

Integrated Analysis of Gut Microbiota Dysbiosis and microRNA-155/microRNA-20a Expression Across Multiple Sclerosis Disease subtypes: Comparison with Controls

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**Integrated Analysis of Gut Microbiota Dysbiosis and microRNA-155/microRNA-20a Expression Across
Multiple Sclerosis Disease subtypes: Comparison with Controls**

Running title: Gut microbiota dysbiosis, miRNA expression in MS subtypes and controls

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Abstract

Multiple sclerosis (MS) is a heterogeneous neuroinflammatory disease encompassing four clinical subtypes. Although gut microbiota (GM) dysbiosis and microRNA (miRNA) dysregulation are implicated in MS pathogenesis, their subtype-specific associations remain poorly characterized. This study investigated GM composition and circulating miR-155 and miR-20a across MS subtypes to identify subtype-specific microbiota–miRNA signatures and evaluate their potential to discriminate clinical phenotypes. This case-control study included 240 participants (n=40 each) with clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), other neurological diseases (OND), or healthy controls (HC). GM phyla/genera and butyryl-CoA gene frequency were quantified by qPCR, alongside serum miR-155 and miR-20a. Diagnostic performance was assessed using ROC analysis and Youden index optimization. SPMS showed the most severe dysbiosis, with reduced Firmicutes and Bacteroidetes, increased Proteobacteria and *Akkermansia*, and decreased butyryl-CoA (all $P < 0.01$). miR-155 was increased, whereas miR-20a was decreased across MS subtypes versus HC ($P < 0.0001$). miR-20a positively correlated with beneficial commensals (Firmicutes: $r = 0.384$) and negatively with pro-inflammatory taxa, whereas miR-155 showed inverse patterns. Bacteroidetes robustly discriminated SPMS from HC (AUC=0.993), while miR-20a discriminated RRMS (AUC=0.893) and PPMS (AUC=0.935). Combined microbial-miRNA panels achieved AUC=1.000 for several comparisons, with 85–95% sensitivity and 75–100% specificity. Gut dysbiosis and circulating miR-155/miR-20a profiles are subtype-dependent, with SPMS showing the most pronounced alterations. Integrated microbial-miRNA signatures showed promising cross-sectional discrimination of MS phenotypes, particularly SPMS, but require longitudinal validation before clinical application.

1. Introduction

Multiple sclerosis (MS) is the most common non-traumatic, immune-mediated disabling disease to affect young adults. The characteristic pathological hallmark of MS is perivenular inflammatory lesions, leading to demyelinating plaques [1]. MS follows a clinical continuum from risk through asymptomatic, prodromal, and symptomatic phases. Based on clinical course and pathological features, MS is classified in four subgroups: clinically isolated syndrome (CIS), RRMS, secondary progressive MS (SPMS) and primary progressive MS (PPMS) [2]. MS is typically suspected when a person presents with CIS and one neurological impairment that does not always lead to definitive MS. This can be mono- or polysymptomatic depending on lesion(s) location [3]. Frequent flare-ups of neurological impairment followed by partial or full recovery is a hallmark of RRMS. Vision loss, exhaustion, and numbness are other symptoms [4, 5]. Neurological impairments in SPMS build up without adequate recovery, whereas PPMS follows a progressive path from the onset of clinically evident MS that Less than 10% of patients with MS experience [2].

The underlying etiology of MS and mechanisms driving its increasing incidence remain incompletely understood, although complex gene–environment interactions almost certainly play a significant role. Amongst various risk factors, heredity accounts for about 30%, and other variables have been propounded to play a role in the MS etiology. Moreover, gut microbiota (GM) dysbiosis and its metabolites may contribute to MS pathogenesis. GM dysbiosis can upset the symbiotic interaction between host factors and the GM. In fact, GM alteration has become a subject of debate in MS pathobiology [3, 6, 7]. On the other hand, MicroRNAs (miRNAs) are small non-coding RNAs that regulate many genes post-transcriptionally. MiR-155 and miR-20a are members of the miRNAs family and regulates gene expression post-transcriptionally by binding to the 3'UTR of target mRNA. miR-155 is a well-established pro-inflammatory regulator that promotes Th1/Th17 differentiation and is upregulated in active MS lesions [8], while miR-20a is thought to exert anti-inflammatory effects by repress NLRP3 complex formation and inhibit IL-1 β expression to restore Treg/Th17 cell balance [9], their reciprocal balance may serve as a molecular readout of the host inflammatory state influenced by GM metabolites. Despite growing recognition of the involvement of both GM and miRNAs in MS pathophysiology, studies investigating their simultaneous association across all four clinical subtypes remain scarce. To date, most research has focused on RRMS, with limited data on progressive forms (SPMS and PPMS) and even fewer comparisons involving CIS or non-MS neurological controls. Furthermore, no study has systematically examined whether the expression of miR-155 and miR-20a correlates with specific microbial compositional changes in a subtype-dependent manner. GM and circulating miRNAs are attractive candidates, and reflect systemic immune–metabolic status. Therefore, the primary aim of this study was to simultaneously characterize GM composition and serum expression of miR-155 and miR-20a in four MS subtypes (CIS, RRMS, SPMS, PPMS), compared with both healthy controls (HC) and patients with other neurological diseases (OND).

2. Materials and Methods

2.1. Study design and subjects

This case-control study was performed in the period September 2023 to December 2025. MS cases attending outpatient clinic of the Sheikh-al-Raeis were evaluated by a neurologist and included in the study. Inclusion criteria were individuals diagnosed with each type of MS including CIS, RRMS, PPMS and SPMS, Iranian ethnicity, and age 16–

60 years; exclusion criteria included presence of other autoimmune diseases, cancer, gastrointestinal disorders, metabolic syndrome, psychiatric disorders, or recent antibiotic/probiotic use that might affect the GM. MS cases were evaluated at a baseline visit where all patients had a neurological examination by the same neurologist according to the 2017 McDonald diagnostic criteria [10]. HC subjects who reported no acute or chronic disorders were selected among individuals who were age- and sex-matched with multiple sclerosis cases and the OND subjects were selected from individuals who had visited a neurologist's office and had a neurological disease other than MS. The study received ethical approval from the ethics committee of Tabriz University of Medical Sciences (ethical code: IR.TBZMED.REC.1403.745). All research involving human participants was performed in accordance with the relevant guidelines and regulations, including the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrollment. Blood serum and stool samples were collected from all six groups. All specimens transported to -80 °C freezer until the test day.

2.2. Bacterial 16SrRNA analysis

Fecal samples were used for assessment of bacterial frequency and dysbiosis by real-time PCR (qPCR). Total genomic DNA was extracted from frozen fecal samples that were first allowed to thaw on ice, then 35 g of stool samples were used for DNA extraction by the DNA extraction kit (KiaGene Fanavar©, Iran) operating as per the manufacturer's instructions. Before q-PCR all extracted DNA were normalized in term concentration at 30ng/μL by nanodrop, Then four intestinal bacterial phyla, including Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, five bacterial genus; *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Bacteroides*, *Akkermansia muciniphila*, one bacterial family: Enterobacteriaceae and one fatty acid gene: *butyryl-coA* gene frequency were investigated by 16SrRNA analysis using the qPCR reaction previously described [11] by specific primers designated for each phylum and genus (Supplementary data table 2).

Real-time PCR experiments were performed utilizing 72-well strips with a capacity of 100μL, employing SYBR Green Master Mix without ROX (Ampliqon©, Denmark) on a Rotor-Gene RG-6000 Real-Time PCR Analyzer (Corbett©, Australia).

2.3. miRNAs expression profile

We selected and investigated two miRNA, miRNA-155 and miRNA-20a expression profile, in patients and control groups in serum. For this purpose, miRNA was extracted using 500 microliters of serum using the miRNA detection kit (AnaCell Teb©, Iran), then cDNA synthesis was performed using the cDNA synthesis kit (AnaCell Teb©, Iran). Using the miRNA155, miRNA20a and U6 (internal control) specific real-time PCR detection kit (AnaCell Teb©, Iran), expression profile of these miRNAs was investigated using qPCR.

2.4. Statistical analysis

Statistical analysis was performed by Genex 16, graph-pad prism 10, SPSS version 27 and R software, and all groups were compared in terms of frequency and type of bacterial phyla and genera. The mean standard deviation was used to report relative data, and $P < 0.05$ was considered statistically significant.

2.4.1. Diagnostic cutoff analysis

Diagnostic cutoff analysis was performed to evaluate the ability of GM and microRNA markers to distinguish among the study groups. The dataset included 240 participants divided equally into six clinical categories, with 40 individuals

in each group. The original numerical codes were converted into diagnostic group labels before analysis: code 1 corresponded to RRMS, code 2 to CIS, code 3 to SPMS, code 4 to PPMS, code 5 to OND, and code 6 to HC. Thirteen quantitative variables were included as candidate diagnostic markers: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, *Lactobacillus*, *Fecalibacterium*, *Bifidobacterium*, *Akkermansia*, Enterobacteriaceae, *Bacteroidetes*, *butyryl-CoA*, miR-155, and miR-20a.

2.4.2. ROC analysis

Receiver operating characteristic (ROC) curve analysis was conducted for all pairwise comparisons among the six diagnostic groups. Each pairwise analysis included one group as the reference negative class and the comparator group as the positive diagnostic class. This approach generated 15 pairwise diagnostic comparisons, and each of the 13 markers was assessed separately within each comparison, resulting in 195 ROC-based diagnostic models. The area under the ROC curve (AUC) was used as the principal measure of discriminatory performance. AUC values closer to 1.00 were interpreted as stronger diagnostic separation, whereas values approaching 0.50 indicated limited discrimination between the two groups. For each marker, the optimal diagnostic threshold was selected using the Youden index. This method identifies the cutoff that maximizes the combined sensitivity and specificity and is calculated using the following formula:

$$J = \text{Sensitivity} + \text{Specificity} - 1$$

The cutoff calculation was structured according to the pROC package workflow in R. For each pairwise comparison, a ROC object was generated for the selected marker, and the optimal threshold was extracted using the coords() function with x = "best" and best.method = "youden". The R formula used for cutoff estimation was:

For each optimal cutoff, sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratios, and the Youden index were calculated. The classification direction was determined from the ROC model. Therefore, when marker values were higher in the positive diagnostic group, values equal to or greater than the cutoff were interpreted as supportive of the positive class. When marker values were lower in the positive group, values equal to or below the cutoff were interpreted as supportive of the positive class. Diagnostic performance was visualized using an AUC heatmap across all marker–comparison pairs, ROC curve panels for the best-performing markers, distribution

plots showing the Youden-derived thresholds, and a summary figure presenting the optimal marker and cutoff for each comparison.

```
</> R

roc_obj <- pROC::roc(
  response = response,
  predictor = marker,
  levels = c("negative_group", "positive_group"),
  direction = "auto"
)

best_cutoff <- pROC::coords(
  roc_obj,
  x = "best",
  best.method = "youden",
  ret = c("threshold", "sensitivity", "specificity", "youden")
)
```

3. Results

3.1. Characteristics of the participants

This study examined 240 participants across six groups include four groups of MS types (CIS =40, RRMS=40, PPMS=40, SPMS=40) and one group other neurologic disease (OND, n=40), and one group healthy controls (HC, n=40). There was no significant difference between healthy, Neurologic and MS groups in terms of mean age and sex ($p > 0.05$). Gender and age information of MS patients and control groups were showed in Supplementary table 1.

