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Διδακτορική Διατριβή

Διατροφικές παρεμβάσεις σε φλεγμονώδη νοσήματα: κατανόηση των μοριακών μηχανισμών δράσης

Χαραλαμπία Αμερικάνου

Βιολόγος, M.Sc.

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ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ
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Doctoral dissertation

Nutritional interventions in inflammatory diseases: investigating the molecular pathways

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Περίληψη

Ως φλεγμονή ορίζεται η εντοπισμένη απόκριση του σώματος σε βλαβερά ερεθίσματα, όπως τα παθογόνα, οι τοξικοί παράγοντες και ο τραυματισμός, η οποία συμβάλλει στον έλεγχο της μόλυνσης, και επιδιόρθωση της ιστικής βλάβης. Η απορρύθμιση αυτού του μηχανισμού και η χρόνια επιμένουσα φλεγμονή μπορεί να οδηγήσει σε χρόνιες φλεγμονώδεις νόσους, όπως είναι η μη αλκοολική λιπώδης νόσος (ή διήθηση) του ήπατος (ΜΑΛΝΗ) (γνωστή και ως λιπώδες ήπαρ) και οι ιδιοπαθείς φλεγμονώδεις νόσοι του εντέρου (ΙΦΝΕ). Η ΜΑΛΝΗ είναι η πιο συχνή νόσος του ήπατος, που χαρακτηρίζεται από συσσώρευση λίπους στα ηπατοκύτταρα, μη προκαλούμενη από το αλκοόλ. Οι ΙΦΝΕ αποτελούν μία ομάδα νοσημάτων που χαρακτηρίζονται από χρόνια φλεγμονή στον γαστρεντερικό σωλήνα. Και οι δύο διαταραχές έχουν υψηλό επιπολασμό στις Δυτικές κοινωνίες, κυρίως λόγω του δυτικού τρόπου ζωής και απαιτούν νέες υποσχόμενες θεραπευτικές προσεγγίσεις με τις λιγότερες ανεπιθύμητες ενέργειες. Επίσης εμφανίζουν κοινά παθογενετικά χαρακτηριστικά, όπως αυξημένη εντερική διαπερατότητα, εντερική δυσβίωση και χρόνιες φλεγμονώδεις αποκρίσεις.

Η Μαστίχα της Χίου αποτελεί ένα φυτικό προϊόν με πληθώρα βιοενεργών συστατικών, όπως τα τερπένια, τα φαινολικά συστατικά και οι φυτοστερόλες. Οι αντιφλεγμονώδεις ιδιότητές της είναι καλά χαρακτηρισμένες. Σκοπός της συγκεκριμένης διδακτορικής διατριβής ήταν να ερευνηθούν οι μοριακοί μηχανισμοί υπό τους οποίους η Μαστίχα εκδηλώνει τις αντιφλεγμονώδεις ιδιότητές της. Πιο συγκεκριμένα μελετήσαμε την αποτελεσματικότητα της Μαστίχας ως μη φαρμακολογική προσέγγιση τόσο στη ΜΑΛΝΗ όσο και στις ΙΦΝΕ, καθώς και το ποιοι μηχανισμοί συνοδεύουν αυτήν τη δράση.

Προκειμένου να αξιολογήσουμε τη δράση της Μαστίχας στη ΜΑΛΝΗ σχεδιάσαμε μία πολυκεντρική, διπλά-τυφλή, τυχαιοποιημένη, ελεγχόμενη με εικονικό φάρμακο κλινική μελέτη και μελετήσαμε την επίδραση στη φλεγμονή και την ίνωση του ήπατος μέσα από μαγνητική τομογραφία ήπατος σε συνδυασμό με αξιολόγηση βιοχημικών παραμέτρων, φλεγμονωδών δεικτών και εφαρμογή μεταγενωμικών και μεταβολομικών αναλύσεων. Μετά από εξάμηνη χορήγηση του συμπληρώματος με τη Μαστίχα παρατηρήσαμε βελτίωση στην εντερική δυσβίωση, όπως αυτή αποτυπώνεται στο δείκτη Bray-Curtis, ο οποίος αυξήθηκε στους εθελοντές που ελάμβαναν Μαστίχα σε σχέση με αυτούς που ελάμβαναν εικονικό φάρμακο (placebo). Επίσης η Μαστίχα μείωσε τον πληθυσμό του Flavonifractor, ένα βακτηριακό γένος που εμπλέκεται στον καταβολισμό της κερσετίνης, ένα φλαβονοειδές με αντιοξειδωτικές και αντιφλεγμονώδεις ιδιότητες. Τα παραπάνω συνδυάστηκαν με μείωση στα χολικά οξέα και τα φωσφολιπίδια του πλάσματος, πιθανά οφειλόμενη στα βιοδιαθέσιμα τριτερπενικά οξέα της Μαστίχας. Τέλος, στους ασθενείς με σοβαρή μορφή παχυσαρκίας παρατηρήθηκε βελτίωση στην ηπατική φλεγμονή και ίνωση όπως αποτυπώθηκαν στη μαγνητική τομογραφία.

Στις ΙΦΝΕ πραγματοποιήσαμε μία διπλά-τυφλή, τυχαιοποιημένη, ελεγχόμενη με εικονικό φάρμακο κλινική μελέτη με τρίμηνη διάρκεια στους ασθενείς με ενεργή νόσο και

εξάμηνη διάρκεια στους ασθενείς σε ύφεση. Η μέτρηση των φλεγμονωδών δεικτών ανέδειξε μία στατιστικά σημαντική αύξηση στα επίπεδα της ιντερλευκίνης 17Α στον ορό στην ομάδα της Μαστίχας με στατιστικά διαφορετικές μέσες μεταβολές στις ομάδες της Μαστίχας και του placebo. Οι διαφορές αυτές παρατηρήθηκαν στους ασθενείς σε ύφεση και πιο συγκεκριμένα σε αυτούς με νόσο του Crohn. Παρομοίως, η μεταβολομική ανάλυση έδειξε αύξηση μεταβολιτών, όπως τα αρωματικά αμινοξέα φαινυλαλανίνη, τυροσίνη, τρυπτοφάνη μαζί με την αλανίνη, γλυκίνη και το οξικό οξύ, μόνο στους ασθενείς σε ύφεση. Καθως η τρυπτοφάνη εμπλέκεται στη ρύθμιση των βοηθητικών Τ-17 λεμφοκυττάρων, τα παραπάνω προτείνουν μία πιθανή μεταβολή του ρόλου αυτών των κυττάρων σε πιο προστατευτικό για τις ΙΦΝΕ σε ύφεση. Τέλος, η Μαστίχα ρύθμισε τα επίπεδα σε κάποια εντερικά μικρόβια που σχετίζονται με φλεγμονώδεις διαδικασίες, παρά την απουσία επίδρασης στη συνολική μικροβιακή δυσβίωση.

Λαμβάνοντας υπόψη το γεγονός ότι όλα τα παραπάνω ανέδειξαν την αντιφλεγμονώδη δράση της Μαστίχας, μελετήσαμε κατά πόσον αυτή η δράση διαμεσολαβείται από επιγενετικούς μηχανισμούς που σχετίζονται με τη φλεγμονή, όπως η ρύθμιση των microRNAs. Τα microRNAs είναι μικρά μόρια που λειτουργούν ως σημαντικοί ρυθμιστές της γονιδιακής έκφρασης, συμπλεριλαμβανομένων και των φλεγμονωδών μονοπατιών. Το αξιοσημείωτο είναι ότι και στις δύο κλινικές μελέτες παρατηρήσαμε ρύθμιση του ίδιου miRNA, του miR-155 (το οποίο εμπλέκεται τόσο στη ρύθμιση των λιπιδίων όσο και των Τ βοηθητικών λεμφοκυττάρων), αναδεικνύοντας ένα πιθανό κοινό μηχανισμό δράσης της Μαστίχας σε δύο διαφορετικές φλεγμονώδεις νόσους. Στο μέλλον χρειάζονται περισσότερες μελέτες για να αποκαλύψουν περαιτέρω τους εμπλεκόμενους μοριακούς μηχανισμούς και να προτείνουν μονοπάτια που ερμηνεύουν τις αντιφλεγμονώδεις ιδιότητες της Μαστίχας.

Λέξεις κλειδιά: φλεγμονή, μη αλκοολική λιπώδης νόσος του ήπατος, ιδιοπαθείς φλεγμονώδεις νόσοι του εντέρου, Μαστίχα, κλινική μελέτη, μικροβίωμα, μεταβολομική, microRNAs

Abstract

Inflammation is a biological process against harmful stimuli, such as pathogens, damaged cells and toxic compounds which contributes in infection control and tissue repairing. Inflammatory dysregulation and chronic inflammatory responses may lead to various chronic inflammatory conditions, such as Non-alcoholic fatty liver disease (NAFLD) and Inflammatory Bowel Diseases (IBD). NAFLD is the most common liver disease, characterised by excessive fat accumulation in liver, not caused by alcohol consumption. IBD represents a group of intestinal disorders that are characterized by chronic inflammation of the digestive tract. Both conditions show a high prevalence in Western societies, mainly due to the westernised lifestyle, and are in need for new promising options for treatment with the least side effects. Also, they share common pathogenic features, such as increased intestinal permeability, gut dysbiosis and chronic inflammatory response

Mastiha, is a plant product with a plethora of bioactive constituents, such as terpenic acids, phenolic compounds and phytosterols. Mastiha's anti-inflammatory properties are well established. The aim of this dissertation was to investigate the molecular pathways under which Mastiha exhibits its anti-inflammatory action. More specifically, we explored the effectiveness of Mastiha as a non-pharmacological intervention in both NAFLD and IBD, and which molecular mechanisms accompany this effect.

