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Review Article

Chronic Constipation: Mechanisms and Roadmap for Testing Candidate Endotypes

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Abstract

Chronic constipation is common, heterogeneous, and often incompletely controlled. This review evaluates cellular and molecular evidence and proposes a roadmap for testing candidate endotypes. We distinguish established physiology and clinical efficacy from human associations, causal effects in experimental systems, and unvalidated hypotheses. Relevant mechanisms involve enteric neurons and glia, pacemaker and smooth muscle networks, epithelial transport, enteroendocrine signaling, microbial metabolites, and pelvic floor coordination. Five candidate endotypes address neuronal composition, pacemaker dysfunction, methanogen enrichment, neuroimmune activation, and cortical–pelvic coordination; pharmacogenomics provides a cross-cutting strategy. Each proposal links measurable features to appropriate sampling and prospective tests that can exclude clinically meaningful effects. Cellular sequencing, spatial RNA and protein profiling, and multimodal integration can identify candidate signatures. Organoid and animal perturbations test mechanisms within models; clinical usefulness additionally requires reproducible assays, independent validation, randomized treatment-interaction tests, and patient benefit. Rome V criteria provide a cohort framework, with the criteria version recorded explicitly. A staged program should begin with clinical phenotyping and biospecimen banking, add mucosal studies, and develop multicenter full-thickness tissue collections for neuromuscular questions. This approach addresses cost, selection bias, and tissue access while distinguishing mechanistic discovery from useful treatment guidance.

Introduction

Constipation is a common reason for gastrointestinal consultation and impairs quality of life. A historical meta-analysis estimated a pooled community prevalence of chronic idiopathic constipation of 14%, with higher rates in women and older adults.[1] Estimates depend on diagnostic criteria and sampling: a 2026 Rome V internet survey of 28,771 adults across 15 countries found chronic constipation in 8.9%.[2] These estimates describe different populations and definitions and should not be interpreted as a temporal decline. Despite multiple targeted secretagogues, prokinetics, and bile acid modulators, many patients remain dissatisfied with treatment, particularly those with severe slow-transit constipation (STC), persistent defecatory dysfunction, opioid-induced constipation, or irritable bowel syndrome with constipation (IBS-C) and prominent abdominal pain.[3] This review focuses on the cellular mechanisms and study designs needed to explain variation within these clinical groups.

Two developments warrant a translational re-examination of constipation. First, single-cell and spatially resolved atlases of the human and rodent gastrointestinal tract reveal diversity within populations previously identified by sparse markers, including the enteric nervous system (ENS), interstitial cells of Cajal (ICC), and platelet-derived growth factor receptor alpha-positive (PDGFRα+) cells.[4-7] Second, large genetic and microbiome studies identify stool-frequency loci, shared susceptibility to irritable bowel syndrome (IBS) and mood disorders, and microbial states associated with stool consistency and STC.[8-11] These advances provide tools for investigating biological heterogeneity; they do not yet demon-

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strate clinically actionable constipation endotypes.

We organize the field into known-knowns, mechanisms and clinical effects supported by established evidence; known-unknowns, testable hypotheses that could refine classification and treatment; and unknown-unknowns, discoveries that cannot be specified in advance. The graphical abstract summarizes this framework.

We use four evidence labels throughout the text and display items. E1 denotes established physiology or demonstrated clinical efficacy, with the relevant system specified. E2 denotes an association in human observations without demonstrated direction or cause. E3 denotes a causal effect of perturbation within an animal, cell, organoid, or microbiota-transfer model; it does not establish causation in patients. E4 denotes a proposed biological subgroup or treatment-response hypothesis. These labels describe evidence type, not a validated hierarchy of study quality, and different claims within one study can receive different labels. Randomized clinical efficacy does not establish the underlying disease mechanism. All five candidate endotypes and the pharmacogenomic strategy remain E4; none is a validated basis for selecting constipation therapy.

Clinical reviews describe the evaluation and management of chronic constipation.[3] We focus on cellular and molecular evidence that could refine treatment selection while preserving established symptom assessment, evaluation for secondary causes, and effective standard care.

This narrative review prioritizes original research on constipation, STC, IBS-C, opioid-induced constipation, enteric neurons and glia, ICC, mucosal transport, microbial and bile acid signaling, single-cell and single-nucleus sequencing, spatial profiling, and multimodal integration. We retained foundational studies that establish mechanisms; consensus papers and methodological reviews provide diagnostic or technical context. During this revision, we checked cited records in PubMed/Europe PMC and publisher or institutional sources, with targeted searches for disputed claims and relevant newer studies through September 2026. We used available full texts and indexed abstracts based on access. Selection reflects mechanistic relevance and author judgment; this is not a systematic review, and we did not undertake a formal risk-of-bias assessment. Statements about missing evidence are limited to the literature identified and should not be read as exhaustive absence claims. We distinguish proposed study designs from published results.

Classification and the evolving clinical taxonomy

Rome V classifies bowel disorders into irritable bowel syndrome, chronic constipation, functional diarrhea, functional abdominal bloating, unclassified bowel disorder, and opioid-induced constipation.[12,13] Table 1 summarizes the constipation-relevant categories and physiological subgroups.[3,9,12-19] Rome V uses chronic constipation (CC) in place of functional constipation. We use CC for the diagnostic category and retain chronic idiopathic constipation (CIC) when reporting trials that enrolled under that term. This terminology does not eliminate the need to evaluate medication effects and other secondary causes. Rome V places dyssynergic defecation within the anorectal framework and removes the broader "functional defecation disorders" category; rectal sensory disorders are addressed separately.[12,14] Anorectal testing is relevant when constipation persists despite treatment, and delayed transit does not by itself exclude an evacuation disorder.[3,13,14]

The Rome V process reviews developments since Rome IV, including gut–brain interactions, diet, the gut microenvironment, pharmacogenomics, and sociocultural factors.[12,20] For IBS, Rome V defines recurrent, noncontinuous abdominal pain or discomfort averaging at least 3 days per month over the preceding 3 months, with associated defecation or stool changes.[13] This differs from Rome IV's pain threshold of at least 1 day per week.[20] These changes alter the boundary between IBS-C and CC. Therefore, when comparing cohorts, retain the criteria version and individual symptom components; statistical adjustment alone cannot make differently defined populations equivalent.

Physiological assessment distinguishes normal or delayed colonic transit from impaired evacuation, although some overlap exists. Transit can be assessed using radiopaque markers or scintigraphy; anorectal manometry, balloon expulsion, and defecography assess evacuation and rectal sensation.[3,14] With compatible difficult-defecation symptoms, one abnormal evacuation test supports probable dyssynergic defecation, whereas at least two abnormal tests support a definite diagnosis. Rectal hyposensitivity and hypersensitivity are separate sensory disorders defined by symptoms and abnormal sensory thresholds, rather than by preserved sensation.[14]

These clinical categories are useful for care and recruitment but do not identify a unique cellular pathology. Surgical studies report altered myenteric neuronal composition and reduced ICC volume in selected STC specimens, while a small single-cell study reports immune–stromal differences.[15,16,21] Such findings are associative in patients (E2), subject to selection and sampling effects, and cannot establish that every symptom-defined phenotype contains distinct endotypes. Transit delay can coexist with defecatory dysfunction, and normal transit does not exclude disabling constipation.[3] We therefore treat clinical classification as the starting point for stratification and the candidate endotypes as hypotheses (E4) requiring replication, appropriate controls, and evidence that identifying them improves treatment decisions.

Pathophysiology: The known-knowns

Figure 1 depicts interacting physiological systems involved in colonic propulsion and evacuation; the arrangement is conceptual rather than a serial motor pathway.[12-14] Table 2 distinguishes established physiology from patient associations and model-specific causal results.[4-11,14-19,21-51] Within the ENS, neurochemical mapping of colectomy specimens found a higher proportion of nitric oxide synthase (NOS)-only neurons and a lower proportion of choline acetyltransferase (ChAT)-only neurons in severe STC than in controls (E2).[15] This is a relative compositional difference, not proof of neuronal loss. Reference atlases resolve molecularly defined neuronal classes and reveal regional and species differences relevant to disease comparisons.[4,5,52] Mouse studies also show nutrient-specific neuronal activation through epithelial signaling and sex-dependent motility effects of enteric glial ablation (E3).[23,27]

ICC generate pacemaker activity within the smooth muscle–ICC–PDGFRα+ syncytium. Anoctamin-1 (ANO1) supports coordinated slow-wave activity in mouse small intestine, while

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Table 1. Constipation disorders and diagnostic anchors

