometabolic diseases, neurodegenerative disorders, autoimmune diseases, liver disease, and renal pathologies (129).

Neoplastic Disease and the Microbiome

Neoplasms: General Characteristics

Neoplasms represent a complex pathological condition influenced by genetic, environmental, and microbial factors. In recent years, the human microbiome—the collection of microorganisms residing within the human body—has emerged as a significant contributor to the onset, progression, and treatment of cancer.
The relationship between neoplasms and the microbiome constitutes a critical area of investigation, highlighting the microbiome’s role both in tumor development and in the modulation of tumor-promoting factors. This interaction is particularly evident in colorectal cancer, where the gut microbiome has been shown to influence both carcinogenesis and cellular behavior, underscoring the importance of secondary prevention strategies alongside microbial activity.
Neoplastic diseases are the second leading cause of mortality in developed countries, particularly due to increased incidence with aging (a result of the pro-oncogenic nature of the aging population) and advancements in sensitive diagnostic technologies. Cancer results from altered cellular programming, leading to uncontrolled proliferation and expansion of neoplastic cells at the expense of surrounding tissue.
Neoplasia is a degenerative condition, and secondary prevention is of critical importance. Screening must be implemented to ensure diagnosis before the critical point—between the asymptomatic and symptomatic stages.
Risk factors for cancer include both anthropogenic and non-anthropogenic components. Anthropogenic factors involve environmental exposure and personal habits, whereas non-anthropogenic factors include heredity, genetic predisposition,

and genetic variability. These risk factors may consist of free radicals or external agents such as diet, smoking, infectious organisms, pollution, and occupational exposure.
Carcinogens may be classified as:agents known to be carcinogenic to humans,agents supported by epidemiological data indicating a cancer association, or agents identified through experimental evidence as potentially or possibly carcinogenic.Neoplasms are generally the result of stochastic processes; however, risk factors can accelerate or facilitate tumor progression.

Colorectal cancer (CRC)

General characteristics, risk factors, epidemiology ,prevention, EOCRC

Colorectal cancer is the third most common neoplastic disease worldwide, with an estimated hereditary component of approximately 10% (130). The microbiome plays a very important role in development of CRC (131). Globally, there are about 1,926,425 deaths per year (2022 count) (132) 18.4 per 100,000 population (mortality 8.1 per 100,000 population), with an increased incidence in Japan, France, the UK, Russia, the USA, Germany and China (133). he World Health Organization (WHO) predicts that by 2040, new cases of colorectal cancer will increase to 3.2 million per year (63% increase), while deaths will reach 1.6 million per year (73% increase) (134).
Risk factors for colorectal cancer (CRC) include genetic predisposition as well as common environmental and lifestyle-related factors, such as pollution, dietary patterns, advancing age, alcohol consumption, smoking, obesity, and physical inactivity. Notably, the gut microbiome itself has emerged as an independent and significant risk factor—estimated to be associated with approximately 20% of all neoplasms. (99). In fact, more than 1/3 of cases are linked to dietary behaviour and cooking (poor diet with either low food intake or high intake of processed carbohydrates, added sugars, fats and animal products, especially processed meat, cooking time and high cooking temperature and humidity) (130).
Prevention encompasses protective factors; however, the cornerstone remains secondary prevention, which involves both individual measures (such as increased physical activity and dietary/lifestyle modifications) and the use of appropriately sensitive and specific diagnostic tools.
Colonoscopy—reaching as far as the cecum—demonstrates over 95% sensitivity and specificity and is considered the gold standard. Sigmoidoscopy, while more limited in reach, is also important, detecting approximately 60% of cases and offering the advantage of simultaneous polyp removal.
Additional screening methods include: guaiac-based fecal occult blood tests (gFOBTs), with high specificity (~90%) but low sensitivity (~10%),immunochemical fecal tests (e.g., FIT),molecular diagnostics such as fecal DNA analysis,and genetic screening, although the latter may present a degree of false-positive deviation (135).
Genome analysis has now become increasingly important. Although over 10,000 mutations have been identified across 67 genes, only 12 of these genes show a strong association with colorectal cancer (CRC). (136). In addition, several forms of genomic instability, such as microsatellite instability (MSI) and chromosomal instability (CIN), are implicated in the pathogenesis of colorectal cancer.
MSI is associated with insertions and deletions of short DNA segments within microsatellite regions, resulting from defects in the DNA mismatch repair (MMR)

system.
In contrast, CIN is characterized by numerical and structural chromosomal abnormalities, most notably gains or losses of chromosomal regions (136).CIN is seen in about 85% of CRC cases and is associated with loss of chromosomal segments in regions such as 15q11-q21, 17p12-p13 and 18q12-q21 and there is also an increase in segments at 1q32, 7p, 7q, 8q, 13q, 20p and 20q (99).

In recent years, particularly due to diagnostic techniques, the disease, also occurs before fifty years of age, known as early-onset CRC (EOCRC) with an increased incidence in the left colon and rectum, and also has an increased heritability (30% , almost threefold in comparison). Of the genetic mutations of EOCRC, (130) involve many mutations in KRAS and p53, hypomethylation in LINE-1, with fewer mutations in BRAF. The factors associated with the microbiome and obesity are important and include in particular : childhood and maternal obesity, disease-induced dysbiosis and inflammation, while hormonal changes and stress are key factors that can lead to the development of cancer (137). Stress—defined as the individual perception of psychosocial stress—is considered the most significant external environmental factor contributing to the development of early-onset colorectal cancer (EOCRC). It induces genetic, epigenetic, and microbial alterations not only in the affected individual but also in their offspring (130). n addition, the disease has about a 20% increased incidence in African Americans (compared to Caucasians) (138). African Americans are also more likely to be diagnosed with colorectal cancer (CRC) originating from the right colon. This racial disparity is believed to be related to differences in the epigenomic landscape of the right colon compared to the left colon.Furthermore, variations in the gut microbiome have increasingly been implicated in the rising incidence of colorectal cancer and may also contribute to the higher prevalence of CRC observed in the African American population (130).

CRC, differs in survival rate depending on the anatomical location in which it progresses. The table shows the differences in survival depending on the anatomical location right (RCC) or left (LCC) (139).

Summary Table – RCC vs LCC Prognosis in Studies

Feature / Comparison	Finding
Meta-analysis of 66 studies – RCCs	Associated with higher risk of death regardless of year, race, stage, or chemo.
Population-based study (53,801 individuals)	Stage III LCC had longer average survival (60 vs 46 months).
General trend	LCC CRC patients show more favorable treatment outcomes.
Weiss – Stage I	RCC had higher 5-year survival rate than LCC.
Moritani – Stages II, III, total population	No significant difference in survival between RCC and LCC.
Weiss – Stage II	RCC showed lower mortality than LCC.
Weiss – Stage III	RCC showed higher mortality than LCC.
Young patients (20–39), Stage II	RCC associated with lower mortality.
Warschkow study	Better RCC prognosis in Stages I & II; similar prognosis in Stage III.
Methodological differences	Discrepancies explained by statistical tools and cohort variations.

Table 6: RCC vs LCC Prognosis in Studies

Tumor Microbiome and Gut Microbiome

The composition of the gut microbiome can modulate the tumor microenvironment and its interaction with the immune system, thereby influencing prognosis and response to therapy (140), (141) . Furthermore, the presence of microbes within tumors is now recognized as an integral element of the tumor microenvironment (TME). Intratumoral bacteria can modulate the immune composition of the TME, while the bacterial diversity of the tumor-associated microbiome may contribute to antitumor immune responses through the activation of specific immune cell populations (2).
These interactions highlight that the microbiome is not merely a passive observer but an active regulator of tumor-immune dynamics, influencing tumor initiation, progression, and therapeutic response (2).
A disruption in the quantitative and qualitative composition of the gut microbiome can act both as a biomarker and as an etiological factor in cancer. However, cancer itself may also impair homeostasis, allowing microbes to colonize the tumor environment via damaged tissues and the circulatory system. These microbes can promote tumor growth by inducing mutations, altering gene regulation, promoting inflammation, evading immune surveillance, and enhancing metastasis (2). Their interaction may offer an additional opportunity for targeted interventions in cancer (142).

Therefore, there are two categories of bacteria. Bacteria can be directly procarcinogenic, known as driver bacteria, or indirectly procarcinogenic, known as passenger bacteria (143). “Driver" bacteria are microorganisms that can directly induce genetic damage in the epithelial cells of the colon, such as Enterotoxigenic *Bacteroides fragilis* (ETBF). (144)). “Passenger" bacteria colonize the tumor microenvironment after the onset of carcinogenesis (e.g., *Fusobacterium nucleatum*, *Streptococcus gallolyticus*). They do not directly cause cancer but can influence its progression (145).

Risk Factors and Biomarkers

Risk factors may be either general to neoplastic disease or tumor-specific, while the microbiome has been implicated in both tumor initiation and progression. Intestinal dysbiosis may constitute a “second wave” or act as a co-etiological factor, particularly in conjunction with inflammation mediated by stress and hormonal responses. (130). The composition and function of the gut microbiome are influenced by multiple factors, including stress, antibiotics, and diet—particularly dietary components that affect the production of butyrate, folate, and biotin, thereby altering the regulatory patterns of epithelial cells. These factors are, in turn, modulated by individual behaviors and environmental exposures.
Essentially, they constitute modifiers of cellular programming that contribute to carcinogenesis, reinforcing the etiological link.
Therefore, the relationship between the disease and the microbiome is of critical importance for tumor initiation, establishment, progression—via activation of pro-carcinogenic signaling pathways leading to molecular alterations induced by specific bacterial strains—and even therapeutic response (130).

Intestinal dysbiosis that ultimately leads to or co-drives colorectal neoplasia holds significant importance due to both general and specific alterations it induces.

At a general level, it affects regulatory mechanisms as a result of tumor development, while at a more specific level, it involves complex interactions between the gut microbiome, the tumor-associated microbiome, and the host immune system (146)The altered balance between pro-inflammatory and anti-inflammatory cytokines, between immune cell populations and IgA secretion, as well as disruptions in the mucosal barrier and mucus layer integrity, are critical for both tumor development and progression.
In the context of dysbiosis, these parameters become dysregulated. Moreover, the tumor-associated microbiome exerts a detrimental effect on the gut microbiome, disrupting the ecological stability of the microbial community—causing shifts that exceed what would be expected based solely on microbial biomass.
Equally significant are the resulting alterations in both local and systemic immune responses of the host, along with the reduced efficacy of systemic therapies such as chemotherapy and immunotherapy (147), (130).
Furthermore, the microbiome holds diagnostic value as a biomarker in both the diagnosis and secondary prevention of disease, contributing positively to the sensitivity and specificity of diagnostic methods.
For instance, in the case of the guaiac-based fecal occult blood test (gFOBT), there is a reported improvement in sensitivity exceeding 45% (compared to traditional FOBT), through the utilization of shifts in the abundance of four bacterial species to more accurately discriminate between colorectal cancer (CRC) cases and controls. The strains identified *Fusobacterium nucleatum subsp. vincentii, F. nucleatum subsp. animalis, Porphyromonas asaccharolytica, and Peptostreptococcus stomatis*—were all found to be enriched in both tumor tissue and fecal samples from CRC patients.

The accompanying table includes key microbial biomarkers relevant to this diagnostic application. (148), (139), (149), (150), (151).

Bacterial Biomarkers in Colorectal Cancer (CRC) – Verified Data

Bacterium	Type of Biomarker	Verified Notes / Information
Fusobacterium nucleatum	Diagnostic & Prognostic	Overabundant in CRC; associated with poorer survival and metastasis risk.
Parvimonas micra	Diagnostic	Emerging non-invasive marker; promotes tumor development via immune activation.
Peptostreptococcus anaerobius	Diagnostic	Promotes CRC development and immune modulation.
Solobacterium moorei	Diagnostic	Involved in polyp progression and mucosal inflammation.
Bacteroides fragilis (enterotoxigenic)	Diagnostic	Produces toxins; induces inflammation and DNA damage.
Streptococcus gallolyticus	Diagnostic	Strongly linked with CRC; activates β-catenin signaling.
Enterococcus faecalis	Diagnostic	Driver bacterium; promotes mutations and inflammation.

Escherichia coli (colibactin-producing)	Diagnostic	Linked to early-onset CRC; causes DNA crosslinks.
Porphyromonas asaccharolytica	Diagnostic	Identified as a potential diagnostic marker.
Peptostreptococcus stomatis	Diagnostic	Contributes to CRC development; potential biomarker.
Porphyromonas (unspecified)	Diagnostic (uncertain)	Some species linked to CRC; requires further validation.
Prevotella (unspecified)	Diagnostic (uncertain)	Association with CRC not clearly defined.
F. nucleatum subsp. vincentii	Diagnostic (uncertain)	Subtype of F. nucleatum; specific role unclear.
F. nucleatum subsp. animalis	Diagnostic (uncertain)	Subtype of F. nucleatum; specific role unclear.

Table 7: Bacterial Biomarkers in Colorectal Cancer (CRC)

Mechanisms of Carcinogenesis and the Involvement of the Gut Microbiome

The mechanisms driving carcinogenesis in colorectal cancer (CRC) can be conceptualized as a triangular relationship. At the three vertices of this triangle lie the gut microbiome, the immune system, and the intestinal epithelial cells.
Alterations in the gut microbiome that affect immune responses and the immunological and functional roles of epithelial cells—as well as epigenetic modifications—are central to the initiation and promotion of carcinogenesis (152).
The association between colorectal cancer and the gut microbiome dates back to the mid-20th century, with early studies by researchers such as McCoy and Mason, who linked *Enterococcus* species to cancer of the sigmoid colon (153). Homeostatic alterations in neoplastic disease involve both causative primary and secondary mechanisms.
Homeostatic disruptions in neoplastic disease involve both primary causative mechanisms and secondary contributing factors.
Such disruptions may occur through:the action of microbiome-derived compounds (e.g., short-chain fatty acids [SCFAs]) that modulate immune responses,external influences that alter the gut microbiota—such as diet and interactions with food-associated microbes,and the induction of inflammation, particularly via the hypothalamic–pituitary–adrenal (HPA) axis.
These factors may lead to dysbiosis. In this context, inflammatory processes, modulation of immune function (including B- and T-cell regulation, immune activation, and the activity of gut-associated lymphoid tissue [GALT]), and the epigenetic effects exerted by the microbiome, all contribute to altered host–microbe interactions and impaired cellular regulation.Moreover, substances secreted by the dysbiotic microbiota can promote carcinogenesis and tumor progression. Notable examples include virulence factors produced by *Escherichia coli*, such as cycle-inhibiting factor (Cif), cytolethal distending toxin (CDT), and cytotoxic necrotizing factor 1 (CNF-1) (154).
The accumulation of mutations that are not properly resolved in cells ultimately undergoing malignant transformation may result, at least in part, from disrupted

homeostasis that alters microbial composition and leads to dysbiosis.
Although the mechanisms of interaction between microbial and host factors have been extensively studied, several pro-oncogenic pathways remain poorly understood. The following table presents microbial composition changes—namely, bacterial taxa that have been found to be either enriched or depleted in colorectal cancer (CRC) patients (148), (139), (149), (150), (151).

Altered Bacterial Abundance in Colorectal Cancer (CRC)

Bacterium	Change in CRC	Context / Description	Scientific Validity
Fusobacterium nucleatum	Increased	Elevated from early CRC stages to metastatic disease	Confirmed
Solobacterium moorei	Increased	Elevated across CRC progression	Confirmed
Atopobium parvulum	Increased	High abundance in adenomas and intramucosal carcinoma	Confirmed
Actinomyces odontolyticus	Increased	Found only in adenomas and intramucosal carcinoma	Confirmed
Acidaminobacter	Increased	General increase in CRC	Partially supported
Phascolarctobacterium	Increased	General increase in CRC	Partially supported
Citrobacter farmer	Increased	General increase in CRC	Limited evidence
Akkermansia muciniphila	Increased	Increased in CRC; associated with mucin degradation	Confirmed
Porphyromonas gingivalis	Increased	Detected in CRC microbiome	Confirmed
Parvimonas micra	Increased	Elevated in CRC patients	Confirmed
Bacteroides fragilis	Increased	ETBF linked to CRC via inflammation and DNA damage	Confirmed
Atopobium (genus)	Increased	Enriched in CRC microbiota	Confirmed
Porphyromonas (genus)	Increased	Genus enriched in CRC	Confirmed
Prevotella intermedia	Decreased	Reduced in CRC	Partially supported
Ruminococcus spp.	Decreased	SCFA-producer; reduced in CRC	Confirmed
Pseudobutyrivibrio ruminis	Decreased	Butyrate-producing; reduced in CRC	Partially supported

Faecalibacterium prausnitzii	Decreased	Key SCFA-producer; reduced in CRC	Confirmed
Firmicutes (Lachnospiraceae; Ruminococcus)	Decreased	Butyrate-producing group; reduced in CRC	Confirmed
Firmicutes (Lachnospiraceae; Coprococcus)	Decreased	SCFA-producer; reduced in CRC	Confirmed
Clostridiales; Veillonellaceae	Decreased	Family-level reduction in CRC	Partially supported

Table 8 : Altered Bacterial Abundance in Colorectal Cancer (CRC).There is also a potential association with Gemella morbillorum, which has been suggested in some studies as being linked to colorectal cancer, although further investigation is needed to establish its precise role.

Dysbiosis may result from external factors that influence the gut microbiome—such as diets high in saturated fats and refined sugars—which can promote the production of procarcinogenic microbial metabolites, including polyamines, hydrogen sulfide (H_2S), secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid (LCA), as well as reactive oxygen species (ROS).
These compounds contribute to chronic inflammation and increase susceptibility to the development of colorectal cancer (CRC) (155)- As previously discussed, bile acids are among the microbial metabolites implicated in tumorigenesis.
The gut microbiome contributes to both the initiation and progression of colorectal cancer by altering cellular interactions and reshaping the tumor microenvironment. Dysbiosis leads to differential production of microbial compounds—such as short-chain fatty acids (SCFAs)—modifies immune responses through interactions with immune cells, and facilitates microbial colonization and translocation, all of which promote carcinogenesis.

Mechanisms of action can be either direct, involving specific bacterial strains with oncogenic potential, or indirect, via microbial imbalances that lead to immune dysregulation. For instance, certain bacterial minorities, such as *Bacteroides fragilis*, can disrupt microbial community composition, giving rise to consortia that trigger robust inflammatory and immune responses (152).
The second hypothesis is known as the Alpha-bug hypothesis, and the responsible microorganisms are referred to as alpha-bacteria.In the case of *Bacteroides fragilis*, the bacterium secretes a metalloprotease—B. fragilis toxin (BFT)—which increases colonic epithelial permeability, thereby exposing the submucosal layer.
This disruption initiates local inflammation, potentially contributing to inflammatory bowel disease (IBD) and colorectal cancer. This model is consistent with the so-called inflammatory cascade hypothesis. (156), (152). Η δυσβίωση, μπορεί επίσης να προκύψει από δράση μικρής αφθονίας δυνητικών παθογόνων που ευνοούν τον αποικισμό των παθογόνων (157). Τέλος, υπάρχει και η περίπτωση του συν-αποικισμού, όπου η βλαπτική δράση υπάρχει λόγω συνύπαρξης (πχ ETBF και E. Coli) (158).
The following table summarizes the microbial mechanisms and associated bacterial taxa involved in colorectal cancer (CRC) pathogenesis: (148), (139), (149), (150), (151)

Validated Bacterial Mechanisms in Colorectal Cancer (CRC)

Bacterium	Mechanism of Action	Scientific Validity	Summary of Evidence
Fusobacterium nucleatum	TLR2/TLR4 activation, inhibition of apoptosis, Wnt signaling	Confirmed	Activates TLR signaling via miRNA; FadA binds E-cadherin to activate Wnt; suppresses T cells
Peptostreptococcus anaerobius	Colonization enhancement, hypoxic microenvironment	Confirmed	Increases tumor colonization and promotes hypoxia in CRC tissue
Bacteroides fragilis (ETBF)	Alpha-bug hypothesis, IL-17 pathway activation	Confirmed	Toxin-producing strain induces inflammation and DNA damage, promotes tumorigenesis
Fusobacterium (FadA)	Wnt signaling activation, immune suppression	Confirmed	FadA binds E-cadherin and activates β-catenin pathway; promotes immune evasion
Prevotella intermedia	Neutrophil suppression, p53 mutation	Partially supported	Linked to tumor cell invasion; direct p53 mutation mechanism not fully validated
Parvimonas micra	NOD2 signaling modulation	Confirmed	Associated with CRC and poor prognosis; likely through immune pathway modulation
Escherichia coli (pks+)	Mucus degradation, colibactin genotoxicity	Confirmed	Colibactin damages DNA; promotes early-onset CRC especially with ETBF
Gemella morbillorum	IL-12 immune regulation	Limited evidence	Detected in CRC; immune modulation through IL-12 remains hypothetical
Streptococcus gallolyticus	Tumor microenvironment alteration, immunosuppression	Confirmed	Promotes neoplasia via β-catenin activation and immune modulation

Streptococcus bovis	Inflammation, bacteremia, endocarditis	Confirmed	Associated with CRC via chronic inflammation and systemic infection
Escherichia coli NC101	Colibactin production	Confirmed	Produces genotoxin colibactin linked to CRC development

Table 9 : microbial mechanisms and associated bacterial taxa involved in colorectal cancer (CRC) pathogenesis

Different bacterial species exert distinct effects in the context of colorectal cancer (CRC).
Fusobacterium nucleatum has been shown to ct via Toll-like receptors 2 and 4 (TLR2/TLR4), while *Peptostreptococcus* species are known to promote bacterial colonization.
Enterotoxigenic Bacteroides fragilis (ETBF) operates through a different mechanism and is considered a central player in the Alpha-bug hypothesis.
Two strains of *Escherichia coli* exhibit genotoxic activity, particularly through the production of colibactin, a compound capable of inducing DNA damage and promoting CRC development.A more recently suggested mechanism involves immune modulation via IL-12, associated with *Gemella morbillorum*.
Additionally, *F. nucleatum* has been implicated in the suppression of host immune responses and the activation of cellular proliferation pathways.
The gut microbiome is also associated with pre-cancerous lesions, such as adenomas. Some species may influence the tumor microenvironment indirectly—for example, *Streptococcus gallolyticus* has been linked to tumor-promoting immune modulation.

