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JAK2V617F drives gut microbiota differences in patients with myeloproliferative neoplasms

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Abstract

Background: Essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (MF) are myeloproliferative neoplasms (MPN). Inflammation is involved in the initiation, progression, and symptomology of the diseases. The gut microbiota impacts the immune system, infection control, and steady-state hematopoiesis.

Methods: We analyzed the gut microbiota of 227 MPN patients and healthy controls (HCs) using next-generation sequencing. We expanded our previous results in PV and ET patients with additional PV, pre-MF, and MF patients which allowed us to compare MPN patients collectively, MPN sub-diagnoses, and MPN mutations (separately and combined) vs. HCs ($N = 42$) and compare within MPN sub-diagnoses and MPN mutation.

Results: MPN patients had a higher observed richness (median, 245 [range, 49–659]) compared with HCs (191.5 [range, 111–300; $p = .003$]) and a lower relative abundance of taxa within the *Firmicutes* phylum; for example, *Faecalibacterium* (6% vs. 14%, $p < .001$). The microbiota of *CALR*-positive patients ($N = 30$) resembled that of HCs more than that of patients with *JAK2V617F* ($N = 177$). In *JAK2V617F*-positive patients, only minor differences in the gut microbiota were observed between MPN sub-diagnoses, illustrating the importance of this mutation.

Conclusion: The gut microbiota in MPN patients differs from HCs and is driven by *JAK2V617F*, whereas the gut microbiota in *CALR* patients resembles HCs more.

KEYWORDS
essential thrombocythemia, gut microbiota, myeloproliferative neoplasms, polycythemia vera, primary myelofibrosis

Jens Jørgen Elmer Christensen, Xiaohui Chen Nielsen, Hans Carl Hasselbalch and Anna Cäcilia Ingham contributed equally to the project.

Novelty statements

What is the new aspect of your work?

Investigation of the gut microbiota in Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) is a highly neglected area of research with only five published papers. We present the most extensive study of gut microbiota in patients with MPN so far ($N = 227$).

What is the central finding of your work?

The gut microbiota in MPN patients differs from healthy controls (HCs) and is driven by *JAK2V617F*, whereas the gut microbiota in *CALR* patients resembles HCs more.

What is (or could be) the specific clinical relevance of your work?

We believe our findings are important for the understanding of MPN-inflammation-microbiota axis, and based on these findings, further research may lead to new treatment modalities.

1 | INTRODUCTION

Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) encompass the disease entities essential thrombocythemia (ET), polycythemia vera (PV), pre-fibrotic myelofibrosis (pre-PMF), and primary myelofibrosis (MF).[1] Chronic inflammation participates in the initiation and progression of MPN.[2–4]

Most patients with MPN harbor one of the driver mutations *JAK2V617F*, *JAK2* exon 12, *MPL*, or Calreticulin (*CALR*) in the hematopoietic stem cell (HSC). These mutations activate the JAK/STAT pathway resulting in accelerated myeloproliferation.[5] Usually, chronic inflammation results in quiescence and exhaustion of the HSC. Additionally, the driver mutations render the mutated cells more resistant and thus selected for.[6,7]

The gut microbiota (the microorganisms in the gut) is important for the regulation of the immune system and steady-state hematopoiesis.[8–10] Changes in the gut microbiota are associated with the development of several autoimmune and inflammatory-driven disorders.[11,12] Bearing in mind that low-grade chronic inflammation is central in the initiation and progression of MPN,[2–4] it is likely that changes in the gut microbiota are also associated with the pathobiology and progression of myeloproliferative disorders.

We have previously shown that, compared with healthy controls (HCs), patients with PV and ET have a higher gut microbiota richness, and a lower relative abundance of the *Firmicutes* phylum; particularly, we observed a lower abundance of *Faecalibacterium* in ET and PV. Furthermore, we have shown that gut microbiota profile differs according to treatment modality in patients with PV,[13] and the differences are more pronounced in patients harboring the *JAK2V617F* mutation in ET compared with patients without the *JAK2V617F* mutation when compared with HCs.[14]

Few other studies have investigated the gut microbiota in patients with MPN,[13–17] and mostly patients with PV or ET have been studied. Thus, the gut microbiota profiles of patients with MFs remain largely unknown. Furthermore, only one small study ($N = 25$, 11 patients with PV, eight patients with ET, and six patients with MF) investigated the gut microbiota in patients with MPN across sub-diagnoses and HCs.[15]

In this study, we aimed to investigate the overall association of the gut microbiota in MPN across sub-diagnoses and somatic mutations by integrating the results from the two previous studies of gut microbiota in patients with ET ($N = 54$) and PV ($N = 102$) and extending the study with data from additional patients with PV ($N = 12$) and patients with pre-PMF ($N = 13$) and MF ($N = 46$), totaling 227 patients with MPN in comparison with 42 HCs.

2 | MATERIALS AND METHODS

2.1 | Recruitment of patients

Patients ≥18 years of age with ET, PV, pre-PMF, primary MF, or post-PV/ET-MF according to the 2016 World Health Organization (WHO) classification[1] were eligible for inclusion. All patients were included from November 2018 to August 2021 at the Department of Hematology, Zealand University Hospital, Roskilde, Denmark.

Patients were excluded if they had been prescribed antibiotics within 2 months, or if there had been any change in cytoreductive treatment within 3 months. Furthermore, patients were not considered for inclusion in case of pregnancy, glucocorticoid treatment, or inability to understand written and oral information. Patients were divided into groups according to MPN sub-diagnoses: ET, PV, pre-PMF, and MF (primary MF and post-PV/ET-MF). Only 13 patients were diagnosed with pre-PMF, and due to small number, this group was not included in the statistical analyses when MPN sub-diagnoses were compared, but included when the whole MPN group was compared with HCs. Furthermore, patients were grouped according to *JAK2V617F* or *CALR* mutation status. Two patients were positive for both *CALR* and *JAK2V617F* and excluded when *CALR* and *JAK2V617F* positive patients were compared with each other and with HCs. To investigate whether the microbiota differed between patients according to allele burden, patients were divided in patients with very low (<5), low (5–19), medium (20–49), and high (>49) allele burden.

2.2 | Healthy control recruitment

A total of 42 HCs were included from the Danish General Suburban Population Study (GESUS), Næstved, Region Zealand[18] in 2021, as previously described.[13] All HCs were negative for the *JAK2V617F* and *CALR* mutations when enrolled in the GESUS study between 2010 and 2013 and matched according to age and gender.

2.3 | Sample collection

The participants collected the stool samples according to written instructions. The stool samples were collected either just before a visit to the outpatient clinic or at the participant's house and stored at −80°C within 6 h from sampling. One stool sample per patient was used in the study.