Category	Defining features	Mechanistic evidence	Diagnostic anchor
Chronic constipation (CC)	Rome V: ≥2 of six features: straining, hard/lumpy stools (Bristol 1–2), incomplete evacuation, blockage, or manual maneuvers (each >25% of defecations); or <3 SBM/week. Loose stools are rare without laxatives; IBS criteria are not met. Symptoms for 3 months, onset ≥6 months earlier[13]	Heterogeneous physiology. Proposed neuronal, pacemaker, secretory, and microbial endotypes remain E4. CIC is retained when describing trials using that term	Symptom assessment and evaluation for secondary causes; anorectal testing if refractory, followed by transit assessment as indicated[3,13]
IBS with constipation (IBS-C)	Recurrent, noncontinuous abdominal pain or discomfort ≥3 days/month for 3 months, with ≥2 associations: defecation, altered stool frequency, or altered stool form; onset ≥6 months earlier. IBS-C: >25% Bristol 1–2 and <25% Bristol 6–7 stools, assessed off bowel-habit medication[13]	Visceral sensitivity and gut–brain interactions; IBS susceptibility loci are E2 evidence and do not define a constipation-specific treatment biomarker[9]	Rome V symptom criteria; targeted anorectal and transit evaluation when clinically indicated[13,14]
Slow-transit constipation (STC)	Delayed colonic transit demonstrated objectively; a physiological subgroup, not a separate Rome V bowel category. Refractoriness is common in study cohorts but is not the definition	Reduced ICC volume and altered neuronal proportions in severe STC; emerging smooth muscle mitochondrial and fibrotic changes (E2)[15-17]	Radiopaque-marker or scintigraphic transit testing; evaluate coexisting evacuation dysfunction[3]
Evacuation dysfunction (clinical grouping)	Rome V names dyssynergic defecation within anorectal disorders and removes the broader functional defecation category. Difficult-defecation symptoms plus objective dyssynergia are required; rectal sensory disorders are separate[12,14]	Impaired coordination and/or propulsion; altered rectal sensory thresholds can coexist. Cortical contributions remain a candidate mechanism (E4)	With compatible symptoms, one abnormal evacuation test supports probable dyssynergic defecation and ≥2 support a definite diagnosis. Interpret manometry with balloon expulsion and, when indicated, defecography[14]
Opioid-induced constipation (OIC)	New or worsening constipation after starting, changing, or increasing opioids; ≥2 CC symptom features; loose stools rare without laxatives[13]	Peripheral μ-opioid effects on motility and secretion; randomized antagonist efficacy (E1)[18,19]	Medication timing, bowel symptoms, and other contributors

Summary only; use full diagnostic algorithms. CC, chronic constipation; CIC, chronic idiopathic constipation; E1, established physiology or clinical efficacy; E2, human association; E4, hypothesis; IBS, irritable bowel syndrome; IBS-C, irritable bowel syndrome with constipation; ICC, interstitial cells of Cajal; OIC, opioid-induced constipation; SBM, spontaneous bowel movement; STC, slow-transit constipation.

PDGFRα+ cells mediate purinergic inhibitory responses through P2Y1 receptors and small-conductance calcium-activated potassium channel 3 (SK3) in mouse colon (E1 physiology; E3 perturbation evidence).[28-30] Reduced ICC volume has been reported in human STC (E2).[16] Laser-microdissection mass spectrometry identified reduced BIN1 and ALDH1B1 in pathological versus relatively normal smooth-muscle regions of STC colectomies. Knockdown in primary human colonic smooth muscle cells impaired contraction and mitochondrial function, increased reactive oxygen species, and activated profibrotic processes. The tissue comparison is E2 and the cell perturbations E3.[17] These findings broaden the mechanistic agenda beyond neuronal and ICC abnormalities.

At the mucosal surface, guanylate cyclase-C signaling through cyclic guanosine monophosphate and the cystic fibrosis transmembrane conductance regulator promotes secretion, whereas inhibition of sodium-hydrogen exchanger 3 reduces sodium absorption. Trials demonstrate clinical efficacy of agents acting on these pathways, without establishing that nonresponders have a pathway-specific defect.[31-36] Selective serotonin type 4 receptor (5-HT4) agonism improves bowel outcomes.[40,41] Microbial short-chain fatty acids (SCFAs) can increase enterochromaffin serotonin synthesis in experimental systems (E3).[39] Methanogen abundance and breath methane are associated with constipation-related features in humans, while methane infusion slows small-intestinal transit in animals (E2 and E3, respectively).[11,42,43] Bile acids engage G-protein-coupled bile acid receptor TGR5-dependent enterochromaffin and intrinsic afferent neuronal signaling in mice; ileal bile acid transporter inhibition improves bowel outcomes clinically, but its benefit does not isolate a TGR5-mediated human mechanism.[46,47]

Coordinated pelvic floor relaxation is required for evacuation, and biofeedback improves dyssynergic defecation.[14,48,49] Genetic studies identify 14 stool-frequency loci and six IBS susceptibility

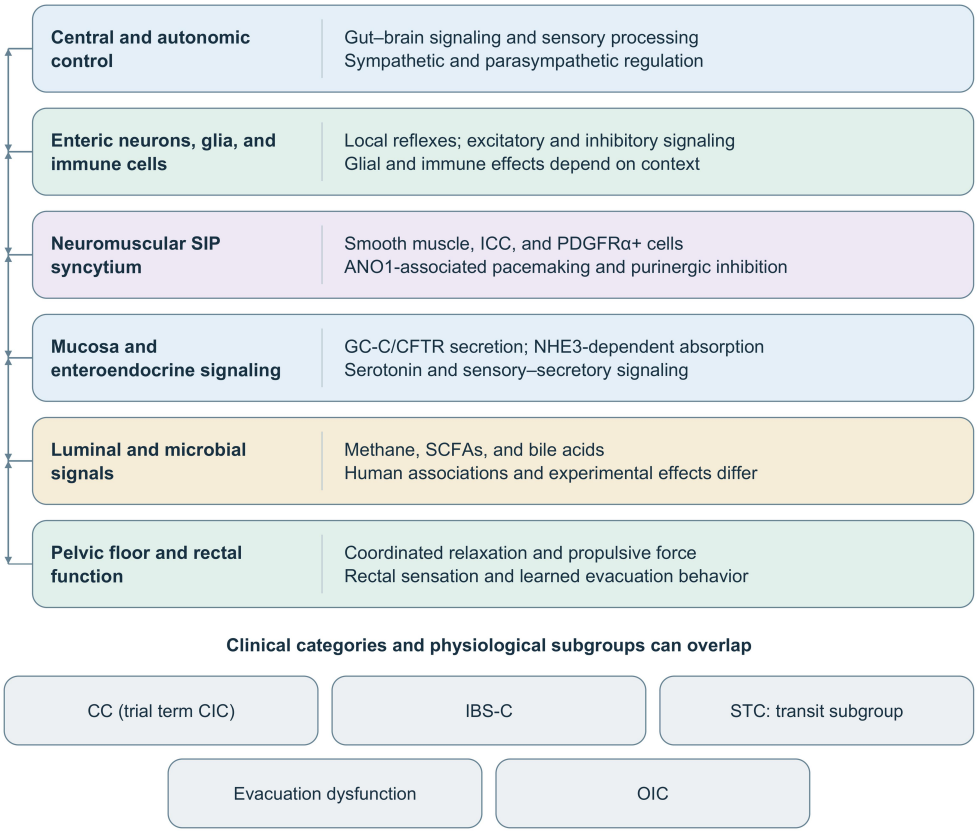


Fig. 1. Interacting systems in constipation physiology. Central and autonomic signaling, enteric neurons and glia, the smooth muscle–ICC–PDGFRα+ (SIP) syncytium, mucosal secretion, luminal signals, and pelvic floor coordination contribute to propulsion and evacuation. Arrows represent conceptual interactions rather than a verified serial pathway. The clinical categories and physiological subgroups can overlap; their depiction does not establish molecular endotypes. Table 2 summarizes evidence and species limitations. Colors separate the labeled domains and have no quantitative meaning. Evacuation dysfunction is a descriptive grouping; Rome V names dyssynergic defecation among anorectal disorders.[12-14] ANO1, anoctamin-1; CC, chronic constipation; CFTR, cystic fibrosis transmembrane conductance regulator; CIC, chronic idiopathic constipation; GC-C, guanylate cyclase-C; IBS-C, irritable bowel syndrome with constipation; ICC, interstitial cells of Cajal; NHE3, sodium-hydrogen exchanger 3; OIC, opioid-induced constipation; PDGFRα, platelet-derived growth factor receptor alpha; SCFA, short-chain fatty acid; SIP, smooth muscle–interstitial cell of Cajal–PDGFRα+ cell syncytium; STC, slow-transit constipation.

loci, with overlap between IBS and mood-related traits.[8,9] Sucrase-isomaltase variants are associated most strongly with diarrhea phenotypes; they should not be presented as established constipation-risk alleles.[50]

Recent therapeutic developments

Table 3 summarizes selected placebo-controlled and exploratory treatment studies.[18,19,31-36,40,41,46,51,53-58] It complements established use of fiber, osmotic or stimulant laxatives, and biofeedback when indicated.[3] The table is not a comparative efficacy analysis: populations, doses, responder definitions, assessment periods, and comparators differ. We calculate absolute response differences and numbers needed to treat (NNTs) within individual randomized comparisons. NNTs use the difference between published response percentages, without intermediate rounding, and are rounded upward to a whole patient; they are approximate point estimates. Direct comparisons exist, including a 4-week trial of polyethylene glycol with electrolytes versus prucalopride, but do not establish a ranking across all agents in Table 3.[59]

