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Original article

Linking the IL-17A immune response with NMR-based faecal metabolic profile in IBD patients treated with Mastiha

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ABSTRACT

Dysregulation of intestinal immune response plays a critical role in the pathogenesis of Inflammatory Bowel Disease (IBD). Mastiha's anti-inflammatory properties are well established. Our aim was to investigate Mastiha's regulatory effect on IL-17A serum levels in IBD patients. Alterations of the faecal metabolome as a functional readout of microbial activity were explored. A randomized, double-blind, placebo-controlled, parallel-group design was applied for a total of 3 months in active and 6 months in inactive IBD patients. Serum IL-17A increased significantly in Mastiha group ($p = 0.006$), and the mean change differed significantly between Mastiha and placebo ($p = 0.003$) even after adjusting for age, sex and BMI ($p = 0.001$) in inactive patients. In inactive UC patients IL-17A decreased significantly only in placebo ($p = 0.033$). No significant differences were detected in active disease. Faecal metabolomics indicated that intervention with Mastiha influenced considerably the metabolic profile of IBD patients in remission exhibiting, in between others, increased levels of glycine and tryptophan. Glycine has been proposed to have a therapeutic effect against IBD, while tryptophan derivatives are involved in immunoregalutory mechanisms, such as the Th17 cells differentiation. Thus, it is quite possible that the immunoregulatory role of Mastiha in quiescent IBD involves the regulation of Th17 cells function and differentiation.

1. Introduction

Inflammatory bowel disease (IBD) is a complex immune condition with two clinically defined entities, Crohn's Disease (CD) and Ulcerative Colitis (UC) [1]. Although, the exact etiology of IBD is unclear, genetic heterogeneity, along with immune dysregulation, imbalance interaction with microbiome and several environmental factors, shape the development of the disease [1,2]. The intestinal inflammation in IBD is controlled by both innate and adaptive immune signals. Cytokines play a key role by determining T cell differentiation of T-helper 1 (Th1), Th2, T regulatory and Th17 cells [3]. Cytokines are key mediators of cellular interactions in the intestine and may regulate the inflammatory processes in IBD. Understanding cytokine networks has resulted in the development of potential biological therapies in IBD [4], the anti-TNF blockers being the most common in clinical practice [5]. In recent years, more biological therapies that target different molecular pathways aside from TNF blockade have been developed, such as those targeting Interleukin (IL)-6, IL-12/IL-23 pathway and IL-17. As regards IL-17 blocking agents, they have been applied in several inflammatory diseases, however in IBD their clinical benefits are not established whereas several adverse events have been reported [6].

The diagnosis of IBD relies on clinical and endoscopic evaluation, diagnostic tools such as blood and stools testing and radiological imaging [7]. Although endoscopy is still considered the gold standard in

Abbreviations: AhR, aryl hydrocarbon receptor; BCAAs, branched-chain amino acids; CD, Crohn's Disease; CRP, C-reactive protein; HBI, Harvey-Bradshaw Index; HMDB, Human Metabolome Database; IBD, Inflammatory Bowel Disease; IBDQ, Inflammatory Bowel Disease Questionnaire; IL, Interleukin; ITT, intention-to-treat; NMR, Nuclear magnetic resonance; OPLS DA, Orthogonal Projections to Latent Structures Discriminant Analysis; PCA, principal component analysis; PMS, Partial Mayo Score; SCFAs, short-chain fatty acids; Th, T helper; TSP, trimethylsilyl propionic acid sodium salt; UC, Ulcerative Colitis.

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IBD diagnosis, it is highly invasive and costly. Therefore, there is an increasing need for non-invasive tests with high sensitivity and specificity in detecting the disease [8]. To this extent, metabolomics can be used for identification of disease biomarkers in biological samples of IBD patients acquired via non-invasive processes (i.e. urine, plasma, stools) [9]. Furthermore, the application of metabolomics analysis has contributed to the improvement of diagnosis and differentiation of IBD subtypes, as well as to the understanding of the association of treatments with the metabolic fingerprint in patients [10].

Current therapeutic strategies in IBD are accompanied by numerous side effects, as well as significant health care costs which has led to the seeking of safer, cheaper, and more efficacious approaches in managing IBD with nutraceutical compounds, such as bioactive phytochemicals, being under investigation [11]. Mastiha, is the dried resinous exudate from stems and branches of Pistacia lentiscus and consists of a plethora of bioactive constituents, including phenolic compounds and phytosterols, and is rich in terpenic acids [12]. Mastiha has been shown to possess several beneficial effects in the gastrointestinal system [13,14], as well as antioxidant [15], and anti-inflammatory properties [16]. Its effect on patients with IBD was proven in several studies, with reduction in disease activity indices and cytokine levels in plasma and in blood mononuclear cells [17], as well as in faecal inflammatory markers [18] and in serum oxLDL [19] having been reported.