2.4 | Clinical and laboratory data

As described before[13] clinical and biochemical data were collected retrospectively. Biochemical data included data on leukocyte and differential count ($\times 10^9$/L), hemoglobin concentration (mmol/L), platelet count ($\times 10^9$/L), lactate dehydrogenase (LDH) (U/L), and estimated glomerular filtration rate (eGFR) (mL/min/1.73 m^2).[19] Information on mutation status (*JAK2V617F*, *JAK2* exon 12, *CALR*, and *MPL*) was obtained using quantitative PCR, fragment analysis, or next-generation sequencing as previously described.[14]

Clinical data included data on comorbidities (by the Charlson Comorbidity Index [CCI]),[20] smoking status, hypertension, body mass index (BMI, kg/m^2), time since diagnosis, use of statins and metformin, type of cytoreductive treatment (current and previous), and MPN diagnosis.

2.5 | DNA extraction and 16S rRNA gene sequencing

For each patient, 100 mg fecal sample was mixed with lysis buffer (260 µL) and 1.4-mm Zirconium beads (OPS Diagnostics LLC, Lebanon, USA). For bead-beating, the TissueLyser II (QIAGEN, Germany) was used for 2 min at 30 Hz. For DNA extraction, an automated eMAG platform (bioMérieux, France) was used, following the manufacturer's protocol.

A modified version of the 341/806 universal prokaryotic primers targeting the V3–V4 region of the nuclear small subunit ribosomal RNA gene was used for 16S amplicon-based sequencing. For further details on primers and sequencing, see Ring et al.[21]

2.6 | Preprocessing, taxonomic classification, and decontamination

FastQ files obtained by ILLUMINA sequencing were trimmed with a quality cutoff of 98% using a sliding window as described previously.[13] DADA2[22] (version 1.12.1), was used for Inference of high-resolution amplicon sequence variants (ASVs), and the R package *decontam*[23] was used to remove contamination, for further details, see Supplementary Methods.

2.7 | Statistical analysis

R (v.4.0.3.)[24] was used for statistical analysis and visualization. The R code used for statistical analysis is available at 10.6084/m9.figshare.24871614. A phyloseq object was created by merging clinical, paraclinical, and sequence data using the *phyloseq* package.[25] For illustrations, as previously described,[14] the packages ggplot2, *cowplot* and *factoextra* were used. Analysis of variance (ANOVA) was used for normally distributed continuous data (mean, SD), whereas pairwise Wilcoxon rank-sum tests were used for non-normally distributed data (median, range). For categorical data, Fisher's exact test were used. To adjust for multiple testing, Benjamini-Hochberg correction was used. Probability (p) values ≤.05 were considered to indicate statistical significance.

Alpha diversity (here measured by the Inverse Simpson index) and observed bacterial richness (ASVs) were calculated on untransformed bacterial counts.

Principal component analysis (PCA) was based on Hellinger-transformed counts and used to visualize differences in bacterial composition (R package *FactoMineR*).[26]

To assess potential differences in bacterial composition between the groups (ET, PV, MF, *JAK2V617F*-positive patients, and *CALR*-positive patients), analysis of similarities (ANOSIM, *vegan* package)[27] was used based on Bray-Curtis dissimilarities. To analyze differential abundance analysis across several taxonomical levels, Linear discriminant analysis (LDA) effect size (LEfSe) analysis was applied (*microbiomeMarker* package)[28] on proportional data with an LDA cutoff of 0.005.

To assess whether the relative abundance of any taxa correlated with specific continuous variables, such as either (i) allele burden or (ii) leukocyte count, platelet count, hemoglobin level, sedimentation reaction (SR) BMI, LDH, eGFR, and neutrophil-to-lymphocyte ratio (NLR), we calculated Spearman rank correlations with package corrr (function correlate)[29] on the top 25-most abundant bacteria of each respective taxonomic level. The package corrplot was used for significance testing with Benjamini-Hochberg correction (cor.mtest), and visualization of correlations.[30]

The anonymized sequence data of the patients with MPN are available at the European Nucleotide Archive (ENA) (PRJEB63403) and the data of the HCs on request.

2.8 | Ethics approval and dissemination

The project was approved by the Regional Ethics Committee (SJ452), the National Committee on Health Research Ethics (SJ-698, SJ-452), and the Danish Data Protection Agency (REG-050-2015, REG-054-2018) and

TABLE 1 Baseline characteristics, that is, demographic data, mutation status, clinical and biochemical data of the healthy controls (A), patients with MPN (B), *JAK2V617F*-positive patients (C), and *CALR*-positive patients (D) included in the study.

Characteristics[a]	HC (A)	All (B)	MPN *JAK2V617F* (C)	*CALR* (D)	*p*
N	42	227	177	30	
Female (%)	17 (40.5%)	116 (51.1%)	90 (50.8%)	15 (50%)	ns
Age	71 (66–74)	68 (31–88)	69 (31–85)	64.5 (48–88)	A vs. B + D, <0.05
Diagnosis					
ET		54 (23.8%)	36 (20.3%)	7 (23.3%)	
PV		114 (50.2%)	111 (62.7%)	0	
Pre-PMF		13 (5.7%)	6 (3.4%)	5 (16.7%)	
MF		46 (20.3%)	24 (13.6%)	18 (60%)	
Mutation					
JAK2V617F	0	177 (78%)			
JAK2 exon 12	NA	3 (1.3%)			
CALR	0	30 (13.2)			
MPL	NA	4 (1.8%)			
JAK2V617F + *CALR*	0	2 (0.9%)			
Triple-negative	NA	11 (4.8%)			
BMI, kg/m^2 median (range)	24.95 (19.2–29.5)	25.2 (16.6–47.4)	25.3 (16.6–47.4)	24.70 (19.5–37.5)	ns
Hypertension, *N* (%)	16 (38.1%)	121 (53.3%)	101 (57.1%)	11 (36.7%)	ns
Charlson Comorbidity Index[b]					
Median (range)	0 (0–4)	1 (0–9)	1 (0–9)	0.5 (0–7)	A vs. B + C, =0.02
No comorbidities (CCI = 0)	22 (52.4%)	75 (33%)	53 (29.9%)	15 (50%)	A vs. B + C, <0.05
Low burden (CCI ≤ 2)	16 (38.1%)	95 (34.3%)	77 (43.5%)	9 (30%)	ns
Moderate to high (CCI > 2)	4 (9.5%)	57 (25.1%)	47 (26.6%)	6 (20%)	A, vs. B, =0.3
Laboratory tests median (range)					
Leukocytes	6.2 (4.5–9.4)	5.9 (2.0–42.3)	5.8 (2.5–42.3)	6.4 (3.0–27.4)	ns
Hematocrit, %	43% (35–49)	40% (30–51)	41% (30–51)	37% (30–48)	A vs. B + C + D, <0.001 C vs. D, <0.01
Platelets	227 (125–355)	327 (20–903)	316 (73–884)	351.5 (142–796)	A vs. B + C + D, ≤0.001
eGFR	73.6 (39–93.7)	81.4 (24.3–121.2)	80.7 (24.3–121.2)	86.2 (42.0–103.1)	A vs. B + D < 0.05
Smoking					
Current smoker (%)	1 (2.4%)	21 (9.3%)	18 (10.2%)	2 (6.7%)	ns
Former smoker (%)	13 (31%)	92 (40.5%)	75 (42.4%)	8 (26.7%)	ns
Never smoker (%)	28 (66.6%)	111 (48.9%)	82 (46.3%)	19 (63.3%)	A vs. B, <0.05
Unknown (%)	0	3 (1.3%)	2 (1.1%)	1 (3.3%)	
Disease length, wk[c] median (range)		295.8 (0.1–2124)	302.7 (0.1–2424)	286.1 (5.2–1339)	ns
Treatment					
IFN, *N* (%)		48 (21.1%)	37 (20.9%)	9 (30%)	
HU, *N* (%)		71 (31.3%)	56 (31.6%)	8 (26.7%)	
No treatment, *N* (%)		19 (8.4%)	15 (8.5%)	1 (3.3%)	
Treatment naïve, *N*(%)		32 (14.1%)	19 (10.7%)	7 (23.3%)	
COMBI, *N* (%)		32 (14.1%)	30 (16.9%)	0	
Other, *N* (%)		25 (11%)	20 (11.3%)	5 (16.7%)	

