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RESEARCH ARTICLE

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Alterations of circulating free fatty acids in patients with pemphigus vulgaris

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Abstract

Free fatty acids (FFA) have gained research interest owing to their functions in both local and systemic immune regulation. Changes in the serum levels of anti-inflammatory short chain fatty acids (SCFA), primarily derived from the gut microbiota, and pro-inflammatory medium (MCFA) and long (LCFA) chain fatty acids, derived from either the gut microbiota or the diet, have been associated with autoimmunity. Circulating FFA were retrospectively analysed by a gas chromatography–mass spectrometry method in the serum of 18 patients with pemphigus vulgaris (PV) at the baseline and 6 months ($n=10$) after immunosuppressive treatments, and 18 healthy controls (HC). Circulating FFA were correlated with the Pemphigus Disease Area Index (PDAI) and serum concentrations of interferon-gamma (IFN-γ), Interleukin (IL)-17A, IL-5, IL-10 and IL-21. Principal Component analysis computed on FFA abundances revealed significant differences in the profile of SCFA ($p=0{,}012$), MCFA ($p=0.00015$) and LCFA ($p=0{,}035$) between PV patients and HC, which were not significantly changed by immunosuppressive treatments. PV patients showed a significantly lower serum concentration of propionic ($p<0.0005$) and butyric ($p<0.0005$) acids, SCFA with anti-inflammatory functions, while hexanoic ($p<0.0005$) and hexadecanoic ($p=0.0006$) acids, pro-inflammatory MCFA and LCFA respectively, were over-represented. Treatments induced a significant decrease of hexanoic ($p=0.035$) and a further increase of hexadecanoic ($p=0.046$) acids. Positive correlations emerged between IFN-γ and acetic acid (Rho = 0.60), IFN-γ and hexanoic acid (Rho = 0.46), IL-5 and both hexadecanoic acid (Rho = 0.50) and octadecanoic acid (Rho = 0.53), butyric acid and PDAI (Rho = 0.53). PV was associated with a remarked imbalance of circulating FFA compared to HC. The serum alterations of SCFA, MCFA, and LCFA may contribute to promoting inflammation in PV. Deeper insights into the immunomodulatory functions of these molecules may pave the way for personalized dietary interventions in PV patients.

Roberto Maglie, Simone Baldi and Giulia Nannini were contributed equally to this work and share first authorship.

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KEYWORDS
cytokines, free fatty acids, long chain fatty acids, medium chain fatty acids, pemphigus vulgaris, short chain fatty acids

1 | INTRODUCTION

Pemphigus vulgaris (PV) is a prototype of organ-specific autoimmune disease, characterized by IgG antibodies against desmosomal cadherins, including desmoglein-3 (Dsg3) and desmoglein-1 (Dsg1). Antibody–antigen binding induces keratinocyte dissociation, a phenomenon referred to as acantholysis, and intra-epidermal blistering.[1,2] The immune modulation of PV involves a complex molecular interplay between antigen-specific T-and B-cells. In detail, autoreactive T helper (Th) cells, mainly polarized towards the Th-type 2 phenotype, support autoantibodies' secretion in an HLA-dependent manner.[3] Moreover, a dysregulated function of Th17 cells and regulatory T cells (Tregs), leading to altered amounts of their related cytokines, further contribute to PV progression.[4,5]

To date, B-cell depletion represents the mainstay of PV treatment[6]; however, novel strategies to modulate T-cell functions towards a tolerogenic profile against Dsg antigens are under development, with the aim to induce durable remission or even PV resolution.[7]

It is well established that intestinal bacteria and their metabolites exert an active role in modulating adaptive immune responses[8,9]; alterations of the gut microbiota (GM) composition and function have been shown to influence systemic inflammation in several autoimmune diseases, including psoriasis, atopic dermatitis, vitiligo and PV.[10]

There is evidence that alterations of the GM influence pemphigus pathology: in one study, enriched *Flavonifractor* spp. were shown to correlate positively with serum levels of interleukin (IL) IL-17A and IL-21; in the same study, serum levels of IL-17A correlated negatively with both Lachnospiracea_incertae_sedis and Coprococcus genera.[11] One of the primary mechanisms by which the GM interacts with the host is by producing metabolites such as short chain fatty acids (SCFA).[12] These molecules, mainly derived by microbial saccharolytic fermentation of complex resistant carbohydrates, regulate several biological processes, including host metabolism and immunity functions.[13]

SCFA can stimulate the differentiation of colonic Treg cells by binding cell-surface receptors such as G protein-coupled receptor (GPR) 109A, GPR41, and GPR43 and/or inhibiting histone deacetylases (HDAC).[14,15] However, given that SCFA can enter systemic circulation, their beneficial and immunomodulatory effects extend far beyond the gut.[16]