Linaclotide and plecanatide improve bowel outcomes in CIC and composite pain–bowel outcomes in IBS-C; diarrhea is an important adverse effect.[31-34,53,54] For the selected doses and endpoints in Table 3, linaclotide's placebo-adjusted response differences are 8.7–19.8 percentage points, corresponding to NNTs of 6–12; plecanatide's differences are 7.3–12.4 points, with NNTs of 9–14. These ranges summarize individual trials and do not establish a between-drug difference. In the 72-µg linaclotide CIC trial, abdominal discomfort did not improve significantly, despite improvement in the primary bowel endpoint.[31] Prucalopride increases complete spontaneous bowel movements (CSBMs) over 12 weeks in predominantly female and male trial populations, with NNTs of 5–6 for the selected comparisons (Table 3).[40,41] An observational cardiovascular safety study found no evidence of increased major-event risk relative to polyethylene glycol, but the adjusted incidence rate ratio of 0.64 (95% confidence interval (CI) 0.36–1.14) does not establish cardiovascular superiority.[60]

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Table 2. Cellular and molecular mechanisms and levels of evidence

Axis	Evidence and interpretation	Evidence type	Key references
Enteric nervous system	Atlases resolve molecular neuron classes in mouse and human intestine. Severe STC specimens show more NOS-only and fewer ChAT-only neurons proportionally, without proving neuronal loss. Nutrient-specific activation is demonstrated in mouse jejunum	E1 architecture; E2 STC association; E3 model perturbation	Drokhlyansky *et al.*[4]; Morarach *et al.*[5]; Wattchow *et al.*[15]; Lasrado *et al.*[22]; Fung *et al.*[23]
Enteric glia and immune signaling	Glial ablation alters mouse motility with sex-dependent effects. Experimental glia–ILC3 and glia–macrophage signaling can support defense or inflammatory resolution. A small STC study identifies immune–stromal associations	E2 human association; E3 model perturbation	Drokhlyansky *et al.*[4]; Chi *et al.*[21]; McClain *et al.*[24]; Ibiza *et al.*[25]; Stakenborg *et al.*[26]; Rao *et al.*[27]
SIP syncytium	ANO1 supports coordinated ICC pacemaking in mouse small intestine; PDGFRα+ cells mediate purinergic inhibition in mouse colon. Human STC shows reduced ICC volume. Smooth muscle BIN1/ALDH1B1 findings combine patient associations with cell perturbation	E1 physiology; E2 patient findings; E3 perturbation	He *et al.*[16]; Singh *et al.*[28]; Baker *et al.*[29]; Kurahashi *et al.*[30]; Liu *et al.*[17]
Mucosal ion transport	GC-C activation promotes cGMP/CFTR-dependent secretion; NHE3 inhibition reduces sodium absorption. Drug efficacy does not establish an underlying pathway defect in individual patients	E1 physiology and randomized clinical efficacy	Schoenfeld *et al.*[31]; Miner *et al.*[32]; DeMicco *et al.*[33]; Brenner *et al.*[34]; Chey *et al.*[35,36]
Enteroendocrine and serotonin signaling	Enterochromaffin cells are the major peripheral source of serotonin. SCFAs stimulate serotonin synthesis experimentally; prucalopride improves CSBM outcomes. Intestinal atlases describe enteroendocrine heterogeneity	E1 physiology and efficacy; E3 SCFA experiments	Berger *et al.*[37]; Gershon[38]; Reigstad *et al.*[39]; Camilleri *et al.*[40]; Yiannakou *et al.*[41]; Hickey *et al.*[7]
Microbiota and metabolites	Stool consistency, methanogens, and STC metagenomic profiles show human associations. Methane slows small-intestinal transit in animals. Fecal-transfer and butyrate studies support mechanisms within their experimental systems	E2 human associations; E3 experiments	Vandeputte *et al.*[10]; Ghoshal *et al.*[42]; Han *et al.*[11]; Pimentel *et al.*[43]; Ge *et al.*[44]; Wang *et al.*[45]
Bile acid signaling	TGR5-dependent enterochromaffin and intrinsic afferent signaling affects mouse motility. Elobixibat improves human bowel frequency; its trial does not isolate a human TGR5 mechanism	E1 clinical efficacy; E3 TGR5 experiments	Nakajima *et al.*[46]; Alemi *et al.*[47]
Pelvic floor function	Symptoms and anorectal testing characterize evacuation dysfunction. Biofeedback improves dyssynergic defecation	E1 randomized clinical efficacy	Rao *et al.*[14,48,49]; Lacy *et al.*[20]
Genetic susceptibility	Stool-frequency GWAS identifies 14 loci; IBS GWAS identifies six loci. Sucrase-isomaltase associations are strongest for diarrhea phenotypes and do not predict constipation treatment	E2 genetic associations	Bonfiglio *et al.*[8]; Eijsbouts *et al.*[9]; Henström *et al.*[50]
Opioid receptor effects	Peripheral μ-opioid receptor antagonists improve SBM responses in OIC trials. These findings do not apply automatically to other constipation categories	E1 clinical efficacy	Chey *et al.*[18]; Hale *et al.*[19]
Microbiota-directed treatment	An open-label randomized FMT trial in STC reports benefit but has substantial blinding and safety limitations. Human-to-mouse transfer used antibiotic-depleted recipients, not a defined gnotobiotic model	Randomized clinical signal with limitations; E3 transfer experiment	Tian *et al.*[51]; Ge *et al.*[44]

E1 denotes established physiology or demonstrated clinical efficacy, not a validated treatment-selection biomarker; E2 denotes human association; E3 denotes effects established by experimental perturbation. Labels classify claims and do not replace study-quality appraisal. ANO1, anoctamin-1; CFTR,

cystic fibrosis transmembrane conductance regulator; cGMP, cyclic guanosine monophosphate; ChAT, choline acetyltransferase; CSBM, complete spontaneous bowel movement; FMT, fecal microbiota transplantation; GC-C, guanylate cyclase-C; GWAS, genome-wide association study; IBS, irritable bowel syndrome; ICC, interstitial cells of Cajal; ILC3, type 3 innate lymphoid cell; NHE3, sodium-hydrogen exchanger 3; NOS, nitric oxide synthase; OIC, opioid-induced constipation; PDGFRα, platelet-derived growth factor receptor alpha; SBM, spontaneous bowel movement; SCFA, short-chain fatty acid; SIP, smooth muscle–interstitial cell of Cajal–PDGFRα+ cell syncytium; STC, slow-transit constipation; TGR5, G-protein-coupled bile acid receptor.

Tenapanor improved the combined pain–CSBM endpoint in both T3MPO trials, with NNTs of 8–13 for the selected comparisons (Table 3); the primary response window was the first 12 weeks even in the 26-week T3MPO-2 study.[35,36] A vibrating capsule improved prespecified CSBM responder outcomes over 8 weeks.[55] In the Japanese elobixibat phase 3 study, the primary efficacy endpoint was change in spontaneous bowel-movement frequency during week 1, not transit acceleration; the companion 52-week study primarily assessed safety.[46] For opioid-induced constipation, naloxegol and naldemedine improve spontaneous bowel-movement responses through peripheral μ-opioid receptor antagonism.[18,19] Regulatory indications and access vary by agent, population, and jurisdiction; these studies should not be interpreted as evidence that every listed therapy applies to every constipation category.

Microbiome-directed approaches have entered controlled evaluation. Ge and colleagues transferred fecal microbiota from patients with STC into antibiotic-depleted, "pseudo-germ-free" mice. Recipients showed reduced fecal output, delayed transit, and weaker colonic contractions.[44] Butyrate and lithocholic acid were significantly reduced; deoxycholic acid showed a nonsignificant downward trend. Separate butyrate and deoxycholic acid supplementation arms improved selected fecal and contractility outcomes, but the study did not establish normalization of transit after supplementation. The recipient mice were not germ-free and did not have a fully defined microbiota. This experiment provides preclinical causal evidence (E3) for a transferable phenotype under those conditions, but it does not establish microbial causation in the donors or a clinically validated human endotype.

Human metagenomic findings remain associative. Han and colleagues compared 24 STC cases with 24 matched controls and identified compositional differences and predicted metabolic differences; these predictions were not direct metabolomic measurements.[11] Ghoshal and colleagues found higher *Methanobrevibacter smithii* abundance in IBS-C and an association with breath methane.[42] A study combining metagenomics in 460 women, serum SCFA measurements, and butyrate experiments in mouse enteric neuronal cells also linked microbial metabolism to candidate mechanisms.[45] These observations and model experiments do not determine whether microbial changes initiate human constipation.

Small antibiotic studies provide preliminary evidence of treatment. A neomycin trial subanalysis found a constipation benefit in 12 methane-producing participants, and a retrospective series favored rifaximin plus neomycin for methane eradication.[56,57] A subsequent randomized study of 31 methane-positive IBS-C participants found lower constipation severity with the combination than with neomycin alone.[58] The latter enrolled only methane-positive patients and therefore did not test a methane-by-treatment interaction against a methane-negative group. An open-label trial of fecal microbiota transplantation (FMT) in 60 patients with STC found improvements over conventional care, but also more treatment-related adverse events.[51] Small samples, limited blinding, donor selection, and uncertain durability constrain interpretation. These results support further research, not routine microbiome-directed treatment of unselected constipation or a validated methane-defined therapeutic category.

In the selected CIC and IBS-C trials, stringent primary responder rates were below 40%; other endpoints and populations produce different values. For example, naldemedine's opioid-induced constipation responder rates reached 47.6% and 52.5%.[19] Trial response is not equivalent to normalization or treatment-free remission. Longer-term comparative data and durable outcomes after treatment cessation remain limited. Biological heterogeneity is one plausible contributor to incomplete response, alongside endpoint stringency, adherence, symptom severity, overlapping disorders, and treatment tolerability. The trial results alone cannot establish an endotype mismatch as the principal explanation.