Note: Pairwise Wilcoxon Rank Sum test, or exact fishers test.

Abbreviations: COMBI, IFN + ruxolitinib; eGFR, estimated glomerular filtration rate; HU, hydroxyurea; IFN, interferon-α; ns, not significant.

[a]At the time of fecal collection.

[b]Comorbidity scores were calculated using Charlson Comorbidity Index (CCI).

[c]Weeks since diagnosis of MPN.

was carried out following the Declaration of Helsinki. All participants signed written consent and were informed in person.

3 | RESULTS

3.1 | Baseline characteristics

In total, we included 42 HCs and 227 patients with MPN: *JAK2V617F* ($N = 177$), *CALR* ($N = 30$), ET ($N = 54$), PV ($N = 114$), pre-PMF, ($N = 13$), and MF ($N = 46$). Compared with HCs, patients with MPN were slightly younger (median [range]: 68 [31–88] vs. 71 [66–74] years; $p = .03$) and had a higher CCI score (Table 1, Figure 1). Patients with MPN had lower hematocrit (median [range]: 40% [30%–51%] vs. 43% [35%–49%], $p < .001$), higher platelet count (median [range]: 327 [20–903] vs. 227 [125–355], $p < .001$), and higher eGFR (median [range]: 81.4 [24.2–101.2] vs. 73.6 [39–93.7], $p = .03$) compared with the HCs (Table 1, Table S1).

3.2 | Gut microbiota alpha diversity

The median observed richness was higher in patients with MPN (245; range, 49–659) compared with HCs (191.5; range, 111–300, $p = .003$) (Figure 2A); the alpha diversity measured by the Inverse Simpson index did not differ (Figure 2B).

Comparing patients with ET, PV, and MF with each other and HCs, the median observed richness was only different in ET (283.5; range, 75–535, $p = .001$) compared with HCs (Figure 2C). When comparing patients with ET, PV, and MF with each other and HCs, the Inverse Simpson index was not different (Figure 2D).

Comparing patients by mutation the median observed richness was 232 (range, 49–659, $p = .01$) in *JAK2V617F*-positive and 273 (range, 99–527; $p = .001$) in *CALR*-positive patients compared with HCs. Observed richness did not differ between the two mutation groups (Figure 2E).

MPN patients with the *JAK2V617F* mutation had a significantly lower median Inverse Simpsons Index compared with the HCs (median Inverse Simpsons index 21.8 vs. 26.3, $p < .05$) and with *CALR*-positive patients (median Inverse Simpsons index 29.0, $p < .05$) (Figure 2F).

Comparing *JAK2V617F*-positive patients with ET, PV, and MF with each other and HCs, the median observed richness only differed in *JAK2V617F*-positive patients with ET compared with HCs with an observed richness at 299.5 (range, 102–535, $p < .001$) (Figure 2G).

Comparing *JAK2V617F*-positive patients with ET, PV, and MF with each other and HCs, the Inverse Simpsons index did not differ after correction for multiple testing (Figure 2H).