Besides SCFA, circulating free fatty acids (FFA) also include medium chain fatty acids (MCFA) and long chain fatty acids (LCFA), which are mainly derived from the diet or adipose tissue stores, and act by promoting the differentiation of both Th1- and Th17-type lymphocytes or by inhibiting the differentiation of Treg cells.[17]

As far as we are aware, no studies have already investigated the role of FFA in PV; hence in this preliminary investigation, we profiled the expression of circulating SCFA, MCFA and LCFA in PV patients, and explored their association with disease activity and serum inflammatory cytokines.

2 | MATERIALS AND METHODS

2.1 | Patients

We enrolled 18 adult patients with PV and 18 age-and gender-matched volunteers as healthy controls (HC). Diagnosis of PV was made according to the following: (i) presence of acantholysis at histopathology; (ii) detection of keratinocyte-binding antibodies in the perilesional skin/mucosa at direct immunofluorescence; (iii) detection of circulating keratinocyte-binding antibodies at indirect immunofluorescence and (iv) determination of the serum concentration of anti-Dsg3/Dsg1 IgG antibodies using Enzyme Linked Immunosorbent Assay (ELISA).[18]

Exclusion criteria were: (i) pregnancy, (ii) known history of chronic gastroenteric disorders or malabsorption; (iii) known intake of antibiotics and/or pre- or probiotics during the 8 weeks before enrolment (iv) prednisone dose >/= 0.3 mg/Kg/day or use of steroid-sparing immunosuppressive agents during the 4 weeks before enrolment.

Serum samples from both PV patients ($n = 18$) and HC ($n = 18$) were collected at enrollment. For PV patients ($n = 10$), an additional serum sample was taken after 6 months of immunosuppressive treatment, while patients were under clinical remission. Data including demographics (sex, age), serum concentration of anti-Dsg3/Dsg1 antibodies, disease severity as assessed by Pemphigus Disease Area Index (PDAI), therapeutic management and outcomes were retrieved from all the patients and summarized in Table 1.

All patients and controls gave written informed consent before enrolment and all the study procedures were performed following the Declaration of Helsinki and received approval from the local Ethic Committee (CEAVC 2023–08313725_bio).

2.2 | Evaluation of serum FFA by gas chromatography–mass spectrometry (GC–MS) analysis

Circulating SCFA (acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids), MCFA (isohexanoic, hexanoic, heptanoic, octanoic, decanoic and dodecanoic acids) and LCFA (tetradecanoic, hexadecanoic and octadecanoic acids), were analysed using an Agilent GC–MS system composed of an HP 5971 single quadrupole mass spectrometer, an HP 5890 gas chromatograph, and an HP 7673 autosampler, according to our previously described protocols.[19,20] Briefly, just before the analysis, each sample was thawed and the

TABLE 1 Demographic and clinical characteristics of patients with pemphigus vulgaris.

			Antibody titre pre-treatment			Antibody titre post-treatment			
N.	Sex	Age at diagnosis	Anti-Dsg1 (UI/mL)	Anti-Dsg3 (UI/mL)	Baseline PDAI	Anti-Dsg1 (UI/mL; cut off >9 UI/mL)	Anti-Dsg3 (UI/mL; cut off >9 UI/mL)	Post treatment PDAI	Treatment (other than oral steroids)
1	F	66	130.5	32.8	9	2.3	11.2	0	AZA
2	F	39	>150	16	30	N/A	N/A	N/A	N/A
3	M	38	7.2	143.2	5.6	N/A	N/A	N/A	N/A
4	F	72	100	>150	67	3.2	25	0	AZA, RTX
5	F	57	8.5	>150	15.9	28.6	127.1	2	AZA
6	F	28	108	116	17	8	10.4	0	RTX
7	F	57	96.9	136.7	10	116.6	132.7	1	AZA
8	M	77	45.5	>150	15	7.2	66.4	0	None
9	F	35	29.9	9.9	6	N/A	N/A	N/A	N/A
10	F	32	>150	125	28.3	4	6.2	3	RTX
11	F	46	19	147.8	N/A	3.4	24.5	N/A	AZA
12	M	73	150	150	29	5	150	0	AZA
13	F	54	25.4	129.2	4	12.6	142.9	N/A	N/A
14	F	20	26.6	150	12	2.7	26.7	3	AZA + RTX
15	F	62	14.2	150	16.6	2.2	23.3	1	AZA + RTX
16	M	44	85.3	127.4	21	5.2	59.3	2	AZA + RTX
17	F	73	126.2	150	25	6.7	81.8	0	MPA
18	F	57	69.8	125.7	14	N/A	N/A	N/A	N/A

Abbreviations: AZA: azathioprine; F, female; M, male; MPA, mycophenolic acid; N/A, not assessed; PDAI, Pemphigus Disease Area Index; RTX, rituximab.