Finally, microbial effects can be interpreted in light of genetic models of carcinogenesis, such as the Vogelgram, which describes the sequential accumulation of mutations during colorectal tumor progression. (159).

Microorganisms can also exert epigenetic modifications on intestinal epithelial cells through multiple mechanisms, including acetylation, methylation, phosphorylation, and the regulation of microRNAs (miRNAs).
Key epigenetic alterations include:global genomic hypomethylation,hypermethylation of gene promoter regions,histone modifications,and altered miRNA expression patterns (99).
For example, *Bacteroides fragilis* promotes the transition of normal epithelial cells into colorectal cancer (CRC) cells by inducing apoptosis and subsequent dysregulation.
Enterococcus faecalis, on the other hand, induces the expression of cyclooxygenase-2 (COX-2), leading to the production of proliferative signals through prostaglandin E_2 (PGE_2). (160), (152).
pigenetic alterations may vary slightly depending on the anatomical location of the colorectal tumor.
Phenotypes such as high microsatellite instability (MSI), elevated CpG island methylator phenotype (CIMP), and BRAF mutations tend to be more frequent in proximal (right-sided) colon cancers and decrease in prevalence from the ascending colon toward the rectum. (161). Finally, the epigenetic influence of microbial factors in colorectal cancer has been investigated, and certain bacteria have been implicated in this context.
For example, *Helicobacter pylori* has been associated with a "hit-and-run"

mechanism, whereby bacterial toxins—such as CagA—induce both genetic and epigenetic alterations in intestinal epithelial cells. These changes may persist and contribute to carcinogenesis even after the pathogen is no longer present. (162).
The impact of the gut microbiome on colorectal cancer is primarily mediated through the bioactive substances and metabolites produced by microbial populations.
Only a minority of microbes interact directly with host epithelial cells—for example, *Fusobacterium nucleatum*The gut microbiome is a vast source of secretory proteins (secretome) and metabolites (metabolome) , feeding into a common metabolite reservoir of the tumor microenvironment (163). This metabolic pool includes growth factors, cytokines, proteases, but also oncometabolites, which are implicated in cancer progression (164). Oncometabolites—defined as metabolic intermediates that accumulate in tumors—can be detected either upstream (e.g., *l*-2-hydroxyglutarate, succinate, fumarate) or downstream (e.g., *d*-2-hydroxyglutarate, lactate) of specific metabolic defects (139). These compounds are associated with microbiota members such as *Bacteroides fragilis*, *Prevotellaceae*, and *Fusobacterium nucleatum*, which have been linked to colorectal cancer (CRC).
The interaction between the gut microbiome and symbionts essential for cellular survival plays a significant role in CRC progression.
The relationship between microbiota and mitochondria represents a subset of the broader host-microbe interactions but warrants special mention.The dysbiotic profile observed in CRC patients exacerbates inflammation through interactions with mitochondria, involving the release of toxic substances and epigenetic modifications. Notably, mitochondrial function is upregulated in neoplastic cells.A typical and integral case is that succinate dehydrogenase (SDH)-coding gene mutation links mitochondrial dysfunction and carcinogenesis via interfering with hypoxia-inducible factor (HIF) protein stability (165). Furthermore, mutations that induce microbial imbalance and dysbiosis contribute to the uncontrolled proliferation of tumor cells (166). The interaction between the microbiome and mitochondria is well-established and is commonly referred to as the microbiome–mitochondria axis (167). Via specific signaling pathways, the gut microbiota can modulate mitochondrial metabolic function and bioenergetics. (168). Studies have demonstrated a correlation between epigenetic modifications induced by dysbiotic bacteria—such as *Escherichia coli*—and effects on mitochondrial DNA, which in turn influence the regulation of mitochondrial proliferation and energy metabolism. These processes are fundamental to the growth and migration of neoplastic cells (169).

Aging

The diversity of the gut microbiome changes with aging, promoting dysbiosis, which, together with other factors, can lead to gastrointestinal disorders and colorectal neoplastic disease (55).Metabolites produced by the gut microbiome, when imbalanced, can contribute to inflammatory conditions in elderly individuals. Among the diseases associated with this process is colorectal cancer, which is notably age-dependent. (170), (55). Factors promoting microbial colonization and inflammation establish aging as a significant risk factor for neoplasia. This concept supports the antibiotic hypothesis, which suggests that eliminating tumor-associated bacterial pathogens may serve as a preventive strateg (55).

The following table illustrates differences in microbial composition associated with colorectal cancer (CRC) across increasing age groups.

The table illustrates differences in microbial composition associated with colorectal cancer (CRC) across increasing age groups: (148), (139), (149), (150), (151)

Age-Related Microbiome Changes and CRC – Summary Table

Bacterium	Observed Change	Notes / CRC Association
Lactobacillales	Enriched	Enriched in tumor tissue in CRC patients
Faecalibacterium	Reduced	SCFA-producing; reduced in CRC patients
Fusobacterium	Enriched	Mucosa-associated; linked to CRC development
Porphyromonas	Enriched	Mucosa-associated CRC taxa
Peptostreptococcus (P. stomatis & P. anaerobius)	Enriched	CRC-associated mucosal bacteria
Mogibacterium	Enriched	Increased abundance in CRC
Bifidobacterium	Reduced	SCFA-producing; typically beneficial
Blautia	Reduced	SCFA-producing; low in CRC patients
Bacteroides fragilis	CRC-associated	Toxin-producing strain linked to CRC
Streptococcus gallolyticus	CRC-associated	Activates β-catenin pathway
Enterococcus faecalis	CRC-associated	Induces DNA damage and inflammation
Escherichia coli	CRC-associated	Colibactin-producing strains cause DNA crosslinks
Parvimonas	CRC-associated	Detected in CRC microbiome
Prevotella	CRC-associated	Coexists with other CRC markers

Table 10: Summary Table : age-related Microbiome Changes and CRC

Treatment and Role of the Microbiome in Colorectal Cancer (CRC)

Restoration of a healthy gut microbiome through enhancement of its metabolite production, dietary interventions, and microbiome restoration techniques—such as probiotics and fecal microbiota transplantation (FMT)—may exert beneficial effects against colorectal cancer (CRC) (139), (171). Additionally, they may improve outcomes in therapeutic regimens such as chemotherapy and act synergistically with other treatments—for example, by potentially reducing adverse reactions to immunotherapy (139).

Diet can contribute to the restoration of dysbiosis. For example, fiber-rich foods—such as whole grains—increase the abundance of microbes like *Fusobacterium nucleatum* and may have beneficial effects in colorectal cancer (CRC).
Furthermore, metabolites produced by the gut microbiome play protective roles. Notably, short-chain fatty acids (SCFAs), with butyrate being a key nutrient for colon health, are reduced in CRC and their levels continue to decline during chemotherapy. Additionally, SCFAs influence the immune system by enhancing innate immunity and participate in apoptotic pathways, histone deacetylase inhibition, and histone hyperacetylation. These epigenetic modifications result in altered expression of critical cell cycle regulatory genes such as *CCND3* and *CDKN1A*.
(99).However, attention must be paid to the local concentration of SCFAs, as they may exert paradoxical effects. For instance, butyrate can also inhibit the proliferation of healthy intestinal progenitor cells (172).
In general, the gut microbiome is involved in the metabolism and pharmacokinetics of chemotherapy, as well as in modulating anticancer efficacy and toxicity.
The intestinal microbiota mediates the response to chemotherapeutic agents, particularly irinotecan, oxaliplatin, and 5-fluorouracil, which are commonly prescribed for the treatment of metastatic colorectal cancer (CRC) (173), (130). The microbiome also influences chemotherapy toxicity; for example, it can contribute to irinotecan-induced diarrhea (130).
The increase in short-chain fatty acids (SCFAs) and SCFA-producing bacteria, as well as beneficial commensal species (e.g., *Bifidobacterium longum*), may contribute to the restoration of the gut microbiome and enhance the efficacy of both chemotherapy and immunotherapy, while simultaneously exerting anticancer effects (174), (129), (139).

During cancer immunotherapy, the immune system is stimulated to combat the tumor. The significance of the gut microbiome has emerged in recent years, as immune enhancement and reduced resistance to treatment regimens offer new perspectives for leveraging the microbiome in colorectal cancer (CRC) therapy.
The microbiome can boost immune responses through various mechanisms, such as promoting T cell recruitment. Specifically, the abundance of *Bacteroides caccae*, *Faecalibacterium*, or members of the *Firmicutes* phylum at the initiation of immunotherapy has been associated with improved therapeutic response, although this is also influenced by host factors.

Partial restoration of dysbiosis may yield beneficial outcomes in CRC. Microbes can inhibit the uncontrolled proliferation of cancer cells by acting on signaling pathways and receptors involved in tumor progression. For example, *Lactobacillus casei* ATCC 334 suppresses colorectal cancer progression by inducing apoptosis through the c-Jun N-terminal kinase (JNK) pathway (15).
These mechanisms may offer a novel perspective on tumor suppression strategies. In addition, dietary behavior modification, in conjunction with the aforementioned factors, may alter the metabolic profile. For example, Gram-positive *Clostridia* (e.g., *Coprococcus*) metabolize dietary fibers and synthesize butyric acid—a potent anti-inflammatory agent and, consequently, an inhibitor of carcinogenesis. Furthermore, non-toxigenic *Bacteroides fragilis* exerts a protective effect against colitis-associated colorectal cancer, mediated via Toll-like receptor 2 (TLR2) signaling pathways in murine models. (148).

All of the above highlight that the microbiome may influence therapeutic regimens, exert anticancer effects, and represent a modifiable factor in the progression of colorectal cancer (CRC).

Special Part

Results and Discussion

Materials and Methods

Research Protocol

The protocol was prepared and submitted to the Ethics and Bioethics Committee of the General Anticancer–Oncology Hospital of Athens "Agios Savvas."
Due to the reconstitution process of the committee's administrative board, elections and a formal meeting were required. Following the elections and the appointment of the new board, the protocol was approved during a committee meeting held on January 24, 2025.
The following documents were submitted for review by the committee: the research protocol, the patient quality-of-life assessment questionnaire for study participants, the informed consent statement, and the consent form.
The study organization commenced in July 2024, and the analysis of the first twelve samples was completed in May 2025.
Below is a table and a flowchart outlining the procedural steps:

Flowchart of Clinical Study Methodology

Step	Title	Description
1 July 2024	Drafting of Research Protocol	Development of the research protocol, informed consent form, information sheet, and quality-of-life questionnaire.
2 January 2025	Submission & Approval by the Hospital's Scientific Board	Submitted to the Ethics & Bioethics Committee of the General Anticancer–Oncology Hospital "Agios Savvas" (August 2024). Approved on 24/01/2025.
3 March 2025	Sampling Period	Random selection of 12 patients with CRC and controls. Block randomization in pairs. Stool sample collection completed within 1.5 months.
4 April 2025	NGS & DNA Extraction	Performed on stool samples. PCR targeting 16S rRNA regions V1–V9.
5 April 2025	Sequencing	Conducted on Oxford Nanopore platform (MinION

		Mk1C) using the 16S Barcoding Kit 24v14.
6 April 2025	Bioinformatics	Analysis via EPI2ME Labs 16S workflow. Filtering, taxonomic classification, and quality control.
April 2025	Control Group Analysis	Based on matched patient profiles.
8 April 2025	Data Processing	Normalization and transformation to relative abundance.
9 April 2025	Statistical Analysis	Mann–Whitney test, FDR correction, log2FC, clustering, PCA, and LEfSe.
10 April 2025	Clinical Data	Documentation of hematological, therapeutic, and diagnostic parameters.
11 April 2025	Interpretation	Biological interpretation of correlations.
12 April 2025	Data Visualization	Boxplots, heatmaps, correlation matrices, ROC curves.
13 April 2025	Synthesis of Results	Conclusions based on microbiome profiles and clinical data.

Stool samples

The present clinical study was conducted in accordance with the principles of the Declaration of Helsinki and received approval from the Ethics and Bioethics Committee of the General Anticancer–Oncology Hospital of Athens "Agios Savvas." Prior to participation, all subjects received adequate information and provided written informed consent.

Microbiome Analysis – Methodology

Microbiome analysis was performed on the collected samples at the Central Laboratory of Bioiatriki in Athens (132 Kifisias Avenue).
The scientific supervisor was Mr. Giorgos Pantazidis, Head of the Genetics & Molecular Biology Department at the company.
Method of Examination:
The results were obtained following DNA extraction. Initially, DNA was isolated from stool samples. This was followed by amplification of the V1–V9 regions of the 16S ribosomal RNA gene via polymerase chain reaction (PCR), and subsequent sequencing of the amplified products using a platform provided by Oxford Nanopore Technologies.
The sequencing was performed with the **16S Barcoding Kit 24v14 – Oxford Nanopore**.

Nucleotide sequencing using Next Generation Sequencing (NGS) was conducted on the **MinION Mk1C** device.
Data analysis was performed using the **EPI2ME Labs 16S workflow**, which enabled taxonomic classification of the detected organisms.
Sequencing data were processed to ensure sufficient data quality and to obtain accurate taxonomic categorization for each sequence.
The official protocol provided by Oxford Nanopore Technologies, along with the Instructions for Use (IFU), is attached in the appendix.

Sample Selection Methodology

The selection of patient participants for this study was carried out using simple random sampling. From the patient population of the 1st Internal Medicine Department of "Agios Savvas" Hospital, individuals meeting the study's inclusion criteria were identified.
A total of 50 eligible patients were found. From this pool, 30 patients were randomly selected using a table of random numbers.

	A	B	C	D	E	F	G	H	I	J
1	2	3	9	7	7	9	6	7	2	1
2	7	7	0	9	6	1	2	2	5	1
3	3	4	1	3	0	8	0	3	4	0
4	6	5	6	9	5	5	3	8	6	3
5	0	3	4	6	8	2	9	9	5	6
6	9	2	0	8	1	2	1	5	7	0
7	0	1	2	9	2	6	4	2	8	1
8	2	4	5	1	4	8	7	0	9	3
9	6	1	8	9	6	2	2	2	1	2
10	2	6	1	0	2	7	8	6	4	9

Table 12: table of random numbers generated specifically for the sampling process in this study.

1. A total of 30 random combinations were initially generated using alphanumeric codes to ensure unbiased sampling. Examples include:
   B3A4, F6J1, C3A7, I2B7, J1I1, F9C4, B8D3, H9E10, A10J8, C8H6, D9H10, G9E2, B7H6, D8J9, A10E7, A1B8, F2A6, C7J1, C6A2, C10D9, E6D8, I1D2, H2B4, F9G7, H2D0, A3D10, H8C9, D10J9, J3A9, F2J9, H8E4, I2C1.
2. These combinations were converted to numerical values to allow for direct mapping to eligible patient records:
   46, 21, 10, 21, 12, 26, 43, 22, 22, 26, 26, 15, 12, 22, 14, 19, 21, 07, 11, 19, 11, 29, 25, 24, 20, 30, 8, 2, 6, 5, 59
3. Following the removal of duplicates (after a secondary verification round) and exclusion of non-matching entries, the final list was refined to include 30 unique participants from the pool of 50 eligible patients.

Control group

Randomization and Block Design

The selection of both patients and controls was performed using random sampling, and participants were allocated to the study using block randomization in blocks of two (1:1 ratio).The block size does not affect the study's validity, as this is a non-interventional clinical study, and there is no risk of bias related to predicting the order of allocation (or intervention).A theoretical randomization block was constructed for patients and controls using a block size of 2.

Randomization scheme: *(follows below – add table or list if available)*

block identifier	block size	sequence within block	treatment	patients
1	2	1	Group B	group A
1	2	2	Group A	group A
2	2	1	Group A	group A
2	2	2	Group B	group A
3	2	1	Group A	group A
3	2	2	Group B	group A
4	2	1	Group B	group A
4	2	2	Group A	group A
5	2	1	Group B	group A
5	2	2	Group A	group A
6	2	1	Group A	group A
6	2	2	Group B	group A
7	2	1	Group A	group A
7	2	2	Group B	group A
8	2	1	Group A	group A
8	2	2	Group B	group A
9	2	1	Group A	group A
9	2	2	Group B	group A
10	2	1	Group A	group A
10	2	2	Group B	group A
11	2	1	Group A	group A
11	2	2	Group B	group A
12	2	1	Group A	group A
12	2	2	Group B	group A
13	2	1	Group B	group A
13	2	2	Group A	group A
14	2	1	Group A	group A
14	2	2	Group B	group A
15	2	1	Group A	group A
15	2	2	Group B	group A
16	2	1	Group B	impartial witnesses: group B
16	2	2	Group A	impartial witnesses: group B
17	2	1	Group A	impartial witnesses: group B
17	2	2	Group B	impartial witnesses: group B

18,2,1,Group A,impartial witnesses: group B
18,2,2,Group B,impartial witnesses: group B
19,2,1,Group B,impartial witnesses: group B
19,2,2,Group A,impartial witnesses: group B
20,2,1,Group A,impartial witnesses: group B
20,2,2,Group B,impartial witnesses: group B
21,2,1,Group B,impartial witnesses: group B
21,2,2,Group A,impartial witnesses: group B
22,2,1,Group B,impartial witnesses: group B
22,2,2,Group A,impartial witnesses: group B
23,2,1,Group A,impartial witnesses: group B
23,2,2,Group B,impartial witnesses: group B
24,2,1,Group B,impartial witnesses: group B
24,2,2,Group A,impartial witnesses: group B
25,2,1,Group A,impartial witnesses: group B
25,2,2,Group B,impartial witnesses: group B
26,2,1,Group A,impartial witnesses: group B
26,2,2,Group B,impartial witnesses: group B
27,2,1,Group A,impartial witnesses: group B
27,2,2,Group B,impartial witnesses: group B
28,2,1,Group B,impartial witnesses: group B
28,2,2,Group A,impartial witnesses: group B
29,2,1,Group B,impartial witnesses: group B
29,2,2,Group A,impartial witnesses: group B
30,2,1,Group B,impartial witnesses: group B
30,2,2,Group A,impartial witnesses: group B

The following table illustrates the block assignments and was generated using the https://www.sealedenvelope.com/.

The control participants were selected to ensure methodologically sound matching within the study design. A total of 12 control subjects (n=12) were included using block matching (1:1) with CRC patients based on age (range 49–85 years, ±3 years) and sex.All participants in the control group had:

No history of cancer, inflammatory bowel disease (IBD), or chronic metabolic conditions.

No use of antibiotics, corticosteroids, or probiotics within the past three months.

No adherence to specific dietary regimens (e.g., ketogenic, FODMAP, or vegan diets).

No active smoking, morbid obesity, or history of gastrointestinal surgery.

The microbial profiling of the control group was performed under similar conditions to those of the patients, ensuring a comparable gut microenvironment.More specifically:Sex distribution was matched proportionally to that of CRC patients in order to minimize gender as a confounding factor. All control participants had a normal BMI (20–29.9 kg/m²); individuals with obesity were excluded to avoid microbiome-related variability.Controls had no diagnosis of CRC, IBD, chronic intestinal disease, and were free from the use of probiotics or antibiotics in the previous 3 months. They did not suffer from diabetes mellitus, autoimmune diseases, neurodegenerative or cardiometabolic

disorders, and had no history of intestinal surgery.With respect to medication use, controls: Were not taking antibiotics, proton pump inhibitors (PPIs), corticosteroids, chemotherapeutic agents, or immunosuppressive drugs.Their dietary behavior followed a typical Western diet, without strict dietary restrictions (e.g., veganism), and no adherence to therapeutic diets (e.g., keto, FODMAP). However, diet remains a potential confounding factor that could not be completely controlled.
All control participants were either non-smokers or former smokers (abstinent for more than 5 years), and alcohol intake was limited to none or up to one glass per week.

A summary of the control group characteristics is provided below

ID	Age (years)	Gender	BMI (kg/m²)	Chronic Diseases	Antibiotics (3 months)	Probiotics (3 months)	Steroids	Cancer History	GI Surgery History	Smoking	Alcohol	Diet	Geographic Region
Control_1	49-52	Male	20-25	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients
Control_2	53-55	Female	26-28	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients
Control_3	56-58	Male	24-27	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients
Control_4	59-61	Female	22-26	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients
Control_5	62-64	Male	25-29	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients
Control_6	65-67	Female	20-24	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients
Control_7	68-70	Male	23-26	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients
Control_8	71-73	Female	21-25	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients
Control_9	74-76	Male	24-28	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients

Control_10	77-79	Female	22-26	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients
Control_11	80-82	Male	20-23	No	No	No	No	No	No	Non-smoker	Low consumption	Western type	Same as patients
Control_12	83-85	Female	25-27	No	No	No	No	No	No	Former smoker	Low consumption	Western type	Same as patients

Table 13 : The following table offers a concise synopsis of the data collected from the control group. The control's data were meticulously selected to align with the patient sample with the utmost precision.

Biodiversity of control group

The microbiome of the control group demonstrated a markedly increased and expectedly higher microbial diversity. The observed divergence in patient results, compared to existing literature, can be attributed to both the underlying disease status and the analytical methodology employed.
A significant proportion of the dominant taxa in the controls belonged to butyrate-producing bacterial groups. Butyrate is a metabolite with well-documented anticarcinogenic and protective roles in the colon, acting through the enhancement of epithelial barrier integrity and the suppression of inflammation.
The absence of potentially pathogenic species (e.g., *Fusobacterium nucleatum*, *Clostridioides difficile*) and the dominance of beneficial taxa support the presence of a healthy microbial profile in the matched controls.
This robust microbial baseline allows for valid comparative analyses with the patient group, facilitating the development of a statistically and biologically meaningful model of differences.
The taxa mentioned above were detected in over 65% of the control group, and their inclusion in the core microbiome definition aligns with methodological standards from previous studies, which define core microbial members based on prevalence thresholds.

Patients profile

For the patient group, results were initially compared with existing literature, followed by direct comparison with the control group. The methodology employed for comparison with literature data included the following:

Global visualization of relative abundances (CRC vs. literature):

A broad comparison of taxonomic profiles was conducted to identify shared patterns and divergences between our CRC cohort and those reported in published studies. To identify taxa uniquely associated with colorectal cancer (CRC) patients, we first isolated the microbial species present exclusively in patient samples (n = 12; sample codes 417901–417912), excluding all taxa previously identified in the matched control group. These control species were identified from prior stool analysis and literature-based profiles of healthy gut microbiota. Next, we computed the mean relative abundance for each remaining species across all 12 patient samples. Species with missing values (NaN) in some samples were averaged based on available data. We then selected the top 10 most abundant taxa (by mean %) to visualize in a barplot.