In order to evaluate the effect of Mastiha in NAFLD we designed a multicenter, randomized, double-blinded and placebo-controlled clinical trial and investigated the effect on liver inflammation and fibrosis through MRI, biochemical, inflammatory and multi-omic analyses. After six months of Mastiha supplementation, we observed a significant improvement on microbiota dysbiosis as depicted in Bray-Curtis dissimilarity index which was significantly greater among patients that received Mastiha, compared to Placebo. Also, Mastiha decreased the proportion of Flavonifractor, a taxa involved in the catabolism of quercetin, a flavonoid with antioxidant and anti-inflammatory properties. The above effects paralleled with a decrease in plasma cholic acid and phospholipids, possibly attributed to the bioavailable triterpenic acids of Mastiha. Finally, in severely obese patients we observed an improvement of the liver fibrosis as assessed via MRI.

In IBD, we applied a randomized, double-blind, placebo-controlled, parallel-group clinical trial with a 3 months duration for patients in relapse and a 6 months duration in patients for remission. Quantification of inflammatory biomarkers revealed a significant increase of serum interleukin-17A (IL-17A) in the Mastiha group and the different mean changes between Mastiha and placebo groups in inactive patients, with this pattern being observed specifically in Crohn's disease (CD) patients in remission. Similarly, metabolomics analysis revealed an increase in metabolites, such as the aromatic aminoacids phenylalanine, tyrosine and tryptophan together with alanine, glycine and acetic acid, only in patients in remission. As tryptophan is involved in T-

helper-17 regulation, the above suggest a possible shift of Th-17 cells to a more protective pathway in quiescent IBD. Finally, Mastiha regulated some gut microbiota related to inflammatory processes, although no evident effect was observed on microbiota dysbiosis.

Taking into account that all the above pointed towards Mastiha's anti-inflammatory activity, we examined whether this action is attributed to epigenetic mechanisms related to inflammation, such as regulation of miRNA levels. MicroRNAs are small molecules that serve as important regulators of gene expression, including inflammatory pathways. Interestingly, the same microRNA, miR-155 (implicated in lipid and Th-17 regulation) was modified by Mastiha in both clinical trials, revealing a possible common mechanism across Mastiha supplementation in two different inflammatory conditions. More studies are needed to further explore this mode of action and introduce other potential pathways that explain Mastiha's valuable anti-inflammatory action.

Keywords: Inflammation, Non-alcoholic fatty liver disease, Inflammatory Bowel Diseases, Mastiha, clinical trial, microbiome, metabolomics, microRNAs

Abbreviations

AGEs	Advanced glycation end products
ALCAs	Anti-laminaribioside carbohydrate antibodies
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANA	Serum antinuclear antibodies
ANCAs	Antineutrophil cytoplasmic antibodies
APC	Antigen presenting cells
APOB	Apolipoprotein b
AR	Androgen receptor
AS	Ankylosing spondylitis
ASCAs	Anti-Saccharomyces cerevisiae antibodies
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BIK	B-cell lymphoma 2 Interacting Killer
BMI	Body mass index
CARD	Caspase recruitment domain
CCL	Chemokine (C-C motif) ligand
CD	Crohn's disease
CLRs	C-type lectin receptors
COX	Cycloxygenases
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNMT	DNA methyltransferase
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	Endoplasmic reticulum
EZH2	Enhancer of Zeste Homolog 2
FC	Free cholesterol
FIB-4	Fibrosis-4
FIH1	Hypoxia inducible factor 1
Foxp3	Forkhead box P3
γ-GT	gamma-glutamyltransferase
GCKR	Glucokinase regulator
GC-MS	Gas chromatography–mass spectrometry
GF	Germ free
GI	Gastrointestinal system
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRAS	Generally Recognised As Safe
GPCR	G-protein-coupled-receptors
GSH	Glutathione
GWAS	Genome-wide association studies
HAECs	Human aortic endothelial cells

HAT	Histone acetyltransferases
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
HMOX 1	Heme oxygenase 1
HOMA-IR	Homeostasis model assessment
HP	Helicobacter Pylori
IBD	Inflammatory bowel diseases
IBDQ	Inflammatory bowel disease questionnaire
ICAM	Intercellular adhesion molecule
IELs	Intestinal intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobines
IkB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
IKK	IkB kinase
IL-	Interleukin-
IRF	IFN regulatory factor
JAK-STAT	Janus kinases-Signal Transducer and Activator of Transcription proteins
LC-MS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoprotein
LIF	Liver Inflammation and Fibrosis
LLC	Lewis lung adenocarcinomas
LRR	Leucine rich repeats
LPS	Lipopolysaccharide
LYPLAL1	Lysophospholipase-like 1
MALDI TOF/TOF	Matrix-assisted laser desorption/ionization-time of flight
MAPK	Mitogen-activated protein kinase
MAPK-JNK	Mitogen-activated protein and c-Jun N-terminal kinases
MBOAT7	Membrane Bound O-Acyltransferase Domain Containing 7
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
miRNA, miR	MicroRNA
MMP-2	Matrix metalloproteinase-2
MNF	Mastic Neutral Fraction
MS	Multiple sclerosis
MTTP	Microsomal triglyceride transfer protein
MTP18	Mitochondrial protein 18
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBD	Nucleotide binding domain
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
NFS	NAFLD fibrosis score
NGS	Next Generation Sequencing Technology

NK	Natural killer
NLRs	NOD-like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
Nox	NADPH oxidase
NSAIDs	Nonsteroidal anti-inflammatory drugs
oxLDL	Oxidized LDL
p53	Tumor protein 53
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PGE	Prostaglandins
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPAR	Peroxisome proliferator activated receptor
PRRs	Pattern recognition receptors
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
ROS	Reactive oxygene species
SCFAs	Short chain fatty acids
SIRT	Sirtuin
SLE	system lupus erythematosus
SMA	Smooth muscle antibodies
SNPs	Single nucleotide polymorphisms
SPF	Specific pathogen free
SS	Sjögren's syndrome
STAT	Signal transducer and activator of transcription
T1D	Diabetes mellitus type 1
TAG	Triacylglycerol
TC	Total cholesterol
TCRs	T cell receptors
TG	Triglycerides
Th-	T helper cell
TIR	cytoplasmic Toll/IL-1R
TLRs	Toll-like receptors
TM6SF2	Transmembrane 6 superfamily member 2
TNBS	Trinitrobenzene Sulfonic Acid
TNF-α	Tumor necrosis factor alpha
Tregs	T regulatory cells
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing-adapter-inducing interferon-β
UC	Ulcerative colitis
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

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1. Introduction

1.1.Inflammation

Immune system exhibits two types of reactions, innate and adaptive [Fig. 1.1]. Innate immunity is a fast, first defense which consists of cells (like macrophages, dendritic cells and neutrophils) that detect, signal the infection and initiate an inflammatory cascade in order to activate the second type, adaptive immunity. The adaptive immunity consists of highly specialized immune cells like antigen-specific B and T lymphocytes that fight the infection and prevent its expansion. In B and T lymphocytes, gene rearrangement results to production of specific antibodies and killer T cell respectively. Adaptive immunity shows immunological memory, giving the host the opportunity to rapidly respond to future invasions of the same pathogen. Inflammation is an adaptive response of the immune system induced either by microbial infection or tissue injury which aims to control the infection or repair the damage and restore homeostasis [Barton et al, 2008].

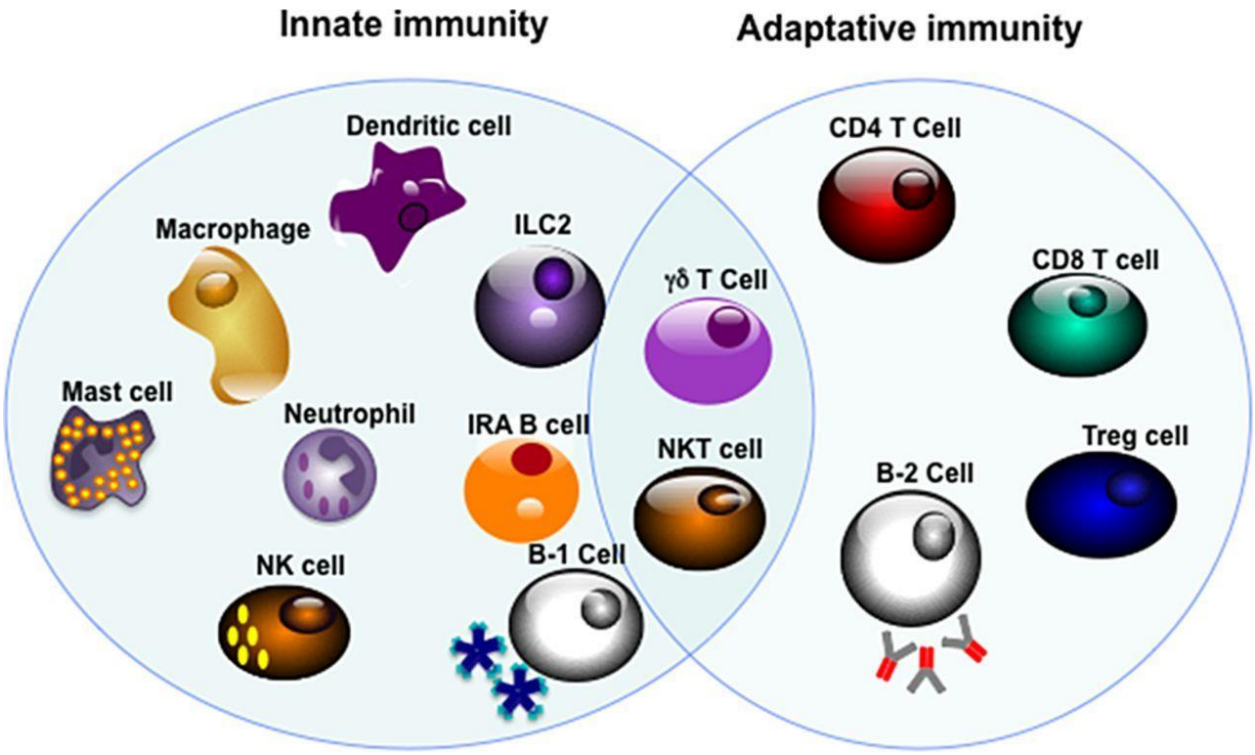


Figure 1.1. Innate and adaptive immunity [Ait-Oufella et al., 2014].