FFA were extracted as follows: an aliquot of 200 μL of serum sample was added to 10 μL of ISTD mixture, 100 μL of tert–butyl methyl ether and 20 μL of 6 M HCl + 0.5 M NaCl solution in a 0.5 mL centrifuge tube. Afterwards, each tube was stirred in a vortex for 2 min and centrifuged at 10 000 rpm for 5 min, and finally, the solvent layer was transferred to a vial with a microvolume insert and analysed.

2.3 | Dosage of serum cytokines

To evaluate the systemic inflammatory response of PV, we used a specifically assembled Milliplex Map kit for the Luminex MAGPIX detection system (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. The following cytokines were analysed: IL-5, IL-10, IL-17A, IL-21 and interferon (IFN)-γ. Cytokine levels were estimated using a 5-parameter polynomial curve (XPonent Software, Luminex Corporation, Austin, TX, USA). The low limit of detection of each cytokine was: IFN-γ =4.67 pg/mL; IL-17A = 5.76 pg/mL; IL-10 = 2.2 pg/mL; IL-21 = 2.12 pg/mL; IL-5 = 2.9 pg/mL.

2.4 | Statistical analysis

Statistical analysis was performed in R 4.2 with the help of the packages stats 4.2.2, vegan 2.6.2, MixOmics 6.20.0 and other packages satisfying their dependencies. The package ggplot2 3.4.0 has been used to plot data and results. Differences in the quantities of FFA and cytokines between PV patients and HC were explored through the non-parametric Mann–Whitney test. Moreover, the monotonic relationships between circulating FFA and serum cytokines or the PDAI score have been tested using the Spearman method. The principal coordinates analysis (PCoA) was performed using the Bray Curtis dissimilarity on proportional abundances and then the differences in centroid position and dispersion between the groups were evaluated through PERMANOVA and Betadisper, with 9999 permutations respectively. The sparse partial least squares discriminant analysis (sPLS-DA), employed as a variable selection tool, was computed on standardized quantities using the MixOmics package, with settings ensuring both reproducibility and prediction power. Every p-value achieved in multiple comparisons was corrected using the Benjamini-Hochberg method and was considered significant if lower than 0.05. Further details are available in the R script (see Data availability statement).

3 | RESULTS

3.1 | Evaluation of serum FFA in patients with pemphigus vulgaris

PCoA of FFA quantities (Table S1) computed using the Bray Curtis dissimilarity index revealed significant differences in the profile of SCFA ($p_{adj} = 0.003$), MCFA ($p_{adj} < 0.0001$) and LCFA ($p_{adj} = 0.005$) between PV patients and HC (Figure 1A–C).

PV samples showed significantly decreased serum concentration of propionic (padj<0.0001) and butyric ($p_{adj} < 0.0001$) acids but higher quantities of isobutyric acid (padj = 0.012) compared to HC (Figure 1D). Regarding MCFA, in comparison to HC, PV patients reported significantly lower quantities of heptanoic ($p_{adj} < 0.0001$) and octanoic ($p_{adj} = 0.002$) acids, but a higher level of hexanoic acid, a well-known pro-inflammatory metabolite ($p_{adj} < 0.0001$) (Figure 1E). Finally, regarding LCFA, the PV patients showed a significant increase of hexadecanoic acid ($p_{adj} = 0.002$) in comparison to HC (Figure 1F).

To better evaluate which metabolites contributed to discriminate PV patients from HC, we applied sPLS-DA, which successfully discerned PV patients from HC along the first latent component using only propionic, butyric and heptanoic acids (Figure S1).

3.2 | Evaluation of serum cytokines

In Figure S2 we reported the serum levels (pg/mL) of the tested cytokines. We did not document significant differences among PV patients and HC. Of note, cytokines were under the low limit of detection in 53% of PV samples and 40% of HC samples.

3.3 | Circulating FFA correlation with disease activity

To get preliminary insights into the FFA significance in pemphigus pathology, we analysed the correlations of their amounts with either the levels of inflammatory cytokines or the PDAI score. With regard to SCFA, positive correlations emerged between IFN-γ and acetic acid (Rho = 0.60) (Figure 2A) and between IFN-γ and hexanoic acid (Rho = 0.46) (Figure 2B). In addition, regarding LCFA, both hexadecanoic (Rho = 0.50) and octadecanoic (Rho = 0.53) acids resulted positively correlated with IL-5 (Figure 2C). All these associations were attenuated after covariate adjustment. Notably, no significant correlations were reported for HC (Figure S3).