With interest to the immunoregulatory role of Mastiha, the aim of this study was to explore Mastiha's effect on IL-17A serum levels in a Greek IBD cohort. Also, we aimed at investigating whether this effect is associated with alterations in stool metabolic profile.

2. Materials and methods

2.1. Study design

The study protocol was reviewed and approved by the Harokopio University Ethics Committee (49/29-10-2015). The trial was conducted according to the rules of the Declaration of Helsinki of 1975 and was registered with ClinicalTrials.gov (Identifier: NCT02796339). Recruitment lasted between May 2016 and September 2017 in Athens, Greece and all subjects gave their informed consent after being provided with a detailed information leaflet describing the study.

Patients with diagnosed IBD either in remission (N = 67) or in relapse (N=62) were enrolled in a randomized, double-blind, placebo-controlled, parallel-group clinical trial. In particular, forty-three UC and eighty-six CD patients were included. The enrollment was based on certain inclusion and exclusion criteria presented in Table 1. Medical history was obtained by experienced gastroenterologist and quality of life was assessed using the validated Greek version of Inflammatory Bowel Disease Questionnaire (IBDQ), Harvey-Bradshaw Index (HBI) and Partial Mayo Score (PMS) [20,21].

After obtaining informed consent and the baseline assessment, patients were randomly allocated to either verum or placebo. Randomization was applied by a computer-generated random number list prepared by an independent investigator, with blinding of all other staff, analysts and participants being strictly maintained. The Mastiha group received natural Mastiha at a dose of 2.8 g daily, while the placebo group received identical placebo tablets for 6 months for patients in remission and for 3 months for patients in relapse, as an adjunct to conventional medical treatment. A different intervention period was chosen as the objective was the induction of remission in patients with relapse, whereas the maintenance of remission in patients with remission. The verum tablets weighed 0.98 g and consisted of 70% Mastiha resin, 14% microcrystalline cellulose, 14% dibasic calcium phosphate anhydrous and 2% magnesium stearate. The placebo tablets weighed 0.99 g and consisted of 49% microcrystalline cellulose with a characteristic off-white to yellowish colour for similarity to verum, 49% dibasic calcium phosphate anhydrous, and 2% magnesium stearate. The verum and placebo tablets had an identical appearance and shared organoleptic characteristics.

Table 1
Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Sex: Male and Female	Positive stool culture for enteric pathogens or Clostridium difficile toxin
Age: 18–67 years old	Antibiotic treatment during and 2 months prior to screening
IBD established by colonoscopy, with consistent histology and clinical course **Active disease;** • **defined by Harvey & Bradshaw Activity Index ≥ 5 in CD,** • **defined by Partial Mayo Clinic Score ≥ 2 in UC** **Inactive disease (>3 months)** • **defined by HBI ≤ 4 in CD** • **defined by PMS ≤ 1in UC**	Bowel surgery ≤ 3 months prior to screening; a planned elective surgery or hospitalisation during the study; clinically significant short bowel syndrome; presence of an intra-abdominal abscess or a fistula with clinical or radiological evidence of an associated abscess; ileostomy; colostomy Any malignancy in the year prior to screening; cardiovascular disease; peptic ulcer
Active disease: Stable treatment with steroids for at least 2 weeks before the start of the trial, mesalamine and mesalamine analogues for 4 weeks and immunosuppressants for 8 weeks **Inactive disease:** Stable treatment with azathioprine or mesalamine and mesalamine analogues	Enteral or Parenteral Nutrition; Alcohol or drug abuse, Vitamin or inorganic supplements, vegan or macrobiotic diet before and during the trial
Stable medication during the trial	Pregnancy, lactation

2.2. Laboratory analyses

2.2.1. Blood and stool sample collection

Standard Blood sampling (20 mL) was performed in all (n = 129) patients after an overnight fast at baseline and post-treatment (after 3 months for patients in relapse and after 6 months for patients in remission). For serum isolation whole blood was collected into serum vacutainers, was mixed 5 times and allowed to clot at room temperature for about 20 min. Then whole blood was centrifuged at 3000 rpm for 10 min at 4 °C for serum isolation and stored at − 80 °C until further analysis. Stool samples were collected with a stool preparation system filled with extraction buffer IDK Extract® (Immundiagnostik, AG, Bensheim, Germany) and extracts were kept for a maximum of 9 days at − 20 °C until further analysis. Stool samples were collected at baseline and post-treatment (after 3 months for patients in relapse and after 6 months for patients in remission) from patients who gave their consent for stool sample collection.