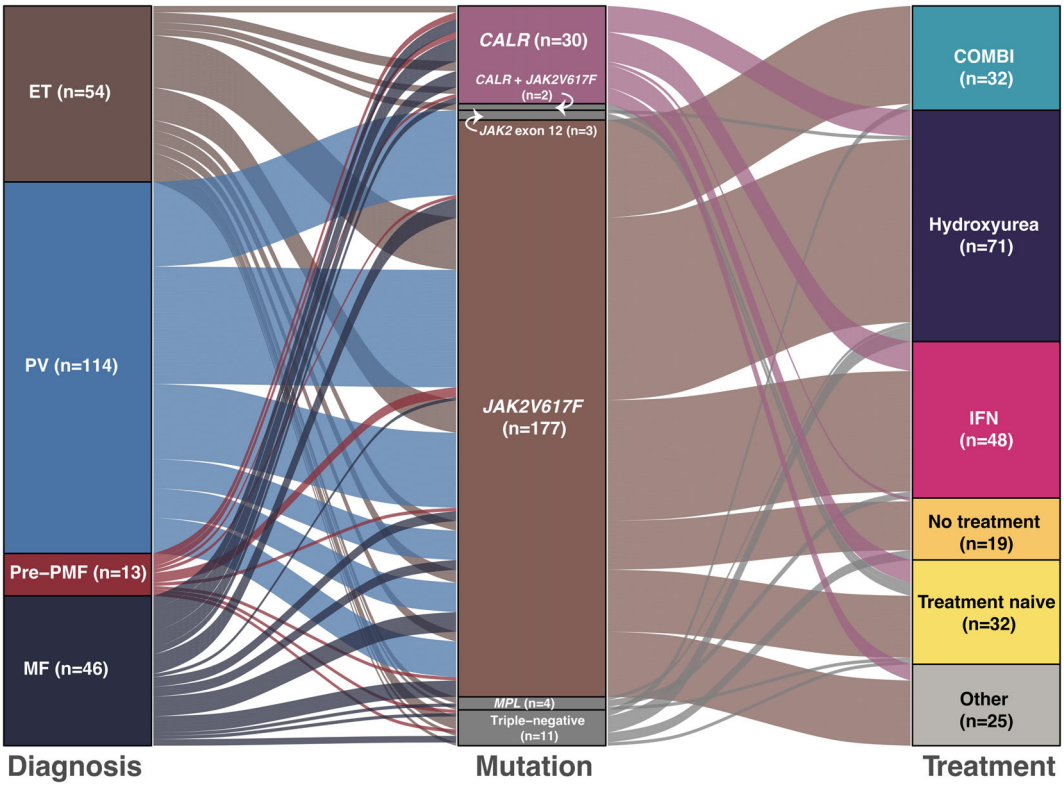

FIGURE 1 Overview of the patients included in the study. Sankey plot illustrating the 227 patients included in the study. In the first column, the sub-diagnoses of the patients are shown. In column two, their corresponding mutations, and in column three, their treatment. The patients can be followed from columns one to three. Patients not positive for the *JAK2*, *MPL*, and *CALR*-mutations are referred to as triple-negative. COMBI, IFN + ruxolitinib; HU, hydroxyurea; IFN, interferon-α.

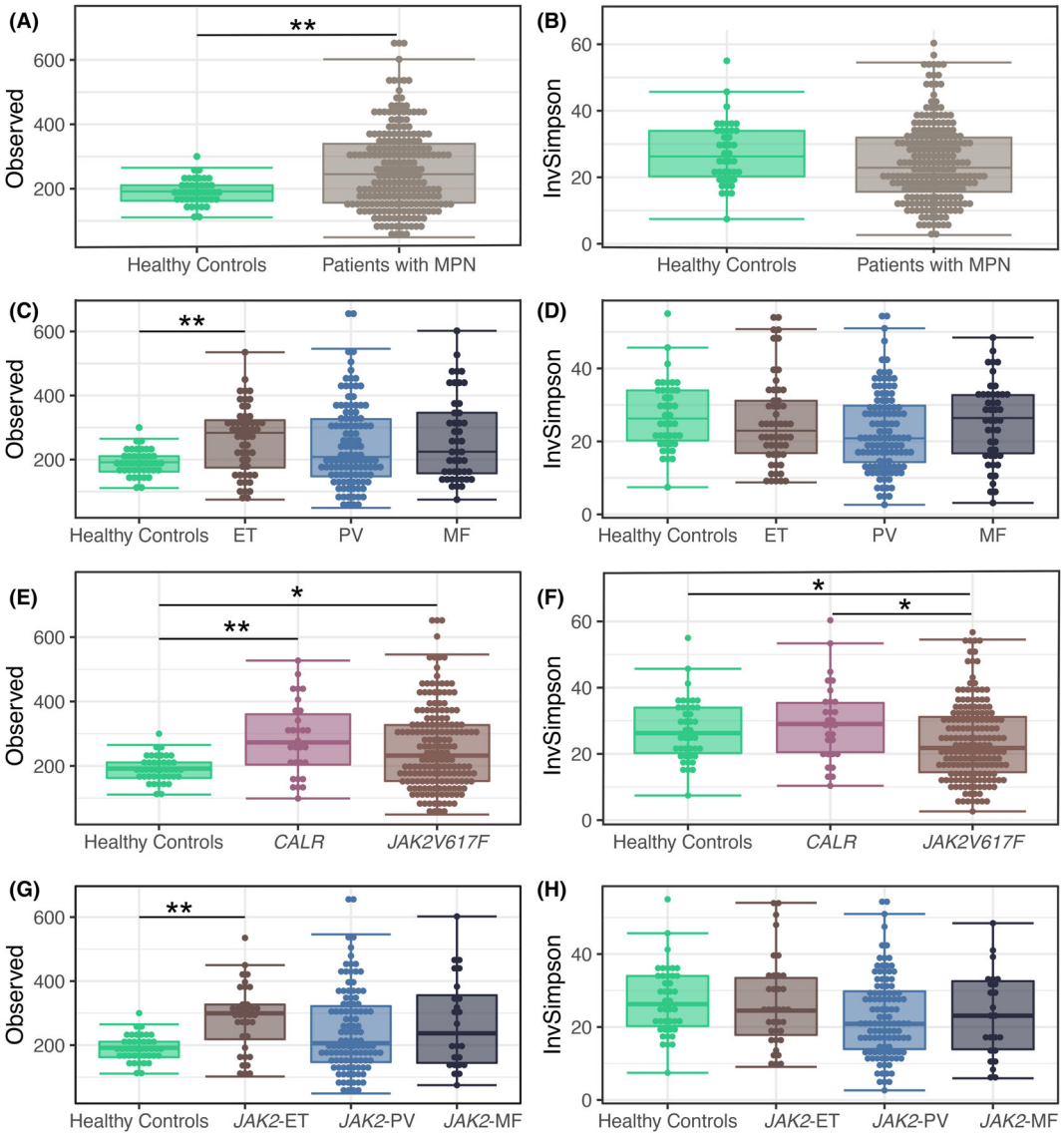

FIGURE 2 Observed gut microbiota richness and alpha diversity in patients with MPN, MPN sub-diagnoses (ET, PV, and MF), and according to mutation and compared with healthy controls (HCs). The observed gut microbiota richness and the median Inverse Simpsons in patients with MPN, (A, B), MPN sub-diagnoses (ET, PV, and MF) (C, D), according to mutation (E, F), and according to sub-diagnosis in the *JAK2V617F*-positive patients (G, H) compared with each other and healthy controls (HCs). Asterisks indicate the following levels of significance: $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

3.3 | Bacterial composition and dissimilarities

Comparing MPN to HCs, the overall bacterial composition did not differ (Figure 3A), but the median Bray-Curtis within-group variations were large (Figure 3B). Comparing patients with ET, PV, and MF with each other and HC, the bacterial composition in patients with ET ($R = 0.06$, $p = .005$) and MF ($R = 0.1$, $p = .001$) differed from HCs and each other ($R = 0.05$, $p = .005$) (Figure 3C; Supplementary Results, Figure S5C). Patients with ET appeared to be the group resembling HCs the most in terms of bacterial composition, followed by patients with MF and then PV.