About the association between FFA and disease activity, only butyric acid, a potent anti-inflammatory metabolite, resulted positively correlated (Rho = 0.53) with the PDAI score (Figure 2D–F). The statistical significance was lost after covariate adjustment.

3.4 | Evaluation of the immunosuppressive treatment on the profile of circulating FFA

Finally, we evaluated the effects of immunosuppressive treatments on circulating FFA profiles. Indeed, PCoA of FFA levels, computed using the Bray Curtis dissimilarity index, revealed no significant differences in the SCFA, MCFA and LCFA profiles of PV patients before and after treatment, while significant differences persisted between PV patients after treatment and HC (SCFA $p_{adj} = 0.0036$; MCFA $p_{adj} = 0.00015$; LCFA $p_{adj} = 0.005$) (Figure 3A–C).

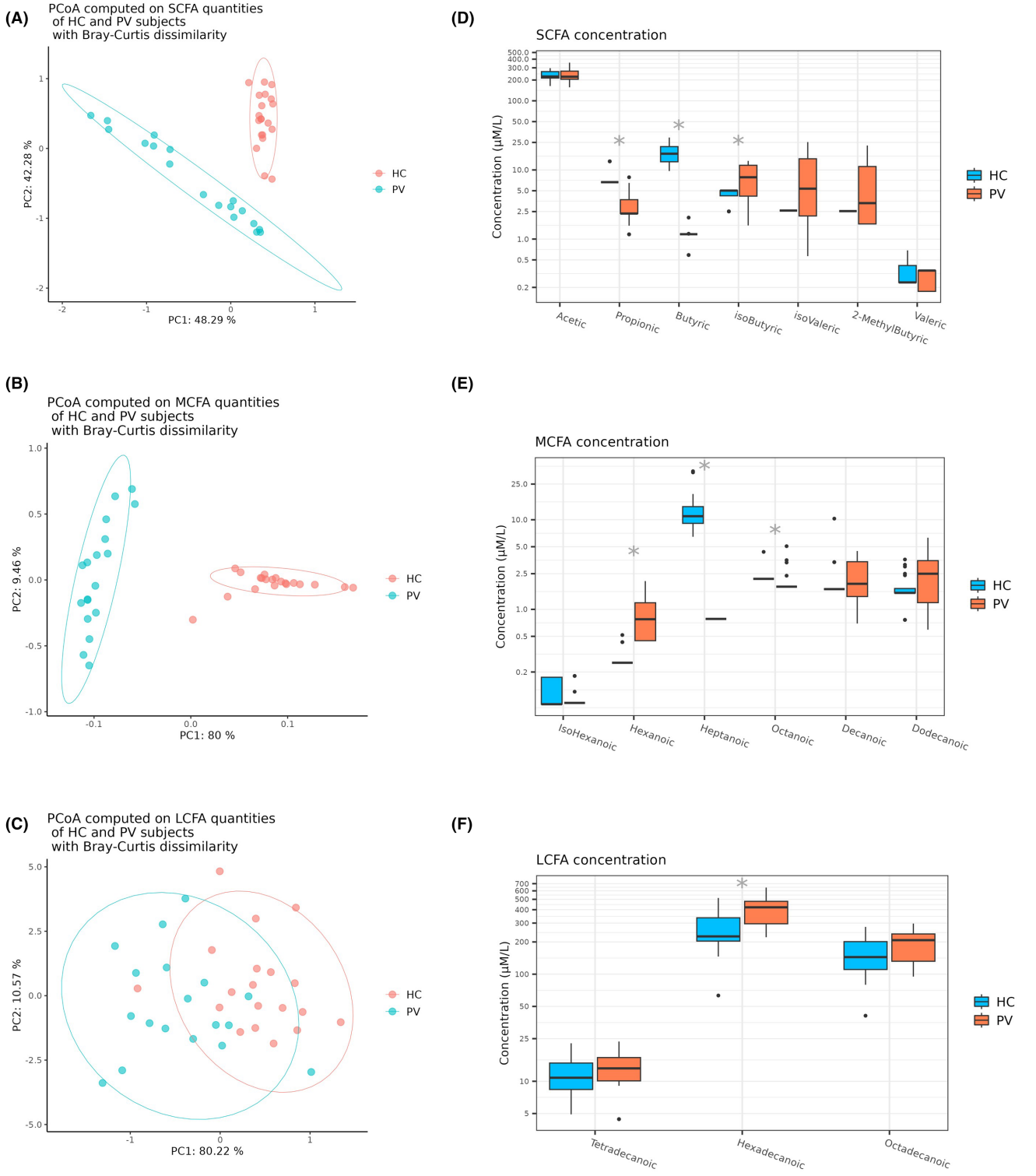

FIGURE 1 Principal component analysis (PCoA) conducted with Bray–Curtis dissimilarity index revealed significant differences in the quantities of short (A) medium, (B) and long, (C) chain fatty acids between sera of patients with pemphigus vulgaris and healthy controls. Box plots represent differences in the serum levels of short (D) medium, (E) and long, (F) chain fatty acids between patients with pemphigus vulgaris and healthy controls. Briefly, patients with pemphigus vulgaris showed significant decrease of short chain fatty acids including propionic and butyric acids (D) as well as of medium chain fatty acids including heptanoic and octanoic acids, (E) Isobutyric acid was the only short chain fatty acid upregulated in the sera of PV patients compared to healthy controls (D). Likewise, sera of patients with pemphigus vulgaris demonstrated increased concentrations of hexanoic acid (E) and hexadecanoic acid (F). Differences in the serum concentrations of circulating free fatty acids between patients with pemphigus vulgaris and healthy controls were assessed using the Mann–Whitney test. The asterisks (*) represent p values <0.05. HC, healthy controls; LCFA, long chain fatty acids; MCFA, medium chain fatty acids; PV, pemphigus vulgaris; PcoA, principal component analysis; SCFA, short chain fatty acids.