3.2. Comparison of the gut microbiota frequency between patients with MS and control subjects

3.2.1. Bacterial analysis at phylum level

Bacterial abundance across the six groups was assessed utilizing the Log2 fold-change, through qPCR, we investigated four major phyla in stool sample of MS and control groups. Based on the qPCR results analysis by one-way Anova (Kruskal-Wallis test), the bacterial phylum and were different in the MS groups and the control group. The qPCR phylum analysis revealed significant alterations in key GM among courses of MS, OND, and HC groups (Fig1). Firmicutes frequency were significantly lower in patients with SPMS and in the OND group compared to HC ($P < 0.0001$ for both), as well as compared to patients with CIS and RRMS (each $P < 0.0001$). In contrast, no significant differences were observed between HC and CIS, RRMS, or PPMS ($P > 0.9999$ for all). PPMS showed significantly higher Firmicutes abundance than SPMS and OND (adjusted $P < 0.0001$), but did not differ from HC, CIS, or RRMS (adjusted $P > 0.9999$). Collectively, these findings indicate that reduced Firmicutes levels SPMS and OND from HC, CIS, and RRMS groups (Fig1-A). Bacteroidetes were significantly lower in patients with SPMS compared to all other groups, including HC, CIS, RRMS, PPMS, and OND ($P < 0.0001$ for SPMS vs. HC, CIS, RRMS, PPMS; SPMS vs. OND, $P = 0.0001$) (Fig1-B). these findings indicate that Bacteroidetes abundance is markedly reduced in SPMS, moderately reduced in PPMS and OND, but unchanged in CIS and RRMS relative to HC. Also, Actinobacteria abundance were significantly higher in patients with RRMS, SPMS, PPMS, and OND compared to HC ($P < 0.0001$ for RRMS and SPMS; adjusted $P = 0.0001$ for PPMS; adjusted $P = 0.0045$ for OND). Furthermore,

RRMS showed significantly higher Actinobacteria frequency than CIS ($P = 0.0075$), these findings indicate that Actinobacteria abundance is elevated in all MS subtypes (except CIS) and in OND relative to healthy controls, with RRMS exhibiting the most pronounced increase (Fig1-C). Proteobacteria abundance were significantly higher in patients with SPMS and PPMS compared to HC (SPMS vs. HC: $P = 0.0005$; PPMS vs. HC: $P < 0.0001$). Both SPMS and PPMS also exhibited significantly higher Proteobacteria abundance than the OND group (SPMS vs. OND: $P = 0.0138$; PPMS vs. OND: $P = 0.0023$). These findings indicate that Proteobacteria abundance is elevated in progressive MS subtypes (SPMS and PPMS) compared to HC and OND (Fig1-D).

3.2.2. Bacterial analysis at genus and family level

We investigated five bacterial genera and one family in MS and control groups that these bacteria could be major bacteria in major phyla and important in gut health and homeostasis. We observed that HC had significantly higher *Lactobacillus* abundances than all other groups (CIS, RRMS, SPMS, PPMS, OND; $P = 0.0055$ to <0.0001), and CIS also showed higher abundances than SPMS ($P = 0.0262$). For *Bifidobacteria*, HC abundances were higher than SPMS, PPMS, and OND ($P = 0.0017$, 0.0107 , and 0.0005 , respectively); additionally, both CIS and RRMS had higher frequency than OND ($P = 0.0198$ and 0.0421). In *Faecalibacterium* frequency, HC exceeded those of CIS, SPMS, PPMS, and OND ($P = 0.0052$ to <0.0001), while RRMS showed higher abundances than SPMS and OND ($P = 0.0030$ and 0.0093). For Bacteroidetes frequency, HC were significantly higher than RRMS, SPMS, PPMS, and OND ($P = 0.0234$ to <0.0001); CIS abundance exceeded SPMS and OND ($P < 0.0001$ for both); RRMS abundances exceeded SPMS and OND ($P = 0.0430$ and 0.0041); and PPMS abundances exceeded OND ($P = 0.0320$). In contrast, Enterobacteriaceae frequency were lower in HC compared to RRMS and PPMS ($P = 0.0069$ and 0.0270), whereas RRMS, SPMS, and PPMS all showed higher abundances than OND ($P = 0.0030$, 0.0330 , and 0.0125). For *Akkermansia* frequency, HC were lower than SPMS and PPMS ($P < 0.0001$ for both); similarly, CIS and RRMS were lower than SPMS and PPMS ($P = 0.0055$ – 0.0071); conversely, SPMS and PPMS were higher than OND ($P = 0.0029$ and 0.0046) (Fig.2). For butyryl-CoA gene frequency, HC were significantly higher than SPMS and OND ($P = 0.0005$ and <0.0001). CIS were higher than SPMS and OND ($P = 0.0433$ and 0.0023). RRMS were higher than SPMS and OND ($P = 0.0215$ and 0.0010). PPMS were higher than OND ($P = 0.0078$). No other comparisons reached statistical significance (Fig.2).

3.3. miRNAs expression profile analysis

For miR-155, HC showed significantly lower expression compared to RRMS, SPMS, and PPMS ($P = 0.0138$, <0.0001 , and <0.0001 , respectively). Additionally, CIS had significantly lower miR-155 than SPMS ($P = 0.0001$), RRMS was lower than SPMS ($P = 0.0033$), and SPMS was higher than OND ($P = 0.0005$). For miR-20a, HC exhibited significantly higher expression than all other groups: CIS, RRMS, SPMS, PPMS, and OND ($P = 0.0022$ to <0.0001). CIS also showed higher expression than SPMS and PPMS ($P = 0.0146$ and 0.0012 , respectively), whereas both SPMS and PPMS had lower expression than OND ($P = 0.0083$ and 0.0006 , respectively). No other pairwise comparisons reached statistical significance for either miRNA (Fig.3).

3.4. ROC analysis

ROC analysis identified several microbial taxa and miRNAs as robust discriminators between patient groups and HC. For CIS vs HC, miR-20a (AUC = 0.7556, 95% CI: 0.6508–0.8604) and *Firmicutes* (AUC = 0.7541, 95% CI: 0.6466–0.8616) performed similarly (both $P < 0.0001$). *Actinobacteria* distinguished relapsing-remitting MS (RRMS) from HC with an AUC of 0.8272 (95% CI: 0.7382–0.9162, $P < 0.0001$). Against HC, SPMS was best differentiated by Bacteroidetes (AUC = 0.9925, 95% CI: 0.9811–1.000), *Lactobacillus* (AUC = 0.9103, 95% CI: 0.8483–0.9723), and miR-20a (AUC = 0.9269, 95% CI: 0.8698–0.9839); PPMS by miR-20a (AUC = 0.9350, 95% CI: 0.8843–0.9857); and OND by Bacteroidetes (AUC = 0.8931, 95% CI: 0.8224–0.9639) (all $P < 0.0001$) (Fig 4).

Among direct disease-versus-disease comparisons, the most striking separation was observed for CIS vs SPMS using Bacteroidetes (AUC = 0.9794, 95% CI: 0.9554–1.000) and for RRMS vs SPMS using the same phylum (AUC = 0.9731, 95% CI: 0.9437–1.000). Bacteroidetes also excelled in distinguishing SPMS from PPMS (AUC = 0.9225, 95% CI: 0.8641–0.9809) and SPMS from OND (AUC = 0.9228, 95% CI: 0.8629–0.9827). Firmicutes achieved the highest accuracy for RRMS vs OND (AUC = 0.8300, 95% CI: 0.7353–0.9247) and for PPMS vs OND (AUC = 0.8516, 95% CI: 0.7668–0.9363). For CIS versus RRMS, *Actinobacteria* showed moderate but significant discrimination (AUC = 0.7119, 95% CI: 0.5910–0.8328, $P = 0.0011$). *Akkermansia* (AUC = 0.7425, 95% CI: 0.6255–0.8595) and miR-20a (AUC = 0.7425, 95% CI: 0.6339–0.8511) performed best for RRMS vs PPMS and CIS vs PPMS, respectively (both $P = 0.0002$). Finally, Bacteroidetes was the top marker for CIS vs OND (AUC = 0.8653, 95% CI: 0.7855–0.9452, $P < 0.0001$). All reported AUCs were statistically significant ($P < 0.05$ to $P < 0.0001$) (Fig S1-S15 supplementary data).

3.5. Combination ROC analysis

To enhance diagnostic accuracy, forward conditional or Wald logistic regression was used to construct multi-marker ROC panels. For discrimination from HC, the following panels achieved excellent performance: *Actinobacteria* + *Lactobacillus* + *Faecalibacterium* + miR-155 + miR-20a for CIS (AUC = 0.9344, 95% CI: 0.8827–0.9861); *Actinobacteria* + *Lactobacillus* + *Bifidobacterium* + *Enterobacteriaceae* + miR-155 + miR-20a for RRMS (AUC = 1.000, 95% CI: 1.000–1.000); Bacteroidetes + miR-155 for SPMS (AUC = 1.000, 95% CI: 1.000–1.000); *Lactobacillus* + *Akkermansia* + miR-155 for PPMS (AUC = 0.9763, 95% CI: 0.9480–1.000); and *Actinobacteria* + Bacteroidetes + miR-155 + miR-20a for OND (AUC = 0.9900, 95% CI: 0.9754–1.000). All $P < 0.0001$ (Fig 5).

Between patient groups, the most powerful panels were: *Actinobacteria* + *Bacteroidetes* for CIS vs. RRMS (AUC = 0.7531, 95% CI: 0.6455–0.8608); Firmicutes + Bacteroidetes + *Proteobacteria* + *Lactobacillus* + *Akkermansia* + miR-155 + miR-20a for CIS vs. PPMS (AUC = 0.9331, 95% CI: 0.8824–0.9838); Bacteroidetes + miR-155 + miR-20a (backward Wald) for CIS vs. SPMS (perfect AUC = 1.000, 95% CI: 1.000–1.000); Firmicutes + *Actinobacteria* + *Bifidobacterium* + *Bacteroidetes* for CIS vs. OND (AUC = 0.9281, 95% CI: 0.8721–0.9842); Firmicutes + Bacteroidetes for RRMS vs. SPMS (AUC = 0.9881, 95% CI: 0.9711–1.000); a separate six-marker panel (*Proteobacteria*, *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, miR-155) for RRMS vs. SPMS (AUC = 0.7313, 95% CI: 0.6192–0.8433); Firmicutes + Bacteroidetes + *Bacteroidetes* for RRMS vs. OND (AUC = 0.8956, 95% CI: 0.8293–0.9620); *Bacteroidetes* (phylum & genus) + *Akkermansia* + miR-155 for SPMS vs. PPMS

(AUC = 0.9869, 95% CI: 0.9699–1.000); and Firmicutes + *Lactobacillus* + butyryl-CoA + miR-155 + miR-20a for PPMS vs. OND (AUC = 0.9494, 95% CI: 0.9018–0.9969). All models achieved $P < 0.0001$ (except the six-marker RRMS/SPMS panel, $P = 0.0004$). Perfect AUCs of 1.000 should be interpreted with caution pending external validation (Fig 5).

3.6. GM-miRNA correlation in MS subtypes

The GM of MS patients was finally profiled and compared with that of age- and sex-matched HC and OND to verify whether the differences in miRNA-155 and miRNA-20a observed in these patients could be the result of changes in the gut microbial ecosystem. Pearson correlation analysis revealed that miR-20a levels were significantly associated with several gut microbial taxa ($n=240$). Positive correlations were observed between miR-20a and the phylum Firmicutes ($r=0.384$, $R^2=0.148$, $p<0.0001$), *Bacteroidetes* ($r=0.378$, $R^2=0.143$, $p<0.0001$), the genus *Lactobacillus* ($r=0.305$, $R^2=0.093$, $p<0.0001$), and *Faecalibacterium* ($r=0.167$, $R^2=0.028$, $p=0.009$). Conversely, miR-20a showed significant negative correlations with Proteobacteria ($r=-0.278$, $R^2=0.077$, $p<0.0001$), Actinobacteria ($r=-0.225$, $R^2=0.051$, $p=0.0004$), *Akkermansia* ($r=-0.393$, $R^2=0.154$, $p<0.0001$), and Enterobacteria ($r=-0.174$, $R^2=0.030$, $p=0.007$). No significant correlations were found for *Bifidobacterium* ($r=0.089$, $p=0.171$) or butyryl-CoA ($r=0.124$, $p=0.056$). Notably, a positive correlation was also detected with *Bacteroides* ($r=0.278$, $p<0.0001$), which may represent a related taxon but requires cautious interpretation (Fig 6). Overall, these findings indicate that miR-20a is differentially associated with specific commensal and potentially beneficial or pathogenic bacterial groups, though the modest R^2 values suggest that miR-20a explains only a small fraction of the variance in each microbial population.