Comparing patients with ET, PV, and MF to HC, the greatest dissimilarity was observed between patients with PV and HCs (median 0.78), followed by those with MF (0.76) and ET (0.75) (Figure 3D), confirming the PCA (Figure 3C). However, compositional differences among patients with PV (within-group variation) were greater (0.79) than the difference between PV and HCs (Figure 3D), explaining the nonsignificant ANOSIM result.

Comparing patients by mutation group to HCs, the bacterial composition differed in patients with *CALR* ($R = 0.11$, $p = 0.001$) (Figure 3E) but not in patients with *JAK2V617F*. The latter finding is possibly due to large within-group variation (Figure 3E,F).

Comparing *JAK2V617F* allele burden (very low [<5], low [5–19], medium [20–49] and high [>49]) to HC, all four allele burden groups differed significantly from HCs in bacterial composition, but not from each other (Figure S1).

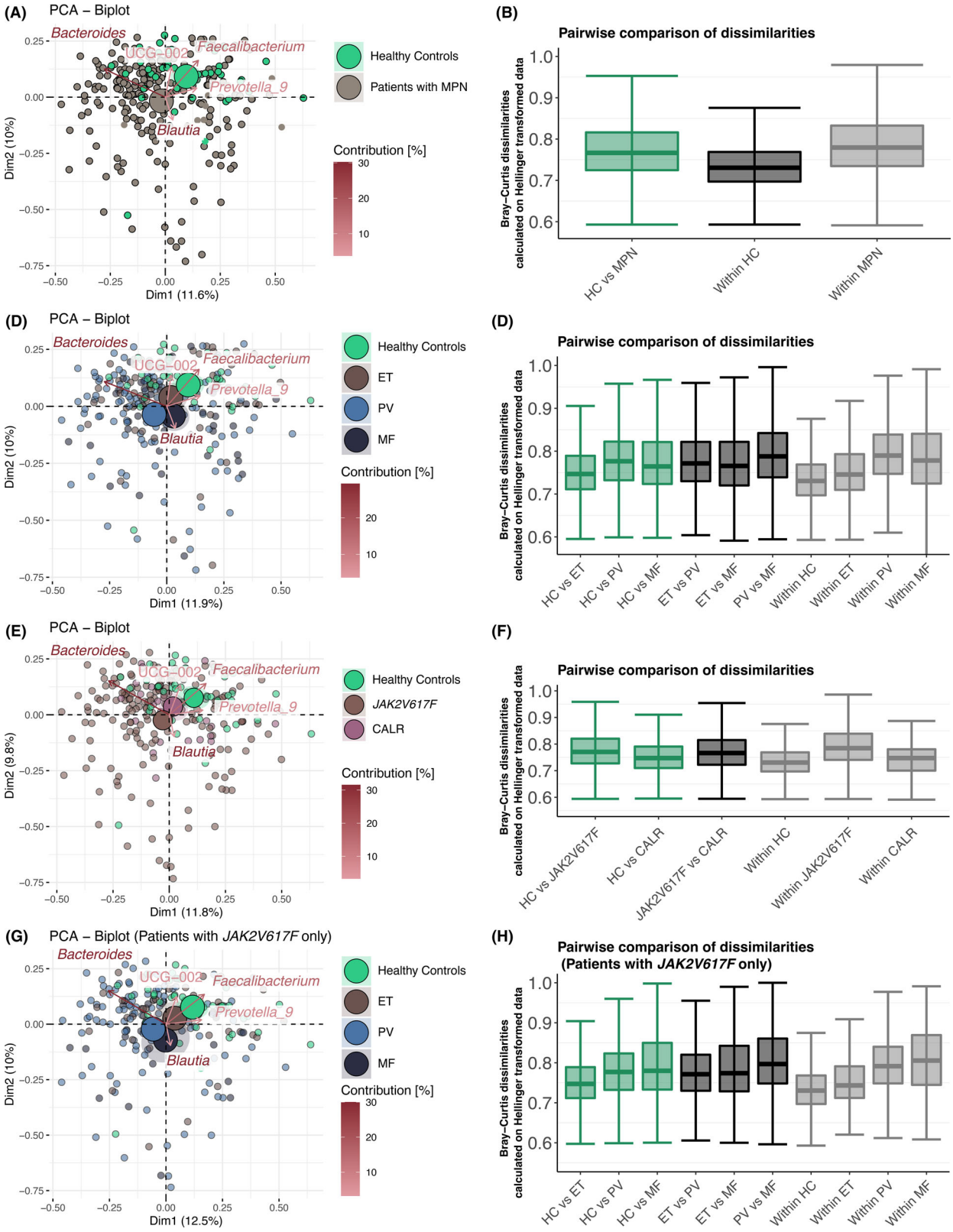

FIGURE 3 Principal component analysis (PCA) and pairwise comparison of dissimilarities in patients with MPN, according to sub diagnoses (ET, PV, and MF), according to mutation status, and according to sub diagnoses in the *JAK2V617F*-positive patients, compared with HCs. Principal component analysis (PCA) and pairwise comparison of dissimilarities in patients with MPN (A, B), according to sub-diagnoses (ET, PV, and MF) (C, D), according to mutation status (E, F), and according to sub-diagnoses in the *JAK2V617F*-positive patients (G, H), compared with each other and HCs. In the biplot, the top five predictors are shown at genus level, the percentage of variance between the groups explained by each genus is visualized by the red arrows.

Comparing *JAK2V617F*-positive patients with ET, PV, and MF with each other and HCs, the bacterial composition of patients with ET and MF remained significantly different from each other ($R = 0.13$, $p < .01$) and remained different from the HCs ($R = 0.1$, $p = .005$; $R = 0.26$, $p = .001$) (Figure 3G).

When pairwise comparison of median Bray-Curtis dissimilarities was performed in *JAK2V617F*-positive patients with ET, PV, and MF compared with each other and HCs, patients with MF differed the most from the HCs (0.78), followed by those with PV (0.78) and ET (0.75) (Figure 3H).

3.4 | Differential abundance of taxa

Several taxa differed between patients with MPN and HCs based on LEfSe analysis; all significantly different taxa with an LDA above 0.005 and a relative abundance above 1% are illustrated in Figure 4.

Patients with MPN had a significantly lower relative abundance of *Firmicutes* compared to HCs (52% vs. 59%, $p = .01$). Furthermore, several taxa within the *Firmicutes* phylum, such as *Clostridia* (45% vs. 52%, $p < .001$), the family *Lachnospiraceae* (7% vs. 9%, $p < .01$), the order *Oscillospirales* (37% vs. 27%, $p < .001$), the family *Ruminococcaceae* (17% vs. 28%, $p < .001$), and the genus *Faecalibacterium* (6% vs. 14%, $p < .001$) were less abundant in patients with MPN compared with HCs. Patients with MPN had a higher relative abundance of the phylum *Actinobacteriota* (4% vs. 2%, $p < .001$) compared with HCs (Figure 4).