Inflammatory response may be acute or chronic depending on the speed and the duration of the reaction. Acute inflammatory response involves the delivery of blood components (plasma, leukocytes and inflammatory mediators, such as cytokines) to the site of infection or injury whereas chronic inflammation occurs when acute inflammatory response fails to eliminate the cause of inflammation. Chronic phase develops specific humoral and cellular immune responses to pathogens. During both phases various soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction [Nevez et al., 2012].

1.1.1. Pathways of inflammation

Inducers and sensors: Inducers of inflammation may be endogenous or exogenous. Endogenous inducers are signals produced by stress or damaging of the tissues and are also known as Damage-associated molecular patterns (DAMPs). For example, in acute inflammation, cellular components like adenosine triphosphate (ATP), K^+ ions and uric acid are released when the plasma membrane is disrupted during cell death. In chronic inflammation, endogenous inducers may include AGEs (advanced glycation end products) and oxidized lipoproteins (such as high-density lipoprotein HDLs and low-density lipoprotein LDLs). These molecules may be detected by macrophages and treated as foreign substances [Nathan, 2002]. Their phagocytosis activates NACHT, leucine rich repeats (LRR) and PYD domains-containing protein 3 (NALP3) inflammasome and production of caspase-1 substrates, such as the interleukin (IL)-1 family. Reactive oxygene species (ROS), produced by those phagocytes, oxidize the lipid and protein components of lipoproteins, converting them into inflammatory signals.

Exogenous inducers can be microbial or not. Microbial inducers consist of pathogen-associated molecular patterns (PAMPs) which are molecules associated with groups of pathogens and factors of viruses. PAMPs are recognised by Toll-like receptors (TLRs) expressed in macrophages and dendritic cells. Non microbial compounds, like allergens, irritants and toxic compounds may also be exogenous inducers of inflammation [Medzhitov, 2008].

Mediators: There are seven classes of mediators namely: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes. Vasoactive amins (histamine and serotonin) and vasoactive peptides (such

as fibrinopeptides) increase vascular permeability. The complement is part of the innate immune system that promotes monocyte recruitment and phagocytosis. Lipid mediators (such as eicosanoids) are derived by phospholipids in the cell membranes which generate arachinoid acid subsequently metabolised either to prostaglandins (PGEs) by cycloxygenases (COX) or to leukotriens and lipoxins by lipoxygenases (LOX). Cytokines (such as Tumor necrosis factor alpha (TNF-a) or IL-6 etc) are produced mainly by macrophages and activate endothelium and leukocytes in the acute phase. Chemokines (Chemokine (C-C motif) ligand, CCL, Chemokine (C-X-C motif) ligand, CXCL) control chemotaxis and proteolytic enzymes (like elastin and metalloproteinases) are involved in host defence, tissue remodeling and leukocyte migration [Medzhitov, 2008].

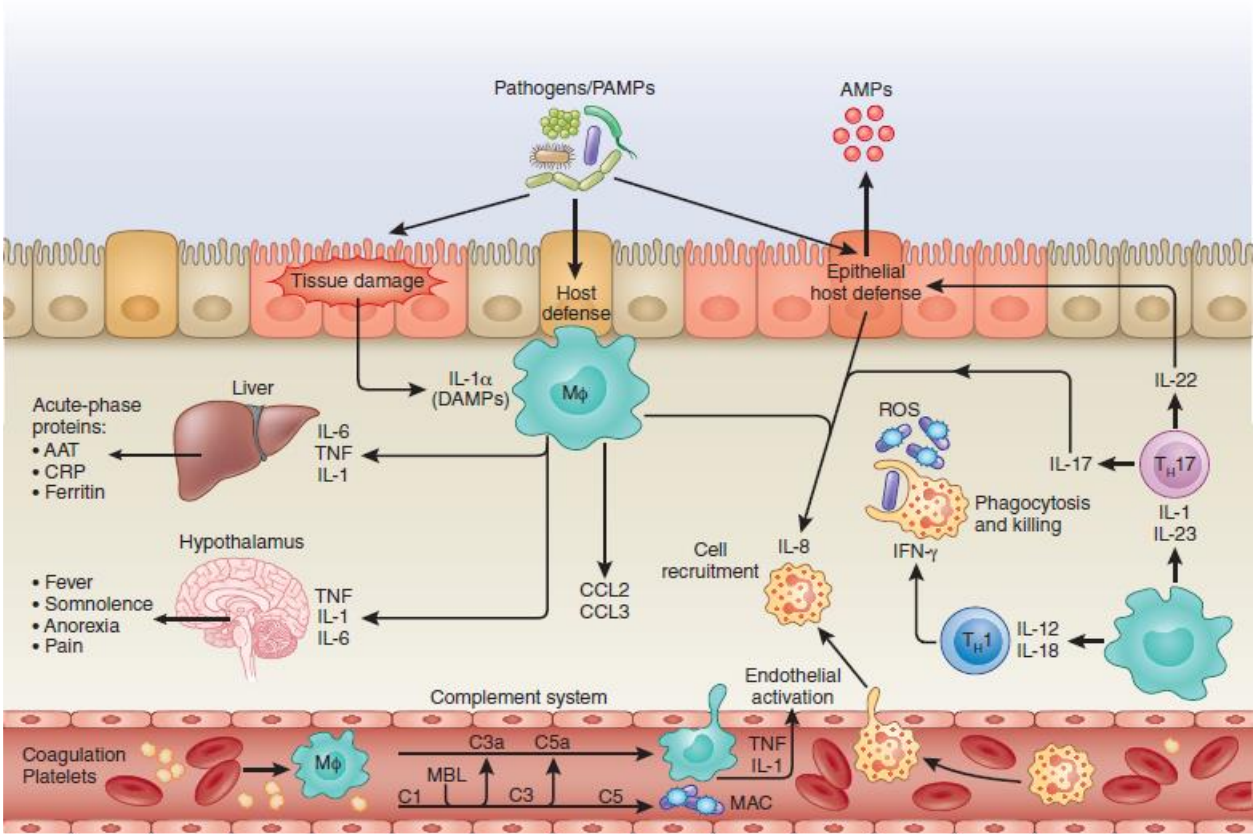


Figure 1.2. Immunological pathways of inflammation [Netea et al., 2017].

Receptors and signaling: Innate immunity cells recognize pathogens (PAMPs) or tissue damage (DAMPs) with pattern recognition receptors (PRRs). Four different classes of PRR families have been recognised, transmembrane proteins (i.e. TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding

oligomerization domain (NOD) -like receptors (NLRs). TLRs have N-terminal leucine rich repeats, a transmembrane regiona and a cytoplasmic Toll/IL-1R (TIR) domain. Different TLRs recognize different patterns, for example TLR-4 recognises lipopolysaccharides (LPS). TLR signaling may be MyD88 or TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent depending on the adaptor and both of them lead to the activation of Nuclear factor kappa light chain enhancer of activated B cells (NFkB) [Newton et al., 2012]. NLRs are cytoplasmic pathogen sensors contacting N-terminal caspase recruitment domain (CARD), a nucleotide binding domain (NBD) and C-terminal LRR. Most NLRs (NOD1 and NOD2) signal NFkB activation or secretion of IL-1b and IL-18. Mediators' receptors include several families. G-protein-coupled-receptors (GPCR) superfamily recognizes lipid mediators, vasoactive amins, complement fragments and chemokines. They are linked to heteromeric G proteins which interact with ion channels or enzymes and stimulate endocytosis and mitogen-activated protein kinase (MAPK) activation which regulates gene expression. TNF receptor family, hematopoietin receptor family, IL-1 family and interferon (IFN) receptor family recognize cytokines and activate signaling pathways, like Janus kinases-Signal Transducer and Activator of Transcription proteins (JAK-STAT), Mitogen-activated protein and c-Jun N-terminal kinases (MAPK-JNK), NFkB and others. Finally, there are some other receptors like Fc receptors which recognize hypersensitivity reactions engaging immunoglobines (Ig) IgE and IgG complexes and transmembrane proteins like selectins and integrines which interact with glycoproteins, and mediate leukocyte circulation across vascular wall [Newton et al., 2012].

1.1.2. Genetics and epigenetics in inflammation

Several genetic linkage and association studies have proved that gene alleles implicated in inflammation processes associate with disease initiation, progression, and severity. Some inflammatory diseases may be caused by heritable mutations, such as Familial Cold Autoinflammatory Syndrome and Familial Mediterranean Fever and others may be complex with single nucleotide polymorphisms (SNPs) in inflammatory genes modifying the disease susceptibility. For example, SNPs in TNF (encoding TNF-a) increase risk for asthma, system lupus erythematosus (SLE), and psoriatic arthritis [Loza et al., 2007]. SLE is strongly associated with an IFN regulatory factor (IRF)-5 allele in four independent case-control studies [Graham et al., 2006]. The major histocompatibility complex (MHC) or HLA (human leukocyte antigen) are

essential proteins of the adaptive immune system expressed in the surface of antigen presenting cells (APC). MHC binds to antigens derived from pathogens and display them on T cell receptors (TCRs) of T lymphocytes in order to activate them. MHC complex is playing a crucial role in several inflammatory/autoimmune diseases. Rheumatoid arthritis (RA) is strongly associated with the class II HLA-DRB1 gene [Matzaraki et al., 2017].