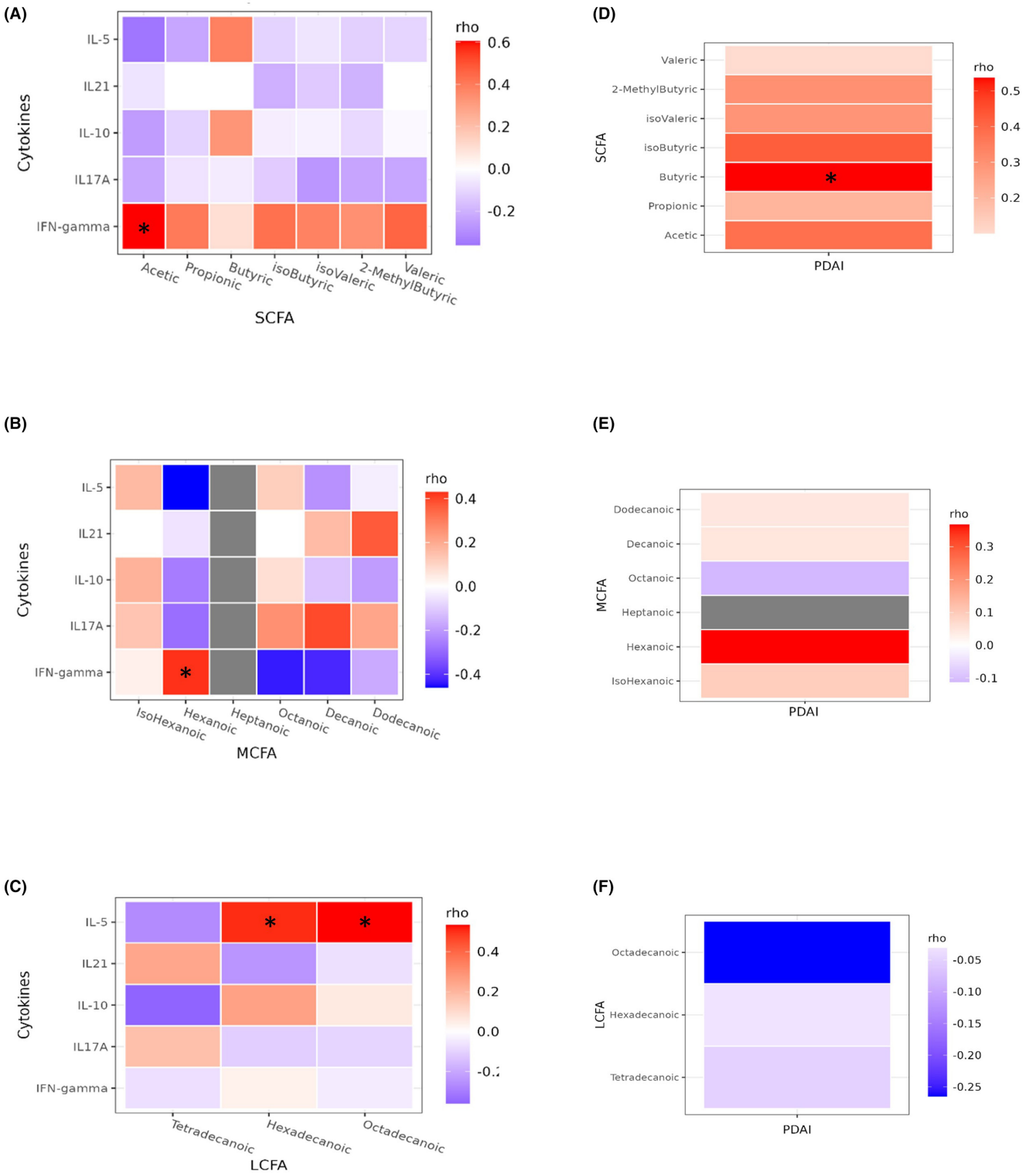

FIGURE 2 Heatmaps of Spearman correlations between serum levels of inflammatory cytokines and circulating short (A) medium, (B) and long, (C) chain fatty acids and between Pemphigus Disease Activity Index score and serum short, (D) medium, (E) and long, (F) chain fatty acids in patients with pemphigus vulgaris. Inteferon-gamma was shown to correlate positively with acetic acid (A) and hexanoic acid, (B) while interleukin-5 was shown to correlate positively with both hexadecanoic and octadecanoic acids, (C) PDAI correlated positively with the serum concentration of butyric acid (D) Red shades indicate positive correlations, whereas blue shades indicate negative correlations; the intensity of colours represents the degree of association. The asterisks (*) represent *p*-values<0.05. LCFA, long chain fatty acids; MCFA, medium chain fatty acids; PDAI, Pemphigus disease activity index; SCFA, short chain fatty acids.

However, at the single FFA level, patients after treatment showed a significant decrease of isovaleric (p_{adj}=0.037) and hexanoic acids (p_{adj}=0.019) and a significant increase of hexadecenoic acid (p_{adj}=0.037) (Figure 3D).

4 | DISCUSSION

Recent evidence suggests the presence of intestinal dysbiosis in patients with PV[10]; however, the mechanisms by which GM alterations influence pemphigus pathology remain elusive. FFA have garnered considerable attention since they represent a pathway utilized by bacterial species in the gut to remotely modulate local and systemic immune responses.[21]

Our group have recently observed changes in the profiles of circulating FFA in various immune-mediated disorders, such as celiac disease,[19] systemic sclerosis[22] and Crohn's disease.[23] Likewise, our data have shown that the serum FFA signature in PV patients is significantly altered in comparison with the 'healthy profile' and displayed a shift towards a pro-inflammatory state. This 'pemphigus signature' could be summarized as follows: (i) a down-regulation of SCFA with anti-inflammatory functions, especially propionic and butyric acids and (ii) an up-regulation of pro-inflammatory MCFA and LCFA, including hexanoic and hexadecanoic acids. Among all the