Compared with PV, ET patients had a higher relative abundance of *Faecalibacterium* (8% vs. 5%, $p = .02$), and MF patients a higher relative abundance of *Firmicutes* and *Clostridia* (55% vs. 49%, $p < .01$ and 50% vs. 40%, $p < .01$) (Figure 5A, Supplementary Results).

Comparing patients by mutation group, the relative abundance differed between the *JAK2V617F*-positive patients and HCs like comparing the entire MPN group and HCs (Figure 5B).

Patients with the *CALR* mutation had a higher relative abundance of the genus *Christensenellaceae R-7 group* within the family *Christensenellaceae* (5% vs. 2%, $p < .01$), the genus *Subdoligranulum* within the family *Ruminococcaceae* (8% vs. 3%, $p = .03$) and a higher relative abundance of *Actinobacteriota* (5% vs. 1%, $p < .01$) compared with HCs. Furthermore, the *CALR*-positive patients, as seen in the *JAK2V617F*-positive patients and patients with MPN in general, had a lower relative abundance of *Lachnospiraceae* (7% vs. 9%, $p = .01$), *Ruminococcaceae* (21% vs. 28%, $p = .01$), and *Faecalibacterium* (8% vs. 14%, $p < .01$). However, the microbiota profiles of patients with the *CALR* mutation were overall more like those of the HCs.

Comparing *JAK2V617F*-positive patients with *CALR*-positive patients, several taxa differed. *JAK2V617F*-positive patients had a significantly lower relative abundance of *Clostridia* (43% vs. 51%, $p < .01$), *Oscillospirales* (26% vs. 32%, $p < .01$), *Ruminococcaceae* (16% vs. 21%, $p < .01$) and *Subdoligranulum* (4% vs. 8%, $p = .01$) (Figure 5B).

Few taxa differed significantly between groups stratified by allele burden (very low [<5], low [5–19], medium [20–49] and high [>49]) (Figure S2). Interestingly, patients with a high allele burden had a higher relative abundance of *Akkermansia* (genus within the phylum *Verrucomicrobiota*) (5% vs. 1%, $p < .01$) compared with patients with low allele burden. Nevertheless, the same gut microbiota profile persisted in all groups when compared with HCs. However, *Firmicutes* did not differ in patients with very low burden, and patients with high allele burden had a higher relative abundance of *Akkermansia* (5% vs. 1%, $p = .04$) and a lower relative abundance of *Prevotellaceae* (0% vs. 4%, $p = .01$) compared with HCs. For all significantly different taxa and *p* values, see Figure S2.

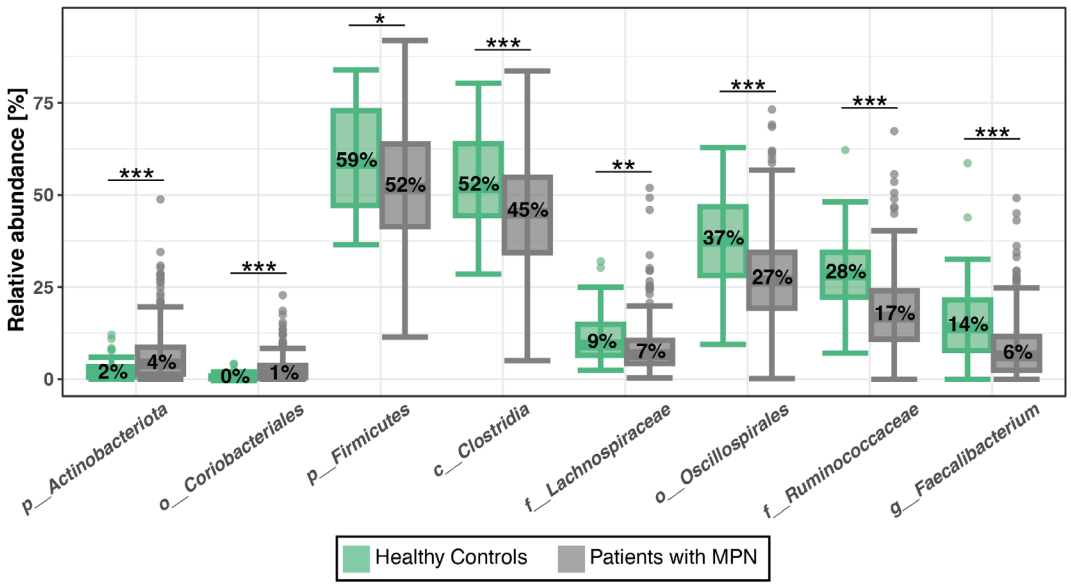

FIGURE 4 Differential abundance analysis of the gut microbiota in patients with MPN compared with HCs. To identify significantly different taxa, Linear discriminant analysis Effect Size (LEfSe) analysis was used. Only taxa with an overall median proportion >1% and an LDA score >0.005 are shown. Asterisks indicate the following levels of significance: *$p < .05$; **$p < .01$; ***$p < .001$. c, class; f, family; g, genus; o, order; p, phylum.

