Mastiha has efficacy in immune-mediated inflammatory diseases through a microRNA-155 Th17 dependent action

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Abbreviations: alanine aminotransferase (ALT); aspartate aminotransferase (AST); Crohn's disease (CD); Harvey-Bradshaw Index (HBI); inflammatory bowel disease (IBD); interleukin-6 (IL-6); iron-corrected T1 (cT1); lipopolysaccharides (LPS); Mastiha Treatment for Obese with NAFLD Diagnosis (MAST4HEALTH); non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH); nuclear factor kappa B (NF-κB); Partial Mayo Score (PMS); peroxisome proliferator-activated receptors (PPARs); sterol regulating element binding protein 1c (SREBP-1c); T helper (Th); Tumor necrosis factor (TNF); ulcerative colitis (UC).

Abstract

Mastiha is a natural nutritional supplement with known anti-inflammatory properties. Non-alcoholic fatty liver disease (NAFLD) and Inflammatory bowel disease (IBD) are immune mediated inflammatory diseases that share common pathophysiological features. Mastiha has shown beneficial effects in both diseases. MicroRNAs have emerged as key regulators of inflammation and their modulation by phytochemicals have been extensively studied over the last years. Therefore, the aim of this study was to investigate whether a common route exists in the anti-inflammatory activity of Mastiha, specifically through the regulation of miRNA levels. Plasma miR-16, miR-21 and miR-155 were measured by Real-Time PCR before and after two double blinded and placebo-controlled randomized clinical trials with Mastiha. In NAFLD, miR-155 decreased in the placebo group ($p=0.054$) whereas it remained unchanged in the Mastiha group. In all IBD and particulary in ulcerative colitis patients in relapse, miR-155 increased in the placebo group ($p=0.054$) whereas this increase was prevented by Mastiha. The mean changes were different in the two groups even after adjusting for age, sex and BMI ($p=0.024$ for IBD and $p=0.042$ for ulcerative colitis patients in relapse). Our results propose a regulatory role for Mastiha in circulating levels of miR-155, a critical player in T helper-17 (Th17) differentiation and function.

Keywords: miR-155, immunity, Th17 cells, Mastiha, Non-alcoholic fatty liver disease, Inflammatory Bowel Diseases, inflammation

1. Introduction

In the course of the last decades, chronic inflammatory diseases are considered the most significant cause of death worldwide and more than 50% of all deaths are caused by inflammation-related diseases such as ischemic heart disease, cancer, diabetes mellitus, chronic kidney diseases, non-alcoholic fatty liver disease (NAFLD) and other [1, 2].

NAFLD is the most common liver pathological condition, with a prevalence of 25% and is characterized by excessive accumulation of fat in the liver not associated with alcohol consumption. It ranges from excessive liver fat (NAFL) to necroinflammation and fibrosis (non-alcoholic steatohepatitis (NASH)), NASH-cirrhosis and eventually to hepatocellular carcinoma [3]. Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, represented mainly by two distinct entities, Crohn's disease (CD) and ulcerative colitis (UC). IBD incidence and prevalence have significantly increased during the last decades and is considered one of the most prevalent gastrointestinal diseases in newly industrialized countries [4].

Both NAFLD and IBD share common features in their pathophysiology, such as increased intestinal permeability, gut dysbiosis and chronic inflammatory response [5]. There is increasing evidence about the co-existence of NAFLD and IBD with more severe IBD promoting the development of liver fat accumulation and severe liver steatosis further aggravating IBD, both sharing most of the defining features of immune-mediated inflammatory disorders. [6]. The great variability in the prevalence of NAFLD in IBD (ranging from 1.5% to even 40%), along with the low prevalence of obesity and diabetes in IBD, suggest that the interrelationship of the two conditions may be attributed to disease specific risk factors associated to underlying chronic

inflammation. Therefore, the increased risk of IBD patients for NAFLD may be related to multiple intestinal disease-related factors, such as disease duration inflammation relapses, metabolic comorbidities and hepatotoxic therapies, i.e steroids, immunosuppressive drugs and biological factors [7, 8].