Pearson correlation analysis ($n=240$) revealed that miR-155 levels were significantly associated with several gut bacterial taxa, though with generally modest effect sizes. Specifically, miR-155 showed negative correlations with Firmicutes ($r=-0.236$, $R^2=0.055$, $p=0.0002$), *Bacteroidetes* ($r=-0.330$, $R^2=0.109$, $p<0.0001$), *Faecalibacterium* ($r=-0.231$, $R^2=0.053$, $p=0.0003$), and *Bacteroides* ($r=-0.142$, $R^2=0.020$, $p=0.028$). In contrast, positive correlations were observed with Proteobacteria ($r=0.413$, $R^2=0.171$, $p<0.0001$), Actinobacteria ($r=0.209$, $R^2=0.043$, $p=0.001$), *Akkermansia* ($r=0.237$, $R^2=0.056$, $p=0.0002$), and Enterobacteriaceae ($r=0.129$, $R^2=0.017$, $p=0.046$). No significant correlations were found for *Lactobacillus* ($r=-0.119$, $p=0.066$), *Bifidobacterium* ($r=-0.126$, $p=0.052$), or butyryl-CoA ($r=-0.115$, $p=0.076$) (Fig 7). Collectively, these results indicate that higher miR-155 expression is associated with a shift away from dominant commensal phyla (Firmicutes/*Bacteroidetes*) and toward Proteobacteria and *Akkermansia*, although the low R^2 values suggest that miR-155 accounts for only a small proportion of the variance in each microbial population.

3.7. Cutoff analysis

Cutoff analysis was employed to identify optimal diagnostic thresholds for each microbial and miRNA marker in specific pairwise comparisons using the Youden index, since no single biomarker uniformly discriminated across all MS subtypes and control groups.

3.7.1. Global diagnostic performance for candidate markers

A total of 195 ROC-based diagnostic cutoff analyses were performed across the 15 pairwise group comparisons and 13 candidate markers. The overall diagnostic performance varied substantially across clinical comparisons, indicating that no single marker provided uniform discrimination across all disease categories. Instead, several markers showed strong performance in specific diagnostic contexts. The most robust results were observed in comparisons involving SPMS, where *Bacteroidetes* consistently achieved high AUC values and strong sensitivity–specificity balance. In contrast, comparisons among clinically related MS phenotypes, such as CIS versus RRMS, CIS versus PPMS, and RRMS versus PPMS, showed more moderate diagnostic separation (Fig 8).

The AUC heatmap demonstrated that *Bacteroidetes* was the most prominent marker in SPMS-centered comparisons. The strongest diagnostic separation was observed between HC and SPMS, where *Bacteroidetes* produced an AUC of 0.993. This value indicated near-complete discrimination between the two groups. *Bacteroidetes* also showed excellent performance in CIS versus SPMS and RRMS versus SPMS, with AUC values of 0.979 and 0.973, respectively. These findings suggest that *Bacteroidetes* may be particularly informative for identifying SPMS-associated biological separation rather than functioning as a general diagnostic marker across all MS-related clinical categories.

3.7.2. Optimal diagnostic cutoffs based on the Youden index

The best-performing marker for each pairwise comparison was selected according to the highest AUC and its corresponding Youden-derived cutoff. Among comparisons involving healthy controls, miR-20a showed strong diagnostic value for distinguishing HC from RRMS and PPMS. In the HC versus RRMS comparison, miR-20a achieved an AUC of 0.893, and the optimal cutoff was 0.1795. Values equal to or above this threshold classified individuals as RRMS, with 92.5% sensitivity and 75.0% specificity. In the HC versus PPMS comparison, miR-20a showed stronger diagnostic performance, with an AUC of 0.935 and an optimal cutoff of 1.439. This threshold identified PPMS with 85.0% sensitivity and 87.5% specificity (Table 1).

Bacteroidetes was the dominant marker for SPMS-related discrimination. In the HC versus SPMS comparison, the optimal cutoff for *Bacteroidetes* was 1.655, with values equal to or above this threshold classifying individuals as SPMS. This cutoff achieved 92.5% sensitivity and 100% specificity, producing a Youden index of 0.925. In the CIS versus SPMS comparison, *Bacteroidetes* achieved an AUC of 0.979 with an optimal cutoff of 1.386, corresponding to 95.0% sensitivity and 92.5% specificity. Similarly, in the RRMS versus SPMS comparison, *Bacteroidetes* showed an AUC of 0.973, with a cutoff of 1.655, 92.5% sensitivity, and 95.0% specificity. These results identify *Bacteroidetes* as the strongest single-marker candidate for SPMS-related diagnostic discrimination in this dataset (Fig 9).

Comparisons involving OND showed a more heterogeneous marker profile. *Bacteroides* was the best-performing marker for distinguishing HC from OND, with an AUC of 0.893 and an optimal cutoff of 1.085. At this threshold, sensitivity was 85.0% and specificity was 80.0%. *Bacteroides* also abundances highest for CIS versus OND, with an AUC of 0.865 and a cutoff of 1.474, producing 77.5% sensitivity and 85.0% specificity. In contrast, Firmicutes provided the best diagnostic separation for RRMS versus OND and PPMS versus OND, with AUC values of 0.829 and 0.852, respectively. These findings indicate that OND-related discrimination was not driven by a single universal biomarker but depended on the specific comparator group.

3.7.3. Diagnostic separation among MS phenotypes

Diagnostic separation among MS-related clinical phenotypes was more modest than the separation observed in SPMS-centered comparisons. CIS versus RRMS was best discriminated by Actinobacteria, with an AUC of 0.712 and an optimal cutoff of 7.708. Values equal to or below this threshold classified individuals as RRMS, with 72.5% sensitivity and 75.0% specificity. CIS versus PPMS was best distinguished by miR-20a, which produced an AUC of 0.743 and an optimal cutoff of 1.078. Although this cutoff achieved high sensitivity of 90.0%, specificity was limited to 52.5%, suggesting considerable overlap between CIS and PPMS when miR-20a was used as a single diagnostic marker. RRMS versus PPMS was best separated by Akkermansia, with an AUC of 0.742 and an optimal cutoff of 15.86. Values equal to or below this threshold classified individuals as PPMS, with 65.0% sensitivity and 90.0% specificity (Fig 10).

The distribution plots supported the ROC findings by showing clear marker separation in the strongest comparisons and greater overlap in moderate-performing comparisons. Bacteroidetes showed distinct distributional separation in SPMS-related comparisons, consistent with its high AUC values. By contrast, markers selected for CIS versus RRMS, CIS versus PPMS, and RRMS versus PPMS showed broader overlap between groups, explaining their lower AUC values and less balanced diagnostic accuracy. This pattern suggests that single-marker thresholds may be insufficient for reliably distinguishing closely related MS phenotypes and that combined biomarker panels may provide better classification performance (Fig 11).

Overall, the diagnostic cutoff analysis identified Bacteroidetes as the most consistent and highest-performing marker for SPMS-associated comparisons, while miR-20a showed notable diagnostic value in distinguishing RRMS and PPMS from HC. *Bacteroides* and Firmicutes were more prominent in OND-related comparisons, indicating that non-MS neurological disease discrimination may reflect a different biomarker structure. Although several single-marker cutoffs achieved excellent diagnostic accuracy, particularly for SPMS, the more moderate performance observed in several MS phenotype comparisons supports the need for future multivariable diagnostic models integrating microbial and microRNA markers.

4. Discussion

MS is a chronic immune-mediated neuroinflammatory disease whose etiology remains incompletely understood. Increasing evidence suggests that alterations in GM composition contribute to immune dysregulation and disease progression in MS, while dysregulated miRNAs have emerged as important regulators of neuroinflammation and potential biomarkers of disease activity [12, 13]. In this case-control study, three principal findings emerged. First, both microbial taxa and miRNAs discriminated MS subtypes from controls, with the strongest separation observed in SPMS. Second, an inflammatory molecular signature characterized by increased miR-155 and reduced miR-20a was associated with depletion of beneficial commensal bacteria and enrichment of potentially pro-inflammatory taxa. Third, combining GM and miRNA markers substantially improved diagnostic performance compared with individual biomarkers, supporting the potential utility of integrated biomarker models for disease stratification.

The distinct microbial and miRNA signatures observed across MS subtypes suggest that each clinical form may be associated with a unique host-GM interaction profile. Notably, RRMS patients displayed microbial alterations that

were intermediate between HC and progressive forms, with Actinobacteria elevation being the most prominent finding. This pattern may reflect the inflammatory relapses characteristic of RRMS, as Actinobacteria species have been associated with pro-inflammatory cytokine production and Th17 differentiation [14]. In contrast, SPMS patients exhibited the most extensive dysbiosis, characterized by depletion of multiple beneficial taxa combined with enrichment of potentially pathogenic groups. This progressive worsening of gut dysbiosis aligns with the clinical trajectory of SPMS, wherein accumulating neurological impairment occurs independent of relapses [15]. SPMS was characterized by reduced abundance of Firmicutes, Bacteroidetes, *Faecalibacterium*, *Lactobacillus*, and *Bifidobacterium*, taxa known to include important short-chain fatty acid (SCFA)-producing microorganisms. These findings are consistent with previous studies reporting depletion of SCFA-producing bacteria in MS patients [12, 16, 17]. SCFAs, particularly acetate, propionate, and butyrate, play critical roles in maintaining intestinal barrier integrity, regulating immune homeostasis, and promoting regulatory T-cell (Treg) responses while suppressing pro-inflammatory Th1 and Th17 pathways [18]. *Butyryl-CoA* gene frequency (butyrate proxy) was lower in SPMS and OND, indicating diminished SCFA synthesis capacity. Therefore, reduced abundance of SCFA-producing bacteria may contribute to systemic immune activation and chronic inflammation associated with progressive MS. Furthermore, SCFAs have been demonstrated to modulate miRNA expression through histone deacetylase inhibition and G-protein-coupled receptor signaling [19]. Reduced abundance of SCFA-producing bacteria may weaken epithelial barrier integrity and promote systemic exposure to inflammatory microbial products, thereby contributing to Treg/Th17 imbalance and autoimmune responses within the central nervous system [20]. This provides a plausible mechanistic link between gut dysbiosis and altered miRNA expression in MS.

Concurrent enrichment of *Akkermansia*, Proteobacteria and Enterobacteriaceae may further exacerbate inflammatory signaling through disruption of mucosal homeostasis and increased immune activation [21-23]. Expansion of Proteobacteria is widely considered a hallmark of microbial instability and chronic inflammation and has been associated with elevated inflammatory cytokines such as IL-6 and IL-8 [24, 25]. Increased abundance of *Akkermansia muciniphila* in SPMS and PPMS is of particular interest. Although *Akkermansia* has been linked to beneficial metabolic effects in some studies, its role in MS remains controversial. Several studies have suggested that excessive mucin degradation, altered gut barrier integrity, and modulation of immune responses may contribute to neuroinflammation in individuals with MS [26, 27], whereas others have reported potentially protective effects [28, 29]. Our findings support the notion that increased *Akkermansia* abundance is associated with progressive MS; however, its precise role requires further investigation. The positive correlation between *Akkermansia* and miR-155 observed in our study further supports a pro-inflammatory role in progressive MS. However, the observed association may also reflect compensatory mechanisms or epiphenomenal changes, underscoring the need for functional studies to establish causality.