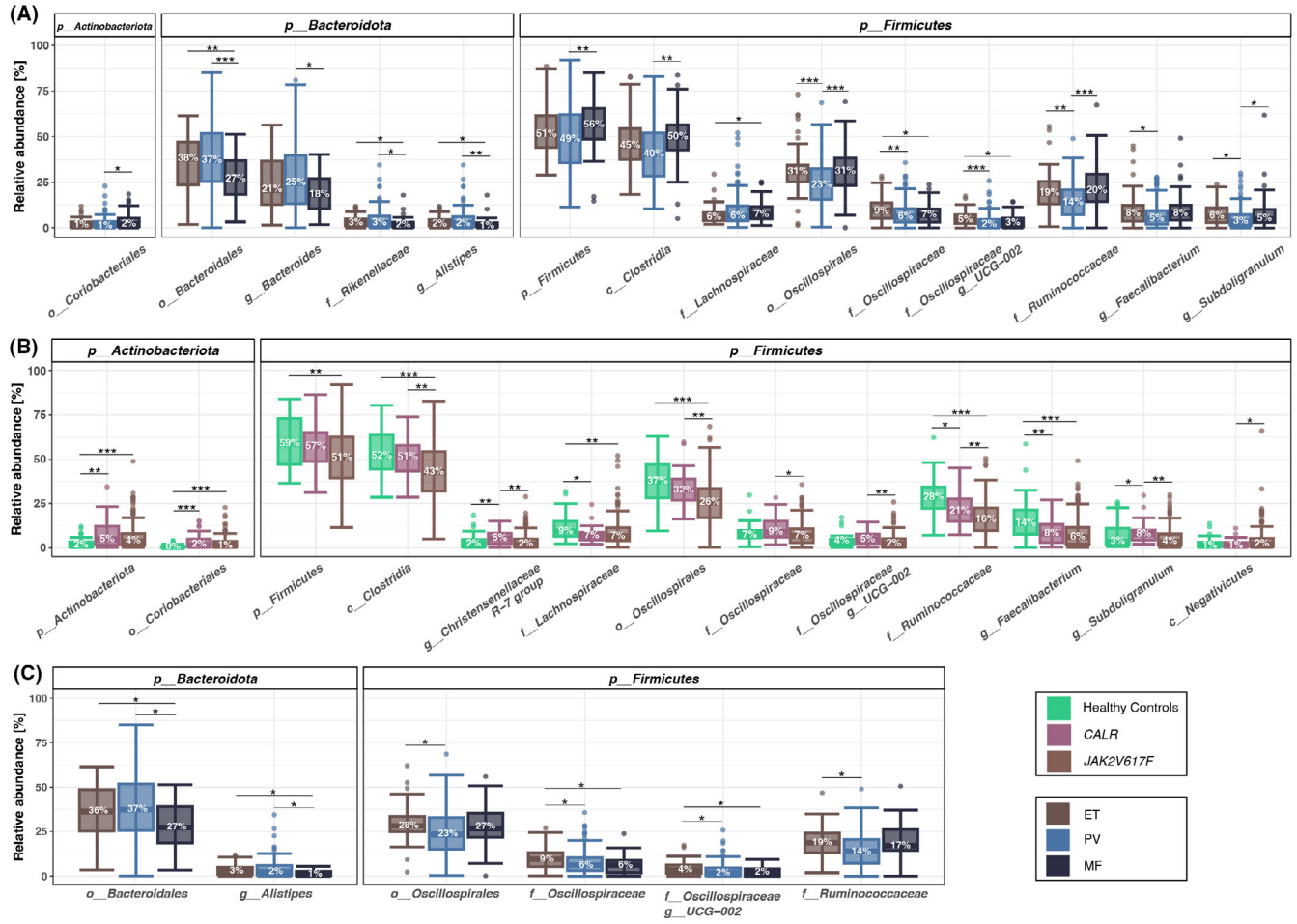

FIGURE 5 Differential abundance analysis of the gut microbiota according to sub-diagnoses in all patients, according to mutation status, and according to sub-diagnoses in the *JAK2V617F*-positive patients only. In (A), differential abundance analysis of the gut microbiota in the different sub-diagnoses (ET, PV, and MF) is shown. Several differences were found. In (B), the two mutation groups (*CALR* and *JAK2V617F*) are compared with each other and HCs. In (C), the *JAK2V617F* positive patients are compared across sub-diagnoses. Although, some differences still persist, fewer differences were found. Only taxa with an overall median proportion >1% and a LDA score >0.005 are shown. Asterisks indicate the following levels of significance: *$p < .05$; **$p < .01$; ***$p < .001$. c, class; f, family; g, genus; o, order; p, phylum.

Comparing the differences in gut microbiota in *JAK2V617F*-positive patients with ET, PV, and MF with each other, considerably fewer taxa differed between the different sub-diagnoses. For further details and *p* values, see Figure 5C.

3.5 | Correlation analysis

When all patients ($N = 227$) were included in the analysis, a few correlations on family and genus level were found (Figure S3). At family level, *Christensenellaceae* and *Oscillospiraceae* negatively correlated with BMI ($r_s = -.23$, $p = .01$, and $r_s = -.19$, $p = .03$), whereas *Lachnospiraceae* positively correlated with BMI ($r_s = 1.9$, $p = .03$). *Akkermansiaceae* negatively correlated with BMI ($r_s = -.19$, $p = .03$) and hemoglobin ($r_s = -.24$, $p < .01$); this was observed at genus level (*Akkermansia*) as well (Figure S3).

When patients were grouped according to mutation status, the negative correlation of *Akkermansia* with BMI ($r_s = -.22$, $p = .02$) and hemoglobin ($r_s = -.21$, $p = .014$) was observed in patients positive for the *JAK2V617F* mutation, but not the *CALR* mutation, or HCs (Figures S3 and S4). Several correlations were found in the *CALR*-positive patients; however, none remained significant after correcting for multiple testing (Figure S4). When testing for correlations between taxa and *JAK2V617F* allele burden, *Akkermansia* was positively correlated; that is, patients with a high allele burden had a higher relative abundance of *Akkermansia* ($r_s = -.24$, $p < .05$). At family level *Oscillospiraceae* were negatively correlated with allele burden ($r_s = -.24$, $p = .04$) (Figure S4).

4 | DISCUSSION

In this study, we observed that the gut microbiota in patients with MPN compared with HCs is characterized by a higher observed richness, and a low relative abundance of specific taxa within the *Firmicutes* phylum (e.g., *Faecalibacterium*), which includes important

producers of short-chain fatty acid (SCFA). These findings were largely driven by *JAK2V617F*-positive patients.

The bacterial composition of *CALR*-positive patients exhibited a closer resemblance to the composition of HCs with a higher relative abundance of *Firmicutes*, *Clostridia*, and *Ruminococcaceae*. Although they did have a higher relative abundance of *Subdoligranulum* compared with HCs and *JAK2V617F*-positive patients.

ET, PV, and MF differed greatly, but not when only *JAK2V617F*-positive patients were compared, illustrating the importance of this mutation.

All *JAK2V617F* allele burden groups differed from HCs in bacterial composition and almost the same gut microbiota profile persisted in all groups when compared with HCs, and few differences were found between the allele burden groups. Of notably interest, patients with high allele burden had an increased relative abundance of *Akkermansia* compared with HCs and patients with low allele burden.

Few other studies have investigated the gut microbiota in patients with MPN.[13–16] We have previously shown that microbiota profiles differ between patients with *JAK2V617F*-positive ET and *JAK2V617F*-negative ET when compared with HCs,[14] with *JAK2V617F*-negative ET patients having a microbiota profile closer to that of HCs. In the present study, we confirm these findings in a larger cohort of 177 *JAK2V617F*-positive and 30 *CALR*-positive MPN-patients. In general, patients positive for the *CALR*-mutation has been associated with better prognosis with regards to survival and thromboembolic complications,[31] making this finding even more intriguing. It could be speculated whether the different *JAK2V617F* /*CALR* microbiota profiles are due to different inflammatory profiles of the diseases, or the microbiota in itself participates in creating the inflammatory environment.[32]

Only one previous study investigated differences in gut microbiota among MPN sub-diagnoses and HCs. The study was small ($N = 25$), and it was found that early-stage MPN (ET/PV) appeared to cluster more densely compared with patients with MF.[15] Moreover, patients with MPN had a lower relative abundance of *Phascolarctobacterium* compared with controls, which we could not confirm in the present study.