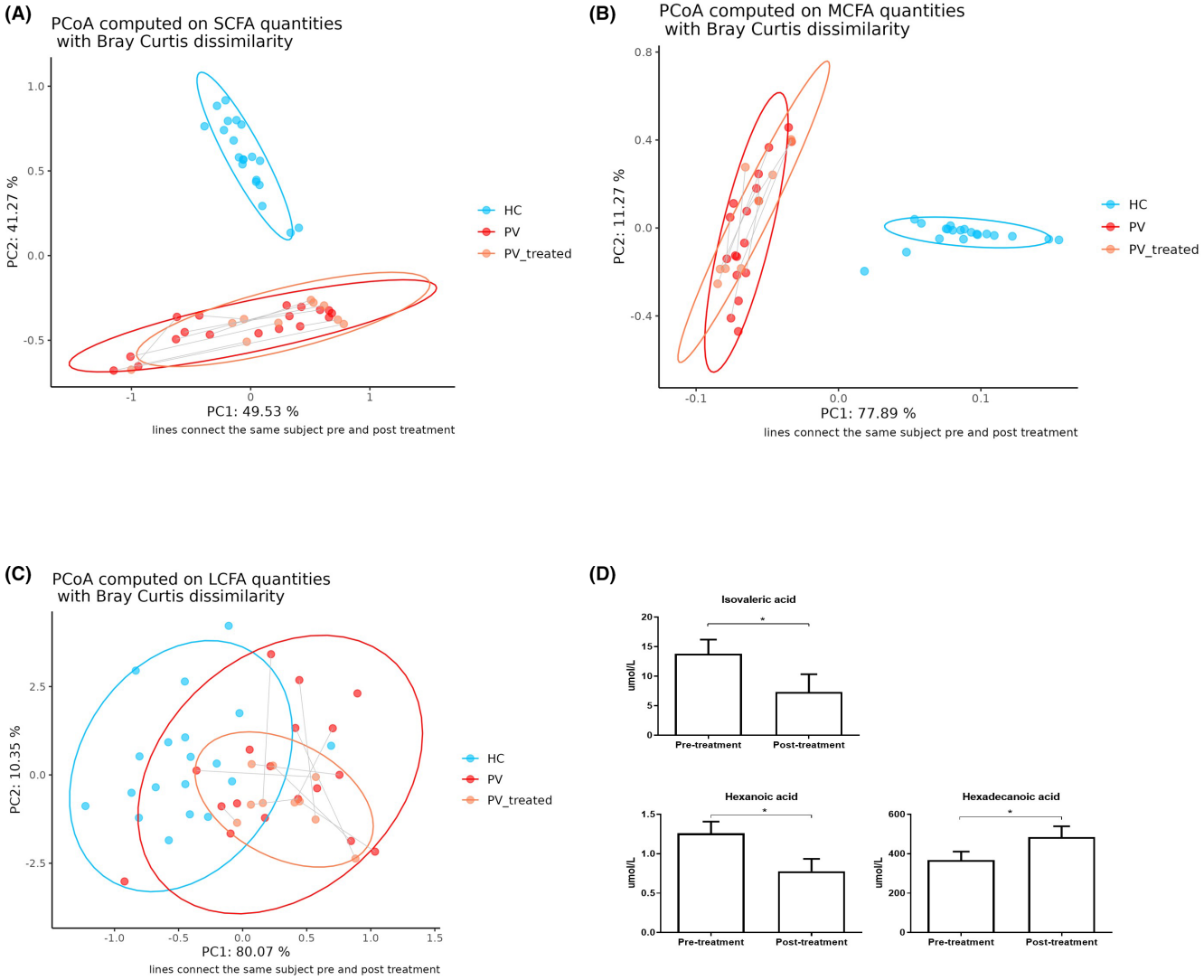

FIGURE 3 Principal component analysis (PCoA) conducted with Bray–Curtis dissimilarity of the short (A) medium, (B) and long, (C) chain fatty acids quantities revealed no significant differences in the profile of patients with pemphigus vulgaris pre- and post-intervention; differences still persisted between sera of pemphigus patients after treatment and healthy controls. Barplots reporting statistically significant differences in free fatty acid serum level among pemphigus vulgaris patients pre- and post-immunosuppressive treatment, (D) hexanoic acid significantly decreased in pemphigus sera after treatment, while hexadecanoic acid showed a significant increase. Comparison of serum levels of free fatty acids between pemphigus patients at the baseline and after treatment was assessed using the paired Wilcoxon signed-rank test; *p*-values less than 0.05 were considered statistically significant. The asterisks (*) represent *p*-values <0.05. HC, healthy controls; LCFA, long chain fatty acids; MCFA, medium chain fatty acids; PV, pemphigus vulgaris; PcoA, principal component analysis; SCFA, short chain fatty acids.

observed changes, our sPLS-DA model identified variation in serum concentrations of propionic and butyric acids as the most influential changes distinguishing patients from controls, thereby suggesting a biological relevance of these metabolites in human PV.