2.2.2. Evaluation of inflammation

IL-6 (R&D Systems, Inc., Minneapolis, USA), IL-10 (OriGene Technologies, Inc., Maryland, USA), IL-17A (Boster Biological Technology, Pleasanton, CA, USA), were measured applying sandwich ELISA. Calprotectin, lysozyme, defensin and lactoferrin were quantified in stool samples applying sandwich ELISA (Immundiagnostik, AG, Bensheim, Germany). C-reactive protein (CRP) in serum was measured by turbidimetry (Cobas 8000 analyser, Roche Diagnostics GmbH). All measurements were performed in duplicate in all patients (N = 129) at baseline and post-treatment (after 3 months for patients in relapse and after 6 months for patients in remission).

2.3. Metabolomic analysis

2.3.1. Sample set and sample pretreatment.

Stool extracts (N = 90) in aliquots of 300uL were lyophilized to dryness using a LABCONCO CentriVap Concentrator and Cold Trap model 800-522-7658. Samples were reconstituted to final volume of 600uL using phosphate buffer (pH = 7.2) in D_2O and trimethylsilyl propionic acid sodium salt (TSP-d4) as internal standard at a final

concentration of 0.5 mM. Samples were vortexed and centrifuged for 10 min at 12,000 rpm at 4 °C. Supernatants were transferred to NOREL UP 5 mm Nuclear magnetic resonance (NMR) tubes for further NMR analysis.

2.3.2. 1 H NMR spectroscopy

All 1 H NMR spectra were acquired using a Varian 600 MHz spectrometer equipped with a triple resonance probe (HCN), at room temperature (25 °C).

1D NOE-PRESAT pulse sequence was used with the following parameters: 128 transients with 32 K data points, 1 s presaturation time, 14 db (106 Hz) presaturation power, 200 ms mixing time, 7184 Hz spectral width, 1 s relaxation delay and 4.45 s acquisition time. Receiver gain was auto set 60 for all acquisitions. All NMR spectra were referenced at TSP chemical shift (0.00 ppm) and processed at 0.3 exponential line broadening.

2.3.3. Data handling and metabolite assignment

All 1 H NMR spectra were preprocessed with MestreNova (v. 10.1) software. Phase correction, baseline correction and sinc apodization were applied to improve spectra resolution. Peak alignment followed total area normalization and binning of 0.001 ppm. The D_2O region (4.68 5.00 ppm) was excluded. Sample normalization was performed by using the dry weight of each sample after lyophilization and incorporating it in the data set as the reference feature to apply the normalization.

Peak assignment and metabolite identification were facilitated by performing a series of 2D experiments namely gCOSY (J-correlation spectroscopy with gradient coherence selection), gHMBCad (heteronuclear multiple-bond J-correlation spectroscopy with adiabatic 180° X-nuclei pulses and gradient coherence selection) and gHSQCad (heteronuclear single-quantum 1-bond J-correlation spectroscopy with adiabatic 180° X-nuclei pulses and gradient coherence selection) at 25 °C on a representative pool sample. Assignment of spectral peaks was assisted by Chenomx database (Chenomx Suite 7.6, Chenomx, Edmonton, Alberta, Canada), an in-house software (Metaboneer) [22] and the online NMR database Human Metabolome Database (HMDB) [23].

2.4. Statistical analyses

Data are expressed as mean ± standard deviation (SD) or with the median and the interquartile range (IQR). Quantitative variables are presented with absolute and relative frequencies. The normality assumption was evaluated using Kolmogorov-Smirnov test. For the comparison of proportions, chi-squared and Fisher's exact tests were used. For the comparison of means, the Student *t*-test and Mann Whitney test were computed. Spearman's correlation tests were used for the correlation analysis. Wilcoxon signed rank tests were used for the comparison of continuous variables among the two time points. Differences in changes of study variables during the follow up period between the two study groups were evaluated using repeated measurements analysis of variance (ANOVA). Analysis was based on logarithmic transformations in case of not normal distribution for repeated measurements analyses. All analyses were conducted on an intention-to-treat basis (ITT). To reduce the bias implicit in utilising only complete cases, multiple imputation procedures for all of the data were implemented. All p values reported are two-tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 23.0).

2.4.1. Multivariate statistical analysis– metabolic pathway analysis

Multivariate statistical analysis was employed to the aligned and normalized spectra, using SIMCA software (v. 14.0, Umetrics, Umea, Sweden). All the extracted models were Pareto (Par)-scaled at a confidence level of 95%. Particularly, the application of Par scaling allows any metabolites of low/medium intensity to affect the analysis only if they represent systematic variation. At first, principal component analysis (PCA) was applied to provide a general insight (trends, clusters, outliers) of samples. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS DA) was applied next, to generate classification models. Model performance has been assessed through the R^2Y (goodness of fit) and Q^2 (goodness of prediction) values. Supervised models have been validated through response permutation testing (999 permutations), analysis of variance (CV-ANOVA) and extraction of ROC curves.