The observed miRNA profiles complemented these microbial alterations. miR-155 was significantly elevated in RRMS, PPMS, and SPMS, consistent with its established role in promoting inflammatory responses through regulation of T cells, macrophages, and cytokine signaling pathways [30]. Conversely, miR-20a expression was elevated in HC and exhibited a stepwise decline across MS subtypes, reaching its lowest levels in SPMS and PPMS,

which points to a possible anti-inflammatory or immunoregulatory role [31]. This reciprocal pattern of elevated miR-155 and reduced miR-20a may reflect the transition from a relatively balanced microbial ecosystem toward a dysbiotic and pro-inflammatory state. miR-20a positively correlated with Firmicutes, Bacteroidetes, *Lactobacillus*, and *Faecalibacterium*, while showing negative correlations with Proteobacteria, Actinobacteria, *Akkermansia*, and Enterobacteriaceae. In contrast, miR-155 exhibited the opposite pattern. Although the observed correlation coefficients explained only a modest proportion of microbial variation implying that additional factors (diet, medications, genetics, environment) also shape the GM. These findings are consistent with emerging evidence indicating bidirectional interactions between GM, microbial metabolites, and host miRNA expression [32-34]. Mechanistically, miR-155 has been shown to target suppressor of cytokine signaling 1 (SOCS1) and Src homology 2 domain-containing inositol phosphatase 1 (SHIP1), leading to enhanced inflammatory signaling through the JAK-STAT and NF- κ B pathways [35]. Conversely, miR-20a, a member of the miR-17-92 cluster, may exert anti-inflammatory effects by targeting C-C chemokine receptor 6 (CCR6) and ROR γ t, thereby modulating Th17 differentiation [9, 36]. Overall, GM and miRNA profiles are MS subtype-dependent and, in combination, may serve as powerful diagnostic biomarkers. This proof-of-concept study demonstrates that GM and miRNA profiling can predict MS progression, though validation in larger longitudinal cohorts is needed. The findings support the gut-brain axis, showing that combined microbial and miRNA markers provide a more comprehensive molecular profile than either alone. Therapeutically, they suggest GM-directed interventions (e.g., probiotics, SCFA, fecal microbiota transplantation) might modulate miRNAs and reduce neuroinflammation, though still experimental, and could guide patient selection in future trials. Overall, these results lay a foundation for multi-omics strategies in MS research and management.

Limitations

The study's main limitations are its low-resolution microbial profiling (necessitating future shotgun metagenomics and metabolomics), cross-sectional single-timepoint design (requiring longitudinal studies for causal inference), restricted Iranian cohort (limiting generalizability), uncontrolled confounders (diet, therapy, activity, comorbidities), and potential overfitting (suggested by AUCs near 1.0, demanding independent validation). Future work should also mechanistically test whether microbiota modulation alters miRNA profiles and clinical outcomes.

5. Conclusion and Future directions

This study reveals a subtype-specific GM-miRNA signature in MS, with Bacteroidetes distinguishing SPMS and miR-20a differentiating RRMS/PPMS from controls. The findings strengthen the link between host-GM interactions and MS pathophysiology, highlighting integrated microbial-miRNA signatures showed promising cross-sectional discrimination of MS clinical phenotypes, particularly SPMS, but require validation in independent longitudinal cohorts before clinical application. However, the authors caution that external validation, longitudinal tracking, and mechanistic studies are still needed to confirm causality and clinical utility. Future work should prioritize multi-cohort validation, multi-omics integration, mechanistic models, clinically evaluated diagnostic panels, and prospective trials testing whether microbiome-modulating interventions (e.g., diet, probiotics) can alter miRNA profiles, clinical outcomes, and treatment responses.

CRedit Author Contributions

All authors contributed to the study conception and design. Farzaneh Rafie Sedaghat: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – Original Draft. Alka Hasani: Conceptualization, Supervision, Project administration, Resources, Validation, Writing – Review & Editing. Javid Sadry-Nahand: Methodology, Formal analysis, Validation, Writing – Review & Editing. Vahdat Poortahmasebi: Methodology, Investigation, Validation, Writing – Review & Editing. Samaneh Hosseini: Investigation, Data curation, Resources. Hossein Samadi Kafil: Conceptualization, Methodology, Supervision, Writing – Review & Editing. Mehdi Meskini Heydarlou: Formal analysis, Software, Visualization, Writing – Review & Editing. Somayeh Ahmadi: Investigation, Data curation, Validation, Writing – Review & Editing. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work.

Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

The study received ethical approval from the ethics committee of Tabriz University of Medical Sciences (ethical code: IR.TBZMED.REC.1403.745).

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Data availability

Data will be available on request.

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Table:

Table 1. Best-performing diagnostic cutoff for each pairwise group comparison

Comparison	Best marker	AUC	95% CI	Optimal diagnostic rule	Sensitivity	Specificity	Youden index
HC vs CIS	miR-20a	0.756	0.640–0.856	CIS if miR-20a $\geq$ 0.177	0.650	0.750	0.400
HC vs RRMS	miR-20a	0.893	0.835–0.955	RRMS if miR-20a $\geq$ 0.1795	0.925	0.750	0.675
HC vs SPMS	Bacteroidetes	0.993	0.978–1.000	SPMS if Bacteroidetes $\geq$ 1.655	0.925	1.000	0.925
HC vs OND	<i>Bacteroides</i>	0.893	0.818–0.957	OND if <i>Bacteroides</i> $\geq$ 1.085	0.850	0.800	0.650
HC vs PPMS	miR-20a	0.935	0.874–0.974	PPMS if miR-20a $\geq$ 1.439	0.850	0.875	0.725

CIS vs RRMS	Actinobacteria	0.712	0.586– 0.819	RRMS if Actinobacteria $\leq$ 7.708	0.725	0.750	0.475
CIS vs PPMS	miR-20a	0.743	0.626– 0.843	PPMS if miR-20a $\geq$ 1.078	0.900	0.525	0.425
CIS vs SPMS	Bacteroidetes	0.979	0.955– 0.998	SPMS if Bacteroidetes ≥ 1.386	0.950	0.925	0.875
CIS vs OND	<i>Bacteroides</i>	0.865	0.781– 0.938	OND if <i>Bacteroides</i> $\geq$ 1.474	0.775	0.850	0.625
RRMS vs SPMS	Bacteroidetes	0.973	0.941– 0.998	SPMS if Bacteroidetes ≥ 1.655	0.925	0.950	0.875
RRMS vs PPMS	<i>Akkermansia</i>	0.742	0.620– 0.845	PPMS if <i>Akkermansia</i> ≤ 15.86	0.650	0.900	0.550
RRMS vs OND	Firmicutes	0.829	0.727– 0.914	OND if Firmicutes $\geq$ 2.997	0.800	0.800	0.600
SPMS vs PPMS	Bacteroidetes	0.922	0.854– 0.976	PPMS if Bacteroidetes ≤ 2.231	0.800	0.875	0.675
SPMS vs OND	Bacteroidetes	0.923	0.860– 0.976	OND if Bacteroidetes ≤ 2.446	0.900	0.850	0.750
PPMS vs OND	Firmicutes	0.852	0.770– 0.932	OND if Firmicutes $\geq$ 3.280	0.725	0.850	0.575

AUC, area under the receiver operating characteristic curve; CI, confidence interval; CIS, clinically isolated syndrome; HC, healthy controls; OND, other neurological diseases; PPMS, primary progressive multiple sclerosis; RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis. Optimal thresholds were selected using the Youden index. The diagnostic rule indicates the classification direction for the positive group in each pairwise comparison.

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542 Figures caption and legend

543 **Figure 1. Relative abundance of 4 major bacterial phyla in courses of MS, HC and OND.** **A:** Relative abundance
544 of Firmicutes phylum in different types of MS that showed slightly decrease in MS courses compared to HC. **B:**
545 Relative abundance of Actinobacteria phylum in different types of MS that showed increase in CIS compared to HC.
546 Also, significantly different between CIS and RRMS, PPMS, SPMS and OND groups. **C:** Relative abundance of

Bacteroidetes phylum in different types of MS that showed slightly decrease in MS courses compared to HC. Significantly different between. **D:** Relative abundance of Proteobacteria phylum in different types of MS that showed slightly increase in MS courses compared to HC. Significantly different between HC vs PPMS and SPMS were observed.

Figure 1 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 2. Relative abundance changes (Log2 Fold Change) of selected bacterial taxa; including *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Bacteroides*, *Enterobacteriaceae*, *Akkermansia*, and the functional gene *Butyryl-CoA* among the study groups. Each dot represents an individual sample, while horizontal lines indicate the mean values. Statistically significant differences between groups are denoted by the corresponding *P*-values. These results highlight notable alterations in gut microbial composition and butyrate-producing potential across the experimental groups.

Figure 2 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 3. Differential expression of (A) *miR-155* and (B) *miR-20a* across the study groups, presented as Log2 fold change relative to the reference group. Each dot represents an individual sample, and horizontal lines indicate mean expression levels. Significant pairwise differences between groups are shown with the corresponding *P*-values. The results demonstrate distinct expression patterns of both microRNAs among the investigated clinical groups, suggesting their potential involvement in disease progression and immune-related regulatory pathways.

Figure 3 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 4. Receiver operating characteristic (ROC) curve analysis. ROC evaluating the diagnostic performance of *miR-155*, *miR-20a*, and selected gut microbiota-related biomarkers, including *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Bacteroides*, *Akkermansia*, *Enterobacteriaceae*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and the *Butyryl-CoA* gene across different study groups. Panels A–O represent pairwise comparisons among healthy controls (HC), clinically isolated syndrome (CIS), relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS), and other neurological diseases (OND). The area under the curve (AUC) and corresponding *P*-values are reported for each biomarker, while the diagonal dashed line indicates random classification performance (AUC = 0.5). Biomarkers with higher AUC values exhibited greater discriminative power and may serve as potential diagnostic indicators for multiple sclerosis subtypes and disease progression.

Figure 4 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 5. Combination receiver operating characteristic (ROC) curves for distinguishing among multiple sclerosis clinical subtypes and control groups.

ROC analyses were performed using stepwise logistic regression (forward or backward, conditional or Wald as indicated) to identify optimal panels of microbial taxa and microRNAs for discriminating between groups. The combinations included various phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria), genera (*Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Akkermansia*, *Bacteroides*, *Enterobacteriaceae*), and microRNAs (miR-155, miR-20a), as well as the metabolite butyryl-CoA. Areas under the curve (AUC) ranged from 0.75 to 1.00, with all models showing statistically significant discrimination ($P < 0.0001$). Abbreviations: HC, healthy controls; CIS, clinically isolated syndrome; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary-progressive multiple sclerosis; PPMS, primary-progressive multiple sclerosis; OND, other neurological diseases.

Figure 5 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 6. Correlation analysis between miR-20a expression and relative abundance of gut microbial taxa.

Scatter plots (A–K) depict the relationships between circulating miR-20a levels and the abundances of major bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria) and key genera (*Lactobacillus*, *Faecalibacterium*, *Bifidobacterium*, *Akkermansia*, *Enterobacteriaceae*, *Bacteroides*), as well as the microbial metabolite butyryl-CoA. Spearman/Pearson correlation coefficients (r) and corresponding p -values are shown for each comparison. Significant positive correlations were observed between miR-20a and Firmicutes ($r = 0.384$, $p = 0.009$), Bacteroidetes ($r = 0.377$, $p < 0.0001$). These findings suggest that miR-20a may selectively associate with distinct components of the gut microbiota, potentially implicating it in microbiota-host crosstalk in the context of multiple sclerosis.