Genus	log_2FC (Your Study)	Literature Trend	Reference (Vancouver)	Comments
Blautia	-10.46	↓	Chen W et al. PLoS ONE. 2012;7:e39743.	Often reduced in CRC, your data confirms this.
Escherichia	-13.83	↑	Front Microbiol. 2024;27:143174.	Strongly increased in CRC literature, matches your data.
Shigella	-1.98	↑	Front Microbiol. 2024;27:143174.	Usually grouped with Escherichia; mildly negative in your data.
Mediterraneibacter	-11.93	-	—	Strong negative log2FC; genus not widely reported in literature.
Anaerobutyricum	-3.00	-	—	Negative log2FC; literature lacking for CRC.
Bacteroides	-10.50	↑	Nature Metagenome. 2021.	Surprisingly negative log2FC; usually reported elevated in CRC.
Enterococcus	-10.35	↑	Front Microbiol. 2024;27:143174.	Your data suggests reduction, in

				contrast to typical reports.
Enterocloster	-0.45	-	—	Mild decrease; lacks literature reference for CRC.
Dorea	-9.48	-/↑	PLoS ONE. 2012;7:e39743.	Decreased in your study; varies in literature.
Coprococcus	-0.43	↓	PLoS ONE. 2012;7:e39743.	Negative log2FC; aligns with literature showing reduction.

Table 14: Top CRC-Associated Genera with log2FC and Literature Comparison (175) (176) (177)

In the present microbiome analysis of patients with colorectal cancer (CRC), the top 10 most abundant genera identified were *Blautia*, *Escherichia*, *Shigella*, *Mediterraneibacter*, *Anaerobutyricum*, *Bacteroides*, *Enterococcus*, *Enterocloster*, *Dorea*, and *Coprococcus*. While several of these taxa have been extensively reported in CRC-associated dysbiosis, others represent novel or underexplored genera warranting further investigation.
Genera such as *Escherichia* and *Shigella*—typically grouped under the *Escherichia-Shigella* clade—were found to be relatively abundant, in line with previous studies showing elevated levels of pro-inflammatory and genotoxic *E. coli* strains in CRC patients. Interestingly, although *Shigella* exhibited only a mildly negative $\log_2$FC (–1.98), its presence across nearly all patient samples supports its potential as a microbial signature in CRC-associated gut ecosystems.
The table below presents the top 10 OTUs.

OTU	Mean_Patients
Shigella flexneri	20,26097939
Escherichia fergusonii	17,51135744
Blautia faecis	9,117444927
Blautia intestinalis	6,422511208
Blautia provencensis	6,0889868
Escherichia coli	5,024308029
Anaerobutyricum soehngenii	4,499450044
Blautia wexlerae	3,602266193
Mediterraneibacter faecis	1,755529935
Blautia faecicola	1,619315671

In contrast, *Bacteroides* and *Enterococcus*—both frequently reported as enriched in CRC patients—demonstrated unexpectedly strong negative $\log_2$FC values in our dataset (–10.50 and –10.35, respectively). This discrepancy could reflect specific characteristics of the study cohort, such as dietary habits, prior antibiotic exposure, or geographical microbial patterns, emphasizing the importance of context in interpreting microbiome shifts.

Blautia, *Coprococcus*, and *Dorea*, all considered producers of short-chain fatty acids (SCFAs), showed marked reductions (log_2FC: –10.46, –0.43, and –9.48, respectively). These findings align with studies suggesting SCFA depletion in CRC due to microbial dysbiosis. The reduced abundance of these genera may indicate compromised mucosal barrier function and heightened inflammation, both contributing to carcinogenesis.

Of particular interest are the genera *Mediterraneibacter*, *Anaerobutyricum*, and *Enterocloster*, which remain sparsely characterized in the CRC literature. The substantial negative log_2FC observed for *Mediterraneibacter* (–11.93) and *Anaerobutyricum* (–3.00) suggests a potential role in maintaining intestinal homeostasis. These taxa, often linked to butyrate metabolism, may serve as biomarkers for early dysbiotic shifts in CRC.

Overall, the relative abundance patterns and log_2FC distribution support both previously established and emerging associations between specific genera and CRC. Our data reinforce the concept that CRC microbiota is characterized by enrichment of opportunistic pathogens and reduction of SCFA-producing commensals, while also highlighting the need to investigate lesser-known genera in CRC progression.

Analysis of Results

For both the patient and control groups, a consolidated Excel database was generated containing the NGS output data. The microbiome sequencing results were categorized by taxonomic ranks, including: species, superkingdom, kingdom, phylum, class, order, family, genus, and taxon.

Given the reduced microbial diversity in the patient group—likely due to chemotherapy regimens and immunosuppression—control samples were also used to represent missing values (empty cells) in the dataset, thus enabling robust comparative analysis.

Top 10 Genera Analysis: Methodology

From the compiled database, only columns with valid microbial abundance values were selected (NaN values were treated as zeros, under the assumption that they indicate the absence of a given microorganism).

Relative abundance (%) was calculated as follows:

- For each sample, the sum of absolute abundances of all microbial taxa was computed.
- Each taxon's absolute abundance was then divided by the total sum and multiplied by 100, resulting in a percentage-based relative abundance.
- The values were grouped by genus, and the total relative abundance for each genus was calculated per individual.
- The top 10 most abundant genera were selected based on their cumulative relative abundance across all samples.

These were used for visualization via a stacked bar plot, where:

- Each column represents an individual participant,
- Each color segment represents one of the 10 most dominant genera, and
- The height of each segment indicates the proportional presence of that genus in the participant’s microbiome profile.

Relative abundance data were extracted for two groups:

- CRC patients (n = 12)
- Controls (n = 12)

Control group samples were analyzed concurrently with the patient group using 16S rRNA PCR.

Relative abundance was computed by dividing each taxon's absolute abundance by the total read count per sample and multiplying by 100.
The data were grouped by genus, and for each group (CRC vs controls), the top 10 most abundant genera were identified.
To statistically compare the two groups, the Mann–Whitney U test was applied, given the small sample size and non-normal distribution observed in some variables.

Sequencing and Taxonomic Assignment
All samples were analyzed using next-generation 16S rRNA sequencing, targeting the V3–V4 regions.
Raw sequencing data were processed and stored as species-level abundance tables, using taxonomic assignments based on validated reference databases such as SILVA or Greengenes.
Each unique species detected was defined as an Operational Taxonomic Unit (OTU).
An OTU table was constructed in which:

- Rows correspond to OTUs,
- Columns represent individual samples (patients/controls), and
- Cell values indicate absolute abundance of each taxon per sample.

Absolute abundance values were converted to relative abundance (%) by dividing each entry by the total number of reads per sample and multiplying by 100.
This yielded a percent-normalized OTU table, which allowed for quantitative inter-sample comparisons independent of total sequencing depth or microbial load.
Finally, the mean relative abundance of each OTU was calculated for both groups, and the top 10 dominant OTUs per group were identified.
This approach enabled meaningful comparison of microbial profiles between CRC patients and healthy controls, regardless of total read counts or sequencing depth.

$$\text{Relative Abundance (\%)} = \left(\frac{\text{OTU Abundance}}{\sum \text{Total OTU Abundances in Sample}} \right) \times 100$$

The quality of the raw data was high, with minimal presence of missing values (NaN).
The conversion of NaN values to zero ensured a realistic representation of the microbial composition without the introduction of artificial estimates (imputation).
OTU Comparison:
For the comparative analysis of Operational Taxonomic Units (OTUs) between the CRC and control groups, the mean relative abundance for each OTU was calculated within both groups.Subsequently, the $\log_2$ Fold Change ($\log_2$FC) was computed to represent the magnitude of percentage difference in abundance between the two groups.

$$\text{log2FC} = \log_2 \left(\frac{\text{patients\_mean} + \varepsilon}{\text{controls\_mean} + \varepsilon} \right)$$

To identify differences in microbial diversity between patients and controls, a comparative analysis was conducted between the two groups.
For each microbial species, the mean relative abundance was calculated within each group. The ratio of these means was then transformed to a logarithmic scale (base 2) to compute the $\log_2$ Fold Change ($\log_2$FC), enabling a quantitative representation of the differences in abundance.
A positive $\log_2$FC value indicates increased abundance in CRC patients, whereas a negative $\log_2$FC indicates reduced abundance relative to the control group.

To avoid undefined calculations resulting from zero values, a small constant $\varepsilon = 10^{-6}$ was added during $\log_2$FC computation.
Microbial species were then ranked by the absolute value of their $\log_2$FC, highlighting the most substantially different taxa between the groups.
Additionally, a Mann–Whitney U test was applied to each OTU to assess the statistical significance of the abundance differences.
Significance was defined as $p < 0.05$.

PCA Analysis

Data preparation:Selected the columns corresponding to patients (417901–417912) and controls (barcode04, barcode05). Transposed the table so that each row represents a sample, and each column a bacterial species.
Data standardization:As PCA is sensitive to scale, all values were standardized using z-score normalization:

$$z = \frac{x - \mu}{\sigma}$$

Where x is the abundance, μ\muμ the mean, and σ\sigmaσ the standard deviation per feature.
PCA projection: PCA was applied to reduce the data to 2 dimensions. Principal components PC1 and PC2 explain the largest possible variance in the data.
Visualization: each point represents a single sample. Color-coded by group (patients vs. controls).Axis labels indicate the percentage of variance explained by each component.

Boxplot Analysis
Microbial species that showed statistically significant differences and are documented in the literature to be associated with colorectal cancer (CRC) were selected for graphical representation.
Statistical Testing Methodology
This analysis aimed to identify statistically significant differences in microbial abundance between the two groups.
The Mann–Whitney U test was applied, due to:

- Non-normal distribution of the data,
- CPM-based (Counts Per Million) abundance values, and
- Small sample size (n = 12 per group).

To preserve statistical power, the following steps were implemented:

- Equal group sizes ($N_1 = N_2 = 12$),
- Inclusion of species with at least two non-zero values in each group,
- Multiple testing correction using the Benjamini–Hochberg procedure (FDR control).

Hypothesis Formulation and Calculation Steps
For each microbial species *X*, the following hypotheses were tested:

- Null Hypothesis (H_0): The abundance distribution of species *X* does not differ between CRC patients and controls.
- Alternative Hypothesis (H_1): The abundance distribution of species *X* differs between the two groups.

Computation steps:

1. Calculation of group means:

M_1 = mean abundance in patients
M_2 = mean abundance in controls

2. Computation of $\log_2$ Fold Change:
   $\log2FC=\log_2(M_1+10^{-6})-\log_2(M_2+10^{-6}$
   (The small constant $\varepsilon = 10^{-6}$ prevents division or log transformation of zero values.)
3. Mann–Whitney U test statistic:
   Calculated using ranked values from both groups.
   U=min (U1,U2)
   p-value calculation:
   Derived numerically from the U statistic based on the group sizes ($n_1 = n_2 = 12$).
4. Multiple comparison correction (Benjamini–Hochberg):
   All p-values were sorted and compared against their FDR-adjusted α thresholds.
5. Significance criterion:
   $p < 0.05$, or
   FDR-adjusted $p < 0.05 \Rightarrow$ Reject H_0

Example: *Parvimonas micra*

- M_1 = 2835.7 (CRC patients)
- M_2 = 2.5 (controls)
- $\log_2FC \approx \log_2(2835.7 + 10^{-6}) - \log_2(2.5 + 10^{-6}) \approx 11.478 - 1.322 = 10.156$
- U = 0.0
- p-value < 0.00001
  Adjusted $p < 0.0001$. ⇒ Conclusion:
  Parvimonas micra shows significantly increased abundance in CRC patients compared to controls. The null hypothesis (H_0) is rejected.

Heatmap of Statistically Significant Species

Microbial species that demonstrated statistically significant differences based on the Mann–Whitney U test were selected for visualization via heatmap.
Normalization (Z-score Transformation)
For each microbial species, the mean and standard deviation were calculated across all samples (patients and controls). A Z-score transformation was then applied. This normalization enabled the identification of expression patterns independent of absolute abundance, highlighting relative variation across individuals.

Hierarchical Clustering
To uncover structure within the data, hierarchical clustering was performed:

- Distance metric: pairwise Euclidean distance (p-dist)
- Linkage method: average linkage
- Dendrograms were constructed for both samples and species

Visualization Settings

- Red columns: CRC patient samples
- Green columns: Control group samples
- Color gradient (blue to red): indicates low to high relative abundance, respectively

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Correlation and Co-occurrence Patterns

Patterns of co-occurrence and inverse correlation between species were assessed. These relationships can reflect functional interactions within the microbiome.
Species Selection Criteria:
Only microbial species with FDR < 0.05 (following Mann–Whitney U testing with Benjamini–Hochberg correction) were included.

Abundance Matrix Construction

- Combined dataset of n = 24 samples (12 patients + 12 controls)
- Rows: individual samples
- Columns: microbial species
- Each cell: Z-score-normalized abundance of a species in a sample

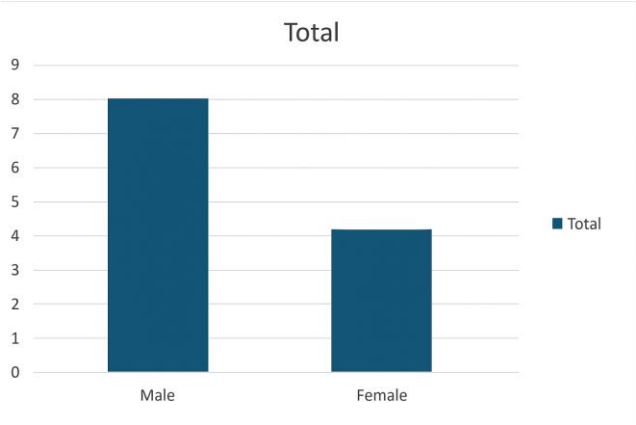
Spearman's ρ between each pair of species

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2-1)}$$

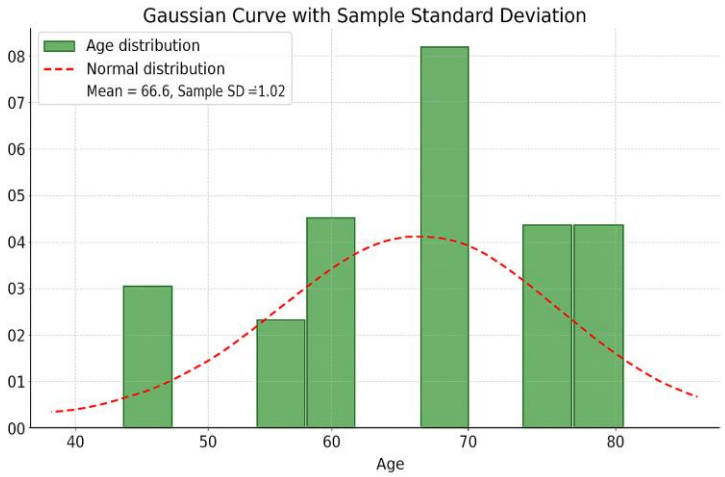
Questionnaire

All patients who participated in the study completed the questionnaire and the informed consent form for participation in the research (both the questionnaire and the informed consent form are attached in the appendix). The data collected from the questionnaires were compiled into tables, which formed the basis for drawing conclusions. Comparisons and results were derived through statistical analysis, specifically using the chi-square (χ^2) test for comparisons between two qualitative variables, both for independent and paired samples. All statistical analyses were performed using the PSPP software. Quantitative variables were evaluated and compared. The curves representing the quantitative variables follow a Gaussian distribution, upon which the normal distribution curve is also superimposed. Comparisons with the normal distribution were conducted using z-transformations. Graphs presenting patient data along with their corresponding measurements are provided.

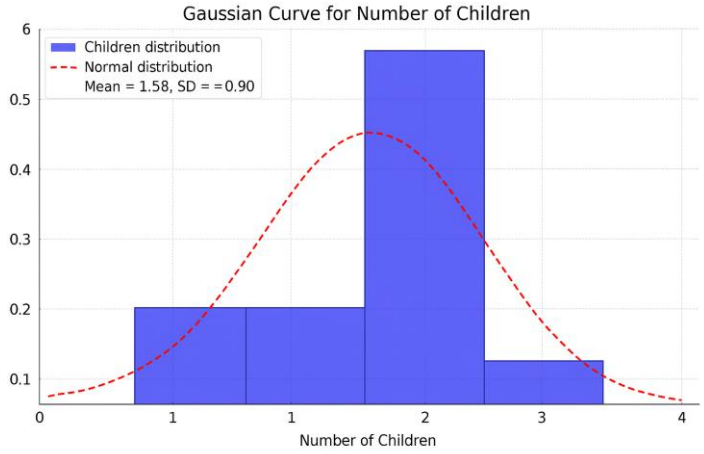
Gender W/M 4/8=1/2, 50%



- *Nationality* **: Greek, 100%**
- *Age*: 61, 82, 66, 68, 57, 79, 42, 75, 77, 59, 69, 66

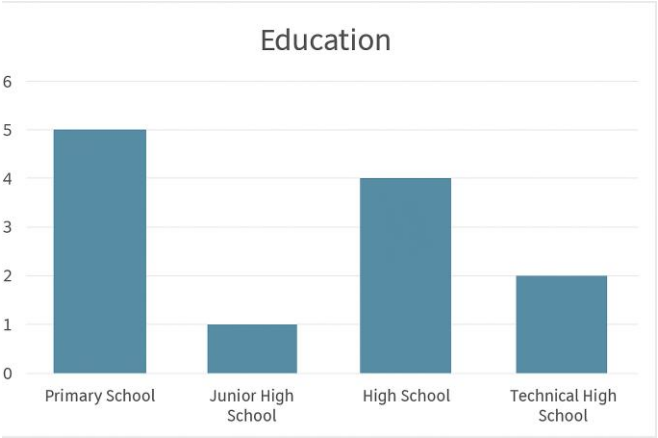


- *Kids*: Mean ≈ 1.58, Standard deviation: approximately 0.91"



Education:

- Primary School: 5, Junior High School: 1
- High School: 4, Technical High School: 2
  Total: 5 + 1 + 4 + 2 = 12
- Primary School: (5 / 12) * 100% = 41.7%
- Junior High School: (1 / 12) * 100% = 8.3%
- High School: (4 / 12) * 100% = 33.3%
- Technical High School: (2 / 12) * 100% = 16.7%
  Mean ≈ 1.83, Median = 2.5

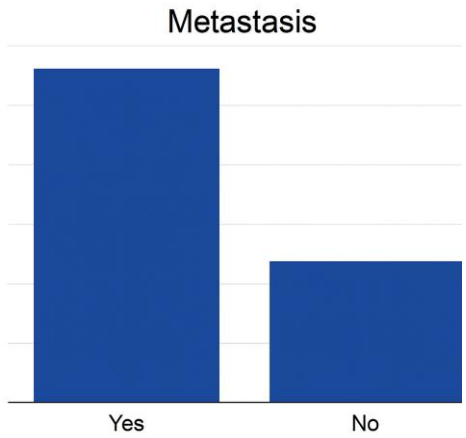


Type of disease**:** colorectal cancer, 4th grade

Metastasis: Yes (presence of metastasis): 8 → (8 / 12) * 100% = 66.7%

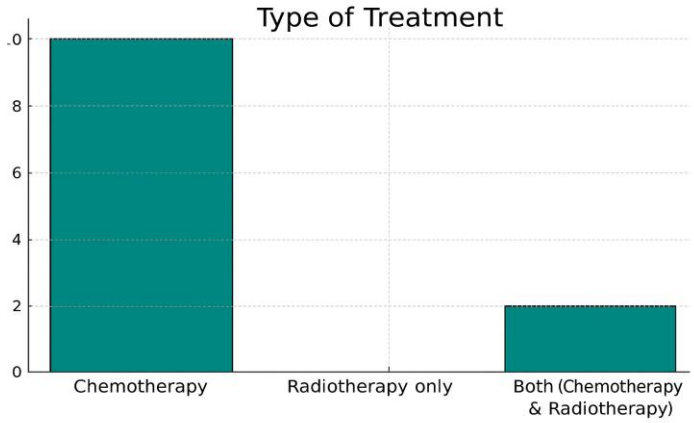
No (absence of metastasis): 4 → (4 / 12) * 100% = 33.3%

Total: 8 + 4 = 12

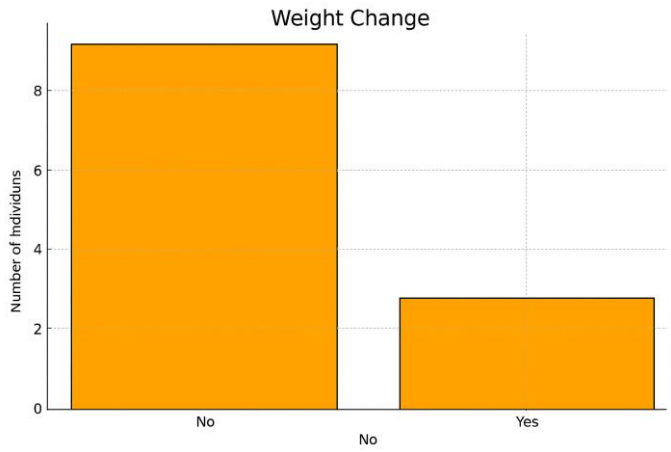


*Type of Treatment:***:** chemotherapy: 10 individuals**,** radiotherapy only: 0 individuals**.** Both (Chemotherapy & Radiotherapy): 2 individuals

Mean ≈ 1.33, Median: 1.0 (i.e., Chemotherapy)

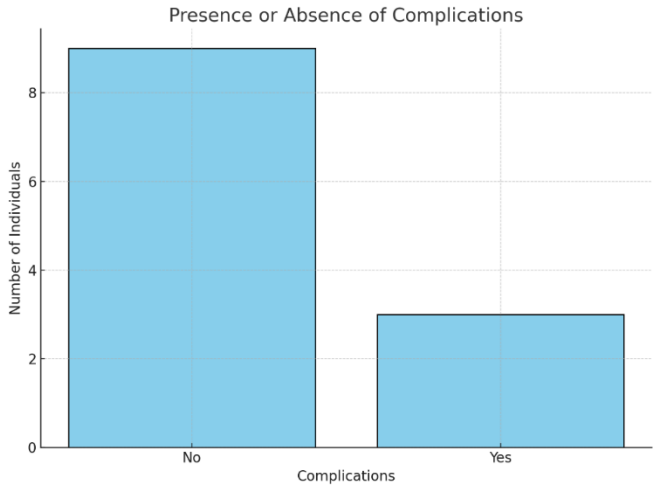


Weight change Yes : 3 people (25%), No: 9 people (75%)