Loading and contribution plots were extracted to reveal the variables that bear class discriminating power. Moreover, in order to improve model visualization and interpretation, the color coded S-line plots were extracted to detect the metabolites that influence most the group membership. MetaboAnalyst 4.044 (http://www.metaboanalyst.ca) (Quebec, Canada) was utilized for sample normalization, biomarker discovery, classification, pathway mapping and applied to discriminant metabolites for enrichment analysis providing altered metabolic pathways. Respectively, it is a hypergeometric test using overrepresentation analysis and pathway topology analysis–related metabolites to metabolic pathways.

3. Results

One hundred and twenty (n = 129) nine patients met our criteria. A total of 62 patients were in relapse and 67 in remission. Sixty-eight were randomised to the Mastiha group and 61 to the placebo group. Eighty-six were diagnosed with CD and 43 with UC. Baseline characteristics in IBD patients in relapse and in remission are given in Table 2. IBDQ was higher and HBI and PMS were lower in remission comparing to relapse, as expected ($p < 0.001$). Correlation analyses were conducted for serum IL-17A and other inflammatory markers. In UC patients in relapse significant correlations were found between levels of IL-17A and IL-6 ($r = 0.714$, $p = 0.006$) and between IL-17A and calprotectin ($r = -0.553$, $p = 0.050$). No significant correlation was observed in IBD patients in total, in patients in remission and in CD patients in relapse.

Serum and stool inflammatory markers were significantly altered between active and inactive IBD patients. More specifically, in serum, IL-6 ($p = 0.001$) and CRP ($p = 0.023$) were higher in IBD patients in relapse, whereas IL-10 ($p = 0.016$) and IL-17A ($p = 0.050$) were higher in IBD patients in remission. Interestingly, when comparing IL-17A levels in relapse and in remission in the two entities, there was no statistically significant difference in UC ($p = 0.350$), whereas there was a trend towards statistical significance in CD ((CD in relapse (18.1 (9.6–26.3)) vs CD in remission (24.4 (14.7–39.4), $p = 0.075$)). In stools, calprotectin ($P = 0.002$), lysozyme ($P = 0.026$) and lactoferrin ($P = 0.024$) were higher in active IBD.

Table 3 describes serum IL-17A levels at baseline and at follow up. In inactive IBD, serum IL-17A increased significantly in Mastiha ($p = 0.006$), and the mean change differed significantly between the groups ($p = 0.003$) even after adjusting for age, sex and BMI ($p = 0.001$). A similar pattern was followed in inactive CD patients, whereas in inactive UC patients IL-17A decrease significantly only in placebo group ($p = 0.033$), although the mean change was not different between the groups. In active disease we detected no significant differences within or between groups at follow up.

Regarding the faecal metabolic profiling, ninety samples before and after Mastiha intervention were included in a multivariate initial PCA model, as presented in Supplementary Fig. 1, without clear differentiation trends. Consequent models were generated for different sample subcategories. Specifically, samples from patients with IBD in relapse and remission before and after Mastiha intervention are presented in Supplementary Figs. 2 and 3.

Specifically, for the case of IBD patients in remission (Fig. 1), the model's goodness of fit (0.88) and its predictive ability (0.68) indicate a statistically significant discrimination of patients before and after Mastiha intervention. Permutation testing further validated the generated

Table 2
Baseline characteristics of the study participants.

	IBD in relapse	IBD in remission	P
Sex (F/M)			0.190[c]
Females	34 (54.8)	29 (43.3)	
Males	28 (45.2)	38 (56.7)	
Age (years) mean (SD)	41 (15.7)	37.5 (10.7)	0.136[a]
BMI (kg/m^2) mean (SD)	23.8 (5.8)	24.4 (3.9)	0.469[a]
Disease duration (years) mean (SD)	11.4 (9.1)	9.4 (6.7)	0.150[a]
Disease location			
Ileal	18 (29.0)	18 (26.9)	0.784[c]
Ileocolonic	14 (22.6)	15 (22.4)	0.979[c]
Colonic	3 (4.8)	7 (10.4)	0.328[d]
Pancolitis	12 (19.4)	12 (17.9)	0.833[c]
Left-sided	7 (11.3)	6 (9.0)	0.660[c]
Other	7 (11.3)	14 (20.9)	0.140[c]
Medication			
Mesalamine	24 (39.3)	34 (51.5)	0.169[c]
Azathioprine	14 (23.0)	21 (31.8)	0.264[c]
Corticosteroids	21 (34.4)	13 (19.7)	0.061[c]
IBDQ mean (SD)	144.7 (25.5)	177.1 (27.1)	**< 0.001**[a]
HBI median (IQR)	6 (5.5–8.5)	3 (1 − 3)	**< 0.001**[b]
PMS median (IQR)	4 (2.5–5)	1 (0 − 1)	**< 0.001**[b]
IL-6 (pg/mL) median (IQR)	5.9 (2.2–16.6)	2.1 (1.1 − 7.5)	**0.001**[b]
IL-10 (pg/mL) median (IQR)	4.6 (4.2–5.5)	5.6 (4.5 − 6.6)	**0.016**[b]
IL-17A (pg/mL) median (IQR)	17.8 (10.4–27.9)	25.6 (14.6 − 39.1)	**0.050**[b]
CRP (mg/L) median (IQR)	3.7 (1–9.6)	1.5 (0.3 − 4.2)	**0.023**[b]
Calprotectin (μg/g) median (IQR)	927.2 (372.9–2415.2)	422.1 (180.3 − 915.7)	**0.002**[b]
Defensin (ng/g) median (IQR)	12 (4–35.4)	6.8 (4 − 20.1)	0.310[b]
Lysozyme (μg/g) median (IQR)	8.7 (6.9–15.6)	7.1 (6 − 10.1)	**0.026**[b]
Lactoferrin (μg/g) median (IQR)	39.6 (15.8–192.5)	20.6 (7.1 − 63.5)	**0.024**[b]