One of the most studied epigenetic mechanisms involved in regulation of immune-mediated diseases are microRNAs [9]. In NAFLD, there is increasing evidence that several miRNAs regulate molecular pathways associated with lipid metabolism, oxidative stress and liver inflammation [10]. Similarly, in IBD, microRNAs are implicated in the regulation of intestinal epithelial barrier function, cell membrane trafficking, and interfere with inflammatory signaling pathways, such as the nuclear factor kappa B (NF-κB) and the signal transducer and activator of the transcription (STAT)/interleukin-6 (IL-6) pathways [11]. Circulating miRNAs are considered a useful tool as they are stable and reflect the physiological state of the tissue they are derived from [12].

Nutrimiromics is a new discipline that focuses on the influence of the diet in gene expression due to miRNAs, and their implications in chronic diseases [13]. The health benefits of dietary phytochemicals are linked with regulation of different microRNAs. For example, phenolic compounds have been shown to modulate miRNAs expression [14] with quercetin upregulating miR-155 levels in macrophages activated by lipopolysaccharides (LPS) [15] and resveratrol altering the levels of miRNAs involved in the regulation of inflammatory responses, such as miR-21, miR-181b, and miR-155 [16].

Mastiha is a natural supplement with active phytochemicals that exhibits health benefits in IBD [17, 18] as well as in NAFLD patients [19]. The aim of this study was to investigate the common molecular pathway that mediate its anti-

inflammatory effects. Accordingly, we focused on the regulation of plasma miRNA levels in the Mastiha Treatment for Obese with NAFLD Diagnosis (MAST4HEALTH) [19] and MASTIHA IBD-GR [17, 18] randomized controlled trials with Mastiha.

2. Materials and Methods

2.1 Patients and study design

This study was performed in subsets of patients with inflammatory conditions who participated in two different larger interventions with Mastiha previously described [17, 18, 19]. Briefly, we included 67 patients from MAST4HEALTH study and 60 patients from MASTIHA IBD-GR study.

MAST4HEALTH was a multicentre randomised double-blind, placebo-controlled clinical trial designed to explore the effectiveness of Mastiha as a non-pharmacological intervention in NAFLD, conducted in three clinical trial sites (Athens, Greece, Milano, Italy and Novi Sad, Serbia). MASTIHA IBD-GR was a randomised, double-blind, placebo-controlled clinical trial designed to explore the effectiveness of Mastiha supplement in IBD. Particularly, in patients in mild to moderate relapse and in remission with either CD or UC we evaluated quality of life, clinical course and inflammatory biomarkers.

Both clinical trials have obtained approvals from Ethics Committees [17, 18, 19], were conducted following the Helsinki declaration and the Data Protection Act 1998 and were registered with ClinicalTrials.gov (MAST4HEALTH Identifier: NCT03135873, MASTIHA IBD-GR Identifier: NCT02796339). All patients signed an Informed Consent before participating in each study.

More information on the inclusion and exclusion criteria, primary endpoints of each study and the study design have been described in the main publications of each study [17, 18, 19].

2.2 Medical, anthropometric and disease activity assessment

Detailed medical history was obtained including personal/family anamnestic data and medication. Body weight was measured to the nearest 0.1 kg. Height was measured to the nearest millimeter and BMI was computed as weight (kg) / height $(m)^2$.

In MAST4HEALTH study, disease severity was assessed by iron-corrected T1 (cT1) which is generated via MRI images with LiverMultiScan software [20]. In MASTIHA IBD-GR study, disease severity was assessed via Harvey-Bradshaw Index (HBI) for CD patients and Partial Mayo Score (PMS) for UC patients.

2.3 Blood collection

Standard blood sample collection (25 ml) was performed after overnight fasting. For plasma isolation whole blood was collected in EDTA whole blood tubes and was kept on ice until centrifugation for 10 min at a speed of 3000 rpm in order to isolate plasma. Plasma was chosen over whole blood that provides high number of miRNAs from erythrocytes and over serum that can increase sample-to-sample variations due to coagulation [21, 22].

2.4 MicroRNA quantification

2.4.1. Plasma RNA isolation

Total RNA enriched for miRNAs was isolated from 100 µl of blood plasma using MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. This kit uses magnetic-bead technology that enables reproducible recovery of high-quality RNA. RNA purity and concentration were assessed by measuring its optical density using an Implen P330 nanophotometer (Implen GmbH).