Figure 6 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 7. Correlation analysis between miR-155 expression and relative abundance of gut microbial taxa.

Scatter plots (A–K) show the relationships between circulating miR-155 levels and the relative abundances of major bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria), key genera (*Lactobacillus*, *Faecalibacterium*, *Bifidobacterium*, *Enterobacteriaceae*, *Akkermansia*, *Bacteroides*), and the microbial metabolite butyryl-CoA. Spearman correlation coefficients (r) and corresponding p -values are displayed directly on each respective plot. These analyses reveal distinct association patterns between miR-155 and specific gut microbial taxa, complementing the miR-20a findings shown in Figure 6.

Figure 7 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 8. AUC heatmap of diagnostic ROC comparisons across all candidate markers. The heatmap presents the area under the receiver operating characteristic curve for each candidate marker across all pairwise diagnostic comparisons. Rows indicate microbial or microRNA markers, and columns indicate diagnostic group comparisons. Higher AUC values represent stronger diagnostic discrimination. Bacteroidetes showed the most consistent high-level performance in SPMS-related comparisons, particularly HC versus SPMS, CIS versus SPMS, and RRMS versus SPMS.

Figure 8 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

Figure 9. ROC curves for the best-performing marker in each diagnostic comparison. Receiver operating characteristic curves are shown for the highest-abundance marker in each pairwise comparison. Each curve represents the ability of the selected marker to distinguish the positive diagnostic class from the reference group. AUC values summarize the overall discriminatory performance, and optimal cutoffs were determined using the Youden index.

Figure 9 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

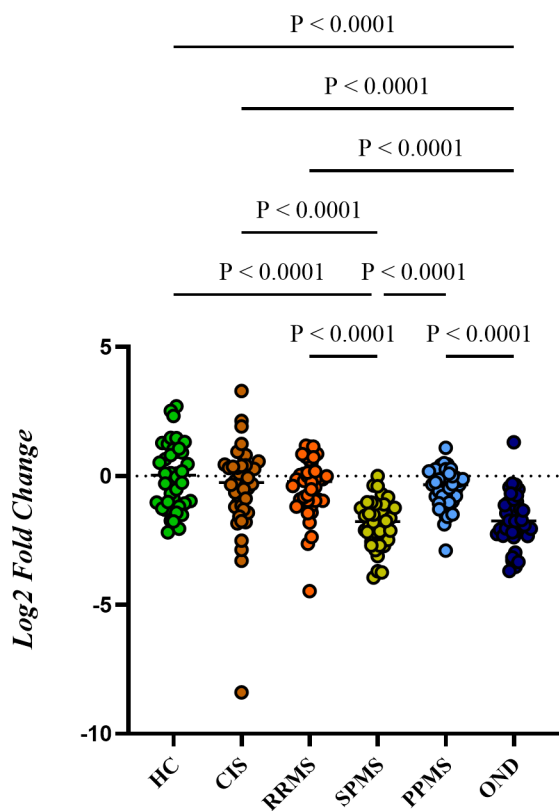
Figure 10. Distribution plots showing Youden-derived diagnostic cutoffs. Distribution plots show the values of the best-performing marker in each pairwise comparison, with the optimal diagnostic cutoff indicated by a reference line. These plots illustrate the degree of separation or overlap between diagnostic groups and provide a visual interpretation of the sensitivity and specificity associated with each cutoff.

Figure 10 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

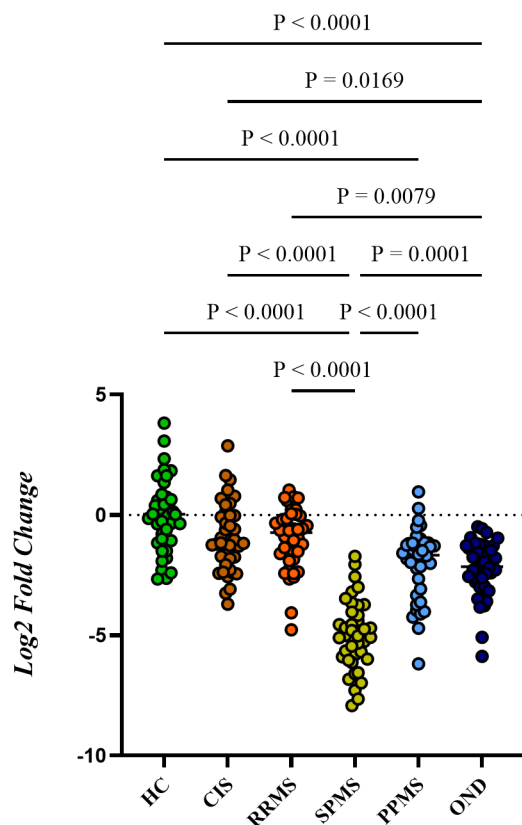
Figure 11. Summary of optimal diagnostic markers and cutoffs across pairwise comparisons. This figure summarizes the best-performing marker, AUC, and Youden-derived cutoff for each pairwise diagnostic comparison. The figure highlights the strong contribution of Bacteroidetes in SPMS-related comparisons, the diagnostic relevance of miR-20a in comparisons involving healthy controls, and the more heterogeneous biomarker profile observed in OND-related comparisons.

Figure 11 legend: CIS: clinical isolated syndrome, RRMS: relapsing-remitting multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PPMS: primary progressive multiple sclerosis, HC: healthy control, OND: other neurological disorder.

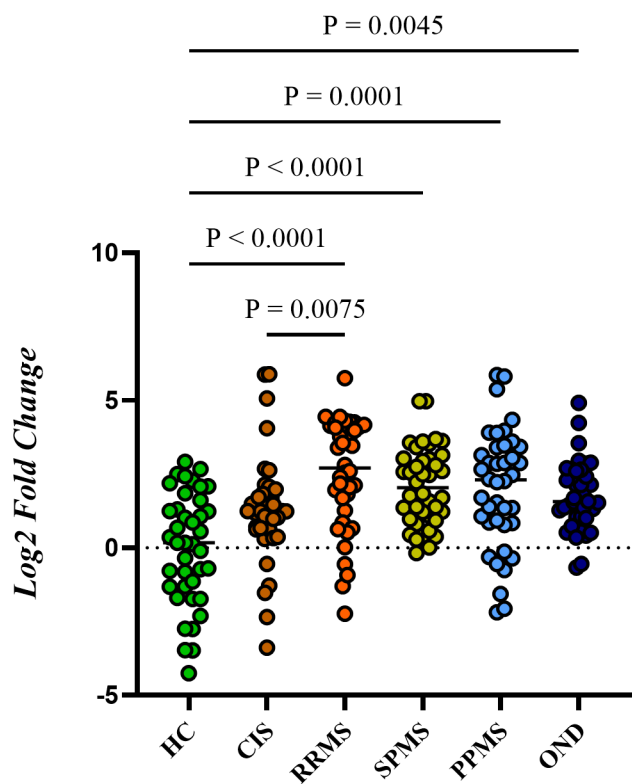
A)

Fermicutes

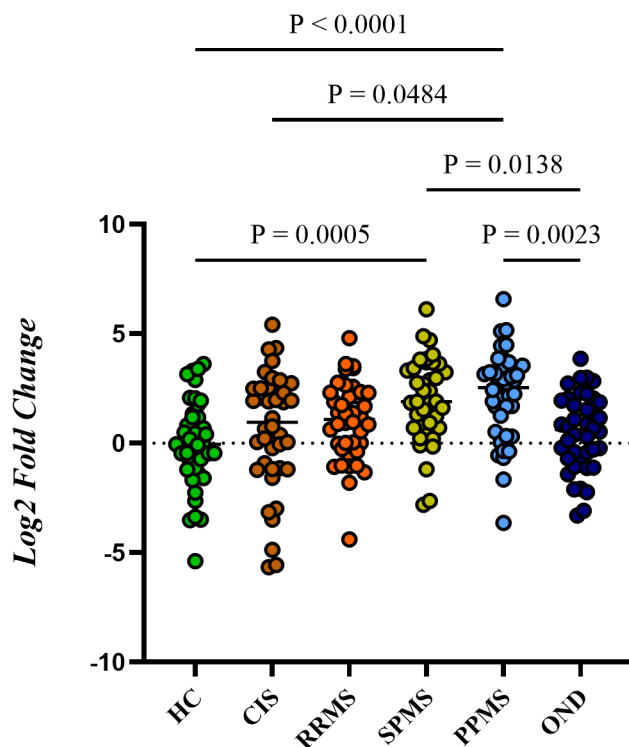
B)

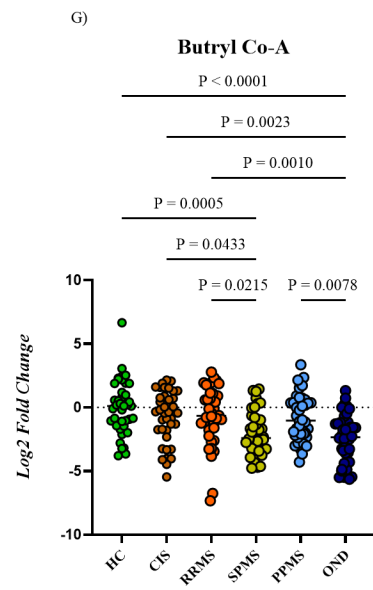
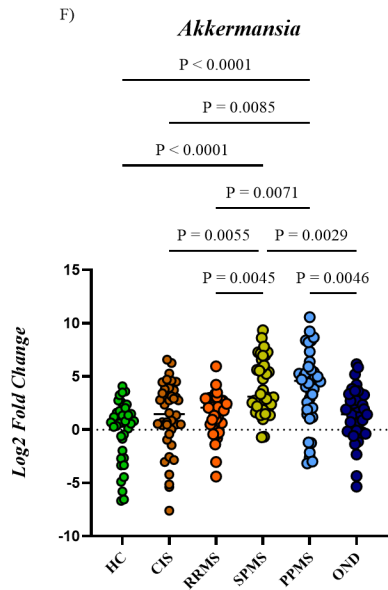
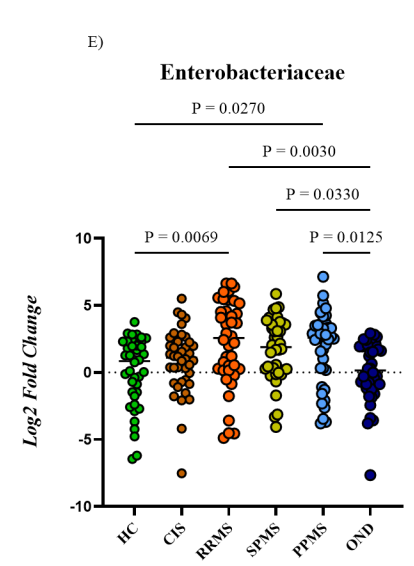
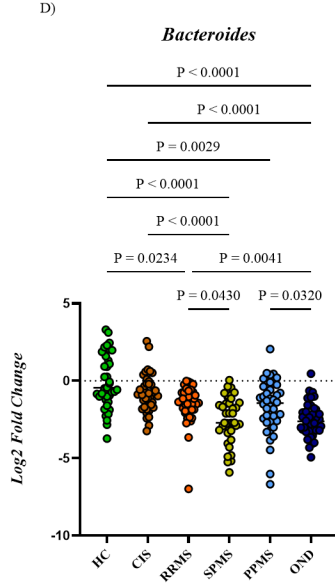
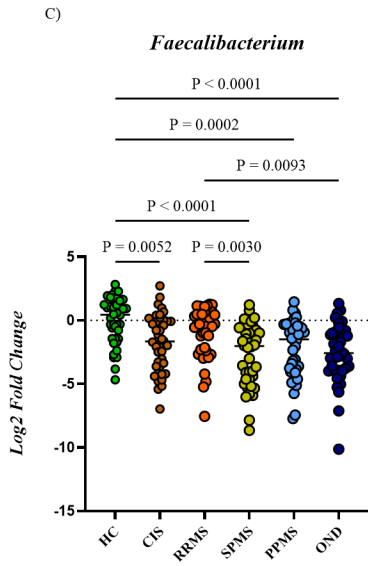
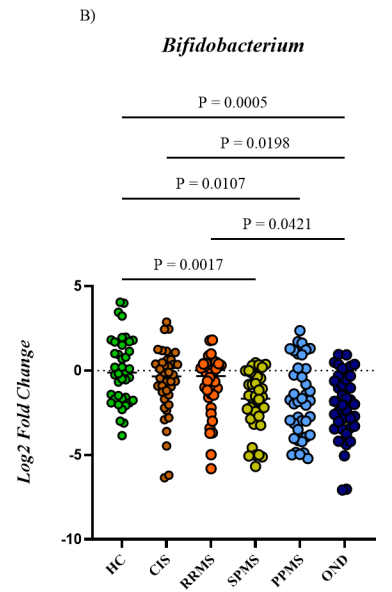
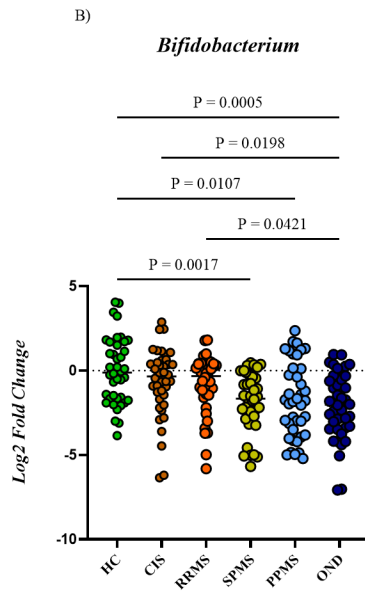
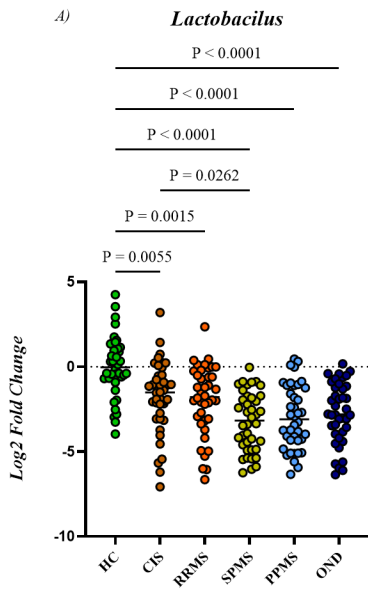
Bacteroidetes