We revealed a low relative abundance of taxa within *Firmicutes* (*Faecalibacterium*) in patients with MPN. A low relative abundance of *Faecalibacterium prausnitzii* has been reported in several inflammatory and autoimmune diseases, such as type-2 diabetes, multiple sclerosis, and neurodegenerative disorders.[33] Moreover, a low relative abundance of *Faecalibacterium* has been reported in other myeloid malignancies such as acute myeloid leukemia (AML).[34]

Taxa within the *Firmicutes* phylum, especially *F. prausnitzii*, are essential producers of butyrate and other anti-inflammatory metabolites.[35–37] Butyrate is a well-known modulator of inflammation and is important in regulating the gut barrier integrity and permeability,[38,39] and a link between damaged intestinal barrier and AML progression, due to increased inflammation, has been proposed.[34] The low relative abundance of *Faecalibacterium* across the entire biological continuum of sub-diagnoses, regardless of mutation, diagnosis, and allele burden is therefore highly intriguing, especially as chronic inflammation is considered a major driver of the development and disease progression in MPN.[2–4] Moreover, the two most prevalent MPN driver mutations (the *JAK2V617F* and the *CALR* mutation), are highly "inflammatory" mutations and associated with increased production of reactive oxygen species (ROS) and increased levels of inflammatory cytokines.[6,7,40] This, together with the role of the microbiota in the regulation of the immune system and steady-state hematopoiesis via microbial components (microbial-associated molecular patterns [MAMPs]),[8–10] renders studies of gut microbiota profiles in patients with chronic blood cancers particularly relevant. A link between the gut microbiota and MPN, where the gut microbiota participates in the inflammatory circle, possibly via damaged intestinal barrier functions and lipopolysaccharide leakage[16] could be proposed.

A recent study investigated the effect of Mediterranean diet on gut microbiome and inflammation in patients with MPN. No changes in gut microbiome and inflammatory biomarkers were found, the study however, was small ($N = 28$).[17] Other studies have shown an increase in *F. prausnitzii*, and a lowering of inflammatory cytokines in participants reviving a high fiber diet.[41] Since inflammation is a hallmark in MPN, larger studies investigating high fiber diets effect on gut microbiota and inflammation in MPN should be pursued.

Interestingly, an increased abundance of *Akkermansia*, a Gram-negative, strictly anaerobic genus within the phylum *Verrucomicrobiota*, was seen in patients with high *JAK2V617F* allele burden, and correlated with allele burden. *Akkermansia* is a mucus-degrading bacterium and normally considered beneficial, assisting with the regulation of gut permeability.[42] However, it has also been linked to increased tumor burden in mice after antibacterial treatment.[43] It might aggravate inflammation and mucus degradation in certain situations[44] and has been associated with colorectal cancer in humans.[45] Moreover, a higher abundance has been reported in patients with multiple sclerosis[46]; however, whether *Akkermansia* aggravates MPN or the increase is a result of the aberrant immune regulation of MPNs remains to be investigated.

Patients positive for the *CALR* mutation had a higher relative abundance of *Subdoligranulum*, a genus generally considered beneficial for metabolic health[47]; however, recently, a specific strain of *Subdoligranulum* stimulating the production of rheumatoid arthritis-related autoantibodies causing joint swelling in mice was identified,[48] the implication in patients with *CALR* should be further investigated.

Despite the large number of MPN patients included in the study, we still did not have statistical power to investigate specific microbiota traits of patients in the triple-negative, *MPL*-positive or pre-PMF subgroups. Moreover, we did not have any information in regard to other clonal hematopoiesis of indeterminate potential (CHIP) mutations or additive mutations among HC or MPN patients, respectively. However, the lack of genetic information was equal between groups. Furthermore, we cannot completely rule out misclassification since some patients with *JAK2V617F*-positive ET might in fact have masked PV and some patients with pre-PMF might be misclassified as ET[49] obscuring a potential difference in the gut microbiota between sub-diagnoses. Indeed, several studies have shown that about 30%–40% of *JAK2V617F*-positive "ET" patients have PV, if a test for red cell mass estimation is performed.[50] We have previously demonstrated that the

gut microbiota composition differs depending on treatment regimen in patients with PV,[13] in this study we did not take treatment into account potentially leading to overestimation or underestimation of differences in gut microbiota between the groups. However, there was not enough power to test the effect of treatment in patients with MF and ET. For this a larger and more homogeneous cohort of patients would be required. It would have been intriguing to compare the gut microbiota of patients with ET and PV with post ET/PV MF, but only seven post PV-MF and two post ET-MF patients were included in the study, this aspect should be perused in further studies.

In addition, a higher resolution for metabolic functions and the archaeome could possibly have been achieved with shotgun sequencing.

In conclusion, the gut microbiota of patients with MPN differs significantly from that of HCs. Regardless of diagnosis, mutation, and allele burden, a lower relative abundance of *Faecalibacterium* was observed across the entire biological continuum of MPN. Importantly, *JAK2V617F*-positive patients and *CALR*-positive patients had distinct gut microbiota profiles, with the bacterial composition of *CALR*-positive patients having a higher resemblance to the composition of the HCs, with a higher relative abundance of *Clostridia* and *Ruminococcaceae* compared with the *JAK2V617F*-positive patients.

AUTHOR CONTRIBUTIONS

The design of the study was made by: JJEC, HCH, XCN, VS, LK, HVN, KF, and CSED. The patients with MPN were included by HCH and CSED and the HCs were included by MKL, CE, and CSED. The bioinformatics were performed by ACI and LOA. ACI and CSED did the tables, statistical analysis and data visualization. CSD, ACI, JJEC, XCN, HCH, VS, LK, and RUS interpreted the results. CSD and ACI wrote the draft, and all authors revised the draft, and approved the final version. The funding was raised by JJEC, HCH, HVN, XCN, LK, TAK, KF, VS, and CSD.

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CONFLICT OF INTEREST STATEMENT

H.C.H. is on the data monitoring board for AOP Orphan, and has received research funding from Novartis. All other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The anonymized sequence data of the patients with MPN are available at the European Nucleotide Archive (ENA) (PRJEB63403) and the data of the HCs on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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