For bold values: $p \leq 0.05$
[a] Student' *t*-test;
[b] Mann-Whitney test;
[c] Pearson's chi-square test;
[d] Fisher's exact test. Quantitative variables (sex, disease location and medication) are presented with absolute and relative frequencies. The results are given as N (%) of the total number.

model while ROC curves for samples before and after intervention provided 95.5% of true positives indicating high predictive ability (Supplementary Fig. 4). The color-coded S-line plot (Fig. 2) indicated the spectral peaks responsible for discrimination between the two groups in patients in remission. Although none of the assigned metabolites presented strong effect on discrimination, the Masthiha group exhibited higher concentrations of acetic acid, alanine, glycine and the aromatic aminoacids tyrosine, tryptophan and phenylalanine and lower concentrations of lysine, the branched-chain amino acids (BCAAs) leucine, isoleucine, valine and succinic acid.

In general, ^{1}H NMR spectra assignment revealed secondary metabolites (Supplementary Fig. 5) mainly falling into amino acids, small MW organic acids, alcohols and amines, while some spectral peaks could not be unambiguously determined. Querying HMDB, all identified metabolites have been previously identified in human stool samples.

For comparison reasons, unsupervised and supervised statistical models were generated for IBD patients both in relapse (Supplementary Fig. 6) and remission (Supplementary Fig. 7) receiving placebo. Neither case provided validated models.

MetaboAnalyst platform was utilized to explore the metabolic pathways related to the abovementioned metabolites (Supplementary Table 1). Statistically significant ($p < 0.05$) pathways are related to glycine, serine, threonine, arginine and proline metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, aminoacyl-tRNA biosynthesis as well as butanoate metabolism and synthesis and degradation of ketone bodies. Schematic representation of metabolic pathways' impact is provided in Fig. 3.

4. Discussion

Several studies have described the role of cytokines in the pathogenesis of IBD and their contribution in controlling the inflammatory response. There is growing evidence that their modulation can be used for therapy and may result in new potential therapeutic targets for chronic intestinal inflammation [5]. Herein we observed a significant increase of IL-17A in Mastiha treated patients, as well as a significant difference of the mean changes between intervention and placebo in inactive IBD and CD patients. In inactive UC patients IL-17A levels decreased significantly only in the placebo group, whereas in the Mastiha group remained unchanged. To the best of our knowledge, this is the first study exploring the effect of a natural product in modulation of IL-17A in IBD patients.

The role of IL-17 family members (IL-17A and IL-17F) in IBD pathogenesis remains controversial as both protective and pathogenic functions have been reported. IL-17 is established as a contributor to tissue inflammation by inducing proinflammatory and neutrophil-mobilizing mediators [4]. Recent reports have provided further support for a disease-protective role for IL-17A in intestinal pathology due to its anti-inflammatory nature, as it was found to selectively down-regulate TNF-a induced RANTES secretion in human colonic subepithelial myofibroblasts [24] and induce mucin production in epithelial cells of the airways [25]. Although IL-17 blocking agents have been used in several inflammatory diseases effectively, such as psoriasis and rheumatoid diseases, the results in IBD patients were not promising. Not only there were no clinical benefits, but also adverse events and discontinuation of the treatment occurred [6]. In this study, levels of serum IL-17A are significantly higher in patients with remission suggesting a protective role of IL-17A.

Similar to Mastiha, curcumin upregulated serum IL-17A in a rat model of non-alcoholic steatohepatitis. IL-17A appeared to mediate its effects through MAPK, NF-κB, and AP-1 which have all shown to be inhibited by curcumin. In this study it was hypothesized that perhaps a reduction in feedback and/or stage of the disease state perpetuated greater IL-17A production [26]. This could be hypothesized for Mastiha as well, as it has been shown that its effect in cytokines (IL-5, IL-13) and eotaxin levels in BALF may be due to inhibition of NF-kB activation [27,28].