C)

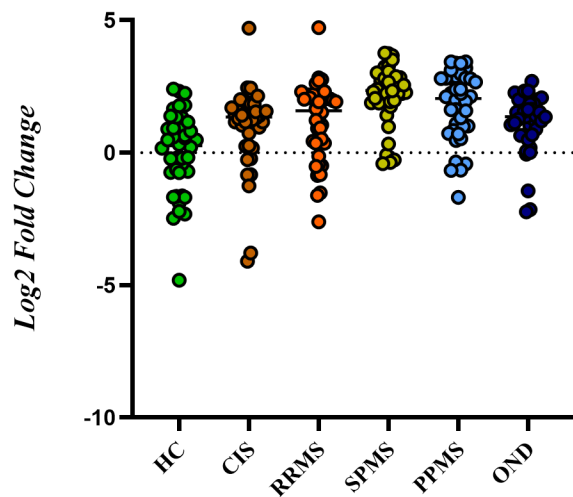
Actinobacteria

D)

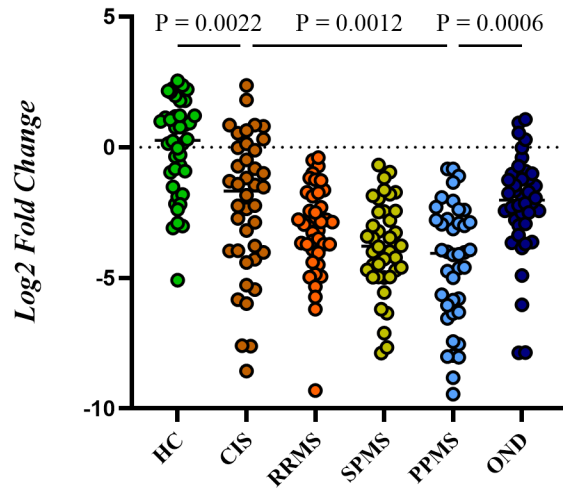
Proteobacteria



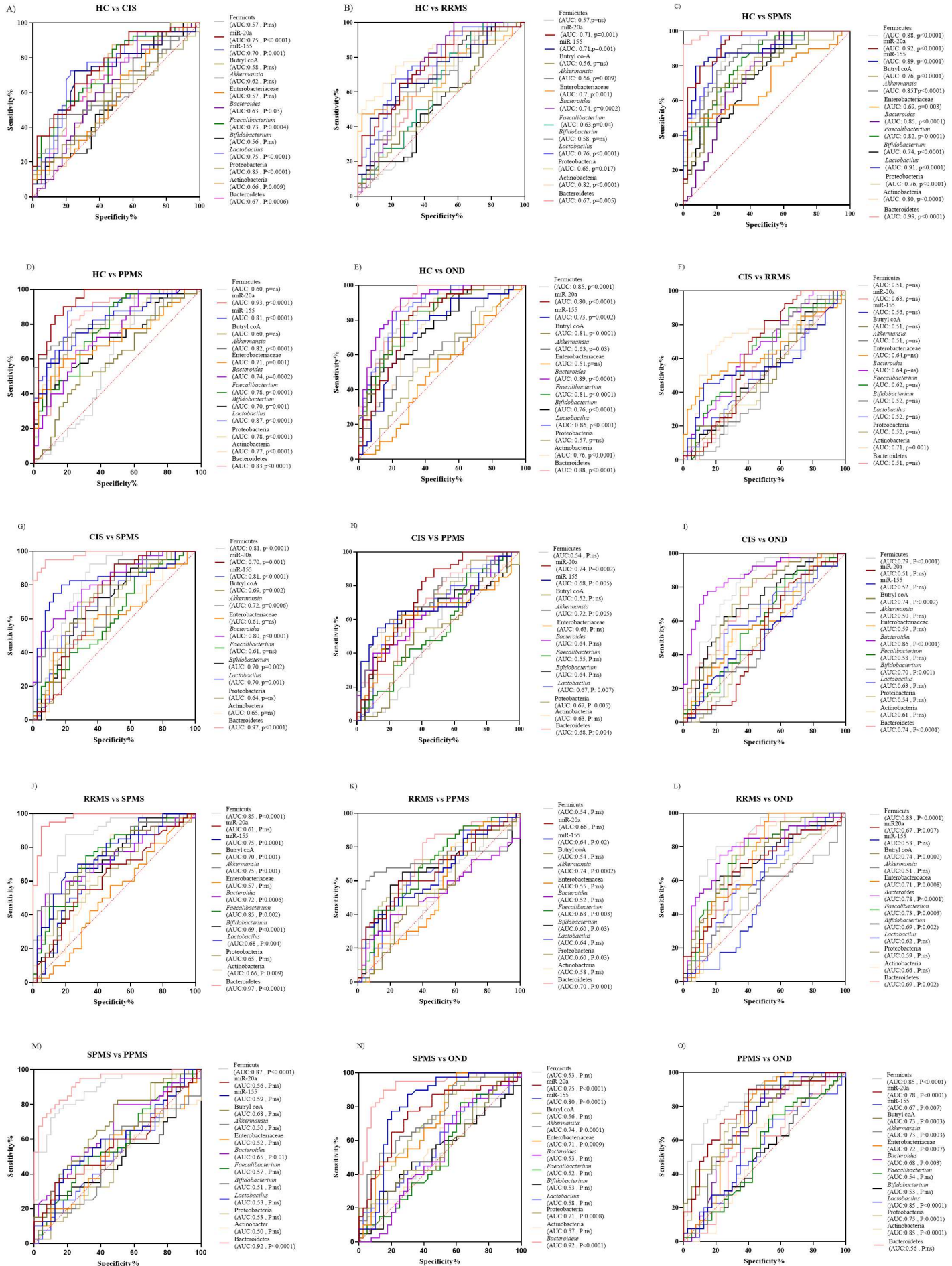
A)

miR-155 $P < 0.0001$ $P < 0.0001$ $P = 0.0001$ $P = 0.0138$ $P = 0.0005$ $P = 0.0033$ 

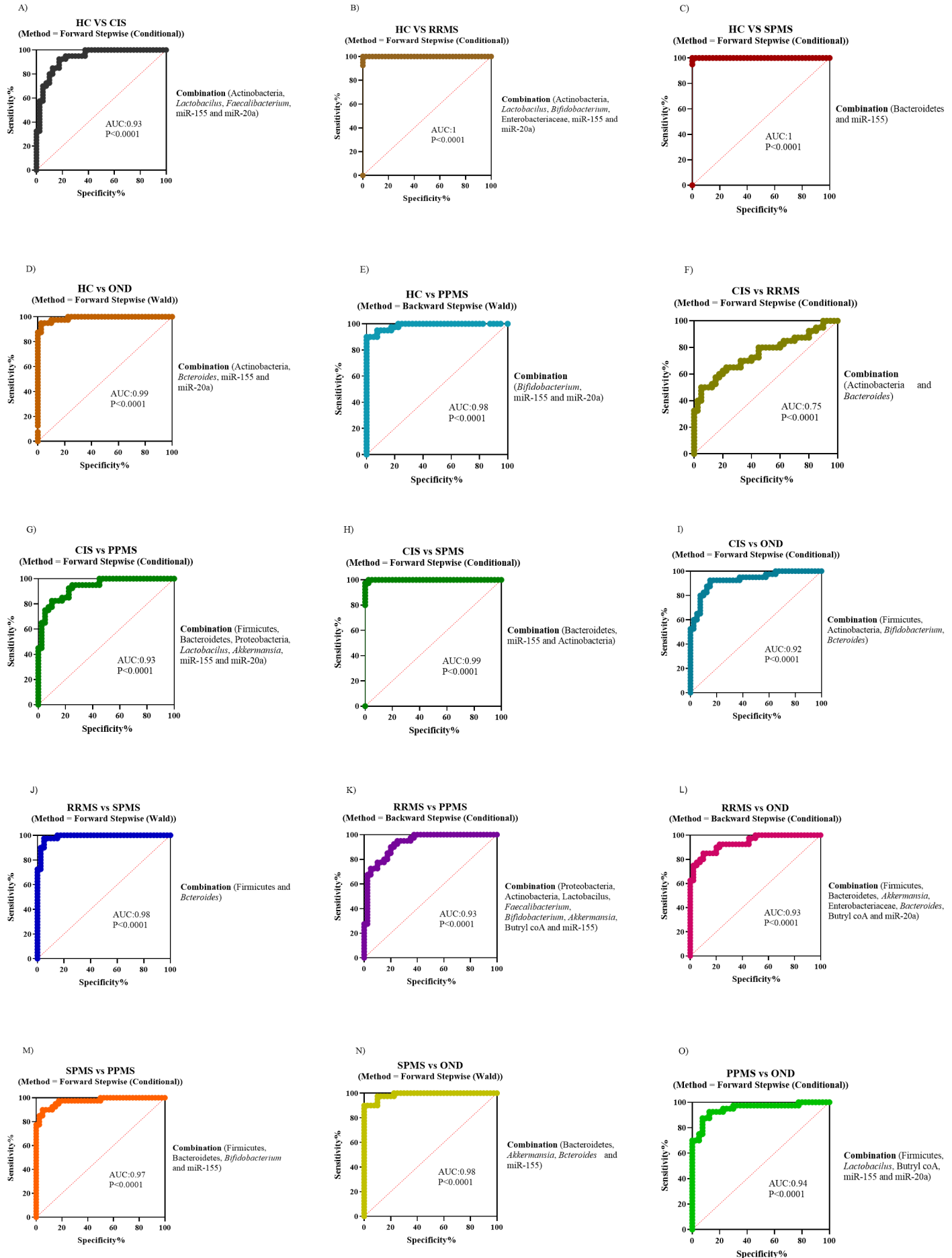
B)

miR-20a $P = 0.0041$ $P < 0.0001$ $P < 0.0001$ $P = 0.0146$ $P < 0.0001$ $P = 0.0083$ 