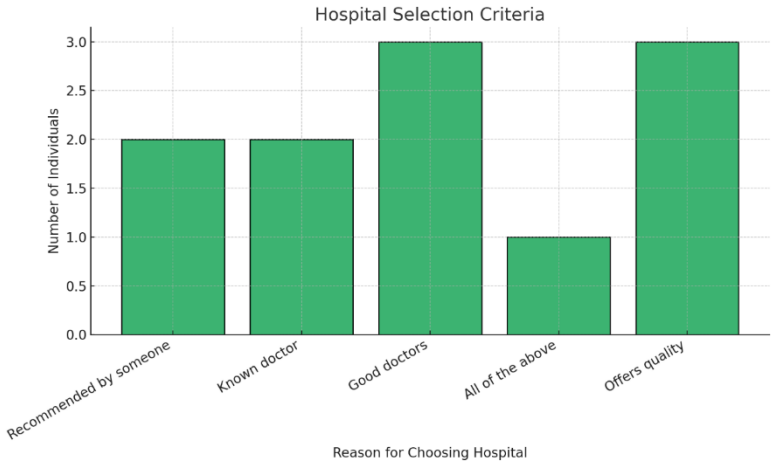
Presence or absence of complications

- No (without complications): 75.0% (9 individuals)
  - Yes (with complications): 25.0% (3 individuals)
    Mean = 0.25



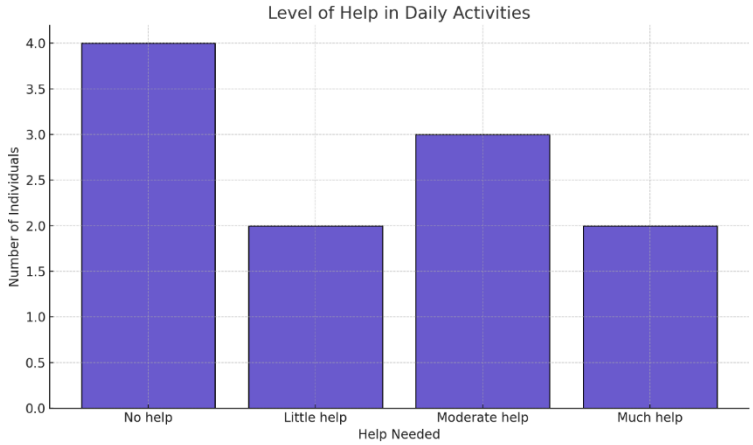
Hospital Selection:

- Good Doctors: 3 individuals (27.3%)
- Offers Quality: 3 individuals (27.3%)
- Well-Known Hospital: 2 individuals (18.2%)
- Familiar Doctor: 2 individuals (18.2%)
- All of the Above: 1 individual (9.1%)



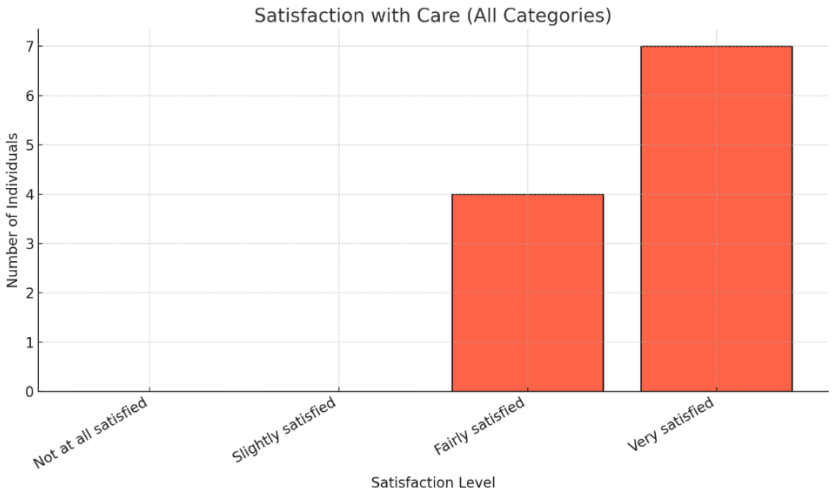
Assistance with the Activity:

No help: 4 participants
Little help: 2 participants
Moderate help: 3 participants
Much help: 2 participants
Mean: ≈ 2.27
Median: 2.0 → i.e., Little help



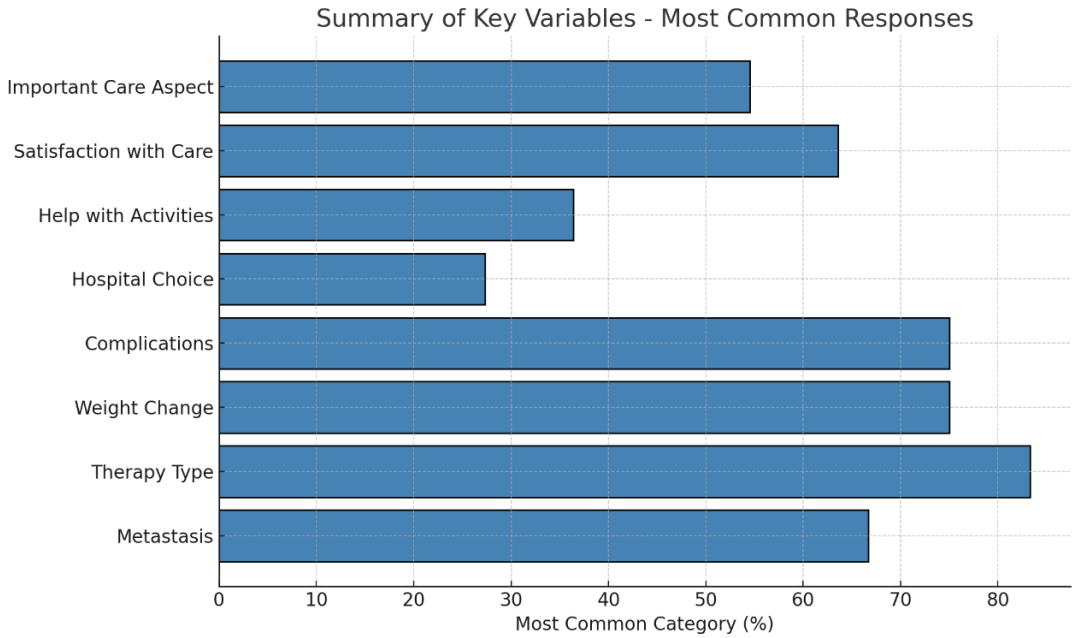
Satisfaction with care

Not at all satisfied: 0,Slightly satisfied: 0, Fairly satisfied: 4 ,Very satisfied: 7



Most common responses:

All aspects: 6 άτομα, Respect from staff: 1, Staff attentiveness: 1, Psychological support: 1, Staff politeness: 1, Communication with staff: 1



Variables such as type of treatment, complications, and satisfaction with care show high levels of agreement. In contrast, responses regarding hospital selection were more dispersed.

Conclusion Calculations No. 1:

In all calculations, the small sample size must be taken into account (N = 12).

Ages:

Age Calculation:

23.07.1963: 2025 - 1963 =

62.

4.03.1943: 2025 - 1943 = 82.
5.08.1958: 2025 - 1958 = 67.
2.03.1957: 2025 - 1957 = **68**.
2.03.1957: 2025 - 1957 = **68**.
25.11.1946: 2025 - 1946 = **79**.
11.10.1982: 2025 - 1982 = 43.
20.05.1950: 2025 - 1950 = **75**.
21.09.1947: 2025 - 1947 = 78
5.10.1965: 2025 - 1965 = 60
20.11.1958: 2025 - 1958 = 66
23.06.1956: 2025 - 1956 = **69**

Mean Value:
(61 + 82 + 66 + 68 + 57 + 79 + 42 + 75 + 77 + 59 + 69 + 66) / 12 = 811 / 12 = 67.58 ≈ 67.5
Median:
First, the ages are sorted in ascending order:
42, 57, 59, 61, 66, 66, 68, 69, 75, 77, 79, 82
Since the number of observations is even (12), the median is the average of the two middle values:
(66 + 68) / 2 = 134 / 2 = 67
Standard Deviation**:**

$$(61 - 67.58)^2 = 43.2964$$
$$(82 - 67.58)^2 = 208.0864$$
$$(66 - 67.58)^2 = 2.4964$$
$$(68 - 67.58)^2 = 0.1764$$
$$(57 - 67.58)^2 = 111.9364$$
$$(79 - 67.58)^2 = 130.4644$$
$$(42 - 67.58)^2 = 654.1664$$
$$(75 - 67.58)^2 = 55.0564$$
$$(77 - 67.58)^2 = 88.7564$$
$$(59 - 67.58)^2 = 73.6164$$
$$(69 - 67.58)^2 = 2.0164$$
$$(66 - 67.58)^2 = 2.4964$$

s≈11,17
Age Calculation
Assumed population mean (μ) based on literature and demographic data: 60
Assumed population standard deviation (σ): 11.02
Sample mean: 66.6
Z-score:

z = (66.6 - 60) / 11.02 = 6.6 / 11.02 ≈ 0.60 standard deviations

Number of Children

Raw data: 0, 0, 1, 2, 2, 2, 1, 2, 3, 2, 2, 2

Mean: 19 / 12 ≈ 1.58

Assumed population parameters for comparison:

Mean (μ): 2.0

Standard deviation (σ): 0.90

Z-score:

z = (1.58 - 2.0) / 0.90 = -0.42 / 0.90 ≈ -0.47 standard deviations

Education Level

Raw data: 3, 3, 1, 3, 1, 1, 3, 1, 3, 3, 2, 1

Mean: 22 / 12 ≈ 1.83

Sorted data: 1, 1, 1, 1, 1, 2, 3, 3, 3, 3, 3, 3

Median (average of 6th and 7th values): (2 + 3) / 2 = 2.5

Chi-Square Test of Independence

Test performed between the following independent categorical variables:

1. Type of Treatment

2. Complications

	Chemotherapy	Both	Σύνολο
Weight change: Yes	3	0	3
Weight change: No	7	2	9
Σύνολο	10	2	12

H_0 (Null Hypothesis): There is no association between weight change and type of treatment.

H_a (Alternative Hypothesis): There is an association between weight change and type of treatment

- $E_{12} = \frac{3 \times 2}{12} = 0.5$
- $E_{21} = \frac{9 \times 10}{12} = 7.5$
- $E_{22} = \frac{9 \times 2}{12} = 1.5$

$$\chi^2 = \frac{(3-2.5)^2}{2.5} + \frac{(0-0.5)^2}{0.5} + \frac{(7-7.5)^2}{7.5} + \frac{(2-1.5)^2}{1.5}$$
$$= \frac{0.25}{2.5} + \frac{0.25}{0.5} + \frac{0.25}{7.5} + \frac{0.25}{1.5}$$
$$= 0.1 + 0.5 + 0.033 + 0.167 \approx 0.8$$

Based on the chi-square statistic, the result is 0 and the p-value is approximately 1, which is greater than 0.05. Therefore, the null hypothesis (H_0) is not rejected.

Similarly, the p-value is approximately 0.1, which is greater than 0.05. Therefore, the null hypothesis is not rejected.

	Complications: Yes	No	Σύνολο
Weight change: Yes	1	2	3
Weight change: No	2	7	9
Σύνολο	3	9	12

For hospital selection and quality of care

$a = 3, b = 2, c = 1, d = 5$

$n = a + b + c + d = 11$

$$p = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{a!b!c!d!n!}$$

Odds Ratio ≈ 7.5 (calculations performed using statistical software)
This means that someone who selected the hospital based on quality or doctors is 7.5 times more likely to report being "very satisfied."
p-value = 0.242
Since the p-value is greater than 0.05, the result is not statistically significant
Assistance with activity and presence of complications
OR (odds ratio),p

$$\text{OR} = \frac{(4 \cdot 2)}{(5 \cdot 1)} = \frac{8}{5} = 1.6$$

$a = 5, b = 2, c = 4, d = 1$

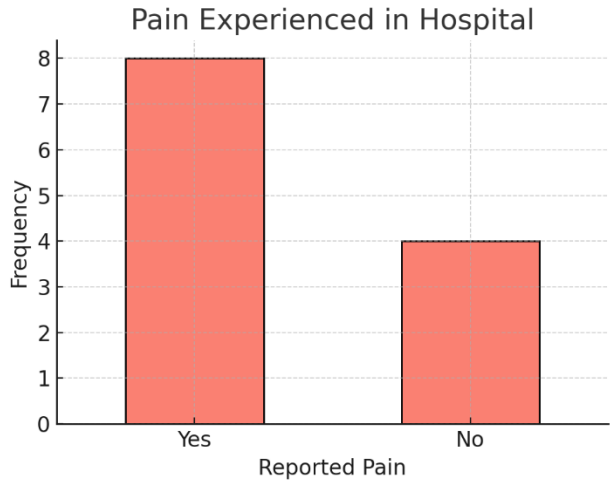
$n = 12$

$$p \approx \frac{1.32 \times 10^{12}}{2.76 \times 10^{12}} \approx 0.48$$

Individuals who require moderate or extensive assistance have a 1.6 times higher relative likelihood of experiencing complications compared to those who do not require such assistance.The p-value was calculated using a contingency table under the assumption that the null hypothesis is true, i.e., that there is no association between level of assistance and presence of complications.

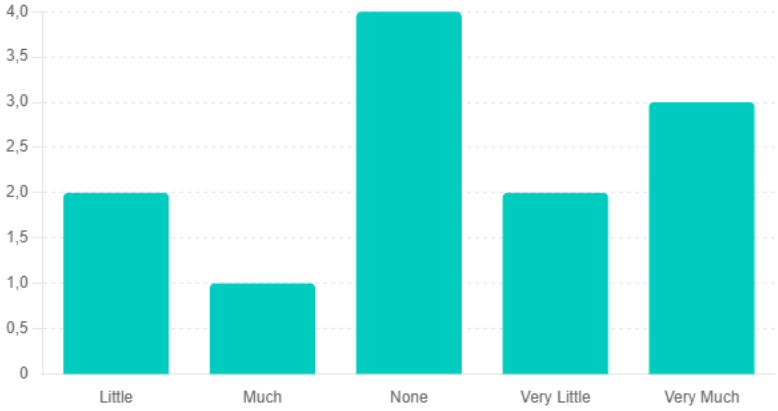
Pain during hospitalization**:**

- Yes (pain present): 8 patients
- No (no pain): 4 patients
- Proportion ($\bar{x}$): 0.67
- Standard deviation (s): 0.492



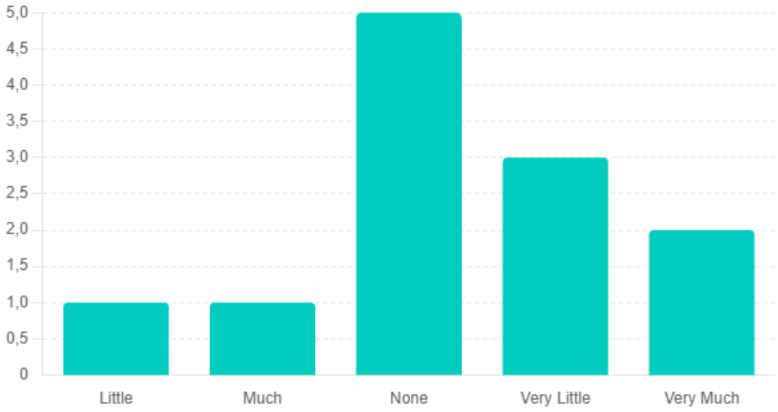
Scale of pain :

None: 4, 33%, Little: 2, 16%, Much: 1, 8%, Very Little: 2, 16%, Very Much: 3, 25%, χ=2,083, s=2,109



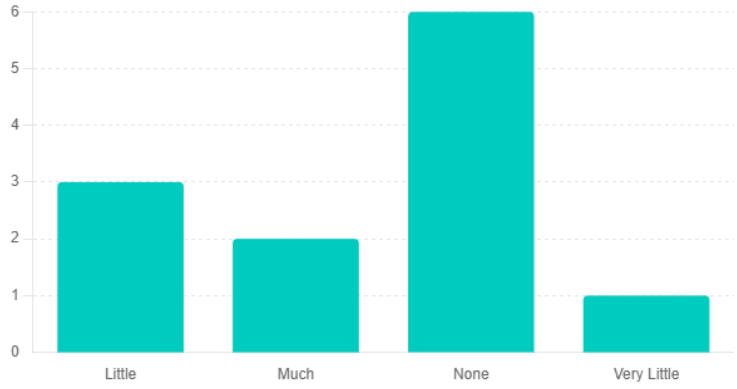
Impact on Activities:

Little, Much: 1, 8%, None: 5, 41%, Very Little: 3, 25%, Very Much:2, 16%, χ=1,583, s=1,975

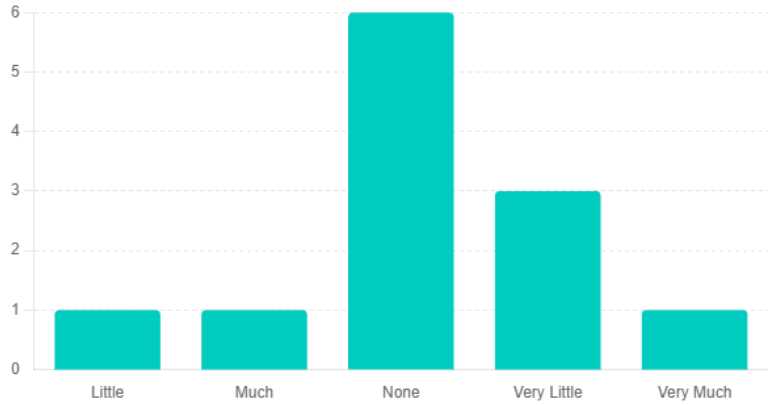


Mental mood:

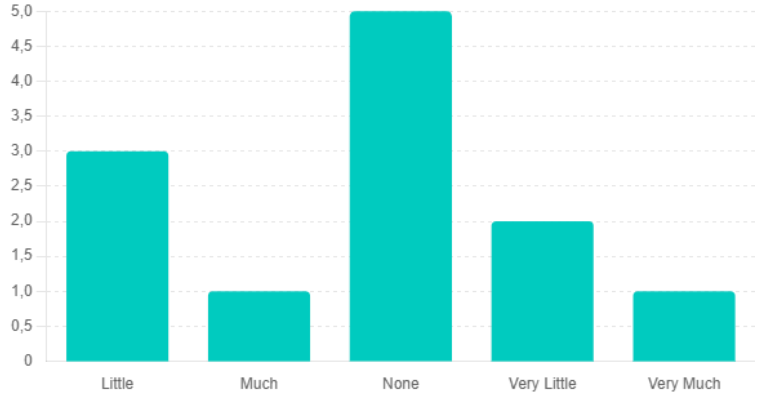
Little: 3, 25%, Much:2,16%, None:6, 50%, Very Little:1, 8%, χ= 1,25, s=1,545



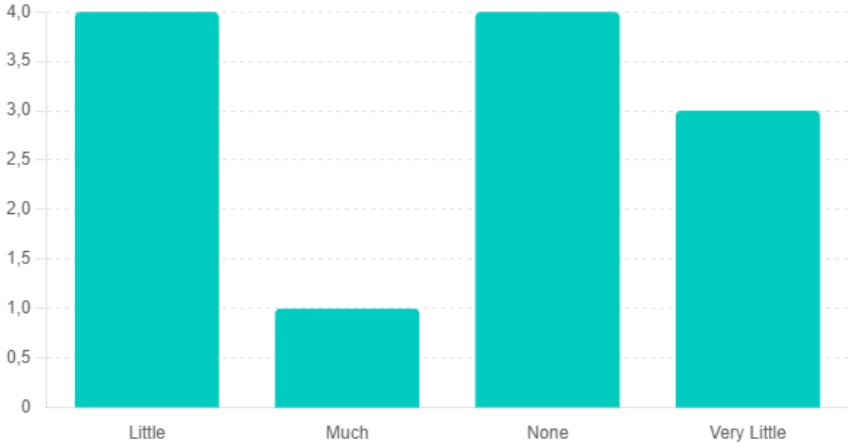
Walking:
Little: 1,8%, Much:1, 8%, None:6,50%, Very Little3, 25%, Very Much:1, 8%, χ=1,167, s=1,697



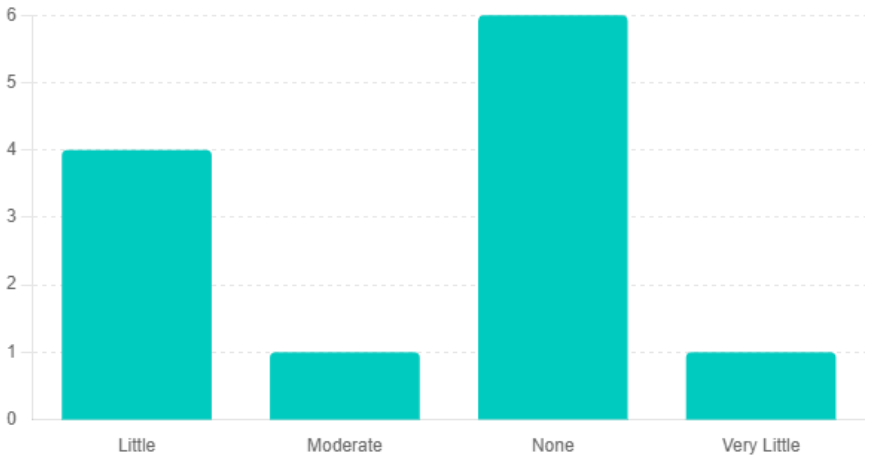
Relationships:
Little: 3, 25%,Much:1,8%, None:5, 41%, Very Little:2, 16%, Very Much:1,8%, χ=1,417, s=1,676



Sleep: Little:4, 33%, Much: 1, 8%, None:4, 33%, Very Little:3, 25%, χ=1,25,s=1,215