Epigenetic mechanisms include DNA methylation, histone modifications, and non coding RNAs which allow the cells to respond quickly to environmental changes. Several epigenetic modifications are associated with various inflammatory disorders. The methylation status of IFN-γ and Forkhead box P3 (Foxp3) genes are vital for the differentiation of CD4+ T cells to T helper (Th)-1, Th2, Th17 or T regulatory cells (Tregs). Altered DNA methylation of MHC II increases the risk of RA. Low levels of DNA methylation is observed in the promoter of IL-6 gene in SLE patients. Changes in DNA methylation exist in CD4+, CD8+ and CD44+ encephalitogenic T cells of MS patients [Jin et al., 2018]. Histone modifications include acetylation, methylation, ubiquitylation and phosphorylation on the N-terminal tails of histones H3 and H4 and may regulate gene transcription in immune response. Monocyte to macrophage differentiation is associated with histone modifications at promoter and enhancer regions. Demethylation of H3K27 is necessary to activate cytokine production in macrophages. H3 acetylation controls LPS stimulated regulation of inflammatory genes [Raghuraman et al., 2016]. HDAC3 inhibition in peripheral blood mononuclear cells (PBMCs) of ankylosing spondylitis (AS) patients is associated with downregulation of TNF-α expression according to Ya Jiang and co-workers [Ya Jiang et al., 2015].

MicroRNAs are small (∼20−30 nucleotide) noncoding RNAs that regulate gene expression, mainly in an inhibitor way (RNA silencing), in all levels of genome function (chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation). MicroRNAs are expressed in innate immune system cells (monocytes, macrophages, dendritic cells (DCs), natural killer, NK cells etc) and control the development of B ant T lymphocytes in the adaptive immune system. They also modulate production of inflammatory mediators and their deregulation has been associated with several immune disorders. For example, miR-146a represses IFN and TLR signaling, miR-23b, miR-30a and miR-125a regulate autoantibody production. Additionally, miR-21, miR-148 and miR-126 which

target Dnmt1, are all upregulated suggesting hypomethylation in lupus [Zan et al., 2014].

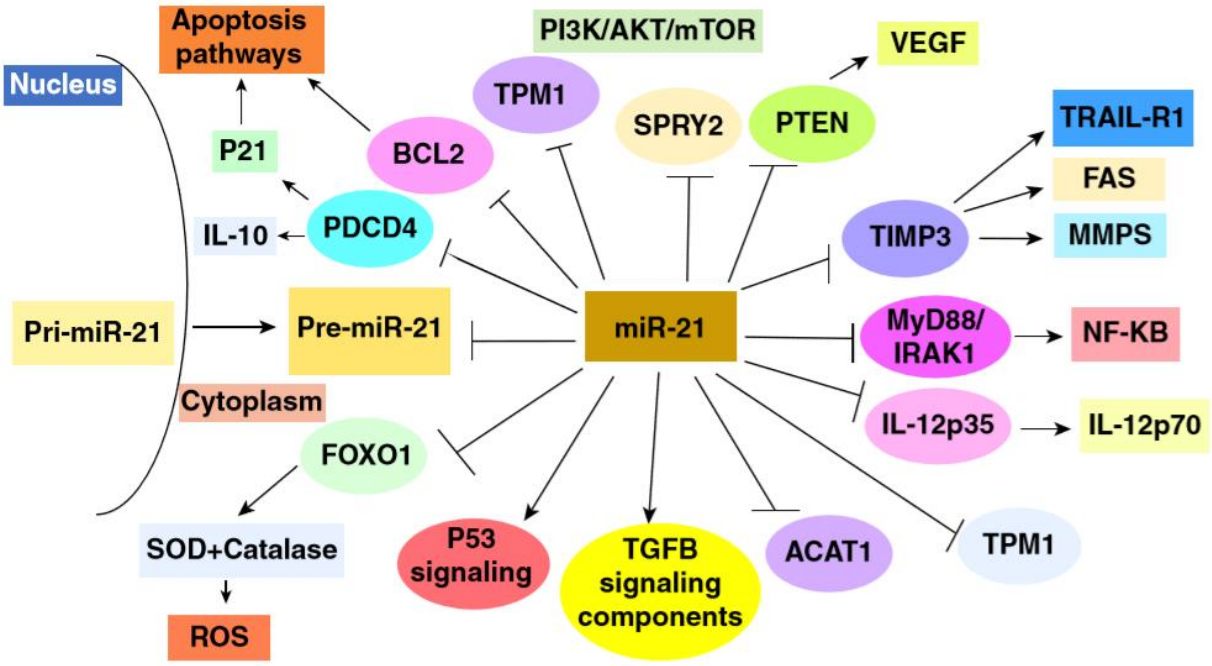


Figure 1.3. Role of miR-21 in inflammation processes [Momen-Heravi et al., 2017].

MiR-21 family controls TLR-signaling pathways, PI3K/AKT/GSK3β and MAPK pathways, induces DNA-hypomethylation and has a prominent role in various inflammatory diseases, such as T1D, MS and psoriasis [Fig. 1.3] [Momen-Heravi et al., 2017]. Two miRNAs (miR-18b and miR-599) are associated with relapse in multiple sclerosis, whereas miR-96 which is involved in interleukin and Wnt signaling pathways is associated with remission. MiR-145, discriminates MS patients from controls with a specificity of 89.5% and a sensitivity of 90.0% [Tufecsi et al., 2010]. In human monocytes, inflammatory activation is associated with increase in miR-125b levels and decrease in B-cell lymphoma 2 Interacting Killer (BIK) and Mitochondrial protein 18 (MTP18), which reduce oxidative phosphorylation and enhanced mitochondrial fusion [Duroux-Richard et al., 2016].

1.1.3. Role of gut microbiome in inflammation

The human epithelium and most of all the lower gastrointestinal tract hosts hundreds of thousands of microorganisms which exhibit an important homeostatic role in the immune

system, with both immune-stimulatory and immune-regulatory effects [Slingerland et al., 2017]. The microbiome protects from infectious pathogens, produces short chain fatty acids (SCFAs) which have anti-inflammatory function by inhibiting HDAC in Tregs and small molecules that interact with the host and shape the development of the immune system. For example, dipeptide aldehydes inhibit cathepsins which are important in antigen processing and presentation and N-acyl amides interact with glycyl radical enzymes which correlate with epithelial adhesion and cellular invasion [Clemente et al., 2018]. Furthermore, disruption of the gut barrier by pathogenic bacteria results to LPS dislocation and transfer to the circulation. This allows macrophages to infiltrate, produce and activate inflammatory cytokines, contributing to local inflammation [Bander et al., 2020]. Finally, several inflammatory molecules interact with gut microbiome. For example, IL-6 is positively correlated with the abundance of *Lactobacillus* species [Cooper et al., 2016] and TNF-α has lower levels in individuals with higher *Bifidobacterium adolescentis* abundance [Schirmer et al.,2016]. C-reactive protein (CRP), an acute-phase reactant, is a downstream inflammatory marker that can be down-regulated through the effects of anti-inflammatory metabolic products of specific gut microbes. This may explain why CRP levels negatively correlate with *Phascolarctobacterium's* abundance, a genus producing propionate, an anti-inflammatory SCFA [Rajkumar et al., 2014, Wu et al., 2017].

Germ free (GF) mice produce fewer intestinal intraepithelial lymphocytes (IELs) and have reduced IgA-secreting plasma cells and Tregs in the lamina propria compared with specific pathogen free (SPF) animals, indicating the critical role of gut microbiome in mucosal immunity [Ostman et al., 2006]. Treatment with prebiotics reduces IgA in feces, upregulates IL-10, CXCL-1 and Mucin-6 genes and downregulates IFN- γ, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1 β genes in the ileum [Carasi et al., 2015]. Enteric microorganisms interacting with epithelial cells in vitro, block IkB ubiquitination and therefore NFkB activation by interference with IkB ubiquitination ligase. Several species of normal human gut bacteria can induce generation of ROS within epithelial cells with significant signaling effects on innate immunity, proliferation, and epithelial movement and restitution. NOX enzymes knockout mice lose rapid commensal-dependent ROS generation [Neish, 2014].

Abnormal interactions between the host and its microbiota may contribute to various chronic inflammatory diseases via several ways. For example, microbial products may act as

continuous stimuli of immune responses, which lead to chronic inflammation. Disturbed microbial development during maturation of the innate immune system results in a failure to induce immunological tolerance, which then induces autoimmunity [Thaiss et al., 2016]. The gut microbiota has a bidirectional relationship with inflammation and depending on its composition, it inhibits or stimulates inflammatory pathways promoting the onset of various inflammatory conditions or reinforcing the disease state [Fig. 1.4] [Bander et al., 2020]. Therefore, variations of microbiota have been associated with different inflammatory diseases, indicating that modulation of gut microbiota may be a novel therapeutic strategy.