The known-unknowns: Testable hypotheses

We propose five candidate endotypes and one cross-cutting pharmacogenomic strategy (Table 4).[4,5,8,9,11,14-16,21,24-30,42,43,48,49,51,55,56-58,61-65] All remain E4. Each links a measurable feature to a prespecified prediction, an appropriate validation method, and evidence that would count against that prediction. These are worked examples rather than an exhaustive classification. Before testing, investigators should define the population, assay threshold, comparator, primary outcome, and minimum clinically important effect, and distinguish discovery from independent validation. Across all six hypotheses, a nonsignificant *P* value is insufficient for refutation: a sufficiently precise estimate must exclude the prespecified effect. Treatment prediction requires a biomarker-by-treatment interaction in randomized data, not merely a correlation with improvement in one treatment arm. Biomarker reproducibility, multiplicity control, and donor-level replication are essential. A negative study challenges the operational hypothesis tested, not every possible biological mechanism in that domain. The proposed strata may overlap, and researchers must measure their stability during treatment rather than assume it.

Hypothesis 1: An altered neuronal-composition endotype. Severe STC specimens show increased NOS-only and reduced ChAT-only neuronal proportions in one regional case-control study (E2).[15] We hypothesize that a reproducible signature identifies patients who derive greater benefit from 5-HT4 agonism than from a comparator (E4). Full-thickness tissue profiling against ENS reference atlases should combine molecular classification with independent counts and marker intensity, because altered expression can mimic a change in cell proportions.[4,5] Mucosal ChAT, glial fibrillary acidic protein (GFAP), PHOX2B, or RET measurements are exploratory correlates, not validated measures of myenteric neuron composition; marker specificity and sampling depth require explicit validation. GFAP does not label all enteric glia.[27] Paired mucosal and full-thickness specimens would establish whether any accessible proxy captures the deeper signature. A

Table 3. Selected treatment studies and trial-specific outcomes

Treatment and mechanism	Population and endpoint	Response or effect	Absolute difference; NNT	References
Linaclotide 72 µg daily; GC-C agonist	CIC; CSBM responder in ≥9/12 weeks[a]	13.4% *vs.* 4.7% placebo. Abdominal discomfort did not improve significantly	8.7 pp; 12	Schoenfeld *et al.*[31]
Linaclotide 290 µg daily	IBS-C; combined pain–CSBM responder in ≥6/12 weeks[b]	33.7% *vs.* 13.9%; 33.6% *vs.* 21.0% in separate trials	19.8 pp; 6 12.6 pp; 8	Chey *et al.*[53]; Rao *et al.*[54]
Plecanatide 3 mg daily; GC-C agonist	CIC; durable CSBM responder[c]	21.0% *vs.* 10.2%; 20.1% *vs.* 12.8% in separate trials	10.8 pp; 10 7.3 pp; 14	Miner *et al.*[32]; DeMicco *et al.*[33]
Plecanatide 3 mg daily	IBS-C; combined pain–CSBM responder in ≥6/12 weeks[b]	30.2% *vs.* 17.8%; 21.5% *vs.* 14.2% in separate trials	12.4 pp; 9 7.3 pp; 14	Brenner *et al.*[34]
Prucalopride 2 mg daily; selective 5-HT4 agonist	CIC; mean ≥3 CSBM/week over 12 weeks. Predominantly female trial and a male trial	30.9% *vs.* 12.0%; 37.9% *vs.* 17.7%, respectively	18.9 pp; 6 20.2 pp; 5	Camilleri *et al.*[40]; Yiannakou *et al.*[41]
Tenapanor 50 mg twice daily; NHE3 inhibitor	IBS-C; combined pain–CSBM response during the first 12 weeks of each trial[b]	27.0% *vs.* 18.7% (T3MPO-1); 36.5% *vs.* 23.7% (T3MPO-2, 26-week treatment)	8.3 pp; 13 12.8 pp; 8	Chey *et al.*[35,36]
Elobixibat 10 mg daily; ileal bile acid transporter inhibitor	Chronic constipation, Japanese phase 3 trial; change in SBM/week during week 1	Least-squares mean change 6.4 *vs.* 1.7 with placebo. Companion 52-week study assessed safety	4.7 SBM/week; NNT not applicable	Nakajima *et al.*[46]
Vibrating capsule; luminal mechanical stimulation	CIC; increase of ≥1 or ≥2 CSBM/week in ≥6/8 weeks (CSBM-1 or CSBM-2)	CSBM-1: 39.3% *vs.* 22.1% sham CSBM-2: 22.7% *vs.* 11.4%	17.2 pp; 6 11.3 pp; 9	Rao *et al.*[55]
Naloxegol 25 mg daily; peripheral µ-opioid antagonist	OIC in noncancer pain; SBM response[d]	44.4% *vs.* 29.4%; 39.7% *vs.* 29.3% in separate trials	15.0 pp; 7 10.4 pp; 10	Chey *et al.*[18]
Naldemedine 0.2 mg daily; peripheral µ-opioid antagonist	OIC in noncancer pain; SBM response[d]	47.6% *vs.* 34.6% (COMPOSE-1); 52.5% *vs.* 33.6% (COMPOSE-2)	13.0 pp; 8 18.9 pp; 6	Hale *et al.*[19]
Rifaximin plus neomycin; microbial modulation	Methane-positive IBS-C; small antibiotic trials with symptom and breath-methane endpoints	A 31-patient randomized study favored combination over neomycin for constipation severity. Earlier evidence includes a post hoc subgroup and retrospective comparison	No NNT calculated; heterogeneous endpoints and designs	Pimentel *et al.*[56,58]; Low *et al.*[57]
FMT; microbial community transfer	STC; open-label randomized trial, *n* = 60; mean ≥3 CSBM/week at 12 weeks	36.7% *vs.* 13.3% with conventional therapy. More reported adverse-event episodes with FMT; blinded confirmation needed	23.4 pp; 5 (unblinded estimate)	Tian *et al.*[51]

Unlike trials should not be ranked; direct comparisons exist.[59] Approximate NNT = 100/(active response % − control response %), rounded upward after calculation from published percentages. These are endpoint- and time-specific point estimates; CIs are not reported. [a] ≥3 CSBM/week and ≥1 above baseline in ≥9/12 weeks. [b] ≥30% pain reduction and ≥1 additional CSBM in the same week, in ≥6/12 weeks. [c] Definition [a] plus response in ≥3 of the last 4 weeks. [d] ≥3 SBM/week and ≥1 above baseline in ≥9/12 weeks and ≥3 of the last 4 weeks. 5-HT4, serotonin type 4 receptor; CI, confidence interval; CIC, chronic idiopathic constipation; CSBM, complete spontaneous bowel movement; FMT, fecal microbiota transplantation; GC-C, guanylate cyclase-C; IBS-C, irritable bowel syndrome with constipation; NHE3, sodium-hydrogen exchanger 3; NNT, number needed to treat; OIC, opioid-induced constipation; pp, percentage points; SBM, spontaneous bowel movement; STC, slow-transit constipation.

Table 4. Candidate endotypes and prospective tests

Hypothesis	Candidate measurement	Validation and intervention test	Evidence against the prespecified claim	Sampling needs
H1 neuronal composition	Higher NOS-only and lower ChAT-only neuronal proportions in severe STC. Mucosal markers are candidate proxies, not measurements of myenteric composition[15]	Validate mucosal proxies against matched full-thickness tissue and ENS atlases. Test a validated proxy in a separate nonsurgical randomized prucalopride cohort[4,5]	Adequately precise exclusion of the specified compositional difference or treatment interaction. Failure of a proxy alone does not refute the tissue mechanism	Tier 3 tissue; Tier 2 proxy; separate clinical trial
H2 ICC pacemaking	ICC volume (CD117/ANO1) and preserved-tissue electrical/calcium activity. Staining alone does not measure function[16,28-30]	Validate tissue measures, then compare stimulation with secretagogue therapy using an independently validated accessible marker. Vibrating capsule and conflicting STC neuromodulation trials are not ICC-stratified[55,61,62]	Precisely exclude structural/functional differences or prespecified biomarker–treatment interactions; interpret assay failure separately	Tier 3; subsequent marker validation and trial
H3 methanogen enrichment	Standardized breath methane, stool methanogen abundance, metabolites, and transit; specify thresholds and sampling conditions[11,42,43]	Randomize treatment within methane-positive and negative strata to test interaction. Antibiotic and FMT findings do not establish methane-guided efficacy[51,56-58]	A sufficiently narrow interaction CI excludes the prespecified clinically meaningful effect. A positive-stratum-only trial cannot establish predictive specificity	Tier 1 samples; research assays and randomized treatment
H4 neuroimmune activation	Analyze IBS-C and STC separately. Candidate mucosal immune activation and deeper neuron–immune relationships require anatomically appropriate samples[21,24-26]	Replicate the exploratory STC signature in independent controls. Validate a population-specific signature before selecting a mechanistically justified intervention; distinguish protective from pathogenic immune activity	Within each population, precisely exclude the specified cellular difference or treatment interaction; no cross-population inference	Tier 2 mucosa; Tier 3 muscle and myenteric plexus
H5 cortical–pelvic coordination	Confirmed defecation disorder plus reproducible fMRI features; altered activation is not anatomical disconnection[14,63]	Validate the signature during standardized biofeedback, then compare adjunctive neuromodulation with a suitable control while maintaining indicated biofeedback in both groups. STC trials do not establish benefit in dyssynergia[48,49,61,62]	Precisely excluded incremental benefit or signature–treatment interaction, with adequate assay reliability	Tier 1 physiology; specialized imaging; randomized trial
H6 genetic response modifiers	Candidate drug-target genes and polygenic scores; susceptibility is not validated response prediction[8,9,64,65]	Randomized trial DNA substudy: prespecify loci, interactions, power, and multiplicity; assess prediction independently	Adjusted interaction CIs wholly within a clinically justified margin (e.g., ±10 pp difference in absolute treatment effects); no meaningful predictive gain in independent data. Limit inference to tested loci, therapies, and populations	Tier 1 DNA; powered trial substudies