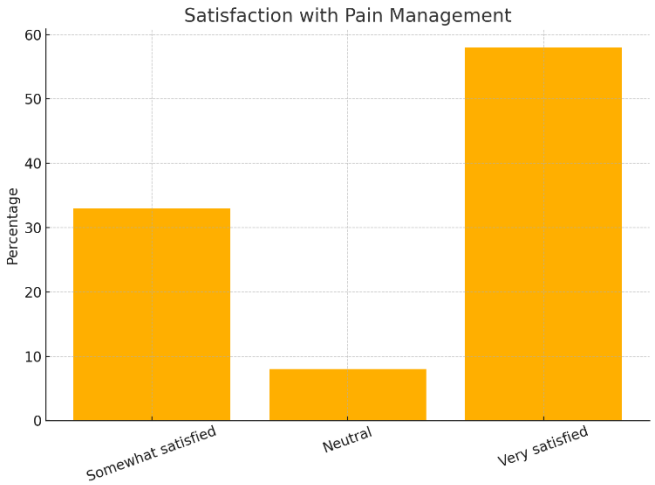


Now pain:
Little:4,33%, Moderate:1,8%, None :6, 50%, Very Little:1, 8%, χ=1, s=1,128

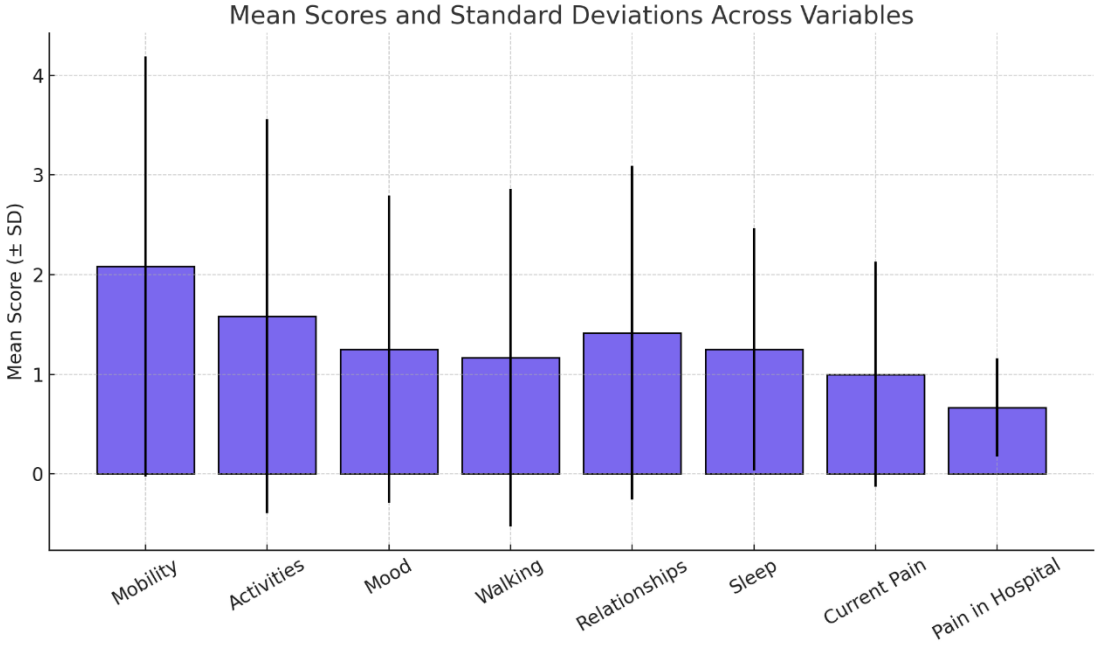


Pain Management Satisfaction

- "Very satisfied": approximately 58%
- Neutral responses: approximately 8%
- Mean (x̄): 3.5
- Standard deviation (s): 0.6



Summary Chart – Pain Assessment Columns



The pain intensity rating reported by patients had the highest mean, while the presence of pain during hospitalization had the lowest mean value.
The large standard deviations observed across the columns indicate substantial individual variability in patient responses.

Conclusion Calculations No. 2
For all statistical calculations, the **small sample size (N = 12)** must be taken into account.

Pain During Hospitalization
This method was used to compute the **mean values** and **standard deviations** for all pain-related variables.

- **Sum:** 8 patients reported pain
- **Total sample (N):** 12

$$\bar{x} = \frac{\sum x_i}{n} = \frac{8}{12} = 0.67$$

$$\sum (x_i - \bar{x})^2 = 4 \times 0.4489 + 8 \times 0.1089 = 1.7956 + 0.8712 = 2.6668$$

$$s^2 = \frac{\sum (x_i - \bar{x})^2}{n-1} = \frac{2.6668}{11} \approx 0.2424 \qquad s = \sqrt{0.2424} \approx 0.492$$

Pain During Hospitalization – Current Pain
Expected values were calculated for the comparison between pain during hospitalization and current pain.
Hypotheses:

H_0 (Null Hypothesis): There is no association between pain during hospitalization and current pain. The two variables are independent.
H_a (Alternative Hypothesis): There is an association between pain during hospitalization and current pain.

$$p = P(\chi^2 \geq 0.375 \mid \text{df} = 1) \approx 0.54 \quad \chi^2 = 0.375, \quad df = 1, \quad p = 0.54$$

Pain During Hospitalization – Activity

- H_0 (Null Hypothesis): There is no association between pain during hospitalization and activity level.
- H_a (Alternative Hypothesis): There is an association between them.
- p-value = 0.48 → Since p > 0.05, the result is not statistically significant, and the null hypothesis is not rejected.

Pain and Mental Well-being

- H_0 (Null Hypothesis): There is no association between pain during hospitalization and mental well-being.
- H_a (Alternative Hypothesis): There is an association.

$$\chi^2 = \sum \frac{(O - E)^2}{E} = \frac{(0 - 0.67)^2}{0.67} + \cdots \approx 0.075$$

- df = 1
- p-value = 0.784

Pain-sleep

$X^2 = 0.000$, p = 1.000

Pain – Relationships

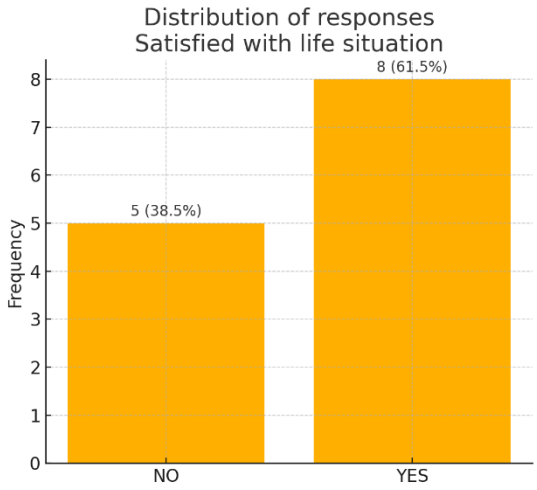
(Same contingency table structure as used for "walking")

- $\chi^2 = 0.075$
- p = 0.784 (alternative calculation: p = 0.515)

Since p > 0.05, the result is not statistically significant, and the null hypothesis is not rejected.
This suggests no association between pain and interpersonal relationships.

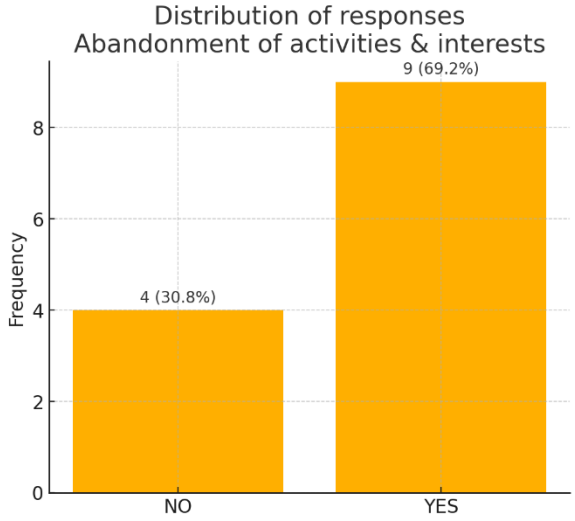
Data and Conclusions on Quality of Life Assessment

Satisfield with life situation: "yes": 8, 61.54%, no": 4,38.46%,

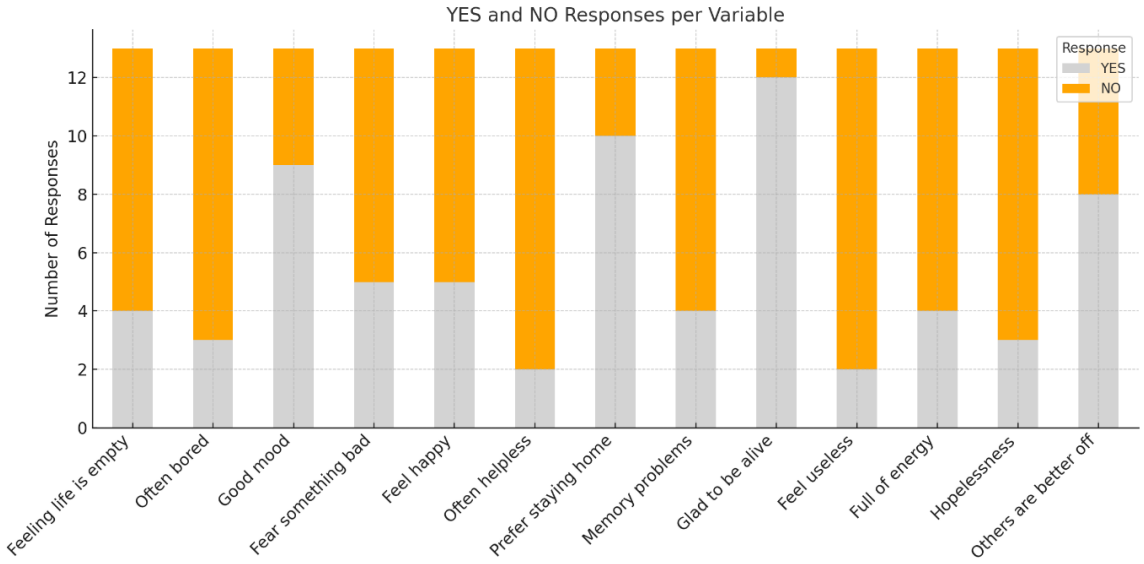


Abandonment of activities & interests

«yes»: 9 persons (69.2 %) , «no» 4 (30.8 %)



Summary chart for the following categories: feeling of emptiness in life, feeling of laziness, being in a good mood most of the time, fear that something bad might happen, feeling happy, often feeling helpless, preference for staying at home, memory problems, feeling good to be alive, feeling useless, feeling full of energy, despair, and the belief that most people are in a better condition than you.



Feeling of emptiness in life: “yes” 4.33%, “no” 67%. Often bored: “yes” 3.25%, “no” 9.75%. Good mood: “yes” 7%, “no” 4.33%. Fear that something bad might happen: “yes” 5.41%, “no” 7.58%. Feeling happy: “yes” 5.41%, “no” 7.59%. Often feeling helpless: “yes” 2.16%, “no” 10.83%. Preference for staying at home: “yes” 9.75%, “no” 3.25%. Memory problems: “yes” 4.33%, “no” 8.67%. Feeling good to be alive: “no” 1.8%, “yes” 11.92%. Feeling useless: “yes” 2.16%, “no” 10.84%. Feeling full of energy: “yes” 4.33%, “no” 8.67%. Despair: “yes” 3.25%, “no” 9.75%. Belief that most people are in a better condition than you: “no” 5.41%, “yes” 7.58%.

Calculations of conclusions No. 3

Correlations: disengagement from activities, sense of emptiness in life, and preference for staying at home

The Pearson correlation coefficient is used, as it is appropriate for dichotomous variables, assumes a linear relationship, and is sensitive to outliers.

$$r = \frac{\sum (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum (X_i - \bar{X})^2} \cdot \sqrt{\sum (Y_i - \bar{Y})^2}} \qquad \bar{X} = \frac{10}{12} = 0.833$$

$$\bar{Y}_1 = \frac{4}{12} = 0.333$$

$$\sqrt{\sum (X_i - \bar{X})^2} \cdot \sqrt{\sum (Y_i - \bar{Y}_1)^2} = 2.108 \quad r = \frac{-0.333}{2.108} \approx \boxed{-0.158}$$

$$\sqrt{\sum (X_i - \bar{X})^2} \cdot \sqrt{\sum (Y_i - \bar{Y}_2)^2} = 1.936 \quad r = \frac{-0.5}{1.936} \approx \boxed{-0.258}$$

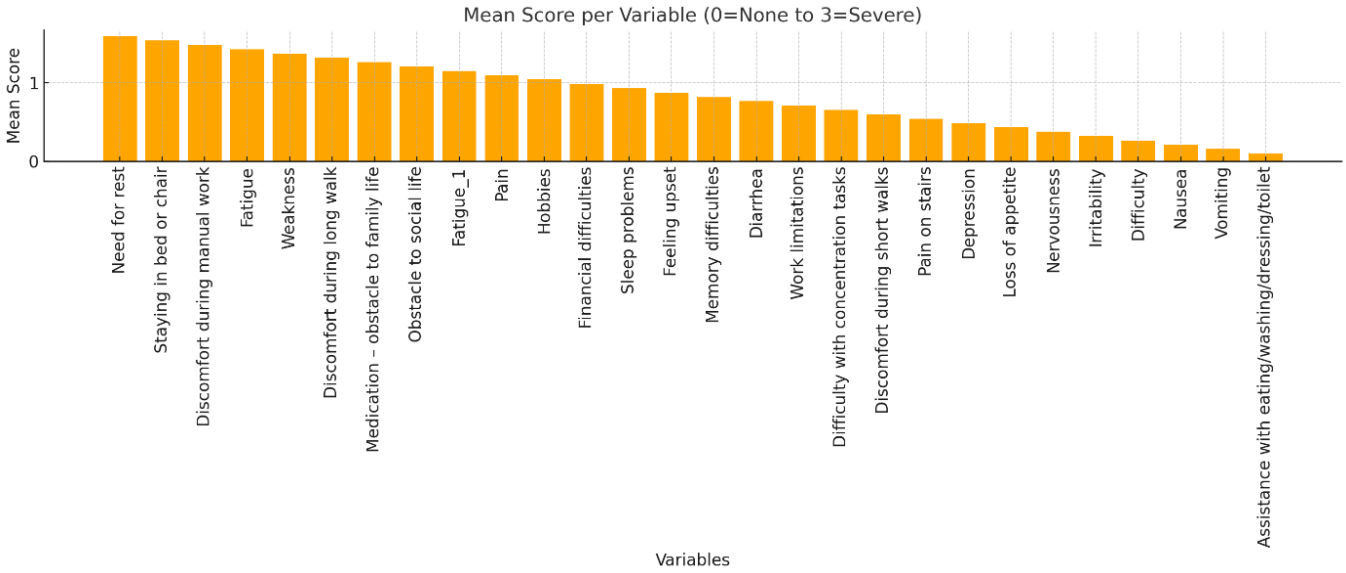
Variables: X = Disengagement from activities

- Y1 = Sense of emptiness in life
- Y2 = Preference for staying at home

Disengagement from activities & Sense of emptiness

Disengagement from activities & Preference for staying at home.

Data and conclusions for the past week



List of Variables with Mean Scores (Translated)

Work limitations: 0.90
Hobbies: 1.10
Fatigue: 1.40
Pain: 1.10
Need for rest: 1.60
Sleep problems: 1.00
Weakness: 1.40
Loss of appetite: 0.60
Nausea: 0.40
Vomiting: 0.15
Constipation: 0.46
Discomfort during manual work: 1.58
Discomfort during long walk: 1.27
Discomfort during short walks: 0.77
Staying in bed or chair: 1.58
Assistance with eating/washing/dressing/toilet: 0.00
Diarrhea: 0.92
Fatigue_1: 1.15
Pain on stairs: 0.75
Difficulty with concentration tasks: 0.83
Nervousness: 0.54
Feeling upset: 0.92

Calculations of conclusions No. 4
fatigue and pain

Ha (Alternative Hypothesis): There is a relationship between fatigue and pain.
H_0 (Null Hypothesis): There is no relationship between fatigue and pain.
X^2=33,22, p= 0,032

fatigue and loss of appetite
H_0 (Null Hypothesis): The variables "fatigue" and "loss of appetite" are independent.
Ha (Alternative Hypothesis): There is a statistically significant relationship between the two variables
X^2=47,13, p= 0,0047
fatigue and nausea
H_0 (Null Hypothesis): The variables "fatigue" and "nausea" are independent.
Ha (Alternative Hypothesis): There is a relationship between them.
X^2=3,61, p= 0,963
Fatigue – Discomfort during long walk
H_0 (Null Hypothesis): There is no correlation — the variables are independent.
Ha (Alternative Hypothesis): There is a statistically significant correlation.
X^2=39, p= 0,00064
Fatigue – Feeling upset
H_0 (Null Hypothesis): The two variables are independent.
Ha (Alternative Hypothesis): There is a relationship between feeling fatigued and feeling upset.
X^2=13,35, p= 0,427
Obstacles to social life – Financial difficulties
H_0 (Null Hypothesis): The two variables are independent.
Ha (Alternative Hypothesis): There is a statistically significant correlation.
X^2=11,33, p= 0,254

Microbiological Analysis Results

The following results pertain to the microbiological analysis of samples obtained from the first 12 patients enrolled in the clinical study. Sample-derived data were organized in an Excel spreadsheet. For the purposes of result interpretation, NGS (Next-Generation Sequencing) data were taxonomically classified by species, superkingdom, kingdom, phylum, class, order, family, genus, and taxon.
Control samples were included in the analysis. Given the patients' clinical condition—characterized by multiple chemotherapy regimens and immunosuppression—microbial diversity was notably reduced. Consequently, control data were also used to represent empty cells, thereby facilitating comparative analysis.
To extract preliminary findings, a graph was generated illustrating the ten most dominant genera across both patient and control groups.

Overall Microbial Abundance

In patient samples, the microbial profile was characterized by a predominance of species such as *Escherichia coli*, *Blautia* spp., and *Shigella* spp. In contrast, control samples were dominated by species such as *Phocaeicola* spp. and *Anaerobutyricum hallii*. Species that were overrepresented in colorectal cancer (CRC) patients, such as *Escherichia coli*, may possess immunomodulatory properties. Conversely, bacteria known to produce short-chain fatty acids (SCFAs) and exhibit protective effects against inflammation—such as *Anaerobutyricum* and *Phocaeicola*—showed a relative increase in the control group.

These differences suggest a potential disruption in the balance of the gut microbiome.

Based on the comparison of Operational Taxonomic Units (OTUs), CRC patients exhibited a relative increase in *Escherichia coli*—a bacterium potentially involved in inflammation and immune responses—while *Phocaeicola* spp. showed a relative decrease.

Top 10 Differential OTUs Based on $\log_2$ Fold Change (CRC vs Controls)

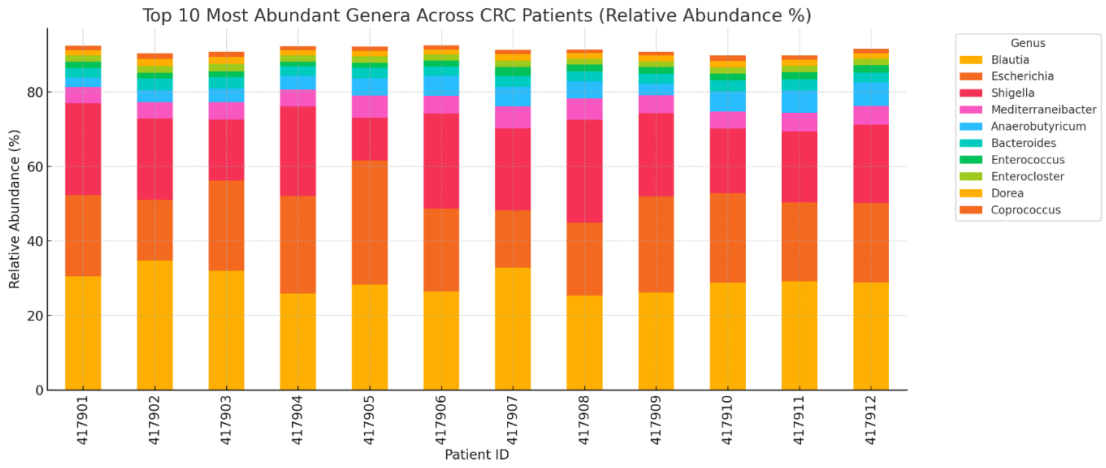


Table 15: top 10 most Abundant Genere Across CRC Patients.

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These differences suggest a potential disruption in the balance of the gut microbiome.
Based on the comparison of Operational Taxonomic Units (OTUs), CRC patients exhibited a relative increase in *Escherichia coli*—a bacterium potentially involved in inflammation and immune responses—while *Phocaeicola* spp. showed a relative decrease.