2.4.2. cDNA synthesis

A systematic literature search for studies involving circulating miRNAs in NAFLD and IBD identified three miRNAs that are implicated in inflammation and have been shown to be dysregulated in both diseases **[Table 1]**. MicroRNA quantification was performed for miR-16-5p, miR-21-5p and miR-155-5p. cDNA was synthesized using TaqMan® Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) which uses universal primers that uniformly amplify all targets even low-expressing miRNA targets and increases the assay sensitivity.

191 **Table 1**. Selected miRNAs and their functions

miRNA	Function	Inflammation	NAFLD	IBD
miR-16	inhibits cell proliferation, invasion, angiogenesis, cell cycle progression, promotes cell apoptosis, regulates tumorigenesis [23, 24, 25]	regulates immune-mediated tissue repair, production of inflammatory mediators, such as tumor necrosis factor (TNF-a), suppresses activation of inflammatory macrophages though mitogen-activated protein kinase (MAPK) and NF-κB signalling, improves inflammation-induced insulin sensitivity [30, 31, 32]	involved in liver fibrosis through autophagy of activated stellate cells, circulating miR-16 levels are increased in NAFLD patients, in correlation with fibrosis stage, in NASH it is negatively correlated to aspartate aminotransferase (AST) and fibrosis prediction scores [12, 38, 39].	activates NF-κB signaling pathway in human colonic mucosa of active UC patients, circulating levels are increased in CD and UC patients compared to healthy controls [44, 45]
miR-21	oncogenic role, targets cancer related genes, regulates cell proliferation, invasion and migration, apoptosis [26, 27]	regulates chronic inflammatory processes and T cell effects, controls toll-like receptors (TLR) signaling, PI3K/AKT/GSK3β, MyD88, MAPK pathways, induces DNA-hypomethylation, activates release of pro-inflammatory cytokines, [32, 33, 34]	involved in liver lipid metabolism through various targets, contributes to NASH, hepatocellular injury, inflammation, fibrosis via peroxisome proliferator-activated receptors (PPARs), is upregulated in NAFLD patients' serum, correlates positively with AST, alanine aminotransferase (ALT) and fibrosis scores [12, 40].	participates in differentiation, apoptosis, and activation of T cells, is upregulated in both intestinal tissues and circulation in association with disease activity in UC patients [44, 46]
miR-155	participates in regulation and differentiation of cells of haematopoietic origin, as well as in type 1 Angiotensin II receptor regulation, maintains the oxygen homeostasis [28, 29]	controls B cell differentiation, antibody production, T helper (Th) 1, Th2 and Th17 differentiation, enhances aryl hydrocarbon receptor (AHR) signaling, mitosis, reduces signaling for toll-like receptors, SOCS, ERK/MAPK, and B-cell receptors [35, 36, 37]	up-regulated in hepatocytes and liver tissue of NAFLD patients, regulates liver X receptor (LXR) α-dependent adipogenic signaling pathways, reduced in the circulation of NAFLD patients [41, 42, 43]	up-regulated in UC and CD, its deficiency protects mice from experimental colitis, plays a key role in the differentiation of B and T cells. miR-155-/- mice express reduced Th17 cells [47, 48, 49]

2.4.3. Plasma microRNA expression quantification by quantitative Real-Time PCR (qRT-PCR)

After cDNA synthesis, we performed qRT-PCR using TaqMan® Advanced miRNA Assays, TaqMan® Fast Advanced Master Mix which provides high specificity and the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc.). QPCR was carried out in duplicate for each sample. Analysis of data was performed using ExpressionSuite™ Software, which allows the calculation of relative gene expression using the comparative Ct (ΔΔCt) method and normalization of sample-to-sample variation to an exogenous control. Caenorhabditis elegans miRNA Cel-miR-39-3p was used as an exogenous control to ensure the reproducible and accurate quantification of circulating miRNA levels. Finally, the relative levels of miRNA in patient samples were compared to a reference sample and the final results were presented as fold change in expression using the 2ΔΔCt formula.

2.5. Statistical analysis

Data are expressed as mean ± standard deviation, mean (SD) and counts for categorical ones. For the comparison of proportions, chi-squared and Fisher's exact tests were used. For the comparison of means, the Student t test was used. Paired sample t test was 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). The covariates used for adjustment were age, sex, BMI for MASTIHA IBD-GR study and age, sex, BMI and center for MAST4HEALTH study. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 22.0).