E4, unvalidated proposals; nonsignificance is not equivalence. Tiers: 1, clinical phenotyping/accessible samples; 2, mucosa; 3, full-thickness tissue. ANO1, anoctamin-1; ChAT, choline acetyltransferase; CI, confidence interval; ENS, enteric nervous system; fMRI, functional magnetic resonance imaging; FMT, fecal microbiota transplantation; IBS-C, irritable bowel syndrome with constipation; ICC, interstitial cells of Cajal; NOS, nitric oxide synthase; pp,

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percentage points; STC, slow-transit constipation.

separate nonsurgical cohort could then test that proxy in a randomized prucalopride comparison. Post-colectomy tissue alone cannot provide a prospective treatment-selection assay for an intact colon. Failure to reproduce the specified composition signature, or a precise absence of the predicted treatment interaction, would argue against this operational endotype.

Hypothesis 2: An ICC-pacemaker endotype. Reduced ICC volume in human STC (E2) and ANO1-dependent coordination in mouse intestine (E3) motivate a candidate pacemaker subgroup; ANO1 dysfunction has not been established as its human cause.[16,28] We hypothesize differential benefit from mechanical or electrical stimulation versus secretagogue therapy (E4). A vibrating-capsule trial supports efficacy in CIC.[55] The open-label No. 2 trial found success at 6 months in 53.7% receiving sacral neuromodulation versus 3.8% receiving conservative treatment for refractory idiopathic STC; outlet obstruction was excluded.[61] However, an earlier sham-controlled crossover trial found no benefit during 3-week active stimulation periods.[62] Different designs and populations, limited blinding, and adverse events temper interpretation; neither trial selected patients by ICC biomarkers.

Testing requires CD117/ANO1 staining and RNA localization in tissue containing the muscularis propria, quantitative ICC assessment, and functional recordings where feasible.[16,28-30] Staining intensity alone cannot establish pacemaker failure. ANO1-directed drugs remain a preclinical proposition requiring direction-specific efficacy and safety studies. Clinical stratification would require a validated accessible proxy or another justified sampling strategy. Replicated absence of the proposed tissue abnormality, or a precise null biomarker-by-treatment interaction, would challenge the specified hypothesis.

Hypothesis 3: A methanogen-enriched microbial endotype. Methane slows small-intestinal transit in animal experiments (E3), while human methanogen and breath findings remain associations (E2).[42,43] We hypothesize that methane-related physiology identifies a subgroup that benefits differently from a microbial intervention (E4). Breath methane, stool *M. smithii* abundance, and transit should be measured separately rather than treated as interchangeable biomarkers.[11,42] Thresholds, substrate, sampling schedule, recent antibiotics, laxatives, and diet should be prespecified. Older antibiotic studies used different methane thresholds and do not validate a universal cutoff.[56-58] A randomized trial including methane-positive and methane-negative strata is needed to test interaction, with methane suppression, transit, symptoms, and adverse events measured longitudinally. FMT or defined microbial consortia would require separate efficacy and safety evaluation; the existing FMT trial was not methane-stratified.[51] A sufficiently precise absence of the predicted methane-by-treatment interaction would argue against the proposed treatment-selection rule, without excluding all microbial contributions to constipation.

Hypothesis 4: An enteric neuroimmune endotype. The STC-specific evidence and broader neuroimmune literature address different questions. An exploratory single-cell study compared cecal tissue from six STC cases with tumor-adjacent tissue from six early-stage colon-cancer controls, identifying immune–stromal differences and inferred signaling networks (E2).[21] Small donor numbers, cancer-associated control tissue, and computationally inferred interactions limit causal interpretation. Separate studies describe histamine-dependent mast cell–glial signaling, glial regulation of type 3 innate lymphoid cells, and glial support of anti-inflammatory macrophages during muscularis inflammation.[24-26] These mechanisms were studied outside constipation cohorts and do not collectively establish a pro-inflammatory constipation signature.

We propose separate IBS-C and STC hypotheses (E4), each requiring its own recruitment, controls, and validation. Mucosal studies could test immune activation, mediator release, and spatial relationships to identifiable nerve fibers. They cannot assess muscularis macrophages or myenteric neuron contacts without deeper tissue. Cell density, activation state, and proximity should be distinguished, and inferred ligand–receptor interactions require experimental confirmation. Initial functional studies should identify whether a pathway is pathogenic or protective before selecting mast cell stabilization or cytokine modulation for a clinical trial. Failure to reproduce the population-specific signature, or a precise absence of the prespecified treatment interaction, would challenge that form of the hypothesis. An IBS-C result should not be transferred to STC, or vice versa.

Hypothesis 5: A cortical–pelvic sensorimotor endotype. Functional magnetic resonance imaging during simulated defecation identified altered activation patterns in 18 women with Rome III functional defecatory disorder versus 15 controls (E2).[63] This does not demonstrate anatomical "disconnection." Biofeedback is an established treatment for dyssynergic defecation, supported by randomized trials and the Rome V framework.[14,48,49] We hypothesize that a reproducible combined anorectal–neuroimaging signature improves prediction beyond standard anorectal tests and identifies differential benefit from an adjunctive intervention (E4). Initial studies should evaluate response to standardized biofeedback and independently validate the signature. A subsequent trial could compare adjunctive neuromodulation with a suitable control while retaining indicated biofeedback. The No. 2 trial's STC findings cannot establish benefit in dyssynergic defecation because outlet obstruction was excluded.[61] Any neuromodulation comparison here remains investigational. The hypothesis would be weakened if the combined signature failed to add reproducible predictive information, or if the prespecified adjunctive treatment showed a precise null interaction.

Hypothesis 6: Pharmacogenomic modification of treatment response. This is an analytic strategy across candidate endotypes. *GUCY2C*, *SLC9A3*, *SLC10A2*, *HTR4*, and bile acid-related *KLB/FGFR4* are nominated by pharmacology rather than validated response predictors. We identified no validated treatment-selection algorithm for the listed constipation therapies based on these loci. The Rome V pharmacology review distinguishes drug-metabolism pharmacogenomics from the more limited evidence concerning therapeutic targets.[64] In 1,568 cancer patients receiving opioids, *COMT* rs2020917 was the only tested variant to pass a 10% false-discovery-rate threshold among 75 variants in 15 genes; this concerned constipation during opioid exposure, not randomized

response to constipation treatment.[65]

Pharmacogenomic analyses should be prespecified within randomized trials and adequately powered using allele frequencies, expected interactions, ancestry, and multiplicity. Stool-frequency and IBS genetic studies offer discovery frameworks, but susceptibility scores are not treatment-response scores.[8,9] For a nominated interaction, evidence against a clinically important effect requires an appropriately adjusted CI lying wholly within a prespecified equivalence margin, for example −10 to +10 percentage points for the difference in absolute treatment effects between genotype strata. The margin requires clinical justification before analysis. This tests only the defined loci, population, treatment, and margin, not the absence of all genetic effects. Any polygenic model must also demonstrate incremental calibration, discrimination, and clinical utility beyond clinical covariates in independent data. Underpowered null findings remain inconclusive.

The unknown-unknowns: A discovery roadmap

The ENS, comprising submucosal and myenteric plexuses, can now be profiled at single-nucleus resolution alongside other intestinal cell populations.[4-7,66] Coordinated clinical, cellular, spatial, and multimodal studies could reveal unanticipated mechanisms (Fig. 2).[67,68] Patient-derived molecular differences remain associations until suitable perturbations establish effects within models, and model validation still requires subsequent testing of clinical relevance. Separate exploration from confirmation so hypotheses emerging from an atlas are evaluated in new specimens or participants.

Single-cell and single-nucleus transcriptomics of the human colon in disease

Mature enteric neurons are difficult to recover intact. RAISIN RNA sequencing profiles intact nuclei with associated ribosome-bound transcripts, and MIRACL-seq enriches rare cell types for droplet profiling; together they enabled foundational adult ENS atlases.[4] Complementary nuclear and cellular studies profiled mouse and human ENS and tested GDNF, neurturin, and TBX3-related mechanisms in experimental systems.[69] In STC, region-matched tissue profiling could identify altered proportions, stress states, and candidate glia–immune interactions. Reference mapping must accommodate disease-specific states rather than force every cell into a healthy category.