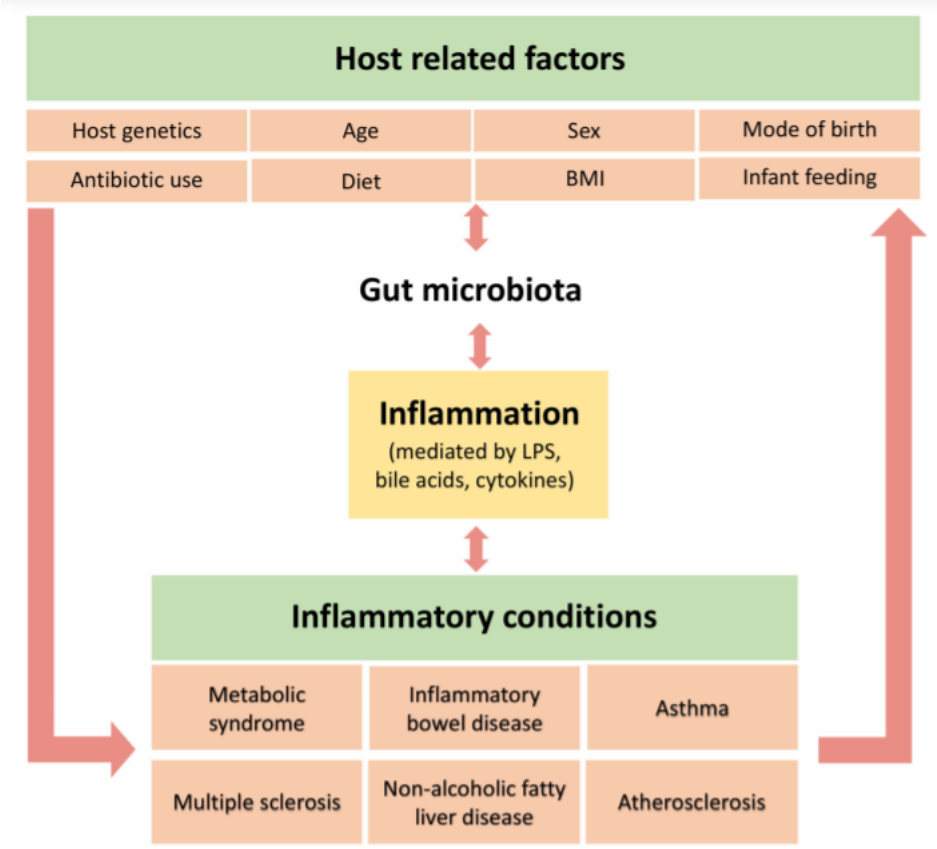


Figure 1.4. Interrelationships between the gut microbiota, inflammation and inflammatory conditions [Bander et al., 2020].

1.1.4. Metabolomics in the analysis of inflammatory diseases

The use of metabolomic analysis facilitates the differentiation between localised and systemic metabolic consequences of inflammation and explores candidate biomarkers for the monitoring of the inflammatory status or novel targets for therapeutic intervention. Many

studies have investigated several metabolites in human disease and animal models proving that the levels of many metabolites are altered by inflammatory process. High energy requirements along with decreasing oxygen supply within the inflammatory environment are the main core of these alterations. For example, immunological responses to tissue hypoxia (such as up-regulation of IL-1, and TNF-a in macrophages) or transcription factor hypoxia-inducible factor (HIF) play a central role in inflammation by regulating cellular metabolism towards anaerobic respiratory pathways and lactate production. Cytokines affect tissue remodeling and provide a feedback mechanism for self-sustaining inflammatory microenvironments, and under circumstances a route to chronic inflammatory disease [Kapoor et al., 2012].

Increased levels of Lyso-phosphatidylcholine (LPC), a class of phospholipids, intermediates in the metabolism of lipids, have been associated with higher risk of developing atherosclerotic cardiovascular disease (ACD) in animal models susceptible to development of ACD [Djekic et al., 2015]. In RA, metabolic disturbances are related to glycolysis metabolism, TCA cycle, amino acid metabolism and lipid metabolism and are mainly reflected in three metabolites: glucose, lactic acid, and citric acid [Li et al., 2020]. Glutamate, a non-essential amino acid which acts as a neurotransmitter in the central nervous system, helps to differentiate people with MS from other neurological and inflammatory conditions. Its excitotoxicity contributes to lesions characteristic of MS in animal models and may be an important mechanism in autoimmune demyelination [Porter et al., 2020]. Arachidonic acid is a polyunsaturated fatty acid (PUFA) that can be oxidized to eicosanoids by COX, LOX, and cytochrome P450 enzymes that modulate inflammatory responses. Skin metabolomics studies found increased concentrations of arachidonic acid in psoriatic skin, suggesting a shift toward LOX-mediated leukotriene production may play a pathogenic role in psoriasis [Yan et al., 2017]

1.2.Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a condition characterized by excessive hepatic triglyceride (TG) accumulation, in the absence of other liver disease etiologies, such as chronic liver diseases, use of medications that induce steatosis or alcohol consumption (>20 g ethanol per day for women, >30 g ethanol per day for men). NAFLD is associated with insulin resistance and is characterized by steatosis in

>5% of hepatocytes [EASL Guidelines 2016]. It is the most common liver disease and consists a serious public health issue in Western societies and around the world [Wree et al., 2013].

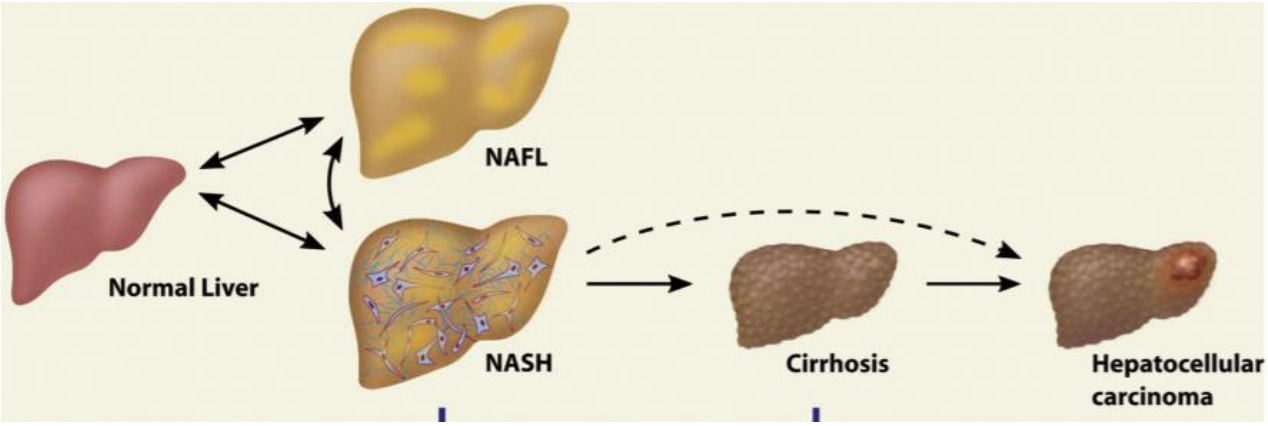


Figure 1.5 NAFLD spectrum [Cohen et al., 2011].

NAFLD includes two pathologically distinct conditions: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) which covers a wide spectrum of disease severity [Fig 1.5]. NAFLD may worsen to NASH (12 to 40 %), further progress to cirrhosis (15 to 25 %) and/or to hepatocellular carcinoma (HCC) (7 %) [Saponaro et al., 2015]. HCC can develop even in the absence of cirrhosis, contrary to other liver diseases of different aetiologies such as alcohol-related or autoimmune liver disease and may contribute to late diagnosis and management. NAFLD is regarded as a manifestation of the metabolic syndrome and obesity and insulin resistance are strongly associated with it. The above lead to chronic inflammation, lipid metabolism dysregulation and a pro-carcinogenic state that promotes HCC [Huang et al., 2020].

1.2.1. Epidemiology

Estimates of NAFLD prevalence vary in the general population. NAFLD is most common in Western societies, with a prevalence of 17–46% in adults, parallel to the prevalence of MetS and depending on the diagnostic method, age, sex and ethnicity [Fig. 1.6] [Younossi et al., 2018]. A meta-analysis of studies from 2006–2014 estimated NAFLD prevalence of 24% (20–29%) in the general population, with a variation ranging from 13% in Africa to 42% in Southeast Asia [Estes et al., 2018, Huang et al., 2020]. NAFLD incidence is 20-86⁄1000 person-years based on liver enzymes and/or on ultrasound, and 34⁄1000 person-years based on 1H-MRS [Younossi et al., 2018]. The rate of NASH has almost doubled during the last years (59.1% in 2010 versus

33% in 2005) [Younossi et al., 2018] and its prevalence is estimated to increase by up to 56% between 2016 and 2030 in China, France, Germany, Italy, Japan, Spain, UK and USA [Estes et al., 2018].

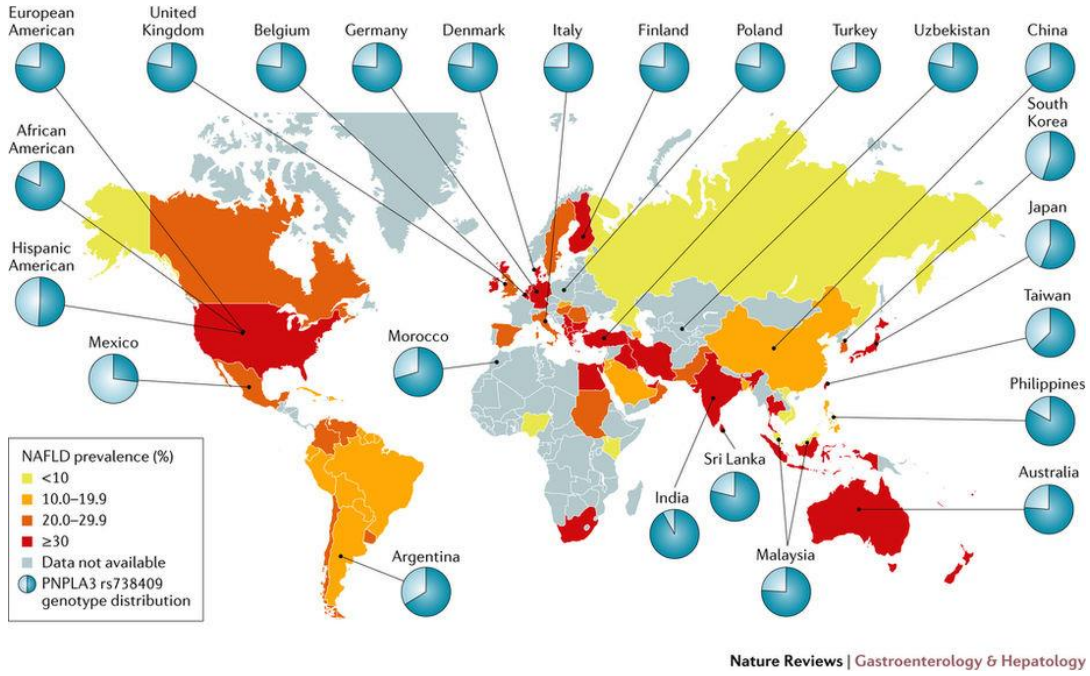


Figure 1.6. Worldwide estimated prevalence of NAFLD and distribution of Patatin-like phospholipase domain-containing protein 3 (PNPLA3) genotypes [Younossi et al., 2018]