Notably, the alterations observed in our PV patients were similar to those previously documented in other autoimmune diseases. For example, patients with multiple sclerosis, a prototype of organ-specific, autoantibody-mediated disease, were shown to have decreased serum abundances of butyrate,[24] but increased concentrations of hexanoic (caproic acid) and hexadecanoic (palmitic) acids in the sera and cerebrospinal fluid, respectively.[25] Furthermore, dietary supplementation with SCFA was shown to improve experimental autoimmune encephalitis in mice.[26] Additionally, decreased concentration of SCFA, especially butyrate, were found in patients with Bechet syndrome.[27,28] Conversely, serum levels of hexanoic and hexadecanoic acids were increased in several autoimmune diseases, including psoriasis and vitiligo.[29,30]

To date, the metabolic functions of several FFA remain not well understood.

Overall, it can be assumed that while SCFA are pro-regulatory molecules, both hexanoic and hexadecanoic acids counterbalance the pro-regulatory effect of SCFA by activating the Th1 and Th17-type immune responses.[31–35] With these premises, we explored if FFA could exert similar functions in pemphigus. First, we investigated potential associations between FFA and disease activity and we found that only butyric acid showed a positive association with the PDAI score (none of the analysed FFA showed correlations with disease duration; data not shown). This finding was unexpected, given the anti-inflammatory function of this molecule. It is known that most butyrate is used within the colonic mucosa and only the unutilized part is transferred into peripheral circulation.[36] We can speculate that, during pemphigus inflammation, blood absorption of butyrate could be up-regulated to dampen disease severity. This hypothesis is merely speculative and deserves further investigation.