Study power should be based on independent donors, not the number of sequenced cells. Consider region, age, sex, medications, disease severity, surgical indication, and processing batch. Regional and species heterogeneity is well documented, while spatial lineage organization supplies additional biological context.[4,22,52] Ligand–receptor inference nominates possible communication; it does not demonstrate signaling or physical contact.[21] Paired chromatin and RNA measurements can relate regulatory states to expression but cannot alone distinguish primary from acquired disease changes.[70,71]

Spatial transcriptomics: Mapping disease to architecture

Spatial profiling preserves information lost during cell isolation. Human intestinal atlases using single-cell sequencing and multiplexed imaging demonstrate regional cell organization, while the spatial-transcriptomics literature provides methodological context.[6,7,66] For constipation, appropriate tissue sections could localize cell-state changes to mucosa, submucosa, plexuses, and muscle layers. Proposed analyses include immune-cell proximity to neurons or ICC and colocalization with ANO1, ChAT, neuronal NOS, and CD117. These measurements should complement, rather than replace, well-controlled quantitative histology.

A multicenter atlas should harmonize anatomical orientation, sampling depth, fixation, preservation, and cell-segmentation quality. Its design must also distinguish local tissue variation from between-patient variation. Pilot estimates of donor-level variation, endotype prevalence, assay failure, and effect size should determine sample size. Treatment-interaction trials will generally require separate and potentially much larger calculations. Full-thickness sampling at this scale depends on coordinated biobanking, whereas mucosal studies are more accessible. Sample the same anatomical site across cases and controls wherever possible, and do not assume cancer-adjacent controls represent healthy community tissue without qualification.

Platform strengths, blind spots, and cross-modal validation

Each platform has distinct strengths and blind spots. Combining cellular, spatial, and functional evidence can strengthen a mechanistic hypothesis, provided the measurements address the same biological question (Fig. 3, Table 5).[4,17,44,52,67,70-73,74-91]

Dissociation-based single-cell RNA sequencing (scRNA-seq) captures cytoplasmic transcripts and cell-state variation, but tissue digestion can alter recovery and induce stress programs. Direct comparisons in mouse kidney and cortex demonstrate advantages of single-nucleus RNA sequencing (snRNA-seq), including access to frozen specimens and reduced dissociation-related artifacts.[74,75] These findings support protocol choice but cannot quantify bias in diseased human colon without direct benchmarking. Nuclear assays also have limitations: depletion of activation-related transcripts was demonstrated in human microglia and should prompt caution when studying inflammatory states, without assuming the same effect size in enteric cells.[76] ENS studies use complementary sampling and profiling methods.[4,5,52] Recovered proportions therefore remain conditional on anatomy, tissue handling, enrichment, and analysis. Independent histological counts and replicate preparations are needed before interpreting an apparent cell deficit as disease biology.

Spatial platforms differ in capture chemistry, effective resolution, sensitivity, and gene coverage. Original spot-based methods capture mixed-cell regions and often require deconvolution.[77] Dense arrays such as Stereo-seq support substantially finer spatial sampling.[78] Capture-element size nevertheless differs from effective biological resolution, which depends on read depth, binning, and segmentation. Imaging methods assay predefined probes; seqFISH+ demonstrated measurement of 10,000 genes and discovery of spatial cell classes.[80] MERFISH also combines molecular identity with spatial organization.[79] Unmeasured transcripts remain inaccessible to a fixed panel, and transcript detection within a segmented cell does not guarantee accurate assignment at tissue boundaries. Record platform versions and panel composition when comparing datasets.

Multiplexed spatial proteomics adds protein localization in intact tissue. Co-detection by indexing (CODEX), imaging mass cytometry, and multiplexed ion beam imaging established high-

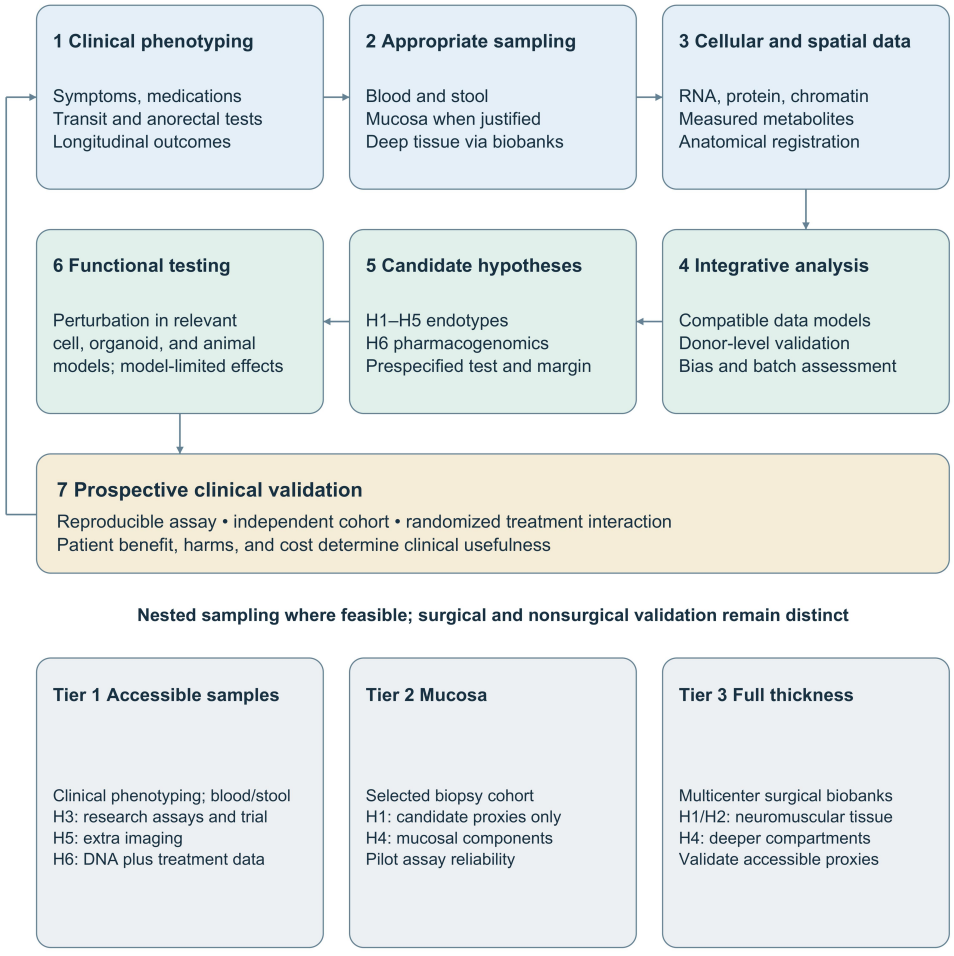


Fig. 2. Proposed roadmap for constipation endotype research. Seven stages connect clinical phenotyping, sampling, cellular and spatial profiling, integration, hypothesis testing, functional models, and prospective biomarker-stratified trials. The feedback arrow denotes iterative refinement, not demonstrated clinical benefit. Sampling tiers indicate access requirements rather than validated cost or accrual estimates. Tier 1 supports baseline phenotyping and accessible samples; Tier 2 adds mucosa; Tier 3 adds deeper neuromuscular tissue. H4 requires Tier 3 for muscularis/myenteric questions, H5 needs specialized imaging for its full signature, and H3/H6 require treatment studies to establish predictive value. H1, neuronal composition; H2, interstitial cell of Cajal pacemaking; H3, methanogen enrichment; H4, neuroimmune activation; H5, cortical–pelvic coordination; H6, genetic response modifiers (Table 4). Clinical utility is an additional empirical test, as illustrated by biomarker-stratified and monitoring-guided Crohn's disease trials.[67,68] Colors distinguish groups of stages and sampling tiers; numbers indicate the proposed sequence. DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

dimensional antibody-based imaging in spleen or tumor tissue.[87-89] In constipation, a validated panel could assess ANO1, neuronal NOS, ChAT, CD117, glial markers, and immune populations, provided antibodies perform reliably in the relevant tissue preparation. A recent STC smooth-muscle study illustrates disease-specific spatial proteomics, but does not establish a general ranking of biomarker performance across platforms.[17] Protein colocalization supports anatomical plausibility; it does not by itself demonstrate signaling or contractile function.

Spatial image analysis requires cycle alignment, background correction, segmentation, marker quantification, normalization, cell phenotyping, and neighborhood analysis. Each step can change apparent cellular relationships, so validation should include manual review of representative regions and sensitivity to segmentation parameters. General discussions of spatial proteomics emphasize these translational challenges.[90] Integration must respect the modalities actually measured. Seurat weighted nearest neighbors (WNN) combines paired modalities; totalVI models RNA and antibody-derived protein measurements; MultiVI integrates RNA and chromatin accessibility, including partially unpaired data; multi-omics factor analysis (MOFA/MOFA+) estimates shared and modality-specific factors.[82-86] These methods are not interchangeable engines for merging every spatial, microbial, and molecular dataset. In particular, models of cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) do not directly ingest arbitrary tissue images. A valid analysis must specify its observational unit, shared features, pairing assumptions, and validation data.