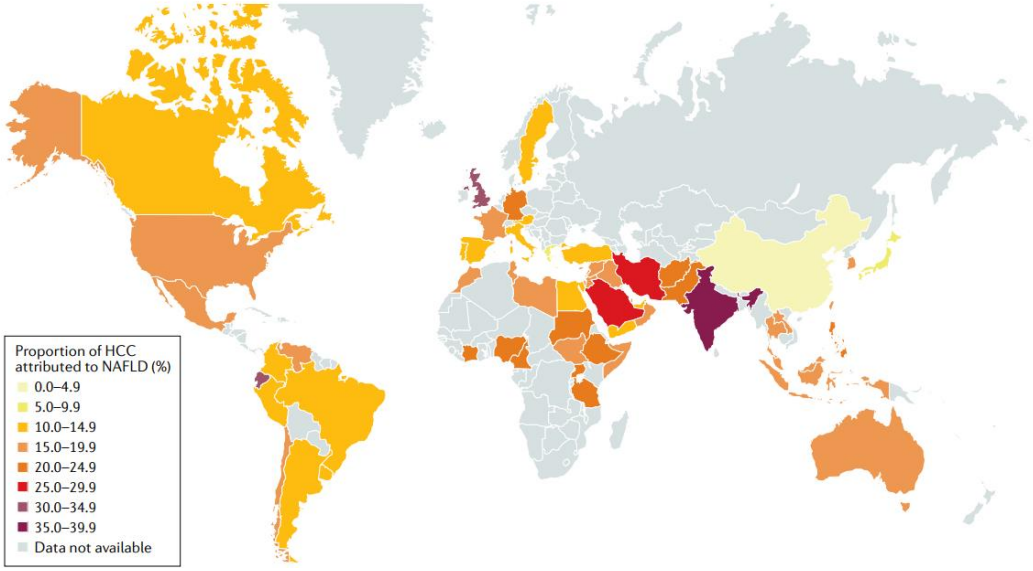


Figure 1.7. The estimated proportion of HCC attributed to NAFLD worldwide [Huang et al., 2020]

The prevalence of NAFLD increases with age, with higher values in males between 40-65 years and associates with body mass index (BMI) with a prevalence of 7%, in normal weight, 65% in class I, II obesity and 85% in morbid obesity. Patients with diabetes mellitus have prevalence between 10-75%, and with hyperlipidemia between 20-92% [Bellentani et al., 2010].

The global prevalence of patients with HCC attributable to NAFLD varies between 1% and 38% [Fig. 1.7] in different countries with those with a higher proportion of NAFLD-related HCC exhibiting a higher prevalence of obesity [Huang et al., 2020].

1.2.2. Pathogenesis

Several theories have been proposed to explain the pathogenesis of NAFLD, the most established being the "Two Hit Theory" and to the "Multiple Parallel Hit Theory". According to the two hit theory, the first hit is lipid accumulation in liver along with environmental factors, obesity and insulin resistance, which sensitize the liver to 'second hits' which activate inflammation and fibrosis. The complexity of NAFLD with factors acting synergistically lead to the multiple-hit hypothesis [Fig. 1.8].

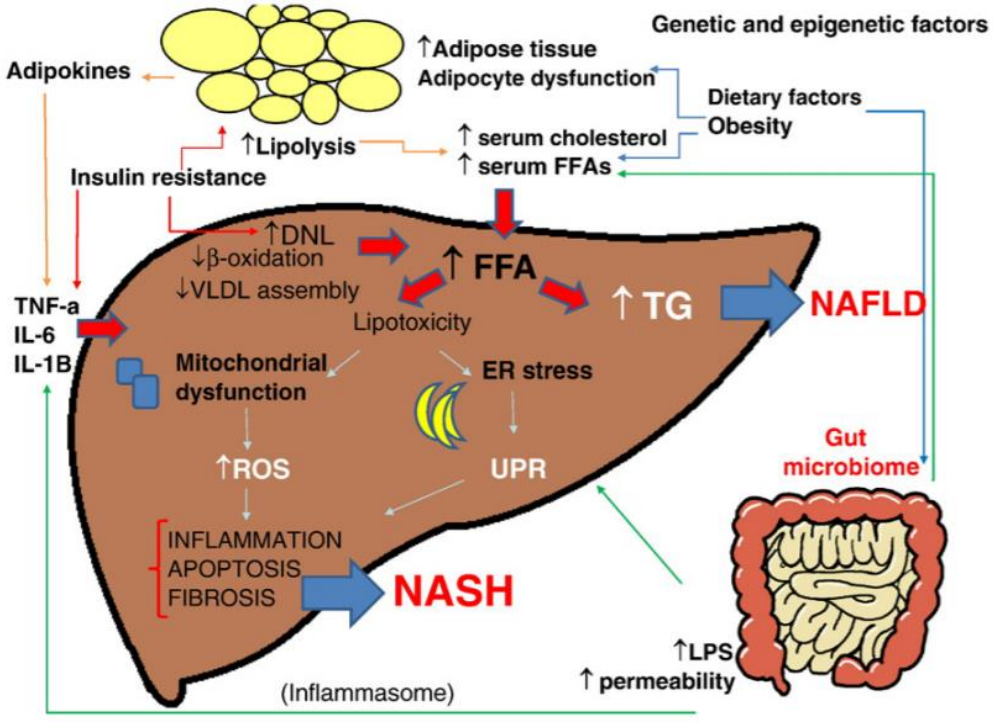


Figure 1.8. Multiple hit hypothesis for the development of NAFLD [Buzzetti et al., 2016]

More specifically, diet, environment and obesity increase the levels of free fatty acids and cholesterol in serum, leading to insulin resistance, adipocyte dysfunction and changes in gut microbiome. The above trigger triglycerides synthesis and accumulation, toxic levels of fatty acids, and other lipid metabolites which cause mitochondrial dysfunction, production of ROS and endoplasmic reticulum (ER) stress, resulting in hepatic inflammation. At the same time, changes in gut microbiome and epithelial permeability, as well as, raised circulating levels of inflammatory molecules, such as LPS, induce further inflammation and trigger ER stress and apoptosis. Finally, genetic and epigenetic factors affect lipid levels, enzymatic processes and inflammatory pathways, finally inducing progression to inflammation and fibrosis (NASH) [Buzzetti et al., 2016].

1.2.2.1. Lifestyle effect in NAFLD

NAFLD is closely linked to lifestyle factors, such as excessive calories accompanied with reduced physical activity. During the last decades unhealthy lifestyle changes in Western world have increased BMI and the prevalence of obesity, which are main pathophysiological drivers of NAFLD [Hallsworth and Adams, 2019].

Increased calorie intake and sedentary lifestyle have been associated with insulin resistance and metabolic disorders and therefore with NAFLD. Macronutrient intake has also been examined in NAFLD with high cholesterol and carbohydrate diet being correlated with NAFLD. Diets enriched in saturated fats and low in omega-3 PUFA increase liver fat accumulation. High fructose consumption increases the risk of NAFLD [Rinella et al., 2016]. Physical activity levels are also associated with the disease. In particular, increased sedentary behavior and lower levels of physical activity, which are higher in people with a predisposition in metabolic disorders, are considered independent risk factors for NAFLD. Although exercise may have a significant effect on liver fat content (up to 30%), weight loss has the most prominent effect in liver fat reduction (up to 80%) [Romero-Gomez et al., 2017].

1.2.2.2. Genetic and epigenetic factors in NAFLD

Before genome-wide association studies (GWAS), various SNPs involved in the metabolic syndrome were thought to be implicated in the pathogenesis of NAFLD as well. Genes related to

a) insulin resistance, b) hepatic free fatty acid metabolism, c) liver fibrogenic pathways and d) endotoxin receptors and oxidative stress responses were reported as candidate genes for NAFLD susceptibility [Li et al., 2012]. GWAS studies changed our understanding on genetics of NAFLD. The first reported GWAS on NAFLD was by Romeo et al. (2008), in a multiethnic population-based study, called the Dallas Heart Study. A single variant in PNPLA3 (rs738409) was strongly associated with hepatic fat content, even after adjusting for BMI, diabetes, ethanol use, global and local ancestry, showing that this association was not attributed to these risk factors or population stratification . PNPLA3 encodes a 481 amino acid protein that belongs to the patatin-like phospholipase family which exhibits lipid acyl hydrolase activity [Romeo et al., 2008]. I148M substitution cancels hydrolase activity by blocking the access of the substrate to the enzyme's active site resulting in TG and retinol accumulation [He et al., 2010].

Other well studied SNPs related to NAFLD/NASH are transmembrane 6 superfamily member 2-TM6SF2 (rs58542926), glucokinase regulator-GCKR (rs780094) and membrane Bound O-Acyltransferase Domain Containing 7- MBOAT7 (rs626283). The TM6SF2 E167K variant associates with lower TM6SF2 function, which results in reduced VLDL secretion, increased hepatic triglyceride content, lower ALP activity and lower expression of some lipid metabolism-related genes [Chen et al., 2015]. GCKR, regulates the activity of glucokinase, a phosphorylating enzyme which regulates hepatic glucose metabolism and activates hepatic lipogenesis [Tan et al., 2013]. MBOAT7 rs641738 T allele is associated with lower expression levels of the protein resulting in changes in the hepatic phosphatidylinositol acyl-chain remodeling and increased hepatic fat content [Campo et al., 2018].