3. Results

The baseline characteristics of the patients included in our analysis are presented in **Tables 2a and 2b**. No significant differences between the Mastiha and the placebo group were observed in either of the inflammatory conditions.

Table 2a. Baseline characteristics of patients of the MAST4HEALTH study that were included in the microRNA analysis. The results are given as mean (SD) for continuous variables and counts for categorical ones.

Baseline Characteristics	**Mastiha (N=27)** mean (SD)	**Placebo (N=40)** mean (SD)	**P***
Age (years)	49.0 (9.8)	49.0 (8.9)	0.972
Sex (M/F)	19/8	28/12	0.511
Centre (GR/IT/SR)	7/10/10	12/15/13	0.842
BMI (kg/m^2)	34.0 (3.2)	33.8 (4.0)	0.765
cT1 (ms)	886.3 (60.4)	869.9 (80.5)	0.345

*Chi-square test for categorical variable; t-test for quantitative variable. BMI: body mass index, cT1: iron-corrected, LIF: Liver Inflammation Fibrosis score, PDFF: proton density fat fraction

Table 2b. Baseline characteristics of patients of the MASTIHA IBD-GR study that were included in the microRNA analysis. The results are given as mean (SD) for continuous variables and counts for categorical ones.

Baseline Characteristics IBD patients in relapse	**Mastiha (N=20)** mean (SD)	**Placebo (N=15)** mean (SD)	**P***
Age (years)	33.6 (8.0)	36.6 (17.6)	0.583
Sex (M/F)	11/9	7/8	0.358
BMI (kg/m^2)	23.5 (4.6)	24.0 (7.6)	0.814
HBI	7.4 (1.7)	6.5 (1.4)	0.273
PMS	3.2 (1.3)	3.3 (1.0)	0.951
Baseline Characteristics IBD patients in remission	**Mastiha (N=10)** mean (SD)	**Placebo (N=15)** mean (SD)	**P***
Age (years)	39.4 (4.8)	38.0 (12.6)	0.705
Sex (M/F)	6/4	6/9	0.384
BMI (kg/m^2)	27.1 (7.6)	23.6 (3.2)	0.282
HBI	2.0 (1.0)	1.9 (1.6)	0.890
PMS	0.8 (0.5)	0.9 (0.4)	0.624

Chi-square test for categorical variable; t-test for quantitative variable. BMI: body mass index, IBDQ: Inflammatory Bowel Disease Questionnaire, HBI: Harvey & Bradshaw Activity Index, PMS: Partial Mayo Clinic Score

3.1. Changes in plasma miRNA levels in NAFLD patients in MAST4HEALTH intervention

The effect of the intervention with Mastiha on miRNA levels in MAST4HEALTH study is presented in **Table 3**. There were no significant differences in the mean changes of the three miRNAs between the Mastiha and the placebo group when examining the whole study population. However, some interesting results were extracted when dividing our population in two different categories according to median of the liver MRI biomarker cT1; the cT1<868.6 and cT1>868.6 ms that correspond to lower and higher liver inflammation accordingly. In particular, in patients with cT1<868.6 ms, a decrease of miR-155 approached borderline significance in the placebo group (p=0.054), whereas in the same category miR-155 remained unchanged in the Mastiha group. In patients with higher liver inflammation and fibrosis no significant changes were observed after the intervention.

3.2. Changes in plasma miRNA levels in IBD patients in MASTIHA IBD-GR intervention

The results of the effect of the intervention on IBD patients in remission and the three-month intervention on IBD patients in relapse, on miRNA levels in the MASTIHA IBD-GR study are presented in Tables **4a, 4b and 4c**. MiRNA-21 increases significantly in both Mastiha and placebo groups in the whole study population in remission (p=0.024 and p=0.012 respectively) and in CD patients in remission (p=0.016 and p=0.050 respectively). In both cases mean changes remained unchainged. In IBD patients in relapse the mean changes of miR-155 differed significantly between the Mastiha and placebo groups (p=0.012) even after adjusting for age, sex and BMI (p=0.024), with a higher increase in the placebo group. A

similar pattern was observed in UC patients in relapse with the placebo having a significant increase (when Mastiha remained unchanged) and the mean changes being statistically significant different ($p=0.012$ and $p=0.024$). Finally, miR-155 increased in the placebo group in patients in remission ($p=0.012$), whereas in the same category miR-155 remained unchanged in the Mastiha group.