Interestingly, IL-17A correlated positively with IL-6 and negatively with calprotectin only in patients with active UC. Recently, it was shown that IL17 + FoxP3 + T cells are only expressed in inflamed intestinal mucosa of patients with CD but not in patients with UC. It is suggested that the microenvironment present in CD tissues (i.e TGF-b and IL-6) may be responsible for this differential expression, as in the presence of TGF-b and IL-6, IL-17 + FoxP3 + T cells expression is induced in UC as well, but not in normal LP CD4 + T cells [29]. Also, it has been shown that IL-6 along with TGF-b are involved not only in the induction of Th17 cells, but also in their regulation, depending on other regulatory signals presented in the microenvironment [30]. The above suggest that different specific microenvironments observed in CD and UC, influence the balance between regulation and inflammation and may explain why the effect of Mastiha was observed mainly in CD patients in remission.

The identified metabolites have been previously reported in IBD faecal samples using NMR spectroscopy. Choline has been previously described as one of the most important metabolic biomarkers, along with carnosine and ribose, associated with Crohn's disease levels of inflammation [31]. Furthermore, the identified metabolite betaine could be originating as an oxidation product of choline or from nutrition and its deficiency has been linked to metabolic syndrome, lipid disorders, diabetes and other diseases [32], while it has been correlated to

Table 3
Changes in IL-17A serum levels in IBD patients after intervention.

		IL-17A serum levels in baseline (pg/mL) Mean (SD)	IL-17A serum levels post-treatment[d] (pg/mL) Mean (SD)	Change Mean (SD)	P[a]	P[b]	P[c]
IBD	**RELAPSE**						
	Placebo	33.6(37.7)	43.1(50.2)	9.5(41.6)	0.374	0.444	0.597
	Mastiha	28.5(34.9)	38(42.3)	9.5(27)	0.072		
	REMISSION						
	Placebo	40.8(31.3)	34.8(54.4)	-6(35.8)	0.083	**0.003**	**0.001**
	Mastiha	23.6(15.7)	47.4(45.5)	23.8(39.3)	**0.006**		
CD	**RELAPSE**						
	Placebo	23.8(17.5)	40.3(44.7)	16.5(37.7)	0.157	0.664	0.561
	Mastiha	24.6(23.3)	32.7(31.4)	8.2(23.9)	0.136		
	REMISSION						
	Placebo	43.3(33.9)	42.6(62.4)	-0.6(41.9)	0.498	**0.019**	**0.010**
	Mastiha	24(16.6)	53.3(46.6)	29.3(39.8)	**0.003**		
UC	**RELAPSE**						
	Placebo	53.2(57.6)	48.9(62.4)	-4.4(47.7)	0.767	0.462	0.371
	Mastiha	36(50.8)	48.3(58.2)	12.2(33.3)	0.308		
	REMISSION						
	Placebo	36.8(27.6)	22.2(37)	-14.6(21.6)	**0.033**	0.117	0.104
	Mastiha	22.4(13.8)	31.7(40.6)	9.2(35.8)	0.859		

For bold values: $p \leq 0.05$.
[a] p-value for time effect (Wilcoxon signed rank tests), comparison of IL-17A serum levels before and post-treatment in each group;
[b] Effects reported include differences in the degree of changes between the groups (repeated measurements ANOVA);
[c] Effects reported include differences in the degree of changes between the groups (repeated measurements ANOVA) after including age, sex and BMI as covariates. Analyses were conducted on an ITT basis and were based on logarithmic transformations.
[d] after 3 months for patients in relapse, after 6 months for patients in remission