Epigenetic modifications are involved in NAFLD pathogenesis and specifically in lipid metabolism, insulin resistance, ER stress, mitochondrial damage, oxidative stress, and inflammation. Epigenetic dysregulations may induce hepatic lipid accumulation and eventually NAFLD and NASH [Sun et al., 2015]. A significant association between the presence of NAFLD and hepatic CpG methylation in promoters of Peroxisome proliferator activated receptor (PPAR-γ) coactivator 1α and mitochondrial transcription factor A was observed in NAFLD patients [Sookoian et al., 2010]. Alterations in the expression of DNMT1 and DNMT3A in the livers of mice fed with lipogenic methyl-deficient diet predetermine susceptibility to hepatic steatosis [Pogribny et al., 2009]. In a study of 100 human frozen biopsies, 69,247 differentially methylated

CpG sites (76% hypomethylated, 24% hypermethylated) were found in patients with advanced NAFLD comparing with those of mild NAFLD, with mainly tissue repairing genes being hypomethylated and genes of metabolic pathways, like one carbon metabolism being hypermethylated [Murphy et al., 2013]. Serine/threonine kinase salt-inducible kinase 2 knockdown mice exhibit overexpression of HAT activator p300 which results in hepatic steatosis, insulin resistance, and inflammation phenotypes. HAT p300 is a regulator of Carbohydrate-responsive element-binding protein, a transcriptional activator of lipogenic and glycolytic genes [Bricambert et al., 2010].

Alterations in micro-RNAs implicated in glucose, cholesterol regulation and lipid metabolism have been associated with the pathogenesis of NAFLD and its progression to NASH. MiR-122 is one of the first microRNAs correlating with lipid metabolism, and its inhibition results in a 25-30% reduction of plasma cholesterol levels and in differentiated expression of hepatic genes involved in cholesterol biosynthesis. Also, it inhibits hepatic stellate cell activation and collagen deposition [Panera et al., 2014]. In mice fed with methyl-deficient diet (animal models for NASH), hepatic over-expression of miR-34a, miR-155, miR-200b and miR-221 and downregulation of miR-29c, miR-122, miR-192 and miR-203 have been reported [Pogribny et al., 2010]. MiR-155 and miR-200 b have been linked with downregulation of EZH2 protein in both in vivo and in vitro models of NAFLD [Vella et al., 2013]. Over-expression of miR-10b increases cellular lipid content acting on PPARα, a nuclear receptor involved in the catabolism of fatty acids [Horton et al., 2002].

Circulating microRNAs can be promising diagnostic and prognostic biomarkers, given that they are easily accessible and structurally stable in almost all body fluids. Yamada et al. [2013] have found increased serum levels of miR-34a, miR-122, miR-21 and miR-451 in NAFLD patients compared to healthy individuals and Cermelli et al. [2011] have shown increased miR-122 and miR-34a serum levels in NASH patients along with a positive correlation with fibrosis and inflammation stage. In the same study, a positive correlation between increased circulating miR-16 and simple hepatic steatosis was highlighted. MiR-34 implication in liver damage may be attributed to induction of pro-apoptotic genes and p53 transcription. In a recent study, aimed at identifying miRNAs that associate with disease severity using 14 published studies, nine

microRNAs (miR34a, -192, -27b, -122, -22, -21, -197, -30c and -16) were altered in different stages of disease severity and fibrosis [López-Riera et al., 2018].

1.2.2.3. The role of gut microbiome in NAFLD

There is a strong interaction between gut-liver axis and microbiota. Due to its anatomical position, liver receives 70% of its blood supply from the gut through the portal vein, so it constitutes the first defense line against antigens. Also it is the most exposed organ to bacteria and bacterial byproducts of the gut [Miele et al., 2009]. This link has been shown in both experimental models and humans. Transplantation of normal microbiota to GF mice induced 60% increase in body fat and a two-fold increase in hepatic triglyceride content [Aron-Wisnewsky et al., 2013]. Furthermore, patients who underwent intestinal bypass, developed NASH which regressed after antibiotic treatment [Drenicke et al., 1982].

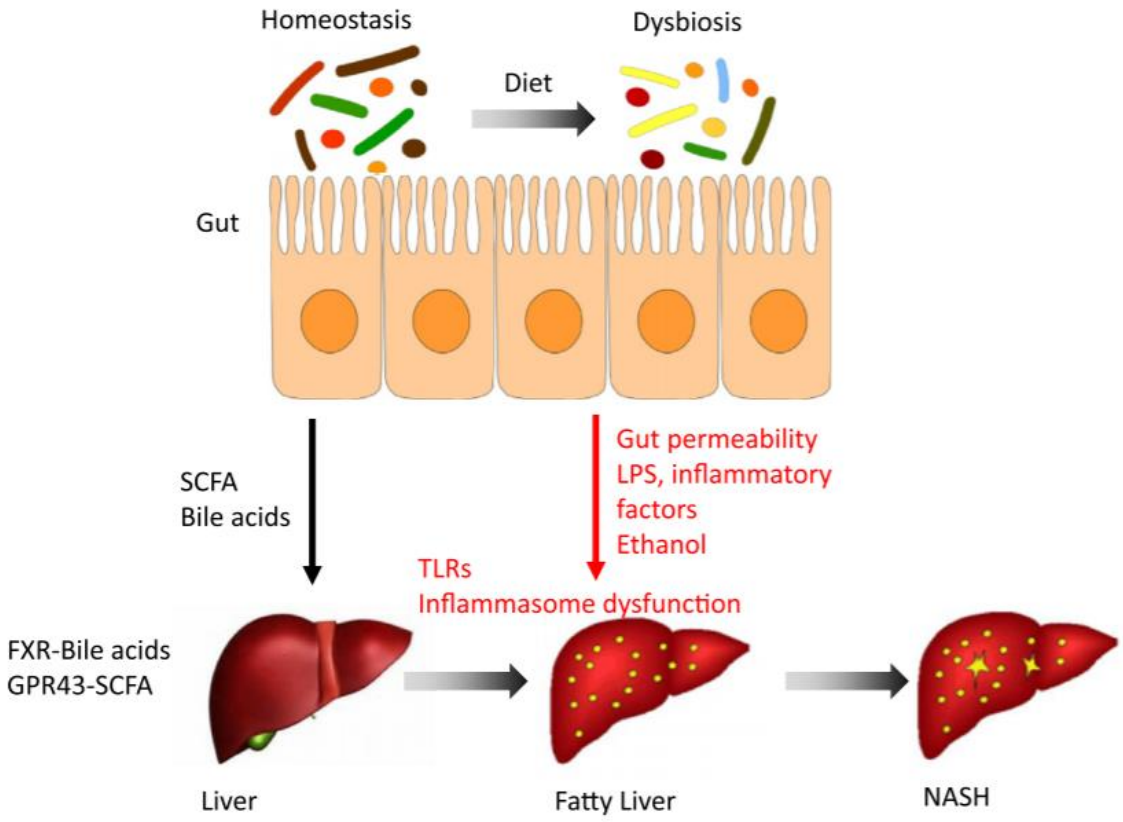


Figure 1.9. Suggested mechanisms for the effect of gut microbiome in NAFLD development and progression to NASH [Bashiardes et al., 2016]

Several mechanisms have been proposed for the role of gut microbiota in NAFLD, including intestinal barrier dysfunction, inflammatory responses and metabolites produced by microbiota such as SCFAs, bile acids, and ethanol [Fig. 1.9] [Bashiardes et al., 2016]. Microbiome dysbiosis is common in NAFLD and is related to increased intestinal permeability, leading to bacterial translocation and contributing to hepatic inflammation [Marra and Svegliati-Baroni, 2018].

Tight junction proteins in intestinal endothelial cells prevent translocation of toxic substances from the gut into the portal system. Dysbiosis disrupts tight junctions, exposing the liver to bacterial products [Leung et al., 2016]. For example, in high-fat diet rats, hepatic steatosis is associated with increased intestinal permeability and translocation of bacterial LPS from Gram-negative bacteria [Mao et al., 2015]. Inflammasome is a multiprotein oligomer, responsible for triggering inflammation, consisting of caspases, NLRs and Interferon-inducible protein AIM2-like receptors. It has been shown that hepatic influx of saturated fatty acids and LPS from gut, may induce inflammasome activation in NAFLD experimental model, namely methionine choline deficient diet mice which are models for NAFLD [Yang et al., 2016].

Several bacterial genera, families and phyla have been found to be differentially expressed in NAFLD patients comparing to healthy control. These microbes are implicated in the disease pathogenesis through different pathways, such as increased intestinal permeability (i.e. Lachnospiraceae), decreased short chain fatty acids (SCFA) production (i.e. *Faecalibacterium*) and elevated serum endotoxin production (i.e. *Bacteroides,* Enterobacteriaceae) [Quesada-Vázquez et al., 2020, Svegliati-Baroni et al., 2020].

In most studies when comparing NAFLD patients with healthy controls, similar altered microbiome signature patterns are observed. At the level of phylum increased Proteobacteria, at the level of family increased Enterobacteriaceae and decreased Rikenellaceae and Ruminococcaceae, and at the level of genera increased *Escherichia, Dorea, Peptoniphilus* and decreased *Anaerosporobacter, Coprococcus, Eubacterium, Faecalibacterium* and *Prevotella*. Similarly, NASH patients compared to healthy controls, show increased Proteobacteria, Enterobacteriaceae and *Dorea* and decreased Ruminococcaceae, Rikenellaceae *Faecalibacterium, Coprococcus* and *Anaerosporobacter*. Although some bacteria exhibit same

patterns in different studies, some of them displays opposite trends in their abundance across the literature, as shown in Fig. 1.10. Discrepancies between studies may be due to the heterogeneity of geographical regions, ethnicity, population characteristics, microbiome sequencing tools, NAFLD diagnostic tools, disease spectrum, drug consumption and circadian rhythm and results should be interpreted with caution [Aron-Wisnewsky et al., 2020].