264 **Table 3.** Changes in miRNA plasma levels in NAFLD patients after intervention

		miR-16 baseline (pg/mL)	**miR-16 post-treatment (pg/mL)**	**Comparison of plasma levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
ALL	Placebo (N=40)	1.706 (1.465)	1.404 (0.874)	0.262	0.648	0.550
	Mastiha (N=27)	1.485 (1.128)	1.390 (0.822)	0.567		
cT1<868.6 ms	Placebo (N=24)	1.448 (1.23)	1.335 (0.758)	0.669	0.506	0.709
	Mastiha (N=10)	1.405 (2.015)	0.880 (0.410)	0.098		
cT1>868.6 ms	Placebo (N=16)	2.092 (1.784)	1.570 (1.046)	0.342	0.481	0.346
	Mastiha (N=17)	1.517 (1.231)	1.594 (0.866)	0.686		
		miR-21 baseline (pg/mL)	**miR-21 post-treatment (pg/mL)**	**Comparison of plasma levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
ALL	Placebo (N=40)	0.316 (0.505)	0.258 (0.335)	0.421	0.804	0.754
	Mastiha (N=27)	0.351 (0.500)	0.269 (0.394)	0.444		
cT1<868.6 ms	Placebo (N=24)	0.375 (0.619)	0.215 (0.255)	0.669	0.506	0.729
	Mastiha (N=10)	0.409 (0.561)	0.298 (0.573)	0.667		
cT1>868.6 ms	Placebo (N=16)	0.247 (0.263)	0.360 (0.450)	0.080	0.975	0.620
	Mastiha (N=17)	0.316 (0.476)	0.252 (0.260)	0.474		
		miR-155 baseline (pg/mL)	**miR-155 post-treatment (pg/mL)**	**Comparison of plasma levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
ALL	Placebo (N=40)	0.229 (0.299)	0.256 (0.427)	0.783	0.705	0.618
	Mastiha (N=27)	0.279 (0.235)	0.266 (0.410)	0.895		
cT1<868.6 ms	Placebo (N=24)	0.258 (0.323)	0.116 (0.123)	**0.054**	0.726	0.581
	Mastiha (N=10)	0.262 (0.121)	0.179 (0.284)	0.462		
cT1>868.6 ms	Placebo (N=16)	0.193 (0.269)	0.562 (0.720)	0.162	0.532	0.519

	Mastiha (N=17)	0.285 (0.255)	0.299 (0.453)	0.913		

[a] p-value for time effect (paired sample t-test), [b] Differences between the groups in the degree of changes (repeated measurements ANOVA), [c] Differences in the degree of changes between the groups in the degree of changes (repeated measurements ANOVA) after including age, sex, BMI and centre as covariates.

Table 4a. Changes in miRNA-16 plasma levels in IBD patients after intervention

		miR-16 baseline (pg/mL)	**miR-16 post-treatment* (pg/mL)**	**Comparison of plasma levels before and post-treatment in each**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	*relapse*					
	Placebo (N=15)	1.625 (1.421)	1.642 (0.776)	0.973	0.498	0.528
	Mastiha (N=20)	2.289 (1.931)	1.662 (1.291)	0.118		
	remission					
	Placebo (N=15)	1.013 (2.056)	2.056 (2.010)	0.144	0.300	0.305
	Mastiha (N=10)	1.564 (1.678)	2.955 (2.201)	0.128		
CD	*relapse*					
	Placebo (N=8)	2.325 (1.529)	1.626 (0.813)	0.438	0.875	0.875
	Mastiha (N=14)	2.321 (2.043)	1.866 (1.340)	0.266		
	remission					
	Placebo (N=7)	1.040 (1.473)	2.039 (1.790)	0.490	0.589	0.188
	Mastiha (N=6)	2.018 (2.491)	2.295 (2.225)	0.307		
UC	*relapse*					
	Placebo (N=7)	0.927 (0.992)	1.659 (0.835)	0.152	0.634	0.718
	Mastiha (N=6)	2.118 (1.739)	0.946 (0.883)	0.347		
	remission					
	Placebo (N=8)	0.995 (0.706)	2.068 (2.312)	0.155	0.421	0.966
	Mastiha (N=4)	1.109 (0.459)	3.615 (2.420)	0.215		