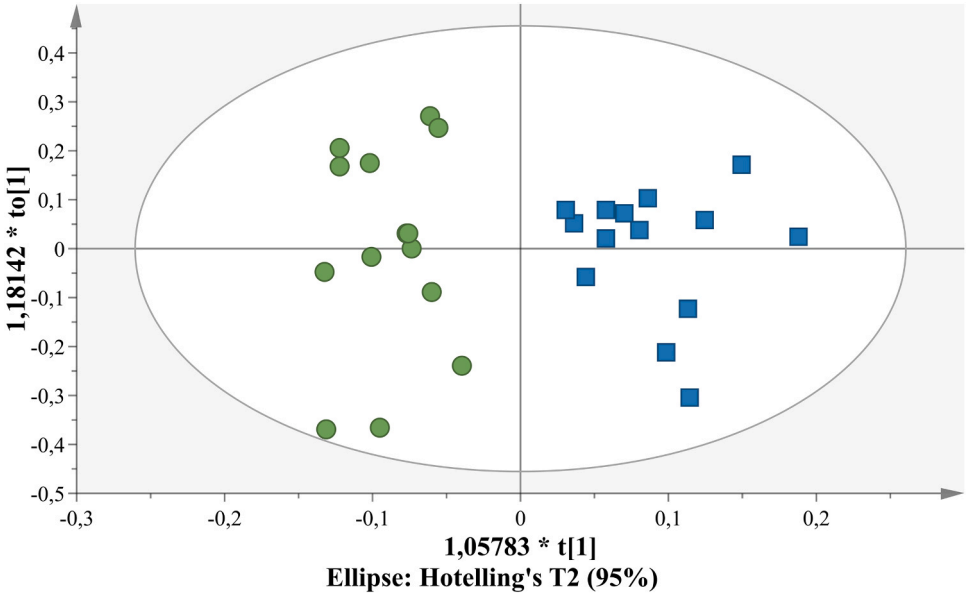

Fig. 1. OPLS-DA model of IBD in remission, A = 1 + 1, N = 28 $R^2X(Cum) = 0.88$, $Q^2(cum) = 0.67$, (circle): green color before and (square) blue color after Mastiha intervention. (colored). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Crohn's disease or ulcerative colitis in children and adolescents from 6 to 18 years old [31].

Marchesi et al. [9] and Bjerrum et al. [33], have presented lower levels of short-chain fatty acids (SCFAs) in stool of IBD patients compared to healthy volunteers. SCFA decrease has been mainly associated to specific bacterial strains producing butyric acid [34,35]. SCFAs act beneficially as they constitute the main source of energy for intestinal epithelial cells [36]. In particular, butyric acid, apart from being a source of energy, has proven anti-inflammatory properties which enhance the protection of epithelial barriers [37].

Our results indicated that Mastiha intervention influenced the metabolic profile of IBD patients in remission exhibiting an increase of the aromatic aminoacids phenylalanine, tyrosine and tryptophane together with alanine, glycine and acetic acid. Interestingly, hippuric acid, although not significantly contributing, it was mainly identified in samples after intervention.

Regarding glycine, although its effect on IBD is not well resolved, it has been suggested to have prophylactic and therapeutic activity against colitis [38]. Phenylalanine has been previously found at higher levels in IBD patients [39] and has been positively correlated with tyrosine even during induction of remission. Hippuric acid has been previously associated with Clostridia populations in gut [10]. The relative abundance of Clostridia has been correlated with intestinal inflammation levels [31] and its populations have been found reduced in patients with UC [40]. In our study, the increased levels of hippuric acid could be partially connected to the phytochemicals present in Mastiha, since its increase in urine samples has been associated with the increased consumption of phenolic compounds from tea, wine and fruit juices [41].

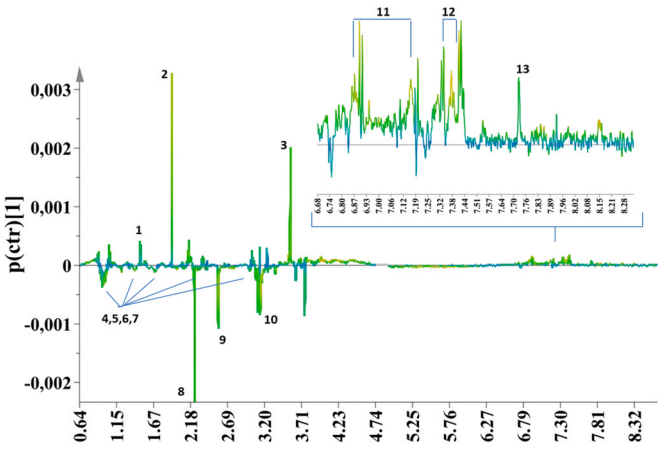

Fig. 2. Color coded S-line plot for the IBD in remission model before and after Mastiha intervention with magnification of the aromatic region of the pseudo NMR spectra. 1. Alanine, 2. Acetic acid, 3. glycine, 4,5,6,7: valine, isoleucine, leucine, lysine, 8: acetone, 9: succinic, 10: unknown, 11: tyrosine, 12: phenylalanine, 13: tryptophan. (colored). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