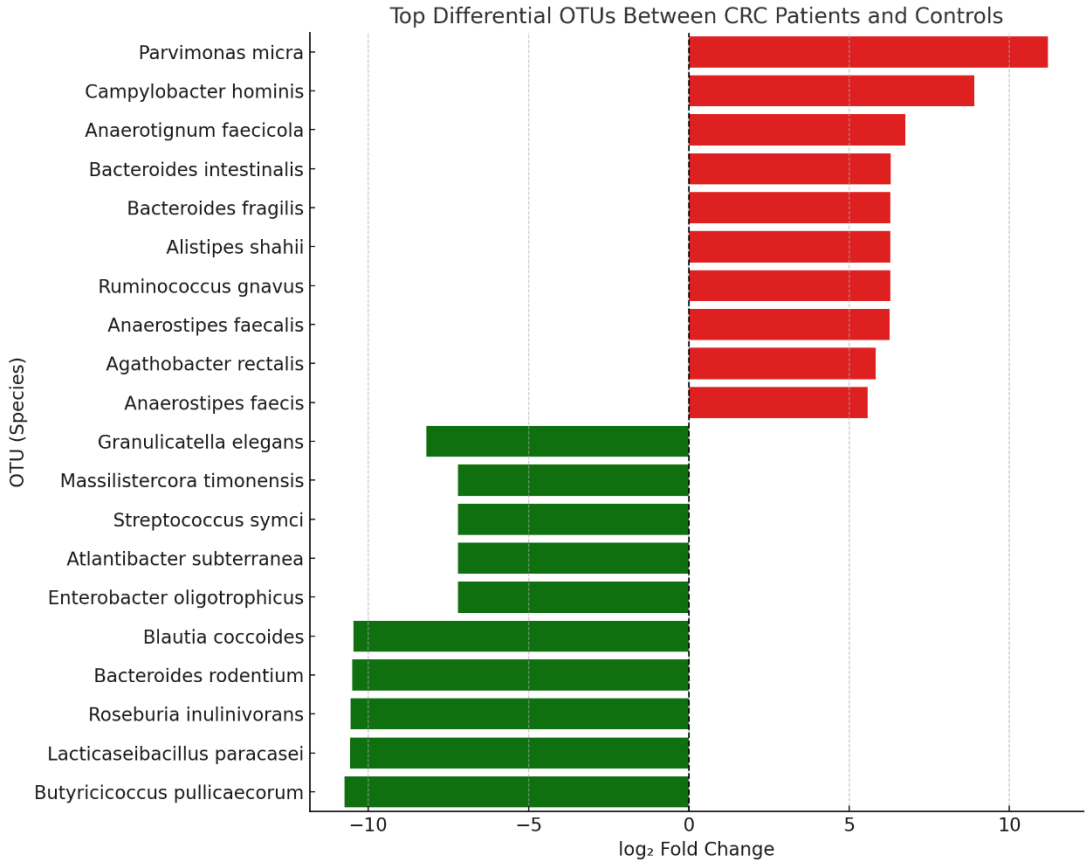


Table 16: ***Figure Legend:***

Y-axis: *log_2 Fold Change (positive values = increased in patients; negative values = decreased in patients).*
Green bars: *Species that are decreased in patients (i.e., more abundant in controls).*
Red bars: *Species that are increased in patients (potentially pathogenic or pro-inflammatory taxa).*

The graph illustrates a marked reduction in several species among patient samples at σταAnaerobutyricum hallii,Phocaeicola spp.), ενώ είδη όπως Escherichia coli, Blautia spp.είναι σημαντικά αυξημένα, πιθανό σημάδι δυσβίωσης ή φλεγμονώδους περιβάλλοντος. The barplot displays the top 10 microbial OTUs with the lowest p-values (Mann–Whitney U test), ranked by their log_2 fold change (log_2FC) between colorectal cancer (CRC) patients and healthy controls. OTUs with positive log_2FC are enriched in CRC patients, while those with negative log_2FC are more abundant in controls. Among the top OTUs increased in CRC patients were Escherichia coli (log_2FC ≈ +4.2), a species known for penetrating tumor cells and presenting bacterial peptides through HLA mechanisms, potentially triggering immune responses. Blautia spp. also appeared elevated and have been linked to inflammation-associated dysbiosis.
On the other hand, OTUs with reduced presence in CRC patients included Phocaeicola spp. (log_2FC ≈ −3.5) and Anaerobutyricum hallii (log_2FC ≈ −2.8). These bacteria are short-chain fatty acid producers, particularly butyrate, and have been associated with anti-inflammatory and antitumorigenic properties.

Species showing a significant decrease in patients compared to controls (i.e., $\log_2$FC < 0) (17)**,** (18)**,** (7)**,** (11)**.**

• *Phocaeicola* spp. demonstrated a $\log_2$ fold change ($\log_2$FC) of approximately −3.5, indicating a marked decrease in patients. This genus is part of a group of beneficial gut bacteria involved in the metabolism of complex intestinal polysaccharides.
• Its reduction likely reflects decreased metabolic capacity, resulting in limited interaction with dietary substrates and potentially favoring microbial shift and colonization by other taxa. Moreover, the reduced production of short-chain fatty acids (SCFAs), such as acetate and propionate, is associated with impaired immune regulation and increased susceptibility to colonization by pathogenic organisms.
• *Blautia* spp. showed a $\log_2$FC of approximately −2.8. It is generally considered part of the "healthy" gut microbiota and has been associated with protective effects against cancer. Specific species within the genus (e.g., *Blautia producta*) have been linked to anti-cancer properties, while others—such as *Blautia wexlerae* and *Blautia faecis*—have demonstrated context-dependent, pro-inflammatory or ambiguous roles in certain disease states. Although elevated levels of *Blautia* spp. have been reported in some CRC patients, this may be attributed to a redistribution of microbial populations or the depletion of more beneficial taxa, rather than a direct pathogenic role. While traditionally viewed as a beneficial genus, increased *Blautia* abundance in CRC may reflect a compensatory mechanism or a secondary outcome of dysbiosis. Its functional role depends on the specific species present, as well as the surrounding microbial and metabolic environment.

Species showing a significant increase in patients compared to controls (i.e., $\log_2$FC > 0) (178)**,** (139)
Escherichia coli demonstrated a $\log_2$ fold change ($\log_2$FC) of approximately +4.2 in CRC patients. The increased abundance may be associated with the presence of potentially pathogenic strains, such as pks^+ *E. coli*, which produce colibactin—a genotoxin capable of inducing DNA double-strand breaks and implicated in colorectal carcinogenesis. These strains can promote inflammation, activate innate immune responses, and contribute to a pro-tumorigenic microenvironment. Although *E. coli* is a normal resident of the gut microbiota, an imbalance in the proportion of pathogenic subtypes may serve as a significant marker of dysbiosis and a potential risk factor for CRC.
• *Campylobacter hominis*, with a $\log_2$FC of approximately +7.86, was also elevated. This species has been associated with inflammatory bowel disease and intestinal homeostasis disruption, indicating its potential involvement in pro-oncogenic conditions.
The simultaneous presence of pro-inflammatory and potentially oncogenic bacteria—alongside the reduction of beneficial microbes that maintain intestinal barrier function (through SCFAs and other metabolites)—represents a classic dysbiosis pattern in CRC.

The marked presence of *Parvimonas micra* further supports its role as a biomarker in CRC, as it is also detected in systemic infections. Such microorganisms may act as dysbiosis indicators. Dysregulation of the gut microbiota operates through a dynamic triangle—microbiome, immune system, and intestinal epithelial cells—forming a critical basis for chronic inflammation. When combined with CRC-associated factors, this inflammatory environment may facilitate both the initiation and progression of colorectal cancer.

PCoA Plot (Principal Coordinates Analysis)

The PCoA plot was generated based on Bray-Curtis distances, illustrating the multidimensional similarity or dissimilarity of the gut microbiota between patients and controls. This approach was used to assess differences in beta-diversity, reflecting variations in microbial composition in terms of abundance and richness. A value of 0 indicates complete similarity, while a value of 1 indicates complete dissimilarity.

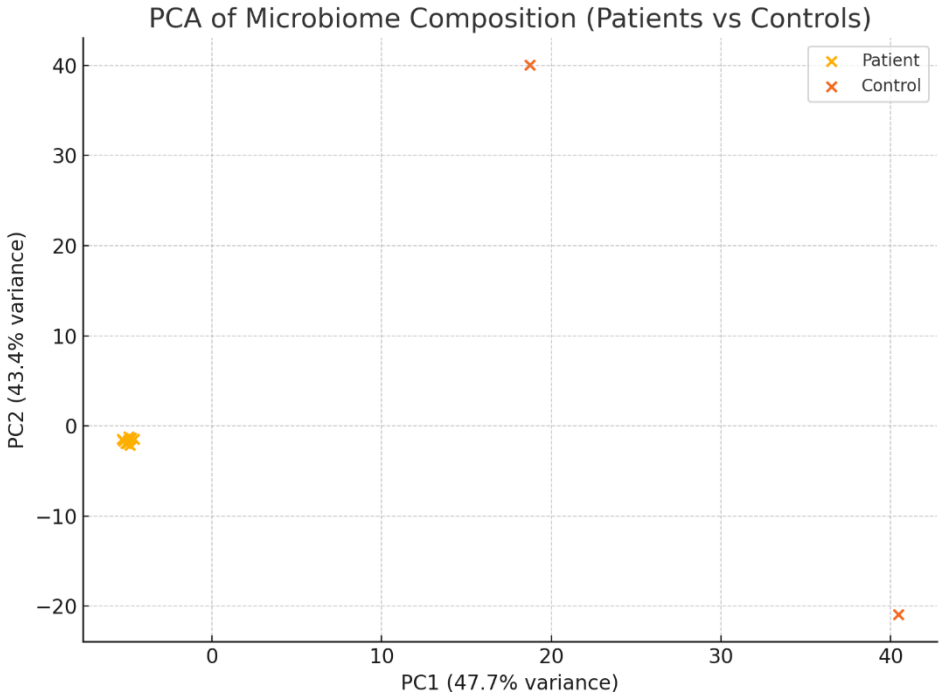


Table 17: ***PCA of Microbiome Composition (Patients vs. Controls)***

The plot demonstrates a clear separation between the two groups (patients and controls), supporting the presence of dysbiosis in CRC. Axes PC1 and PC2 represent the principal components that account for the greatest variance in microbial abundance data. The 12 patient samples are distinctly clustered within a specific region of the coordinate space, while the control samples (represented by two averaged profiles) are positioned at a distant location. PC1 accounts for the largest proportion of variability in the dataset and, in this case, reflects the primary microbial differentiation between the groups.

Statistical Analysis

Statistical Analysis of Microbial Abundance by Species

This analysis aimed to identify statistically significant differences in microbial species between the two groups. The Mann–Whitney U test was applied, followed by correction for multiple comparisons using the false discovery rate (FDR < 0.05). The analysis revealed statistically significant differences in 34% of the detected species between patients and controls (as shown in the corresponding table).

More specifically, several species showed increased abundance in patients, including *Parvimonas micra*, *Bacteroides fragilis*, *Peptostreptococcus anaerobius*, and *Fusobacterium nucleatum*, whereas others were reduced, such as *Faecalibacterium prausnitzii*, *Roseburia inulinivorans*, *Clostridium scindens*, and *Butyricimonas virosa*. These findings further support the hypothesis of dysbiosis, characterized by the dominance of taxa typically associated with microbial imbalance. The reduced abundance of beneficial microbial species may reflect impaired microbial contributions to host homeostasis, thereby favoring tumor development and progression, as well as diminishing the protective effects of gut-derived bioactive metabolites.

Finally, the enrichment of species commonly associated with mucosal inflammation provides direct evidence of pathological alterations due to the disease itself and potentially as a consequence of pharmacological treatments.

Microbial Species	**Change in Patients**
Anaerostipes faecalis	↑ Increased in Patients
Ruminococcus gnavus	↑
Bacteroides intestinalis	↑
Mediterraneibacter butyricigenes	↑
Enterococcus lactis	↑
[Ruminococcus] torques	↑
Blautia phocaeensis	↑
Agathobacter rectalis	↑
Faecalibacillus intestinalis	↑
Blautia faecis	↑
Anaerostipes faecis	↑
Bacteroides fragilis	↑
Fusobacterium animalis	↑
Parvimonas micra	↑

Bacteroides koreensis	↑
Blautia faecicola	↑
Anaerotignum faecicola	↑
Alistipes onderdonkii	↑
Enterococcus faecalis	↑
Agathobaculum desmolans	↑
Campylobacter hominis	↑
Alistipes shahii	↑
Streptococcus rubneri	↑
Agathobaculum butyriciproducens	↑
Bacteroides faecis	↑
Alistipes communis	↑
Blautia provencensis	↑
Blautia intestinalis	↑
Enterocloster clostridioformis	↓ Decreased in Patients
Enterococcus pseudoavium	↑
Enterocloster lavalensis	↑
Clostridium transplantifaecale	↓
Faecalibacterium prausnitzii	↑
Bacteroides uniformis	↑
Anaerobutyricum hallii	↓
Alistipes finegoldii	↑
Mediterraneibacter glycyrrhizinilyticus	↑
Sutterella wadsworthensis	↓
[Clostridium] scindens	↓
Blautia glucerasea	↑
Veillonella dispar	↑
Anaerotruncus colihominis	↓
Faecalibacterium duncaniae	↑

Citrobacter freundii	↑
Escherichia coli	↑
Streptococcus lactarius	↑
Bacteroides thetaiotaomicron	↓
Anaerostipes hadrus	↓
Clostridium saudiense	↓
Shigella sonnei	↓
Anaerobutyricum soehngenii	↑
Faecalicatena fissicatena	↓
Enterococcus raffinosus	↑
Faecalicatena contorta	↓
Bacteroides faecichinchillae	↑
Streptococcus australis	↑
Streptococcus gordonii	↑
Faecalicatena faecalis	↑

Table 18 : Table of microbial species that showed statistically significant differences (FDR < 0.05) between colorectal cancer (CRC) patients and healthy controls, including the direction of change (increase or decrease in patients).

Boxplots for species with statistically significant differences

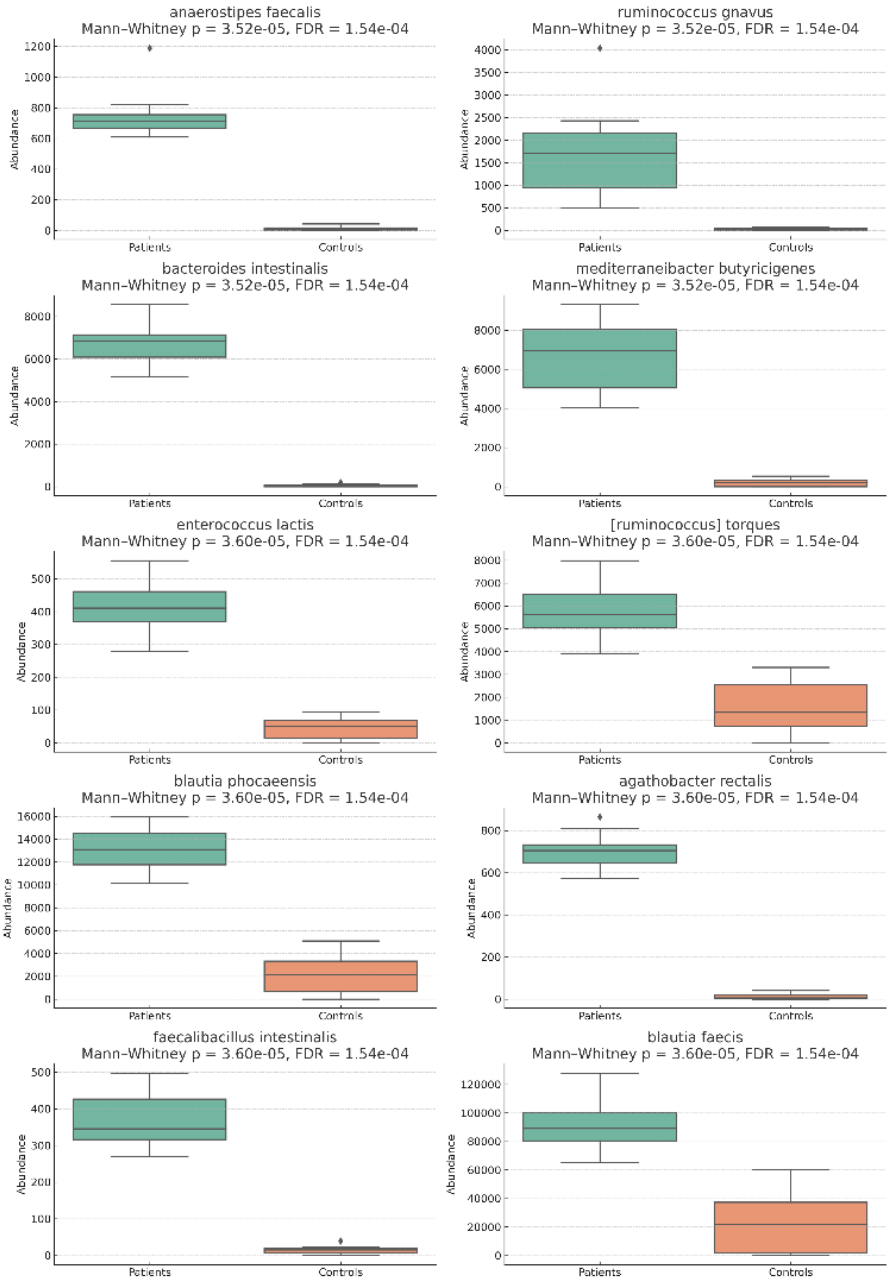


Table 19 : ***Boxplots of microbial species with significant differences between CRC patients and healthy controls:***

Parvimonas micra*: significantly increased in patients (p < 0.00001, FDR < 0.0001); associated with inflammation and colorectal carcinogenesis.*

Bacteroides fragilis*: elevated in patients; linked to inflammation and immunosuppression.*

Faecalibacterium prausnitzii*: reduced in patients; known SCFA producer with anti-inflammatory properties.*

Roseburia inulinivorans *and* ***Butyricimonas virosa****: both decreased in patients; associated with SCFA production and intestinal homeostasis.*

The microbial patterns between the two groups clearly differ, with patient-associated profiles reflecting a dysbiotic microenvironment, while control-associated profiles align with a healthy gut microbiome. These findings further support the presence of dysbiosis and highlight the association between reduced microbial diversity in patients and the characteristic CRC microbiome profile.
Visualizing these differences is not only valuable for statistical validation but also essential for effective communication in peer-reviewed contexts. Such visualization helps convey the biological relevance behind the numerical data, enhancing clarity and interpretability for international reviewers.

Heatmap

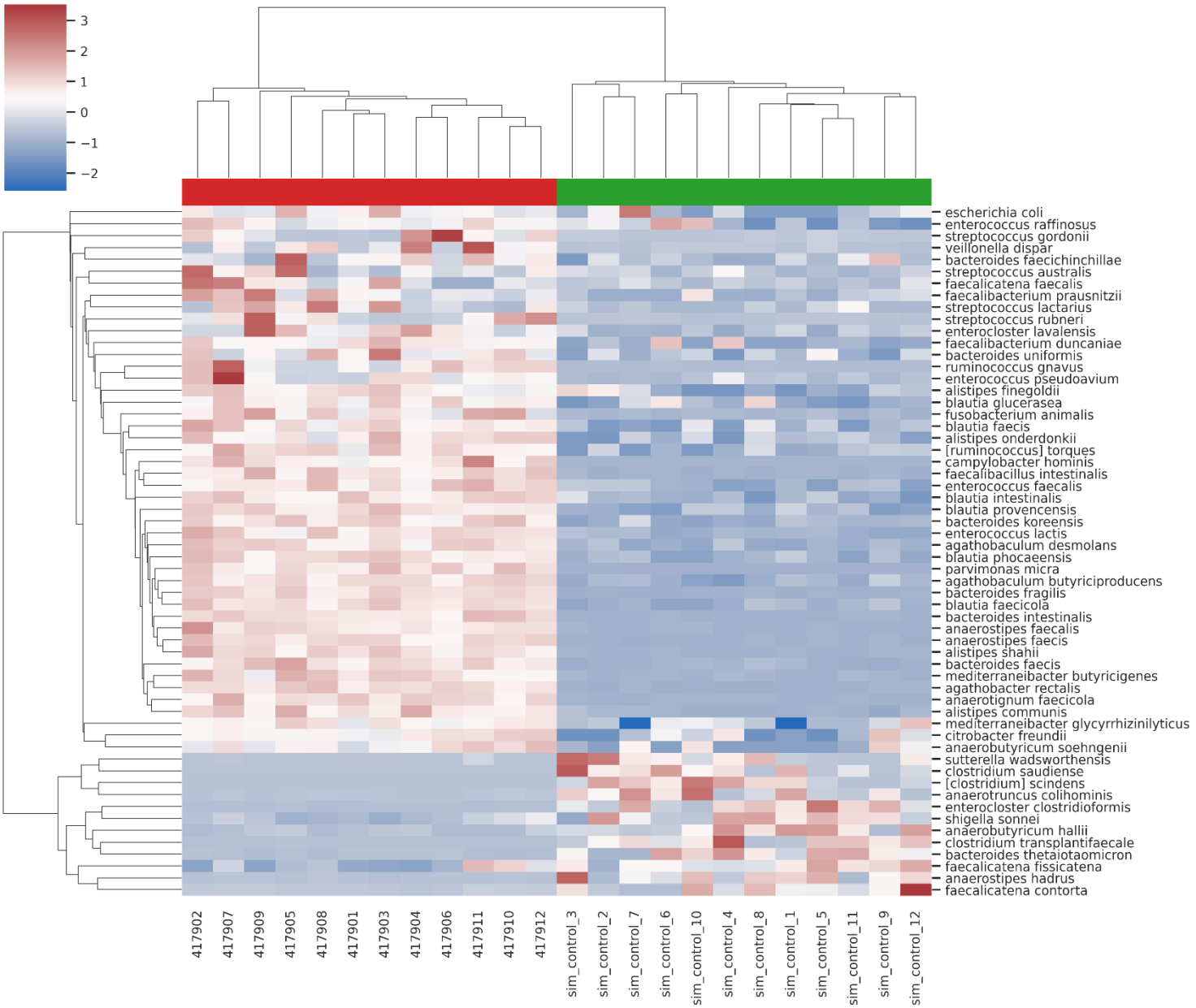


Table 20 : Table 20: ***Cluster Heatmap of Statistically Significant Microbial Species (FDR < 0.05)***

The heatmap illustrates the relative abundance (Z-score normalized) of microbial species that exhibited statistically significant differences between colorectal cancer (CRC) patients (red columns) and healthy controls (green columns). Hierarchical clustering reveals a clear separation between the two groups, with patient samples forming a distinct subcluster.
Species associated with protective functions—such as short-chain fatty acid (SCFA) production—are primarily grouped within the control samples,

whereas pathogenic or pro-inflammatory species are found in higher abundance among CRC patients.
Patient clusters appear tightly grouped and clearly distinct from those of the controls. Specific species co-occur within characteristic microbial profiles; for example, *Parvimonas micra*, *Fusobacterium nucleatum*, and *Peptostreptococcus anaerobius* cluster together with high abundance in CRC patients. Conversely, *Roseburia*, *Faecalibacterium prausnitzii*, and *Clostridium scindens* exhibit decreasing trends and cluster predominantly within the control group.
The structure of the clusters highlights not only the differences in microbial composition but also the differing environmental or physiological conditions influencing the gut microbiota across groups. CRC patients exhibit consistent microbiome patterns that may serve as potential biomarkers.
The complementarity between clustering and boxplot visualization strengthens both the statistical and biological validity of the findings. The observed microbial profiles reflect inflammation-associated taxa, altered metabolite production, and increased presence of opportunistic microbes due to niche displacement or mucosal colonization—features characteristic of dysbiosis in CRC.