[a] p-value for time effect (paired sample t-test), [b] Differences between the groups in the degree of changes (repeated measurements ANOVA), [c] Differences in the degree of changes between the groups in the degree of changes (repeated measurements ANOVA) after including age, sex and BMI as covariates. *after 3 months for patients in relapse, after 6 months for patients in remission

Table 4b. Changes in miRNA-21 plasma levels in IBD patients after intervention

		miR-21 baseline (pg/mL)	**miR-21 post-treatment* (pg/mL)**	**Comparison of plasma levels before and post-treatment in each**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	*relapse*					
	Placebo (N=15)	0.343 (0.319)	0.323 (0.261)	0.884	0.160	0.675
	Mastiha (N=20)	0.176 (0.113)	0.277 (0.358)	0.168		
	remission					
	Placebo (N=15)	0.171 (0.134)	0.977 (1.056)	**0.012**	0.675	0.802
	Mastiha (N=10)	0.169 (0.127)	1.199 (0.748)	**0.024**		
CD	*relapse*					
	Placebo (N=8)	0.433 (0.377)	0.223 (0.157)	0.209	0.418	0.237
	Mastiha (N=14)	0.183 (0.118)	0.298 (0.390)	0.206		
	remission					
	Placebo (N=7)	0.192 (0.182)	1.457 (1.299)	**0.050**	0.658	0.790
	Mastiha (N=6)	0.087 (0.038)	1.135 (0.100)	**0.016**		
UC	*relapse*					
	Placebo (N=7)	0.216 (0.180)	0.464 (0.329)	0.244	0.187	0.303
	Mastiha (N=6)	0.153 (0.106)	0.204 (0.242)	0.659		
	remission					
	Placebo (N=8)	0.152 (0.081)	0.556 (0.589)	0.097	0.178	0.506
	Mastiha (N=4)	0.223 (0.142)	1.224 (0.531)	0.194		

[a] p-value for time effect (paired sample t-test), [b] Differences between the groups in the degree of changes (repeated measurements ANOVA), [c] Differences in the degree of changes between the groups in the degree of changes (repeated measurements ANOVA) after including age, sex and BMI as covariates. *after 3 months for patients in relapse, after 6 months for patients in remission

Table 4c. Changes in miRNA-155 plasma levels in IBD patients after intervention

		miR-155 baseline (pg/mL)	**miR-155 post-treatment* (pg/mL)**	**Comparison of plasma levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	*relapse*					
	Placebo (N=15)	0.090 (0.094)	0.188 (0.177)	0.287	**0.012**	**0.024**
	Mastiha (N=20)	0.052 (0.048)	0.069 (0.104)	0.576		
	remission					
	Placebo (N=15)	0.076 (0.077)	0.469 (0.402)	**0.012**	0.767	0.839
	Mastiha (N=10)	0.069 (0.090)	0.380 (0.445)	0.270		
CD	*relapse*					
	Placebo (N=8)	0.130 (0.116)	0.048 (0.041)	0.293	0.510	0.384
	Mastiha (N=14)	0.055 (0.052)	0.075 (0.118)	0.652		
	remission					
	Placebo (N=7)	0.123 (0.090)	0.204 (0.142)	0.648	0.264	0.722
	Mastiha (N=6)	0.070 (0.088)	0.256 (0.203)	0.570		
UC	*relapse*					
	Placebo (N=7)	0.050 (0.054)	0.328 (0.141)	**0.054**	**0.012**	**0.042**
	Mastiha (N=6)	0.045 (0.038)	0.056 (0.065)	0.490		
	remission					
	Placebo (N=8)	0.101 (0.101)	0.509 (0.545)	0.417	0.309	0.301
	Mastiha (N=4)	0.030 (0.016)	0.146 (0.120)	0.210		

283 [a] p-value for time effect (paired sample t-test), [b] Differences between the groups in the degree of changes (repeated measurements ANOVA), [c] Differences in
284 the degree of changes between the groups in the degree of changes (repeated measurements ANOVA) after including age, sex and BMI as covariates. *after 3
285 months for patients in relapse, after 6 months for patients in remission.