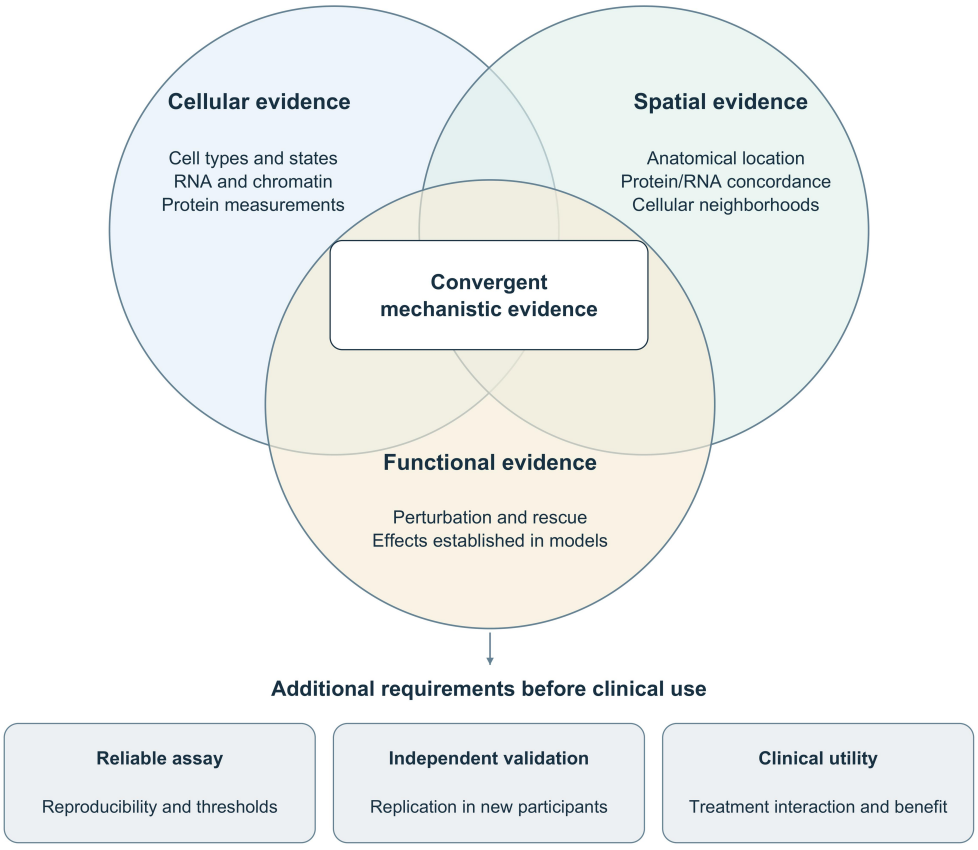


Fig. 3. Convergent evidence and the additional requirements for clinical use. Cellular profiles, spatial measurements, and functional perturbations address different aspects of a candidate mechanism. Their agreement strengthens mechanistic evidence but does not automatically establish a treatment-selection biomarker. Correlated measurements, technical artifacts, and model limitations must be considered; the number of modalities is not a validated grading system. Reproducible assays, independent cohorts, treatment-interaction testing, and demonstrated patient benefit are required before clinical use. Table 5 summarizes platform capabilities and limitations. The translational distinction is illustrated by ENS-containing organoid models and the prospective PROFILE trial.[67,72,73] Colored circles distinguish evidence domains; the downward arrow denotes the subsequent validation requirement. ENS, enteric nervous system; RNA, ribonucleic acid.

No transcriptomic or proteomic modality directly establishes cellular function. Expression, protein abundance, chromatin accessibility, and physiology answer related but distinct questions. Convergent findings strengthen a mechanistic hypothesis only when measurement errors and samples are sufficiently independent. Anatomical constraints matter as much: routine mucosal biopsies do not sample the muscularis propria or myenteric plexus. Mucosal signatures remain candidate surrogates for deeper neuronal or ICC abnormalities until validated against paired tissue.

Multi-omic integration: Linking host, microbe, and metabolite

A longitudinal cohort can connect stool metagenomics and measured metabolites with circulating markers, genotype, symptoms, transit, and selected tissue assays. Collect samples synchronously where feasible, and record diet, medications, rescue treatment, and collection conditions. MOFA/MOFA+ can identify shared axes in aligned cohort-level data; cell-level models such as WNN, totalVI, and MultiVI require their own compatible inputs and cannot simply merge patient-level microbiomes with unmatched single cells.[82-86] Missing-data assumptions and imputation should be stated explicitly. Analyses must distinguish measured metabolites from values predicted from microbial gene content, and associations from intervention effects. Replication should occur in independent participants rather than randomly splitting cells from the same donor across training and test sets.

Mendelian randomization can assess evidence consistent with a causal exposure–outcome effect under assumptions of instrument relevance, independence from confounding, and no effect on the outcome through pathways other than the exposure. Researchers cannot conclusively verify the latter assumptions. Testing whether a microbial taxon or bile acid affects constipation requires exposure-specific instruments, such as microbiome or metabolite quantitative-trait loci, rather than variants associated with stool frequency or IBS. Those genome-wide association studies may supply related outcome data, but stool frequency is a quantitative proxy and IBS includes several stool-pattern subtypes; neither is identical to a constipation diagnosis.[8,9]

Table 5. Cellular and multi-omic platforms for mechanistic discovery

Approach	Strengths	Limitations	Role in validation
Single-cell RNA sequencing	Broad RNA measurement including cytoplasmic transcripts; resolves cell states	Dissociation changes recovery and can induce stress. Kidney/cortex comparisons inform design but do not establish colon-specific performance[74,75]	Pair with nuclei and tissue imaging; quantify donor and cell-recovery biases
Single-nucleus RNA sequencing	Compatible with frozen specimens; enables enteric neuronal profiling and rare-cell enrichment[4,52]	Transcript detection and recovery differ by cell type. Reduced activation-associated RNA detection in human microglia is a context-specific caution[74-76]	Map ENS subtypes while validating proportions and RNA changes in intact tissue
Spatial RNA sequencing	Broad transcript profiling with anatomical coordinates; array resolution varies[77,78]	Original spot-based methods can mix cells; high-density arrays do not guarantee complete single-cell transcript capture	Localize candidates by intestinal layer; validate segmentation and deconvolution
Spatial RNA imaging	High-resolution localization; MERFISH and seqFISH+ illustrate targeted and large-panel approaches[79,80]	Panel size is method-dependent, not universally 100–500 genes; seqFISH+ demonstrated 10,000. Probe design, detection, and segmentation remain constraints	Validate transcript localization and spatial relationships without inferring communication from proximity alone
Joint protein and RNA	CITE-seq links RNA to antibody-detected epitopes in individual cells[81]	Original implementation emphasizes surface proteins; antibody specificity and background constrain inference	Assess cell states and RNA–protein agreement using appropriate antibody panels
Joint chromatin and RNA	sci-CAR and SHARE-seq link accessibility with transcription[70,71]	Paired measurements do not by themselves distinguish primary from acquired changes or prove regulatory causation	Nominate regulatory relationships for longitudinal and perturbation studies
Multimodal integration	WNN: paired modalities; totalVI: RNA/protein; MultiVI: RNA/chromatin; MOFA: shared and modality-specific factors[82-86]	Pairing, observational units, missingness, and model assumptions differ. Batch correction can erase biology; imputation cannot recover unsampled tissue layers	Benchmark with held-out donors, measured concordance, and biological conservation
Spatial proteomics	CODEX, IMC, and MIBI retain spatial protein context. Laser-microdissection mass spectrometry identified region-specific STC smooth-muscle changes[17,87-89]	Antibody imaging requires validated reagents and segmentation. Microdissection mass spectrometry averages proteins within sampled regions. Superior clinical biomarker yield is unproven	Orthogonal validation of abundance and localization; proximity is not functional signaling[90]
Functional models	Organoids, ENS-containing models, and animal perturbations test mechanisms within their systems[72,73,91]	Species, developmental, and reductionist limitations. The cited STC transfer experiment used antibiotic-depleted mice[44]	Establish E3 model effects; require separate analytical and prospective clinical validation before treatment selection

Platform choice should follow the biological question; this paper establishes no hierarchy of clinical biomarker performance. ATAC, assay for transposase-accessible chromatin; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CODEX, co-detection by indexing; ENS, enteric nervous system; IMC, imaging mass cytometry; MERFISH, multiplexed error-robust fluorescence *in situ* hybridization; MIBI, multiplexed ion beam imaging; MOFA, multi-omics factor analysis; MultiVI, multimodal variational inference; RNA, ribonucleic acid; sci-CAR, single-cell combinatorial indexing of chromatin accessibility and RNA; seqFISH+, sequential fluorescence *in situ* hybridization plus; SHARE-seq, simultaneous high-throughput ATAC and RNA expression with sequencing; STC, slow-transit constipation; totalVI, total variational inference; WNN, weighted nearest neighbor. E3 denotes experimental effects in model systems.

The analysis plan should assess instrument strength, ancestry, linkage disequilibrium, sample overlap, and horizontal pleiotropy. Sensitivity analyses and bidirectional models can probe assumptions but cannot guarantee validity. Without credible instruments, causal estimation should not be presented as definitive evidence. Repeated sampling is also needed to test whether microbial and metabolic stratifiers remain stable during a treatment interval. Cross-sectional stool-consistency associations do not establish within-person temporal stability.[10] In a separate mouse spinal-cord-injury model, inulin and SCFA-related interventions supported ENS resilience through diet–microbiome–immune mechanisms; transferability to idiopathic constipation remains unproven.[92]

Selecting and benchmarking integration methods

Benchmark integration methods using held-out donors, preservation of known biology, batch robustness, calibrated uncertainty, and sensitivity to missingness (Table 5). Confirm inferred states with independently measured markers and relevant functional experiments. A well-mixed computational embedding alone does not establish a clinically meaningful endotype.