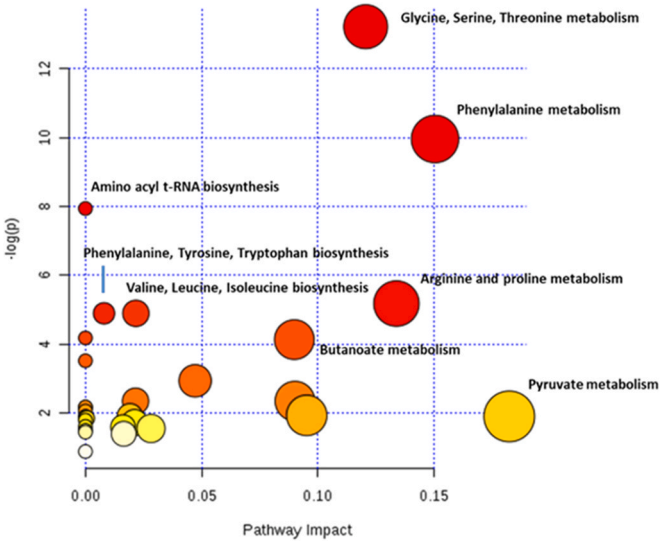

Fig. 3. Schematic representation of perturbed pathways identified in MetaboAnalyst 3.0. The *x*-axis represents the pathway impact and the *y*-axis the pathway enrichment. Larger size and darker color of each node (pathway) represents higher pathway enrichment and higher pathway impact values. (colored). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Finally, tryptophan is considered to have a positive role in IBD reducing gut permeability and expression of proinflammatory cytokines while it has been proposed as a promising treatment candidate for IBD [38,42]. The increased levels in our study indicate the shift of the metabolism to normal levels, possibly due to further remission of IBD which might be partly related to the phytochemical components of Mastiha supplementation. This result coincides with previous findings of our group on active UC patients of the same cohort [9] presenting significantly decreased plasma tryptophan levels in UC patients receiving a placebo compared with their baseline but remained largely unchanged in the UC patients receiving Mastiha.

It has been recently proposed that when Th17 cells lose their ability to secrete IL-17A and turn into IFN-γ producers they express high levels of aryl hydrocarbon receptor (AhR). AhR is a transcription factor which responds to different ligands, including derivatives of tryptophan and its activation is accompanied by reduction of Th1 and Th2 cytokines [43]. For example, indoleamine-pyrrole 2,3-dioxygenase (IDO), a tryptophan catabolism enzyme, is suggested as a true immunoregulatory mechanism, controlling the balance between Th17 and Treg subsets [44]. In the absence of a ligand, there is only a basal expression of AhR in Th17 cells, which are activated in the presence of numerous endogenous agents, such as prostaglandins, bilirubin at high concentration, modified low-density lipoprotein and various modifications of tryptophan [45]. The observed increase in stools tryptophan levels after Mastiha supplementation may be accompanied with a decrease in tryptophan derivatives and the subsequent activation of AhR transcription, which may explain the increase in IL-17A serum levels.

Recent findings suggest additional reason for the contrasting effects of IL-17 blockade in different diseases. More specifically, Th17 cells produce other cytokines than IL-17A (eg, IL-17F and IL-22) and IL-17A is not only produced by Th17 cells and is less influenced by IL-23 family signaling explaining why in some environments (eg, the gut) IL-17A may even have regulatory functions. Thus, although its pro-inflammatory role in some disorders is well described, IL-17A may function as a negative regulator of immunity in the intestinal mucosa, possibly by interaction with the intestinal microbiome, such as fungal elements [46].

While this study has some interesting results it is subjected to some limitations, such as the absent of endoscopy at follow-up and the fact that IL-17A was measured in serum, whereas metabolite profiling was performed in stools. Although, gut microenvironment may be better reflected by mucosal cytokine expression rather than serum, a follow-up endoscopic procedure in such a short period would be burdensome for the patients and would increase the drop-out rate as well. Nevertheless, the absence of follow-up endoscopy was compensated by the study design of a randomised, double-blind, placebo-controlled clinical trial and the very tight control of the groups to ensure compliance to the protocol, along with the use of high sensitivity ELISA kits capable of reflecting the mucosal expression to a satisfactory level.

5. Conclusions

In conclusion, the findings of the present study demonstrate a positive effect of Mastiha supplementation for IBD patients, especially those in remission. The changes in stool metabolic profile detected after Mastiha consumption are accompanied by an increase in IL-17A levels in serum that may be associated with a possible shift of Th17 cells to a regulatory role, more protective for quiescent IBD. More studies are needed to further explore the role of Th17 cells in IBD and confirm whether the above observations also exist in a mucosal level, as well as the possible effect of phytochemicals on Th17 cells differentiation and consequent healing of the colonic ulcerations.

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CRediT authorship contribution statement

Conceptualization: Andriana C. Kaliora; **Data curation**: Charalampia Amerikanou; **Formal analysis**: Charalampia Amerikanou; Panagiotis Zoumpoulakis; **Funding acquisition**: Andriana C. Kaliora; **Investigation**: Charalampia Amerikanou, Eirini Dimitropoulou, Efstathia Papada, Charalambos Fotakis; **Methodology**: Charalampia Amerikanou, Aristea Gioxari, Andriana C. Kaliora, Panagiotis Zoumpoulakis; **Project administration**: Andriana C. Kaliora, Panagiotis Zoumpoulakis; **Software:** Charalampos Fotakis, Panagiotis Zoumpoulakis;

Supervision: Andriana C. Kaliora, Panagiotis Zoumpoulakis; **Visualization**: Andriana C. Kaliora, Charalampia Amerikanou; **Roles/Writing - original draft**; Charalampia Amerikanou; **Writing - review & editing:** Aristea Gioxari, Andriana C. Kaliora, Panagiotis Zoumpoulakis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111535.

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