4. Discussion

In search of the molecular pathway underlying the efficacy of Mastiha in immune-mediated inflammatory diseases, data herein suggest microRNA-155 as the key molecule regulated in IBD and in NAFLD. Over the last few years, miRNAs have emerged as important regulators in various biological processes, including cell proliferation, differentiation, autophagy, metabolism and immune responses [50]. It has been shown that they can influence several molecular signaling pathways associated with inflammatory responses [9]. Their role has been investigated in both NAFLD and IBD. In NAFLD, there is increasing evidence that several miRNAs regulate molecular pathways are associated with lipid metabolism, oxidative stress and liver inflammation [10]. In IBD, they are implicated in T-cell differentiation, Th17 signaling pathway, autophagy, intestinal epithelial barrier function, and inflammatory signaling pathways, such as the NF-κB and IL-6/STAT3 [11, 51].

MiR-16 acts as a regulator of immune-mediated tissue repair and the production of inflammatory mediators, such as TNF-a [25]. It is increased in NAFLD patients and to positively correlate with fibrosis in early fibrosis, whereas negatively in NASH [12, 38]. In IBD, it promotes activation of NF-κB signaling pathway in human colonic mucosa of active UC patients [45] and its circulated levels are higher in CD and UC patients than healthy controls in a Greek IBD population [44]. MiR-21 has a key regulatory role in innate immunity, as it is involved in the differentiation of monocytes, TLR4 activation and is induced by danger signals, such as activators of NF-kB in a negative feedback loop, in order to neutralise damage [33]. In NAFLD, it is involved in liver lipid metabolism and contributes to inflammation and fibrosis via PPAR-α [52]. It is upregulated in the serum of NAFLD patients and correlates

positively with AST, ALT and fibrosis scores [53]. In IBD, miR-21 plays an important role in the differentiation, apoptosis, and activation of T cells that contribute to the pathogenesis of IBD. It is upregulated in both intestinal tissues and circulation and is associated with disease activity in UC patients [46]. In our study, no effect of Mastiha was detected on the levels of miR-16 and miR-21.

MiR-155 is a critical regulator of inflammation, overexpressed in several activated immune cells, responding to many inflammatory stimuli, such as TNF-a, interferons and TLR ligands [54]. It controls inflammation at multiple levels, like B cell differentiation and antibody production, and controls Th1, Th2 and Th17 differentiation [35].

MicroRNA-155 is considered one of the biologically most relevant miRNAs in liver diseases as it is implicated in liver injury, steatosis, inflammation, fibrosis, and carcinogenesis [55]. Although in other inflammatory diseases it is considered a pro-inflammatory miRNA, in NAFLD it offers a protective negative regulatory feedback mechanism aimed at limiting lipid accumulation in lipid macrophages [56]. In contrast, knockout of miR-155 ameliorates hepatic steatosis and fibrosis in mice on a methionine and choline-deficient diet [57]. It is notable that studies on the levels of miR-155 in NAFLD are contradictory, as both up- and downregulated levels have been reported. In most cases, miR-155 seems to be upregulated in hepatocytes and liver tissues [41] and reduced in the circulation of NAFLD patients [43]. Hence, its role may be either protective or exacerbating. In any respect, its implication in NAFLD is through suppressing LXR α-dependent adipogenic signaling pathways [42]. LXRs control immune cell function through direct and indirect mechanisms, either through regulation of genes involved in lipids homeostasis, such as sterol regulating element binding protein 1c (SREBP-1c), or through regulation of Th1,

Th17 polarization and Treg differentiation [58, 59]. The above pinpoint the critical role of miR-155 in lipid regulation and that its deregulation exacerbates hepatic steatosis. In our study miR-155 decreased in the placebo group whereas remained unchanged in the Mastiha group, in patients with cT1<868.6 suggesting a possible regulatory role of Mastiha.

MiR-155 is up-regulated in both UC and CD and its deficiency protects mice from experimental colitis [48]. It has a key role in the differentiation of B and T cells and contributes to the development of regulatory T cells [47]. MiR-155-/- mice express reduced systemic and mucosal interferon-γ-expressing CD4+ T cells, and more specifically, Th17 cells [49]. Furthermore, one of miR-155 targets, LXR is considered as an anti-inflammatory mediator in IBD, with LXR-deficient mice being more susceptible to colitis and activation of LXR receptors accelerating disease recovery [60]. In our study, the mean changes of miR-155 differed significantly between the Mastiha and placebo groups in UC patients in relapse with a higher increase in the placebo group. A similar activity has been proposed for cinnamaldehyde, an active compound from cinnamon that has been shown to reduce inflammation via miR-155 inhibition in colon tissues [61]. Furthermore, a study by Liu et al. [62] showed that miR-155 inhibition TNBS-colitis amelioration was mediated by an impact in the differentiation and function of Th17 cells. The above result come into agreement with our proposed Th17 regulatory role of Mastiha [63].