Pearson and Spearman Correlations

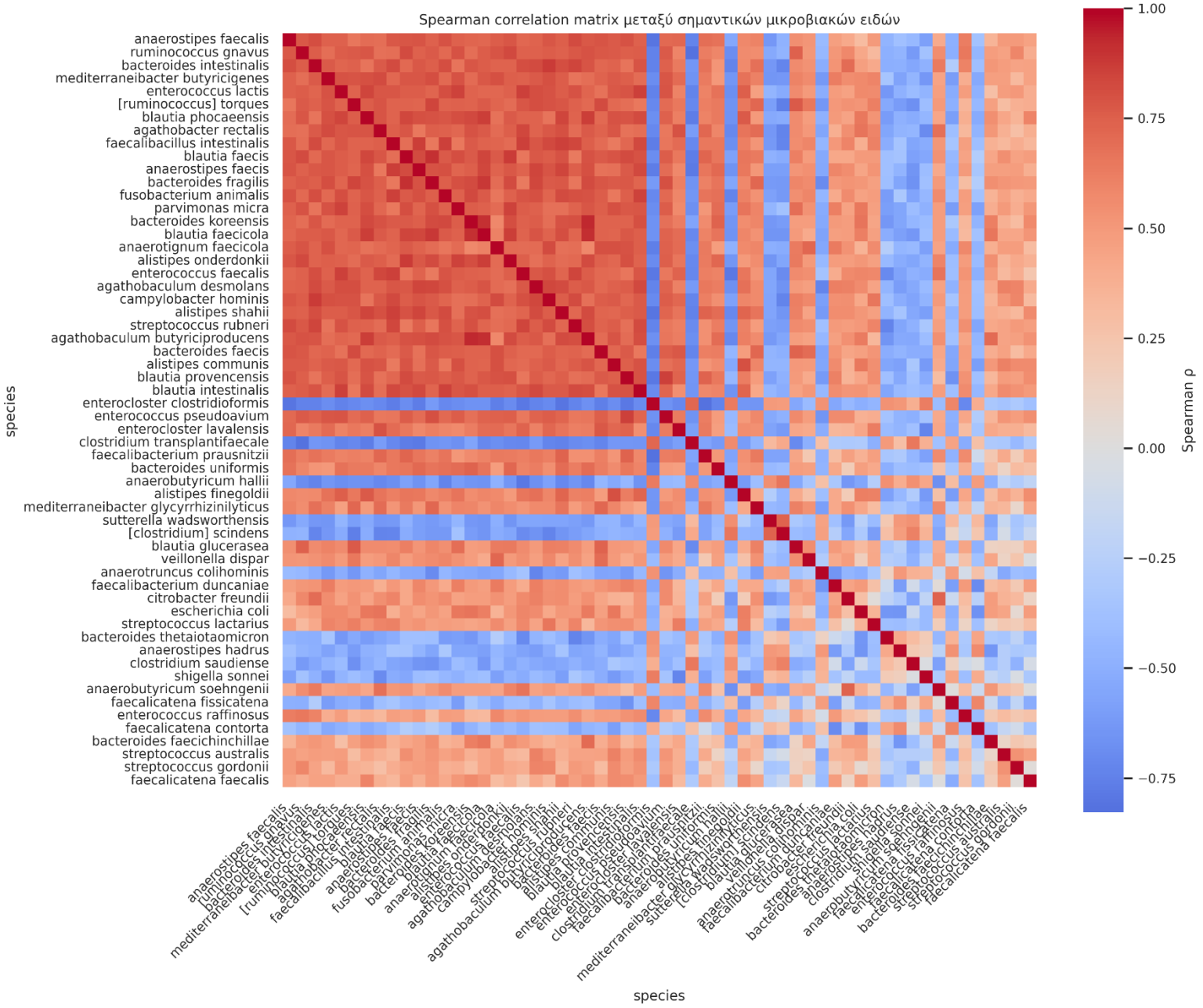


Table 21: ***Heatmap with Color-Coded Correlation Coefficients***

Red = strong positive correlation (ρ → 1)
Blue = strong negative correlation (ρ → -1)

The heatmap displays correlation coefficients (ρ) for each pair of microbial species that showed statistically significant differences in abundance between colorectal cancer (CRC) patients and healthy controls. Strong positive correlations (red) indicate species that tend to co-occur in CRC samples, whereas strong negative correlations (blue) represent species exhibiting mutually exclusive abundance patterns.

Results and Analysis of Quality of Life Questionnaire

The selection of the 30 patients who would participate in the study was conducted using a random number table and according to the eligibility criteria outlined in the research protocol. From the demographic data and general questionnaire responses available for the first 12 patients, the following observations were made:

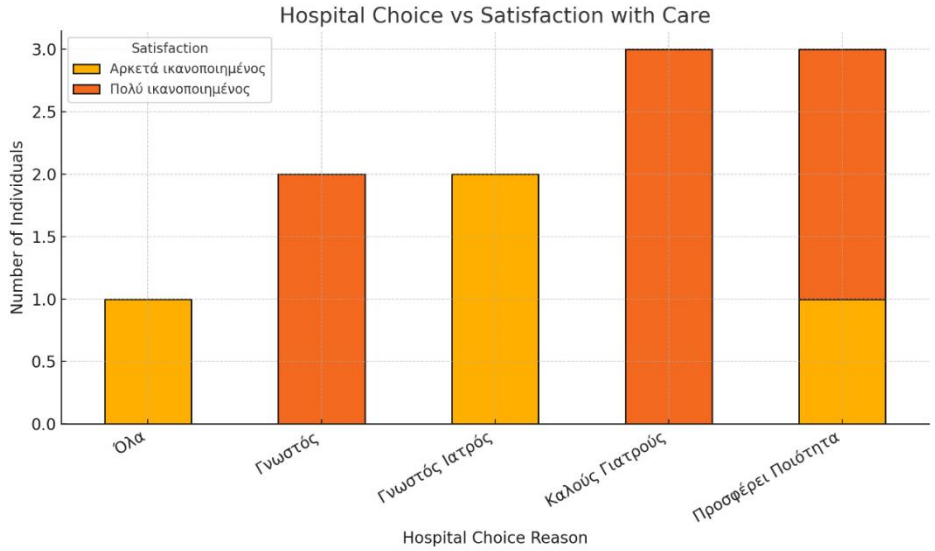
There were twice as many male patients as female. All participants were of Greek nationality. Age distribution was within the expected range, with relatively low standard deviation (age range = 40 years). Ages approximately followed a normal distribution, with a slight positive skew of $z = +0.6$ standard deviations relative to a theoretical normal distribution with a mean of 60 years and standard deviation of approximately 11. Therefore, participants fall within the expected age range for the disease and its stage.

Most patients reported having children. The distribution curve was slightly negatively skewed, by $z = -0.47$ standard deviations, from a theoretical normal distribution with a mean of 2.0 children (calculations included at the end of the section).

Regarding education, the majority (50%) had a low level of formal education (elementary or middle school), with a smaller proportion having completed high school or vocational training. This reflects the older age profile of the sample and is consistent with educational trends for their generation. Thus, the distribution of educational levels mirrors the age structure of the sample population.

The ratio of patients with metastatic disease was 2:1. Most patients had been receiving chemotherapy for more than two years and had undergone more than 15 cycles. Among those with metastasis, nearly all had received chemotherapy, while two patients had also undergone radiotherapy. Weight changes and complications were reported in 3 patients. Patients who reported no weight change also reported no complications and were undergoing chemotherapy. Conversely, complications were reported by patients who had experienced weight changes and were undergoing chemotherapy (or both therapies). Weight changes may be related to either the type of treatment or the presence of complications. To test this, a chi-squared (χ^2) test was applied. The analysis showed no statistically significant association. Therefore, weight loss could not be clearly attributed to either treatment type or complications; it may be influenced by either or both factors equally.

Hospital choice appeared to be strongly influenced by the patient's prior knowledge of the hospital staff, especially the treating physician. However, the perceived quality of care was considered equally important. This indicates that while the physician may be a factor, the quality of care is just as critical in a patient’s choice of treatment center. In fact, patient satisfaction with care was more closely associated with the expectation of receiving high-quality services than with personal reasons such as being treated by a known physician.



It is evident that patients who selected the hospital based on the quality of care and medical staff reported higher levels of satisfaction than those who chose the hospital due to a known physician, who reported being only moderately satisfied.
Participants who selected the option “all of the above” (i.e., care quality, physician, staff) tended to report general satisfaction, but none indicated being very satisfied.
Most patients stated that they do not require assistance with daily activities, while a small number reported needing some help. Statistical analysis (χ^2 test) showed that this association was not statistically significant (p-value = 0.242), likely due to the small sample size.
Only two individuals reported needing assistance. When examining the possible relationship between need for assistance in daily activities and the presence of complications, no statistically significant difference was found. Although the raw data suggest that patients with complications tend to require more help, the result was not statistically significant (p = 0.48), again likely due to limited sample size.
Overall, most patients indicated that every stage of care is important to them.

Conclusions on Pain Assessment Distributions

The selection of 30 patients for this study was based on a random number table and the inclusion criteria defined in the study protocol. From the pain assessment data available for the first 12 patients, the following findings emerged:
8 out of 12 patients reported experiencing pain during their hospital stay, while 7 out of 12 reported no or minimal pain at the time of questionnaire completion. More patients reported pain while hospitalized compared to their current state. However, the difference was not statistically significant (p = 0.54 > 0.05).
Although most patients reported experiencing some pain, only three individuals described the pain as considerable. This likely correlates with the fact that only 24% reported that pain had a significant impact on their activity level, while the remaining patients indicated either no impact or minimal impact. No statistically significant association was found between pain

intensity and activity level (p = 0.48), suggesting that the level of pain did not substantially affect daily activity. It is important to note that the small sample size limits statistical power, and results may differ when analyzing the full sample (N = 30).
Additionally, 58% of patients stated that pain had no or minimal effect on their psychological well-being. No statistically significant correlation was found between pain experienced in the hospital and emotional status (p = 0.515). Changes in routine activities such as walking were also minimal: 75% of patients reported no impact, 18% reported slight or very slight impact, and only one patient (8%) reported considerable difficulty. Again, no statistically significant association was found between pain level and walking ability (p = 0.515).
Similar non-significant associations were observed for sleep quality and interpersonal relationships. Pain appeared to have minimal influence on these domains, as confirmed by statistical results (p = 0.515 for sleep and p = 1.0 for social relationships). While sleep, mood, and activity are typically influenced by pain and general clinical status, no significant associations were observed in this sample—possibly due to the limited number of participants.
Finally, all patients except one reported being either very satisfied or fairly satisfied with their pain management. One patient reported feeling neutral.

Discussion

Conclusions on Quality of Life Assessment Distributions

Overall, patients tend to express an optimistic outlook regarding their quality of life while living with the disease. A subset of patients who report feeling a sense of emptiness tend to prefer staying at home and show signs of withdrawing from activities. However, this group is small, and due to the limited sample size, no statistically significant correlation was found between feelings of emptiness and activity withdrawal, or between feelings of emptiness and preference for staying at home.
More specifically, a weak negative correlation was observed between feelings of emptiness and activity withdrawal, as well as between activity withdrawal and preference for staying at home. A moderate negative correlation was found between feelings of emptiness and preference for staying at home.
Despite these trends, only one patient who reported withdrawing from activities and preferring to stay home also expressed feelings of uselessness, and only two patients believed that others were in a better condition than themselves.
These correlations were assessed using the Pearson correlation coefficient.

Comparison	**Pearson r**	**Interpretation**
Activity Withdrawal & Sense of Emptiness	–0.158	Weak negative correlation

Comparison	Pearson r	Interpretation
Activity Withdrawal & Preference for Staying Home	–0.258	Fairly weak negative correlation
Sense of Emptiness & Preference for Staying Home	–0.408	Moderate negative correlation

Overall, patients demonstrated a positive psychological outlook, with notable indicators of resilience and optimism. The majority of participants reported a good mood (69%) and a strong sense of satisfaction from being alive (92%). Feelings of helplessness, hopelessness, and worthlessness were reported at low levels (15–23%).
Resilience and optimism appeared to be associated with an ability to adapt to lifestyle changes without experiencing combined social withdrawal.
A high percentage of patients (77%) expressed a preference for staying at home, but this was not strongly associated with feelings of emptiness or activity withdrawal. Similarly, although 85% reported withdrawing from certain activities, this was not accompanied by signs of psychological collapse.
A small proportion of patients (1–2 individuals) reported significant emotional distress. Specifically, 8% indicated they often feel fear that something bad might happen. In addition, 61.5% believed that others are in better condition than themselves, suggesting the presence of negative social comparison and potentially lowered self-esteem.
Finally, 31% of participants reported experiencing a sense of emptiness, representing a notable minority.

PCoA

There is strong clustering and compositional homogeneity among patients in terms of both quantitative and qualitative aspects of their gut microbiota. A notable reduction is observed in taxa typically present in a healthy gut microbiome, such as Faecalibacterium prausnitzii, Ruminococcus spp., and Clostridium spp., while species associated with CRC are enriched.More specifically, the significant presence of Parvimonas micra, Bacteroides fragilis (enterotoxigenic strain, ETBF), and Campylobacter hominis—bacteria linked to toxin production, immunosuppression, and activation of pathways such as Wnt/β-catenin and IL-17—supports the hypothesis that dysbiosis exhibits disease-specific profiles, including neoplasm-specific subtypes.
The shared shifts in microbial diversity likely reflect common bacterial markers that are altered in CRC. Furthermore, the enrichment of specific taxa suggests that the dysbiotic profile is not random but rather systematic and potentially driven by shared pathological alterations in the intestinal microenvironment. This consistent patient clustering reinforces the interpretation that dysbiosis is structured, not incidental, and may play a critical role in both carcinogenesis and disrupted intestinal homeostasis (179), (180).
There is also a well-supported hypothesis that the microbiome may be directly implicated in the pathophysiological mechanisms of carcinogenesis. Dysbiosis could

contribute to and accelerate the progression toward malignancy by disrupting the gut environment. A key manifestation of this is the reduction in the concentration—and thus biological activity—of microbial metabolites such as short-chain fatty acids (SCFAs). The decrease in SCFAs has downstream effects on various physiological systems, including impaired homeostasis, reduced apoptosis, increased inflammation, and altered immune regulation. These disruptions correspond to the clinical features observed in CRC patients (e.g., inflammation, immune dysregulation).These microbiome alterations have potential diagnostic and therapeutic applications. Taxa that are enriched under dysbiotic conditions may serve as biomarkers for CRC diagnosis and, when monitored longitudinally, may help predict disease progression. Furthermore, bacterial communities that exhibit systematic clustering and reduced abundance could serve as adjunctive therapeutic targets—for example, to complement chemotherapy. Notably, taxa identified in the literature but absent in the current dataset should also be considered in clinical decisions. Potential therapeutic strategies include probiotic supplementation or fecal microbiota transplantation (FMT), tailored to the individual patient's microbial profile. Finally, the interaction between the gut microbiome and chemotherapeutic agents should be considered in treatment planning, as the microbiome may modulate therapeutic efficacy. Designing treatment regimens that incorporate microbiome-modulating approaches could provide synergistic benefits (181).

Pearson-Spearman

The strong correlation among microbial species found in CRC patients under dysbiotic conditions—such as *Parvimonas micra* with *Fusobacterium nucleatum*, and *Peptostreptococcus anaerobius* with *Bacteroides fragilis*—suggests a potential synergistic interaction within the dysbiotic microbiome. This synergy may play a causal role in colorectal carcinogenesis, particularly given that these associations occur within specific microbial clusters, which may serve as candidate biomarkers. Conversely, correlations between health-associated and dysbiosis-associated taxa tend to be negative—for example, *Roseburia* vs. *Parvimonas micra*, and *Faecalibacterium* vs. *Peptostreptococcus anaerobius*. Species such as *Parvimonas micra*, *Fusobacterium nucleatum*, and *Peptostreptococcus anaerobius* display strong positive correlations, forming a distinct "pathogenic cluster" associated with inflammation, enhanced epithelial adhesion, and potential promotion of carcinogenesis through metabolite production and inflammatory signaling pathways. On the other hand, SCFA-producing species such as *Roseburia inulinivorans*, *Faecalibacterium prausnitzii*, and *Anaerostipes* exhibit positive correlations with each other and negative correlations with pathogenic taxa, highlighting their possible protective and antagonistic role against dysbiosis-associated species (182)The observed correlations may reflect potential interspecies competition—either for nutritional substrates or through immunological mechanisms, such as the anti-inflammatory effects exerted by short-chain fatty acids (SCFAs). (183), (184). Based on the findings, intestinal dysbiosis in CRC patients is clearly confirmed. Moreover, the microbial profile distinctly differentiates these patients within the colorectal cancer context. The increased presence of pathogenic and pro-inflammatory bacteria—such as Escherichia coli, Parvimonas micra, and Bacteroides fragilis—alongside the concurrent depletion of beneficial short-chain fatty acid (SCFA)-producing species, including Faecalibacterium prausnitzii, Anaerobutyricum hallii, and Roseburia inulinivorans, highlights a shift toward a dysbiotic state.When

comparing the top 10 dominant genera, a relative increase in taxa associated with CRC was observed in patients, in agreement with previously published literature. (185), (186), (187), (188), (189), (177).

Species such as Parvimonas micra, Fusobacterium nucleatum, and Peptostreptococcus anaerobius display strong positive correlations with one another, forming a "pathogenic cluster" associated with inflammation, immune modulation, and colorectal carcinogenesis. These patterns reinforce the hypothesis—supported by recent literature—that microbial signatures in CRC are not random but systematic, potentially playing a causative role in disease pathogenesis.

Approximately 46 microbial species were found to be increased and 12 decreased in CRC patients. The dysbiosis observed is associated with bacteria capable of producing epigenetically active compounds (Escherichia coli), pro-inflammatory molecules (Parvimonas micra, Fusobacterium animalis, Enterococcus faecalis, Citrobacter freundii), and immune-modulatory agents (Blautia spp., *Bacteroides fragilis*). This microbial imbalance likely arises from both local inflammation and systemic immune suppression, facilitating bacterial translocation and colonization.

Patients in this study had undergone an average of more than 15 chemotherapy cycles, a factor known to strongly affect gut microbiome composition. Chemotherapy contributes to dysbiosis not only by direct cytotoxicity on the gut epithelium but also through immunosuppressive effects and alteration of host-microbe interactions (e.g., via GALT, SCFA pathways, and epithelial signaling).

About 34% of microbial species showed statistically significant differences between CRC patients and controls (FDR < 0.05). Notably, high $\log_2$ fold-change values were observed for *Campylobacter hominis* (+7.86) and *E. coli* (+4.2), while *Phocaeicola spp.* (−3.5) and *Anaerobutyricum hallii* (−2.8) showed significant reductions—highlighting dramatic shifts in microbial community structure.

The reduction in beneficial species is of particular concern, as it may compromise multiple host systems: immunoregulation (*Sutterella wadsworthensis*), epithelial barrier function (*Anaerobutyricum hallii*, *Faecalicatena spp.*, *Clostridium scindens*), metabolic activity (*Bacteroides thetaiotaomicron*), and overall intestinal homeostasis.

These findings support the idea that dysbiosis may serve not only as a biomarker for CRC but also as a therapeutic target. Restorative interventions such as fecal microbiota transplantation (FMT) or probiotic supplementation could help re-establish microbial balance. Species like *Faecalibacterium* and *Anaerobutyricum* may play a supportive role when integrated into chemotherapeutic regimens. Tailored microbiome-based strategies could enhance treatment outcomes and support personalized oncology.

Multivariate analysis (PCA) and hierarchical clustering (heatmap) revealed clear separation between CRC patients and healthy controls. This distinction was largely driven by enrichment of pathogenic taxa. These patterns highlight the potential of the gut microbiome as a non-invasive diagnostic tool and a complementary therapeutic target, especially through reintroduction of beneficial taxa using personalized approaches.

Among patients with metastatic CRC (n = 4), there was increased abundance of pro-inflammatory species (*Parvimonas micra*, *E. coli*, *Fusobacterium animalis*, *Bacteroides fragilis*), suggesting a possible link between dysbiosis and metastatic potential. In contrast, patients with favorable hematologic profiles (WBC 5–10 $\times 10^9$/L, Hb >12 g/dL) maintained higher levels of *Faecalibacterium prausnitzii* and *Anaerobutyricum soehngenii*, both of which support intestinal homeostasis.

One patient with Lynch syndrome exhibited pronounced dysbiosis, while patients over 75 years of age showed elevated levels of several pathogenic taxa. These individual findings emphasize the influence of host factors—including age, immune status, and genetics—on microbial composition.

While patient data partially diverged from previously published studies (190), (191) (176) , the presence of well-documented dysbiosis markers such as *E. coli* (colibactin$^+$) and *B. fragilis* (ETBF) was confirmed. Divergences may be attributed to patient-specific variables (e.g., disease duration of 1–5 years), pharmaceutical regimens, and methodological differences in microbiome profiling. Geographic, dietary, and technical variation should also be considered when interpreting inter-study discrepancies.

Chemotherapy, combined with adjunctive treatments (antibiotics, PPIs, antiemetics), significantly disrupts microbiome stability. These effects include increased colonization by opportunistic pathogens (*Enterococcus*, *Klebsiella*, *Fusobacterium*) under immunosuppression, reduced α-diversity, and impaired gut barrier function. This further reinforces the need to monitor and, where possible, modulate the microbiota as part of comprehensive cancer care (192), (193), (194). The chemotherapy regimens used in these patients, including Avastin (100 mg) and ALYMSYS (400 mg), may exert both positive and negative effects on the gut microbiota. On the one hand, they can enhance alpha-diversity in patients who respond favorably to treatment and reduce the abundance of pathogens associated with CRC progression—such as *Fusobacterium nucleatum*—while promoting the relative increase of beneficial taxa like *Bifidobacterium*.

On the other hand, their predominant effect appears to be negative, as they contribute to dysbiosis by decreasing alpha-diversity, increasing the prevalence of pathogenic species, and amplifying gut inflammation.

) (195), (196), (197), (198), (199), (200), (201).