A staged implementation path

Cost, tissue access, and achievable recruitment require a staged design. We propose three implementation tiers, distinct from evidence labels E1–E4. When feasible, nest more intensive studies within a shared cohort, with explicit linkage to a surgical biobank when identical sampling is impossible. Document selection into biopsy or surgery because nested sampling does not eliminate selection bias. Pilot studies should measure costs, accrual rates, and assay failure rather than assume them based on a platform's sophistication or apparent simplicity.

Tier 1 establishes standardized symptom diaries, medication and dietary histories, clinically indicated anorectal and transit testing, longitudinal follow-up, and consented biospecimen banking. Breath methane and targeted stool assays can support the microbial hypothesis; shotgun metagenomics, untargeted metabolomics, and broad serum panels are research additions rather than routine low-cost tests. Banked DNA supports later pharmacogenomics but does not by itself test treatment prediction. H3 requires an embedded randomized intervention to test predictive interaction; H5 requires additional neuroimaging for the full proposed signature; H6 requires randomized treatment data and sufficient genotype-stratum sizes. Tier 1 can establish repeatability and candidate prevalence before expensive profiling expands.

Tier 2 adds mucosal sampling during clinically indicated or specifically consented research colonoscopy. A pilot should establish marker specificity and reproducibility before widely applying multiplex immunoassays. Selected specimens may undergo cellular and spatial profiling. This tier addresses the mucosal component of H4 and explores accessible correlates for H1, but cannot validate those correlates without paired deeper specimens. Select the sequencing subset using donor-level power and representativeness, and bank the remaining specimens. Mucosal immune readouts cannot substitute for muscularis macrophage or myenteric circuit measurements. Analyses should remain separate for IBS-C and STC unless a justified pooled model explicitly tests population differences.

Tier 3 uses full-thickness tissue from clinically justified surgery and appropriate comparison resections. It is central to H1 and H2 and to deeper neuroimmune questions in H4. Prospective multicenter biobanking should harmonize consent, anatomical sampling, ischemia time, preservation, and processing. Colectomy specimens represent selected, treatment-refractory disease; comparison tissue from other surgical indications also carries potential confounding. Paired mucosal and full-thickness samples are required to validate proxies, and subsequent treatment-prediction studies should use separate nonsurgical participants where appropriate.

Functional validation: From omics to mechanism

Functional experiments test specific causal steps suggested by molecular observations. Intestinal organoid–ENS co-culture approaches provide a methodological starting point, but their developmental stage, cellular composition, and region of origin limit generalization.[91] Childs and colleagues generated pluripotent-stem-cell-derived intestinal organoids with neuroglial, endothelial, and organized smooth-muscle components through coordinated differentiation, without requiring co-culture; after transplantation, these constructs showed peristaltic-like contractions.[72] This supports model development, not an established patient-specific constipation assay.

For epithelial candidates, proposed readouts include pathway-specific transepithelial ion transport and direct measurement of serotonin release. Calcium imaging measures cellular activity and should not be described as directly measuring serotonin secretion. Neuromuscular constructs require a functional muscle component before contraction or coordinated motor activity can be interpreted. Gene editing, pharmacological perturbation, and rescue experiments can test selected pathways, with appropriate viability and off-target controls. Such experiments establish effects within the model (E3), not an endotype's causality in patients.

Defined gnotobiotic consortia could extend the antibiotic-depleted STC fecal-transfer model.[44] Engrafted human pluripotent-stem-cell-derived nitrergic neurons improved motility in nitric-oxide-neuron-deficient mice.[73] That result should not be used to justify nitrergic replacement in the proposed H1 subgroup, whose reported relative shift is toward more NOS-only neurons. Across these models, convergence of independent perturbation and rescue results can strengthen mechanistic confidence. Clinical biomarker development additionally requires analytical validity, prospective reproducibility, and evidence that using the marker improves patient outcomes; no fixed number of laboratory platforms guarantees those properties.

Clinical phenotyping at the necessary depth

Phenotype quality limits discovery. Cohorts need validated symptom measures, medication exposure, transit testing, and anorectal evaluation appropriate to the clinical presentation.[3,14] Assess psychological symptoms and other gut–brain factors with validated instruments, given their association with IBS.[9] Tests should answer prespecified questions; every participant need not undergo every modality. Longitudinal records should capture pain, stool form, straining, incomplete evacuation, rescue medications, and patient-valued benefit alongside bowel frequency.

Digital approaches are promising but vary in maturity. A smart-toilet feasibility study monitored six healthy volunteers, while a stool-image smartphone study was conducted in IBS with diarrhea rather than constipation.[93,94] Additional engineering demonstrations include a toilet system using simulated excreta, an endoscopic PressureCap capsule tested in phantoms and porcine models, and a mountable toilet combining urine and stool measurements.[95-97] An ingestible thread-based pH device similarly supports sensor development rather than established constipation phenotyping.[98] These technologies require disease-specific validation, reproducibility across users, and practical assessment of missing data before serving as trial endpoints.

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Candidate biomarkers should enter prospective randomized studies that can test treatment interactions. Adaptive designs may be useful when justified, but are not intrinsically superior to conventional trials. The PROFILE trial shows that biomarker-stratified recruitment is feasible and that a plausible molecular signature may still fail to predict differential treatment benefit.[67] This combination of feasibility and falsifiability is the relevant precedent for constipation endotyping.

Limitations and counterarguments

Three limitations shape this program. First, tissue access selects severe disease: findings from colectomy specimens may not apply to community constipation. Accessible proxies require validation against deeper tissue and subsequent testing outside the surgical population. Second, the stability of candidate stratifiers is uncertain. Stool consistency is associated with microbial composition,[10] but the cited cross-sectional study does not establish longitudinal stability or variability of methane-defined groups. Repeated measurement should determine whether a marker remains informative over the interval in which it would guide treatment. A state-dependent marker could still be useful if its timing and interpretation are validated. Third, the added cost must be justified by patient benefit. Expensive molecular profiles will have limited clinical value if simpler measures provide comparable treatment guidance. Cost-effectiveness analyses should include testing burden, false classification, treatment harms, and access, not only discrimination statistics. These limitations favor a staged program with explicit decisions about when to stop an unproductive assay or hypothesis.

The inflammatory bowel disease precedent supports caution. In PROFILE, 386 patients with newly diagnosed active Crohn's disease underwent stratification using a whole-blood assay derived from T-cell transcriptional signatures before randomization to top-down or accelerated step-up treatment. Although top-down treatment produced substantially better outcomes overall, no statistically significant biomarker–treatment interaction was detected: absolute difference 1 percentage point, 95% CI −15 to 15, $P = 0.944$.[67] This did not demonstrate utility for that selection rule; the CI nevertheless included potentially important interaction effects. In TAILORIX, infliximab escalation using symptoms, biomarkers, and drug concentrations did not increase sustained corticosteroid-free clinical remission with no ulcers at week 54 compared with symptom-guided escalation: 33%, 27%, and 40% across the three arms, $P = 0.50$.[68] This was a particular prospective dosing strategy, not a test of every form of therapeutic drug monitoring.

These trials illustrate why promising biology, prognostic association, and predictive clinical utility must be evaluated separately. Interpret a null result with its CI, power, endpoint, treatment contrast, and implementation context. Neither study establishes that all precision approaches fail, but both argue against assuming that an atlas-derived signature will quickly improve care. In constipation, randomized interaction tests and independent validation should determine whether biomarker information adds value beyond clinical phenotyping, rather than the signature's biological appeal or technical complexity.

Conclusions

Constipation research has identified important neuronal, glial, pacemaker, smooth-muscle, secretory, microbial, and sensorimotor mechanisms, while randomized studies support several targeted treatments. Incomplete clinical response supports investigating biological variation, but does not prove that endotype mismatch is its dominant cause. We propose a coordinated program of five candidate endotypes and a cross-cutting pharmacogenomic strategy, each linked to prespecified measurements and informative tests. The Rome V framework provides a clinical basis for cohort definition, with criteria version and overlapping physiological abnormalities recorded explicitly. Single-cell, spatial, and integrated molecular methods can nominate candidate signatures; anatomically appropriate sampling and functional perturbations test their plausibility. Clinical use requires reproducible assays and prospective evidence of added benefit. A staged program beginning with clinical phenotyping and banking, extending to mucosal studies, and building multicenter full-thickness resources can address feasibility without overstating what accessible tissue can reveal. Progress should be judged by replicated mechanisms and improved patient outcomes, including informative negative studies, rather than the scale of molecular data alone.

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Conflict of interest

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Author contributions

Conception and design (JWW, RDG, SS); administrative support (JWW); literature collection and assembly (E Bonora, E Boschetti, VP, SA); analysis and interpretation, manuscript writing, and final approval (E Bonora, E Boschetti, VP, SA, RDG, SS, JWW). All authors have approved the final version and publication of the manuscript.

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