Our results suggest miR-155 as a key regulator in the mode of action of Mastiha as its levels seem to be regulated in the Mastiha group in both RCTs. MiR-155 influences LXR activity and therefore is implicated in the regulation of lipogenic genes, as well as the regulation of Th17 differentiation. Our results parallel with the the Th17 regulatory action in the case of MASTIHA IBD-GR [63] and the lipid

regulatory activity Mastiha in the case of MAST4HEALTH [19] under the common key regulators miRNA-155. The above suggested mechanism is presented in **Figure 1**.

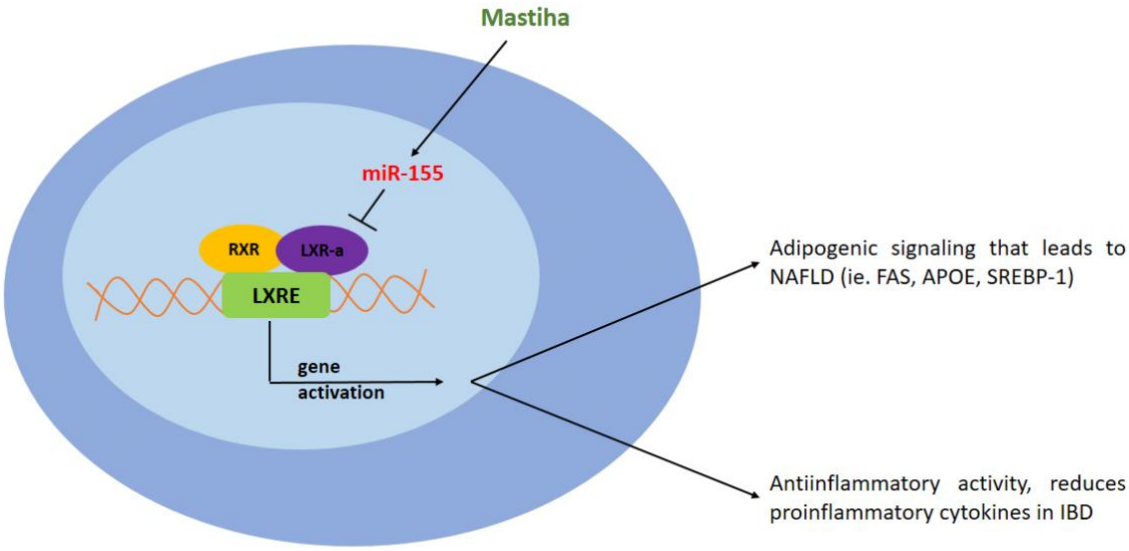

Figure 1 (colored)**.** Mastiha's suggested mechanism of action in miRNA regulation. Mastiha may manipulate the miR155/LXR pathway through regulation of serum miR-155 levels. More specifically, in NAFLD patients it ameliorates a decrease of miR-155, which can be associated with disease progression. In patients with active UC, it ameliorates an increase of miR-155, which is associated with proinflammatory effects.

The findings of this study have to be seen in light of some limitations. The primary limitation to the generalization of these results is the relatively small number of samples. Additionally, the between-subject variability of miRNA levels is quite high precluding the identification of small differences between groups. Also, miRNA levels were detected in the circulation and not in the affected tissue. Thus, the

functional link between plasma miRNAs levels and their effect in specific cell types or cell compartments may differ between individual miRNAs and may not always correlate. The above limitations are counterbalanced by the use of high-sensitivity and specificity methodologies that ensure the reproducible recovery of high-quality material. Also, miRNA expression in peripheral blood has been proven to reflect mucosal changes and alterations in circulating inflammatory cells [64].

5. Conclusions

Circulating levels of miR-155, a critical player in the differentiation of Th17 cells, are regulated by Mastiha administration in IBD and NAFLD that share common pathophysiological features, suggesting this as the key mediator of Mastiha's anti-inflammatory activities. Further studies to confirm this mechanism of action are necessary.

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