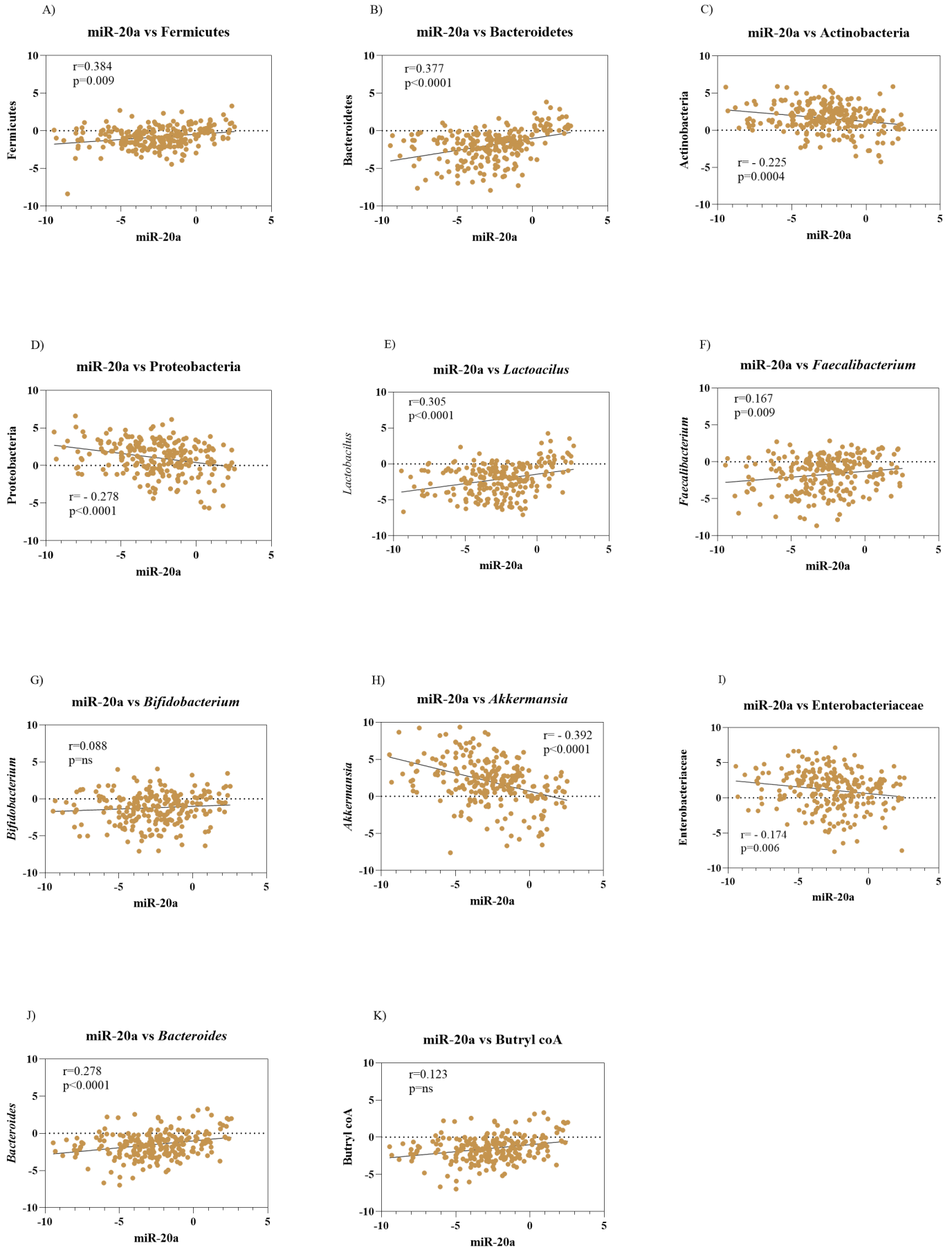
ROC Curves



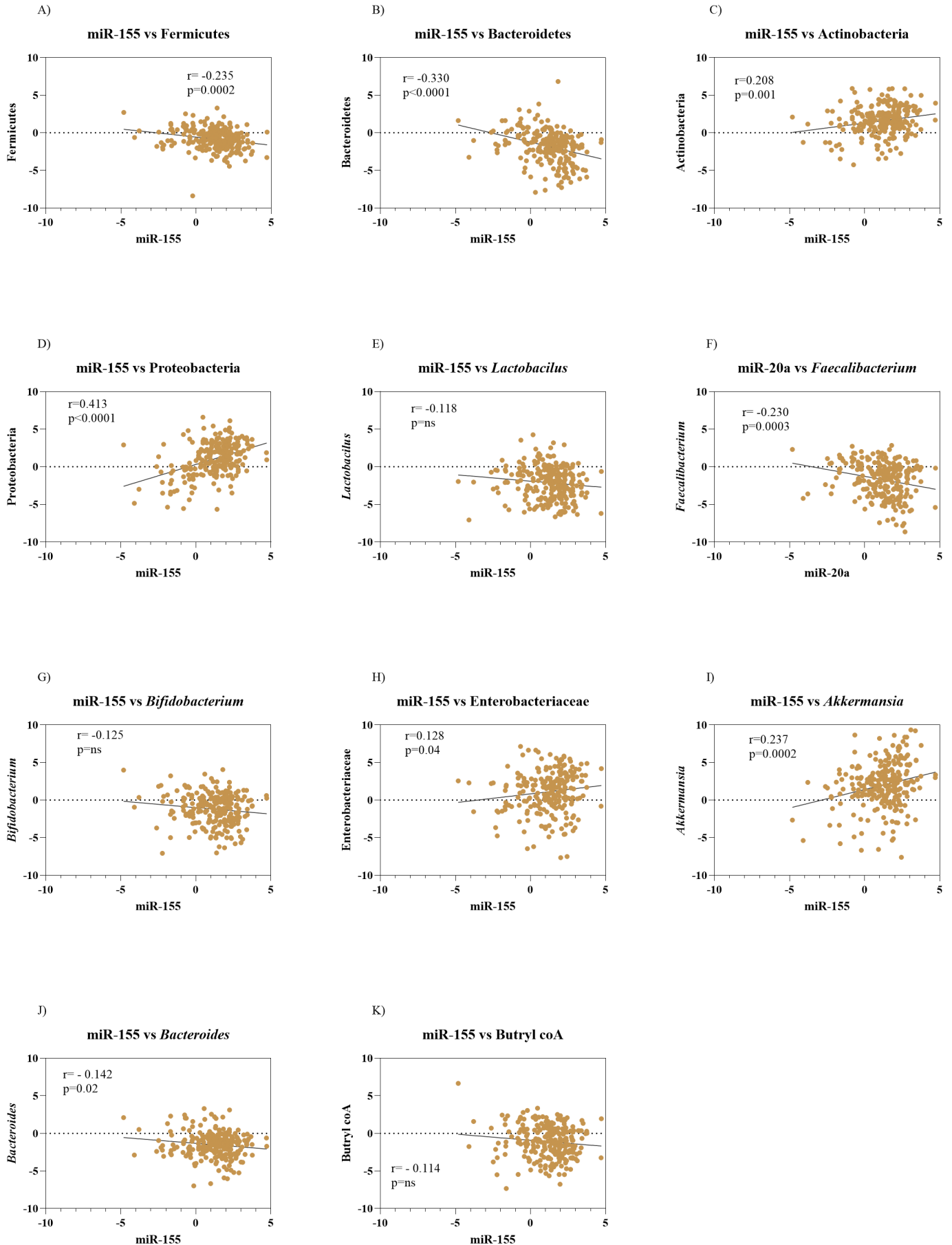
Combination ROC curves



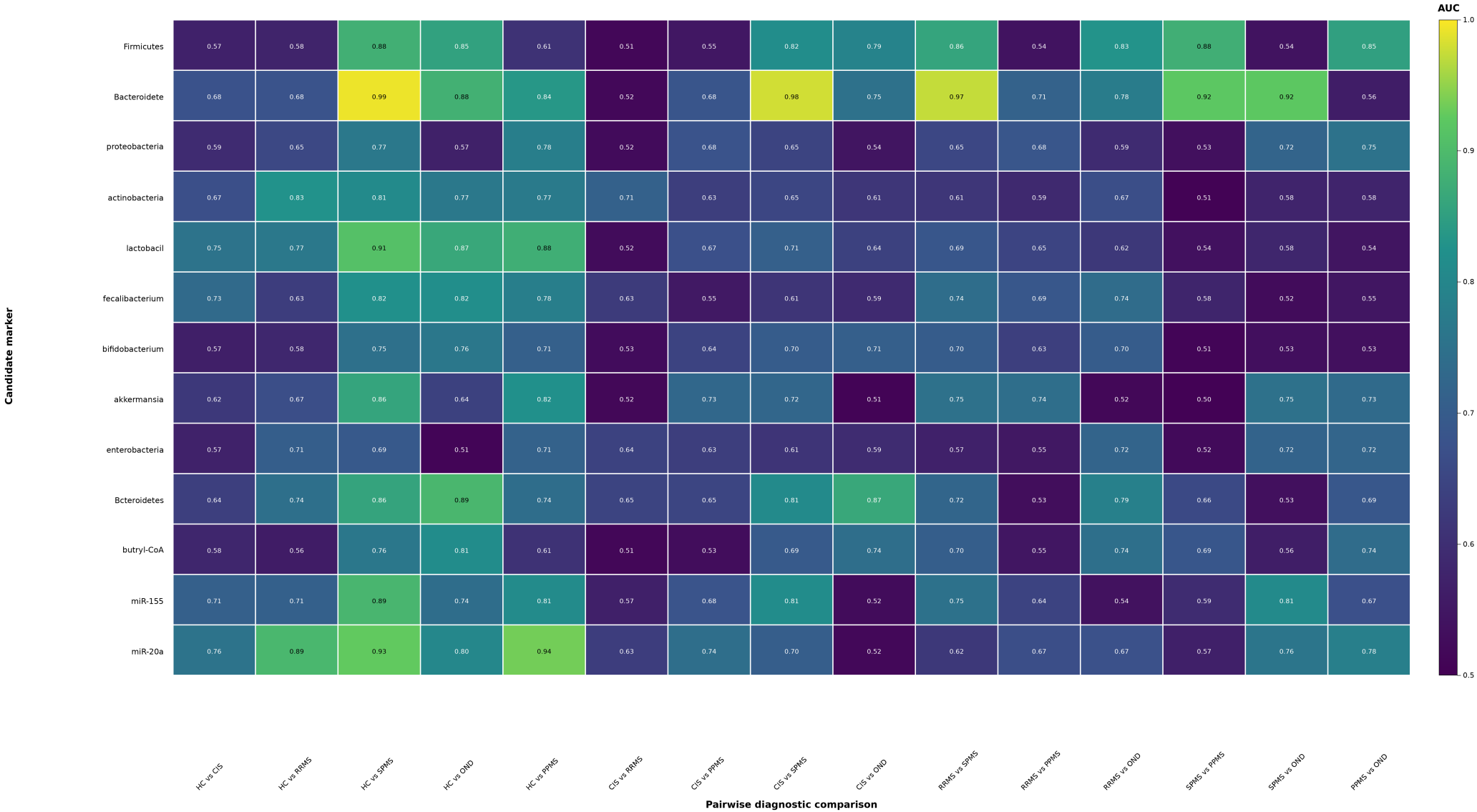
Correlation Analysis: miR-20a and gut microbiota