Regarding the analytical method, differences exist compared to more sensitive approaches such as shotgun metagenomics. The key distinctions are summarized in the table below (202), (203), (204)

Parameter	**16S rRNA (this study)**	**Shotgun Metagenomics**
Scope	Bacteria only	Bacteria, viruses, fungi, archaea
Resolution	Mainly genus/species level	Strain- and gene-level resolution
Functional information	No	Yes (e.g., metabolic pathways, resistance genes)

Parameter	16S rRNA (this study)	Shotgun Metagenomics
Taxonomic accuracy	Moderate to good	Very high
Cost/Requirements	Lower	Higher

While shotgun metagenomics generally offers higher sensitivity and taxonomic resolution, the use of long-read platforms such as Oxford Nanopore—despite enabling full-length 16S rRNA gene sequencing—may introduce a higher per-base error rate compared to short-read technologies like Illumina. This limitation may have influenced the detection or quantification of certain low-abundance taxa in this study.

Additional discrepancies may also be attributed to factors related to the underlying composition of the gut microbiome within different populations. Variables such as geographic origin, cultural dietary habits, and the country in which a study is conducted can significantly influence microbial profiles and lead to divergence between studies.In Greece, such microbiome studies remain relatively rare. However, a notable large-scale clinical study was conducted in 2024 by Messaritakis I. et al., in which both fecal and blood samples were collected for microbiome and immunological analysis. (176). When comparing the results, an overlap was observed in bacterial genera such as *Anaerococcus* and *Peptoniphilus*, whereas other genera—including *Fenollaria*, *Finegoldia*, *Ezakiella*, and *Porphyromonas*—were not detected. These differences may be attributable to the use of a different DNA extraction and sequencing approach (QIAamp PowerFecal Pro DNA Kit [QIAGEN] / 16S Barcoding Kit 24v14 – Nanopore), slight variations in dietary patterns, and patient-specific treatment regimens.

A similar microbial shift toward potentially pathogenic families was also documented in the patients from the present study (e.g., presence of *Peptoniphilus*, *Anaerococcus*), further supporting the hypothesis of shared dysbiosis-related mechanisms within the Greek CRC population.

Finally, it is important to acknowledge a key limitation: the study has not yet been completed, and the current results are based on a small subset of enrolled patients, which may not accurately reflect the overall cohort.

Patients

Patients appear to exist in an immunosuppressive environment, as reflected in clinical data (e.g., extended chemotherapy exposure and gut dysbiosis with immunosuppressive microbial profiles). This state may affect both mood and daily functioning, contributing to fatigue and discomfort. While fatigue emerged in the questionnaire as a central symptom, significantly associated with anorexia ($p = 0.0047$) and discomfort during physical activity, it was not significantly associated with vomiting, indicating a non-therapeutic etiology.

Changes in the gut microbiome—especially the depletion of SCFA-producing species such as *Faecalibacterium prausnitzii*, *Roseburia*, and *Anaerobutyricum hallii*—may contribute to metabolic dysfunction, fatigue, and negative energy balance.

Although pain was reported by 67% of participants, only 25% stated that it had a substantial impact on functionality. Pain was not significantly associated with mood ($p = 0.427$), but did show a moderate association with fatigue ($p = 0.032$). Overall, the sample exhibited strong psychological resilience to both fatigue and pain, which may reflect a supportive environment and the patients' meaning-making around the illness.

Potential microbial profiles, such as increased abundance of *Fusobacterium* and *Streptococcus* spp., may contribute to neuroendocrine activation and influence the perception of pain without a direct impact on mood.

The findings highlight a unique psychological profile among CRC patients, characterized by a high level of resilience and optimism, despite the presence of disease and physical symptoms. The majority of patients maintain a positive psychological state, with 92% expressing satisfaction about being alive and 69% reporting good mood. Feelings of helplessness, hopelessness, or worthlessness were low (15–23%), suggesting effective psychological adaptation and mental strength in facing illness.

Although 77% of patients reported a preference for staying at home, and 85% had reduced activity engagement, these behaviors were not accompanied by psychological distress (e.g., emptiness or emotional collapse). The moderate negative correlation between preference for staying home and feelings of emptiness, along with the lack of statistically significant relationships, suggests these behaviors may reflect personal coping strategies rather than pathological withdrawal.

Nevertheless, a vulnerable minority (1–2 individuals) showed signs of emotional burden, including fear, low self-esteem, and persistent emptiness. Specifically, 31% reported feeling a sense of emptiness, and 61.5% believed others were in better condition, reflecting negative social comparison and possible concerns about self-image and self-worth.

Questionnaire

Integrated Analysis of Quality of Life and Symptom Burden

Despite the fact that 66% of patients presented with metastatic disease, their overall psychological state was relatively stable. Notable findings include: 92% reported feeling good about being alive,69% reported being in a good mood, Only 16% reported frequent feelings of helplessness

Pain was reported by 67% of patients, yet only 25% stated that pain had a substantial impact on their daily functioning.

Fatigue emerged as the primary variable associated with anorexia, limited physical activity, and perceived quality of life. For example, a statistically

significant relationship was observed between fatigue and discomfort during long walks (p = 0.00064).
Most patients acknowledged that pain and fatigue negatively affect their quality of life, with statistically significant associations identified.
Although an apparent relationship was noted between barriers to social life and financial difficulties, the association was not statistically significant (p = 0.254).
The presence of a microbial signature consistent with dysbiosis, the reduction of beneficial species, and the negative correlation with quality-of-life indicators support the hypothesis that gut microbiota disruption in CRC patients has a multifactorial impact, including:

- Biological: immunosuppression, inflammation
- Psychosocial: fatigue, reduced well-being
- Therapeutic: potential influence on chemotherapy response/resistance

Identifying these microbial patterns may form the basis for predictive biomarkers, targeted probiotic therapy, or even fecal microbiota transplantation (FMT) as an adjunctive tool within personalized treatment approaches.
Patients who report pain also tend to experience greater fatigue, which may affect their social engagement, activity level, and sleep quality. Specifically, there is a statistically significant relationship between fatigue and pain (p = 0.032).
Although fatigue may impact sleep, no statistically significant association was found (p = 0.068), possibly due to the small sample size.
Fatigue also correlates with anorexia—the more severe the fatigue, the higher the likelihood of experiencing loss of appetite, or vice versa. This relationship demonstrated stronger statistical power than others (e.g., sleep), with significant association observed (p = 0.0047).
On the other hand, fatigue was not significantly related to vomiting tendency (p = 0.963), suggesting that vomiting may be treatment-related rather than fatigue-induced.
Fatigue also impacts physical activity: a statistically significant association was found between fatigue and discomfort during long walks (p = 0.00064). In other words, the more severe the fatigue, the more likely the patient is to report discomfort with long walks, and vice versa.
However, pain was not significantly associated with mood in this sample (p = 0.427). While some individuals may subjectively link the two, no consistent statistical relationship was observed across the dataset.

Conclusions

The statistically significant intestinal imbalance, primarily dysbiosis, identified in CRC patients, confirms that the neoplastic disease causes alterations in the microbiota, with a predominance of microorganisms that trigger and enhance inflammation and immunosuppression. The result of this

dysbiosis tends to create a characteristic microbial profile, consistent with pathogenic strains, which appears to be common among CRC patients. These hypotheses are emphasized by recent studies and suggest that current research focuses on dysbiosis and microorganisms that systematically – and not randomly – appear in CRC patients (only one early-onset CRC patient participated in this study). These microbial signatures may further support the hypothesis that the microbiome could serve as a biomarker for both diagnosis and disease progression.

The statistically significant difference between patients and controls, along with the specific microbial profile, can support the formulation of the hypothesis that the microbiome may act as a **second wave** in the onset of CRC and contribute to the emergence of the disease.

Overall, the findings portray a patient population that, despite the severity of the disease, maintains a positive attitude and functionality, reinforcing the importance of psychosocial support and mental health monitoring, especially for vulnerable subgroups.

The presentation of preliminary results does not imply the same final outcome. The study will include 30 participants, and the final results may differ from the current analysis and conclusions. Moreover, due to the small statistical power of the sample size, strong hypotheses and generalizations for the entire sample cannot be made, nor can strict qualitative comparisons with the literature.

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Appendix

Questionnaire

The questionnaire is presented in Greek, as it was originally drafted and distributed to the patients in this language.

Δημογραφικά στοιχεία

1. **φύλο:**
Άνδρας
Γυναίκα
2. **Ημερομηνία Γέννησης**: _____
3 . **Αριθμός Παιδιών: _____**
7 . **Εθνικότητα**:
Ελληνική
Άλλη
8. **Εκπαιδευτικό υπόβαθρο**
Δημοτικό
Γυμνάσιο
Λύκειο
Τεχνική σχολή
Πανεπιστήμιο
Μεταπτυχιακό
Διδακτορικό
Άλλο

Σχετικά με τη νόσο

1. Τύπος νεοπλασματικής νόσου :_____

2. Στάδιο της νόσου: _____

Ύπαρξη μετάστασης

ΝΑΙ_____, ΟΧΙ_____

3. **Θεραπευτική αντιμετώπιση** :

ακτινοθεραπεία
χημειοθεραπεία
και τα δύο
άλλο

4. Σημαντική μεταβολή βάρους τις τελευταίες δεκαπέντε μέρες:

ΝΑΙ_____,ΟΧΙ_____

Αν ναι, σημειώστε τα κιλά: _____

5. Περιεγχειριτκές επιπλοκές :

ΟΧΙ_____, ΝΑΙ____

Αν ναι προσδιορίστε:

_____

8. **Σημειώστε τη διάρκεια της νόσου σε μήνες :** _____

9. **Γιατί επιλέξετε αυτό το Νοσοκομείο;**

Γιατί έχω γνωστό γιατρό μου
Για την φήμη του ότι προσφέρει ποιότητα
Γιατί έχει καλούς γιατρούς
Γιατί έχει καλό νοσηλευτικό προσωπικό
Μετά από σύσταση γνωστού μου
Γιατί έχω ξανάρθει
Γιατί αυτό εφημέρευε
Γιατί είναι κοντά στο σπίτι μου
Γιατί είναι το μοναδικό

12**. Κατά τη διάρκεια της παραμονής σας στο Νοσοκομείο πόση βοήθεια χρειάζεστε από το προσωπικό του Νοσοκομείου για τις καθημερινές σας δραστηριότητες (φαγητό, πλύσιμο, τουαλέτα);**

Πολύ βοήθεια
Αρκετή βοήθεια
Λίγη βοήθεια
Καμιά βοήθεια

13. **Πώς αισθάνεστε συνολικά από την φροντίδα σας στο νοσοκομείο;**

Πολύ ικανοποιημένος
Αρκετά ικανοποιημένος
Λίγο ικανοποιημένος
Απλά ικανοποιημένος
Ούτε ικανοποιημένος ούτε δυσαρεστημένος
Καθόλου ικανοποιημένος
Δυσαρεστημένος
Πολύ δυσαρεστημένος

Αδιάφορο

14. **Τι θεωρείτε το πιο σπουδαίο στην φροντίδα σας στο νοσοκομείο;**

Η ενημέρωση από το προσωπικό
Ο σεβασμός από το προσωπικό
Το ενδιαφέρον του προσωπικού
Η ψυχολογική υποστήριξη από το προσωπικό
Οι ανέσεις του Τμήματος (δωμάτιο, καθαριότητα)
Το φαγητό
Η ευγένεια του προσωπικού
Η επικοινωνία με το προσωπικό
Όλα

16. Αξιολόγηση πόνου

16^{α}) Πονέσατε κατά την παραμονή σας στο νοσοκομείο;
ΝΑΙ _____ ΟΧΙ _____
16β) Πόσο πονέσατε;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____
16γ) κατά πόσο ο πόνος σας αυτός επηρέασε τις συνήθεις δραστηριότητες σας;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____
16δ) Την ψυχική σας διάθεση;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____
16^{ε}) Το περπάτημά σας;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____
16στ) Τις σχέσεις σας με τους άλλους;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____
16ζ) Τον ύπνο σας;
Πάρα πολύ _____ Πολύ ____ Λίγο ____ Πολύ λίγο ____ Καθόλου _____

17. **Πώς αισθάνεστε από τον τρόπο που αντιμετωπίστηκε ο πόνος σας;**

Πολύ ικανοποιημένος
Αρκετά ικανοποιημένος
Λίγο ικανοποιημένος
Απλά ικανοποιημένος
Ούτε ικανοποιημένος ούτε δυσαρεστημένος
Καθόλου ικανοποιημένος
Δυσαρεστημένος
Πολύ δυσαρεστημένος
Αδιάφορος

18. πόσο πονάτε αυτή τη στιγμή;

Πάρα πολύ
Πολύ
Αρκετά
Λίγο

Πολύ λίγο
Καθόλου

19.

		ΝΑΙ	ΌΧΙ
1	Είστε ευχαριστημένος από την κατάσταση της ζωής σας;		
2	Έχετε εγκαταλλείψει πολλές από τις δραστηριότητες και τα ενδιαφεροντά σας;		
3	Αισθάνεστε ότι η ζωή σας είναι άδεια;		
4	Βαριέστε συχνά;		
5	Έχετε καλή διάθεση τον περισσότερο καιρό;		
6	Φοβάστε ότι μπορεί να σας συμβεί κάτι κακό;		
7	Αισθάνεστε ευτυχσμένος τον περισσότερο καρό;		
8	Αισθάνεστε συχνά αβοήθητος		
9	Προτιμάτε να είστε στο σπίτι από το να κάνετε έξω διάφορα πράγματα		
10	Αίσθανεστε ότι έχετε προβλήματα με τη μνήμη σας από ότι οι άλλοι		
11	Πιστεύετε ότι είναι όμορφο που είστε ζωντανός;		
12	Αισθάνεστε άχρηστος σε αυτή σας την καταστάση;		
13	Αισθάνεστε γεμάτος ενέργεια;		
14	Αισθάνεστε ότι η καταστασή σας είναι απελπιστική;		
15	Πιστεύετε ότι ο περισσότερος κόσμος είναι σε καλύτερη κατάσταση από εσάς;		

Κατά τη διάρκεια της τελευταίας εβδομάδας	Καθόλου	Λίγο	Αρκετά	Πολύ
Περιοριστήκατε στην εργασία σας ή σε άλλες καθημερινές σας ασχολίες;	1	2	3	4
Περιοριστήκατε σε ασχολίες που κάνετε στον ελεύθερο σας χρόνο;	1	2	3	4
Κουραστήκατε και λαχανιάσατε;	1	2	3	4
Πονέσατε;	1	2	3	4
Είχατε ανάγκη από ξεκούραση;	1	2	3	4
Είχατε προβλήματα κατά τη διάρκεια τοου ύπνου;	1	2	3	4
Αισθανθήκατε κάποια αδυναμία;	1	2	3	4
Είχατε ανορεξία;	1	2	3	4
Είχατε τάση για έμετο;	1	2	3	4
Κάνατε εμετό;	1	2	3	4
Είχατε δυσκοιλιότητα;	1	2	3	4

		Καθόλου	Λίγο	Αρκετά	Πολύ

1	Αισθάνεστε ενοχλήσεις όταν κάνετε χειρωνακτικές εργασίες, όπως όταν μεταφέρετε ένα βαρύ αντικείμενο;	1	2	3	4
2	Αισθάνεστε ενοχλήσεις όταν κάνετε μια μεγάλη βόλτα;	1	2	3	4
3	Αισθάνεστε ενοχλήσεις σε μικρούς περιπάτους;	1	2	3	4
4	Χρειάζεται να μένετε στο κρεβάτι ή σε καρέκλα κατά τη διάρκεια της ημέρας;	1	2	3	4
5	Χρειάζεστε βοήθεια όταν τρώτε, ντύνεστε, πλένεστε ή όταν πηγαίνετε τουαλέτα;	1	2	3	4

Τη τελευταία εβδομάδα	Καθόλου	Λίγο	Αρκετά	Πολύ
Είχατε διάρροια;	1	2	3	4
Αισθασθήκατε κουρασμένος/η;	1	2	3	4
Είχατε πόνο, που να εμπόδιζε τις καθημερινές σας ασχολίες;	1	2	3	4
Είχατε δυσκολία σε ασχολίες που απαιτούν συγκέντρωση (πχ διάβασμα)	1	2	3	4
Αισθανόνασταν υπερένταση;	1	2	3	4
Αισθανόνασταν στενοχωρημένος/η;	1	2	3	4
Αισθανόσασταν ευέξαπτος/η;	1	2	3	4
Αισθανόσασταν ότι έχετε κατάθλιψη;	1	2	3	4
Έχετε δυσκολία να θυμηθείτε διάφορα πράγματα;	1	2	3	4
Τα φάρμακα ή η φυσική σας κατάσταση, σας εμπόδισαν στην οικογενειακή σας ζωή;	1	2	3	4
Η φυσική σας κατάσταση ή τα φάρμακα σας εμπόδισαν στην κοινωνική σας ζωή;	1	2	3	4
Η φυσική σας κατάσταση ή τα φάρμακα σας δημιούργησαν οικονομικές δυσκολίες;	1	2	3	4

Κυκλώστε τον αριθμό που θεωρείτε ότι ταιριάζει καλύτερα από το 1 έως το 7									
Πως θα εκτιμούσατε συνολικά την κατάσταση της υγείας σας, τις τελευταίες εβδομάδες;									
	1	2	3	4	5	6	7		
	Πολύ κακή					*Εξαιρετική*			
Πως θα εκτιμούσατε συνολικά την ποιότητα της ζωής σας τις τελευταίες εβδομάδες;									

	1	2	3	4	5	6	7		
	Πολύ κακή					*Εξαιρετική*			

Summary of clinical protocol

Introduction:
Colorectal cancer is the third most common form of cancer worldwide, with an incidence increasing by approximately 1% annually and a relatively higher frequency in men. The onset, progression, and outcome of the disease are directly associated with the composition of the gut microbiota. Pathological alterations in the composition and abundance of gut microbiota can influence gastrointestinal disorders and neoplastic diseases through mechanisms involving metabolites, epigenetics, signaling, and inflammation. Furthermore, studies suggest that dysbiosis may worsen the prognosis and overall condition of patients with colorectal cancer.
Objectives:
To assess the composition and abundance of intestinal microbiota in patients with colorectal cancer through fecal sample analysis.
Methods:
Primary measurements:
16S rRNA sequencing of fecal samples; bacterial species classification and identification using reagents.
Study design and type:
Cross-sectional, non-interventional.
Indication:
Colorectal cancer and metastatic colorectal cancer.
Study population:
Patients from the "Agios Savvas" hospital who meet the inclusion criteria.
Sample size:
20–30 participants.
Study duration:
Approximately 5 months.
Data Analysis:
Descriptive statistics will be used. For qualitative variables, the percentage of patients in each category will be calculated. For quantitative variables, the mean, median, standard deviation, and 95% confidence interval will be determined.

Main Objective - Secondary Objectives: The aim of the present study is to investigate the human microbiome, specifically the gut microbiota, through stool samples from patients with colorectal cancer and metastatic disease. The study will analyze the quantity, diversity, and changes in the gut microbiota of patients. Additionally, a questionnaire will be administered to

assess patients' quality of life. Patients diagnosed with colorectal cancer and metastatic disease will be included, and potential alterations and dysbiosis in their gut microbiota will be evaluated to establish correlations with health outcomes.

Sample: A single stool sample will be collected from each patient.

Secondary Objectives: The goal is to correlate these microbiota changes, when identified, with the overall impact on the patient's health and quality of life.

Benefits: No direct personal benefit is anticipated for the patients participating in the study in terms of outcome, apart from receiving information. The execution of the study will contribute to the documentation of specific data that will enhance existing knowledge and potentially trigger the application of gut microbiota restoration techniques in patients, in line with international research and clinical protocols.

Study Design:

Type of Clinical Study: This clinical study is a prospective observational study of gut microbiota in patients with colorectal cancer and metastatic disease. It is non-interventional, meaning there will be no changes to the patients' clinical management or therapeutic regimen by the researchers. The study does not replace the treating medical team, which retains responsibility for all therapeutic decisions. The study will be conducted at the Agios Savvas Oncology Hospital. Patient information will be collected retrospectively through review of medical records and possibly interviews, as well as through the completion of a quality of life questionnaire by the patients.

Study Population: Sample size calculation was performed via random sampling from the available patient population. The study will include 20–30 adult patients, aged 50 to 80 years, diagnosed with colorectal cancer and metastatic disease, who have undergone surgery and are receiving treatment at Agios Savvas Oncology Hospital. Participants will remain in the study until its completion.

Participant Enrollment: Inclusion Criteria:

- Age between 30 and 80 years, regardless of gender
- Confirmed diagnosis of colorectal cancer and possible metastatic disease
- Undergoing first-line chemotherapy
- Signed informed consent following detailed information
- Ability and willingness to provide written informed consent and comply with the study protocol requirements
- Ability to read, understand, and complete the study-specific questionnaire

Exclusion Criteria:

- Inability to comply with the study protocol

Withdrawal of Participants: Participation in this study is voluntary. If patients choose to withdraw, they may do so at any time and for any reason. This decision will not affect their relationship with their treating physician or the quality of care they receive, which will continue unchanged.

Study Timeline: The total study duration is estimated at two months, from the data collection of the first participant (date of inclusion) to the completion

of data collection of the last participant. This will be followed by sequencing and sample analysis, lasting approximately two months, and another two months for results interpretation.

Data Collection: Study data will be collected from patient medical records during their clinical monitoring by the study physicians according to standard clinical practice, and from participants through interviews and questionnaire completion. No additional lab tests will be conducted beyond routine clinical assessments. Secondary data will be collected from medical histories and files.

Upon identifying eligible individuals, the following data will be collected: patient history, demographics (name, address, contact details), personal history, medical record number, pathology number, admission date and reason, surgeries undergone for the disease, exam results, comorbidities, diagnosis, co-existing conditions, clinical, radiological, culture/biopsy, biochemical, immunological, hematological findings, treatment plan, outcome, current status, informed consent documents.

Patients will be thoroughly informed and given sufficient time to provide consent. For early withdrawals, the reason must be documented.

Sample Analysis: Collected samples will undergo 16S rRNA sequencing as part of a metatranscriptomic approach. Sample processing includes collection, sorting, filtration, DNA fragmentation, amplification and cloning into vectors, sequencing, assembly, and bioinformatics-based identification. High-fidelity checks, sequence classification, and potentially further analyses will follow.

Questionnaire Administration: The questionnaire aims to evaluate quality of life. Patients will receive instructions and complete it in a quiet and comfortable space, unassisted. Once completed, the study physician will review the responses to ensure all questions are answered. Any omissions will be pointed out to the respondent, although they retain the right to skip questions they are unwilling to answer.

To protect confidentiality, patients' names or identifying information will not be recorded. Instead, each patient will be assigned a unique identification code for study purposes.