In addition, we evaluated the serum levels of some inflammatory cytokines, including IFN-γ, IL-17, IL-5, IL-21 and IL-10 in PV patients and their association with FFA. These cytokines are associated with the activation of various T-cell subsets, including Th1 (IFN-γ), Th17 (IL-17 and IL-21), Treg (IL-10) and Th2 (IL-5) cells. However, in line with previous reports, we documented that these cytokines were not significantly elevated in the sera of PV patients compared to HC.[37] This observation can be explained by several factors including the limited sensitivity of the used methodology, the individual variability among subjects, the presence of baseline alterations in cytokine levels even among healthy subjects (due to factors such as genetics, lifestyle, and environmental influences) and the potential for local rather than systemic cytokine alterations in PV. Interestingly, positive correlations emerged between IFN-γ and hexanoic acid, IL-5 and hexadecanoic and IL-5 and octadecanoic acid. Considering the capability of promoting inflammation of hexanoic, hexadecanoic and octadecanoic acids,[38–40] our results may suggest a potential pathogenic role of these molecules in pemphigus. By contrast, we could not appreciate relevant associations between FFA and cytokines such as IL-10, IL-17 and IL-21, which parallel the Treg/Th17 immune axis. Future studies could directly investigate the association of FFA with T-cell subpopulations, for example, assessed by cytofluorimetry, rather than with their signature cytokines.

Previous studies have investigated the GM composition in PV patients[41,42] Consistent with our findings, these studies reported a decrease of butyrate-producing microbial species in the gut of PV patients.[41,42] However, it is not yet fully elucidated if these alterations can be reversed with the achievement of disease remission after immunosuppression. We documented that disease remission was associated with a significant reduction in hexanoic acid, a known pro-inflammatory MCFA,[43] and a simultaneous increase in hexadecanoic acid. Notably, the cumulative profiles of SCFA, MCFA and LCFA remained almost unchanged following steroidal and immunosuppressive treatments. These findings suggest that abnormal GM composition and changes in related metabolites, including FFA, may contribute to the onset and progression of autoimmunity. However, they may be less affected by treatments.

It is unclear what are the causative mechanisms of the decrease of hexanoic acid and increase in hexadecanoic acid after treatment. While dietary changes that occur during the healing of mucosal erosions could explain the increase in hexadecanoic acid, a LCFA mostly derived from food, it could be possible that immunosuppressive treatments may alter gut permeability and reduce the absorption of pro-inflammatory metabolites, such as hexanoic acid.

The analysis of a large panel of FFA, their modification after treatment and the inclusion of a relatively large cohort of patients despite PV being a rare disease are major strengths of the study. By contrast, limitations include the missing evaluation of the dietary habits and GM characterization in both patients and controls. In particular, the ingestion of certain foods is restricted in PV patients owing to the presence of mucosal erosions and pain and surely, these limitations in food intake could significantly alter the GM composition and function. Future investigations may also lead to dissecting the role of FFA in pemphigus pathology and, beyond, other autoimmune bullous diseases, such as bullous pemphigoid.

To conclude, although further studies are needed to validate our findings, this preliminary report documented for the first time that the circulating FFA profiles are substantially altered in PV patients. Overall, our results suggest that microbial-derived metabolites could play a role in pemphigus pathology. Recent emerging data support the effectiveness of novel butyrate-based strategies for preventing and treating immune-mediated diseases.[44,45] Likewise, specific dietary interventions, for example, butyrate supplementation, might represent a potential aid to the therapeutic approaches of pemphigus vulgaris.

AUTHOR CONTRIBUTIONS

RM, SB GN, EN performed the research. MEB, MB and GP contributed to the execution of the experiments and the collection of patient data. RM, SB, GN, LDG designed the research study. MB and GP contributed essential reagents or tools. SB, GN, LDG analysed the data. RM, SB, GN wrote the paper. AA, EA, FS, EN, MR revised the manuscript. Furthermore, all authors have read and approved the final manuscript.

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FUNDING INFORMATION

None.

CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The cytokine and free fatty acid quantities' tables and the R script used are freely available at https://github.com/LeandroD94/Papers/tree/main/2023_Pemphigus_ffa_cks.

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SUPPORTING INFORMATION

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

Data S1.

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