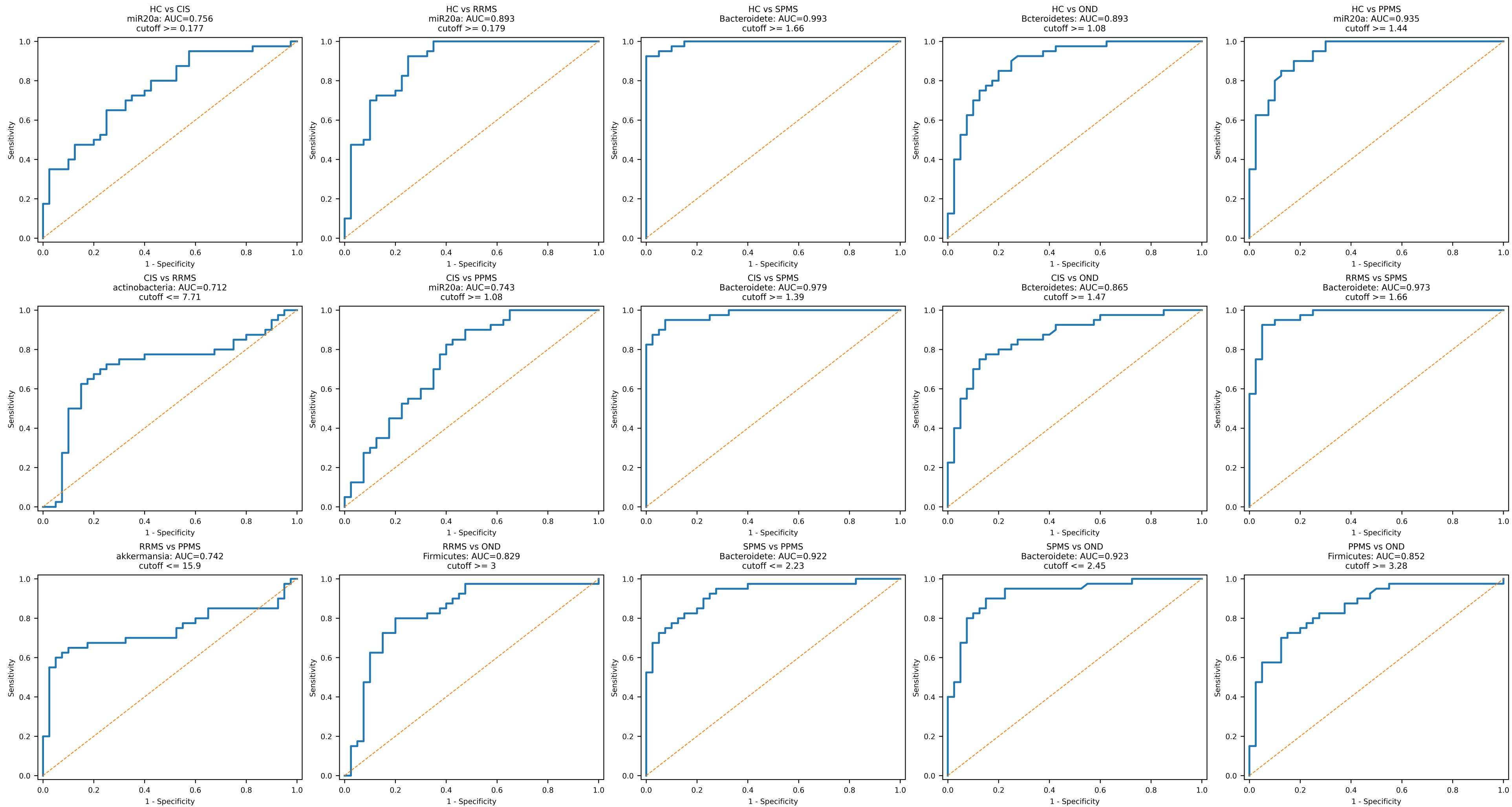
Correlation Analysis: miR-155 and gut microbiota



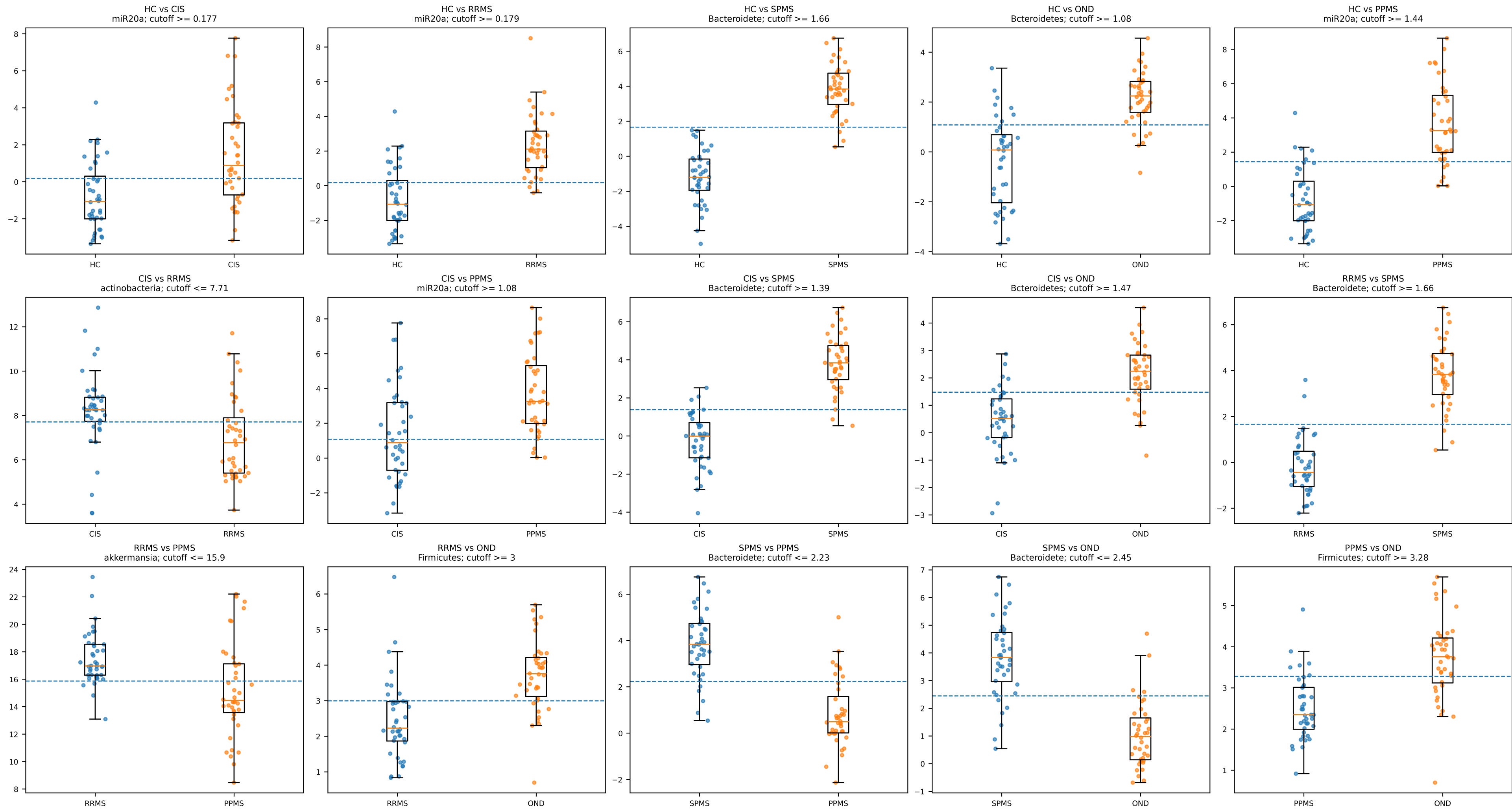
AUC heatmap for diagnostic ROC comparisons



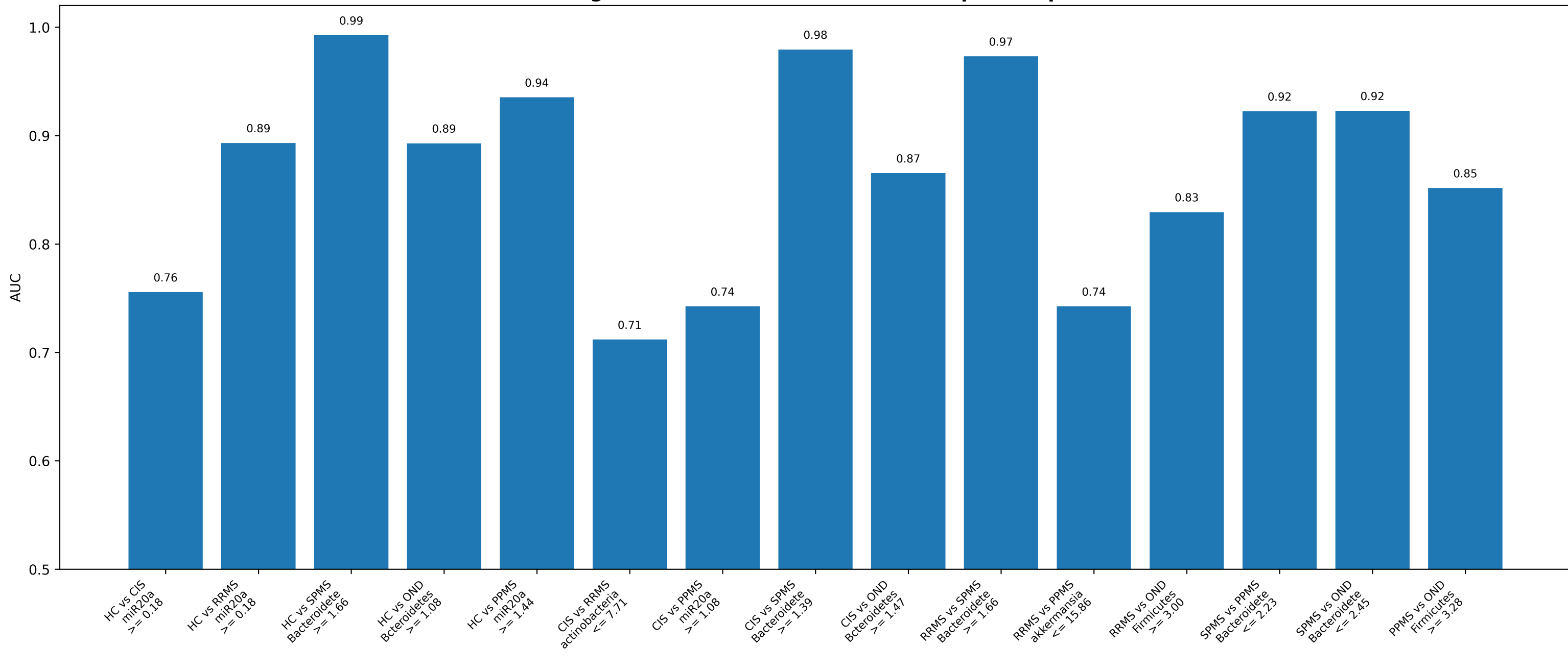
Best ROC curve and diagnostic cutoff per comparison



Distribution of best marker values with diagnostic cutoff



Best diagnostic marker and Youden cutoff per comparison



Supplementary Files

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