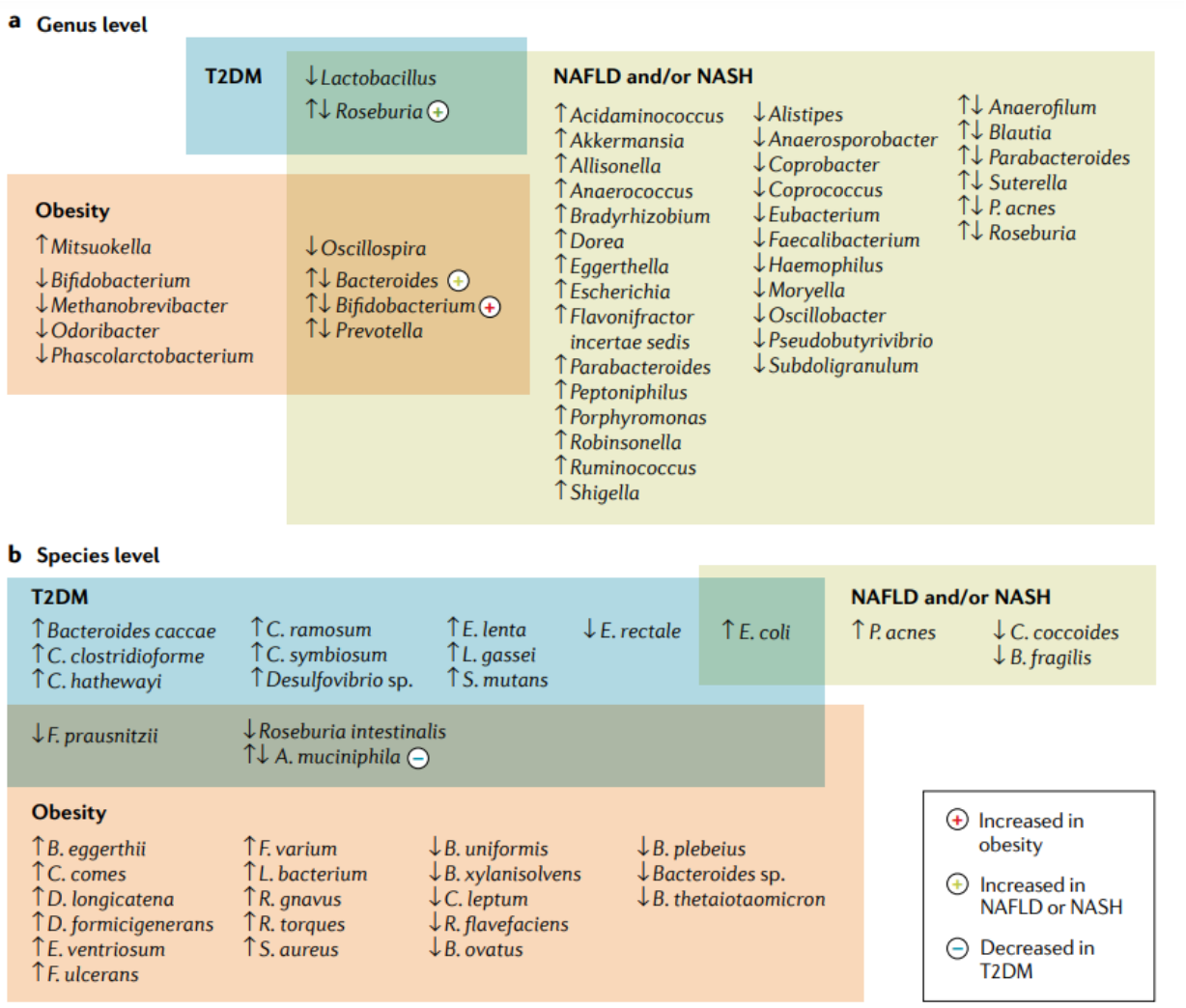


Figure 1.10. Overlapping microbiota species and genera signatures in NAFLD, diabetes and obesity [Aron-Wisnewsky et al., 2020]

1.2.2.4. The role of immunity and inflammation in NAFLD

One of the key factors in the pathogenesis of NAFLD and the progression to NASH are innate immunity activation and inflammation. Liver immune cells recognize pathogens or cell

damage signals derived from the adipose tissue or gut, related to changes in the microbial balance and/or bacterial translocation and promote the inflammatory response. As a result, cell injury and death occurs, promoting disease progression [Arrese et al., 2016].

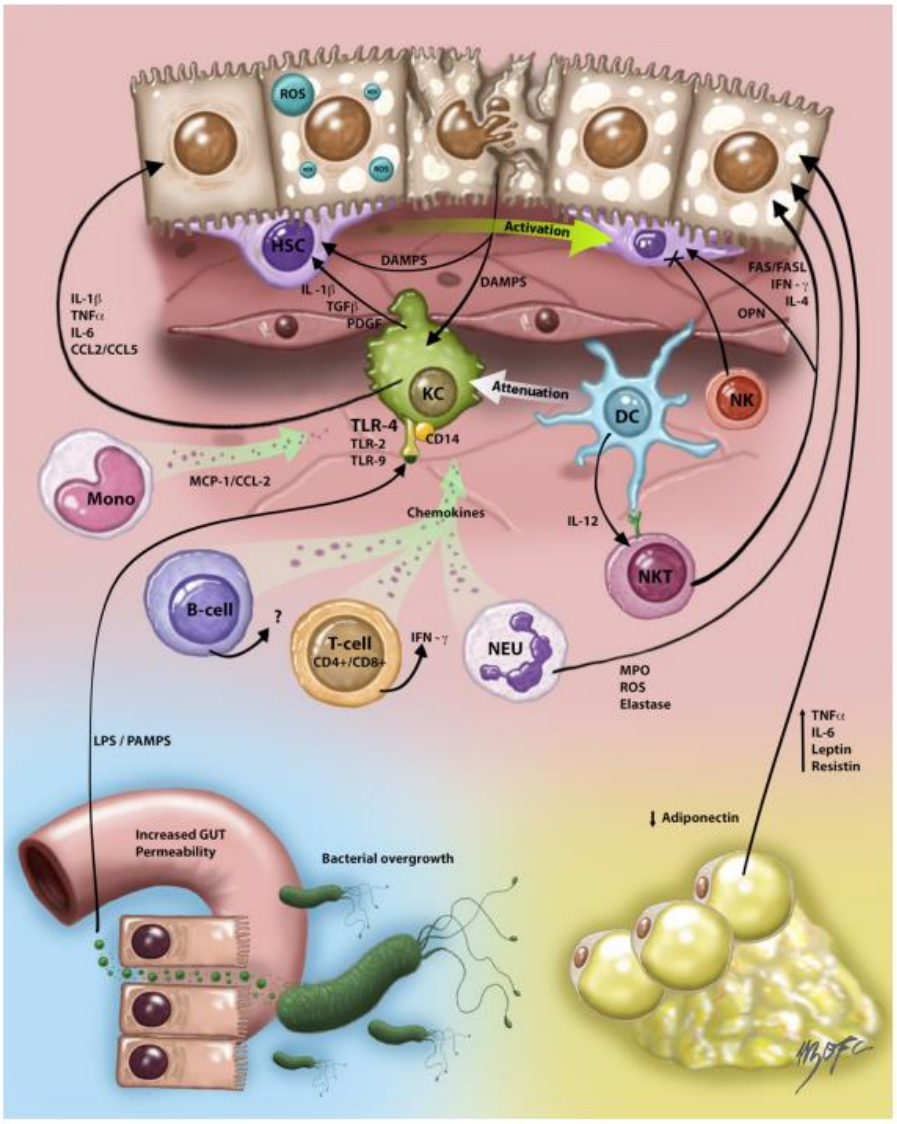


Figure 1.11. Immune and inflammatory responses in NAFLD/NASH [Arrese et al., 2016]

Several endogenous and exogenous inducers have been described in NAFLD. Nuclear factors, nuclear and mitochondrial DNA, purine nucleotides and uric acid have been identified for their role as DAMPs in the setting of the disease and can be released by fat overload which induces lipotoxicity [Ganz et al., 2013]. These DAMPs along with PAMPs (LPS and other gut derived bacterial products via the portal circulation because of the altered intestinal barrier-‘‘leaky gut’’) bind to PRRs, triggering a local inflammatory response and creating an injury

amplification loop [Fig 1.11]. TLRs are the best characterized PRRs in NAFLD and are expressed in most liver cells (hepatocytes, Kupffer cells, hepatic stellate cells and others) [Seki et al., 2008]. Activated Kupffer cells produce proinflammatory cytokines, such as TNFa, IL-1b and IL-6 which contribute to injury and necrosis. TGF-b secretion promotes fibrosis development on hepatic stellate cells and monocytes recruitment amplifies Kupffer cells activation through the production of monocyte chemoattractant protein-1 (MCP-1) [Arrese et al., 2016]. Other immune cells contribute as well, such as NKT cells which are depleted during steatosis development but increase in more progressive stages, contributing to inflammation and fibrosis [Tajiri et al., 2012]. Adipose tissue secretes abnormal level of adipokines, such as high leptin, and resistin, which contribute to inflammation and low adiponectin which has an antiinflammatory and insulin-sensitizing role [Parker, 2018].

Although innate immunity plays a key role in the development of hepatic inflammation in NASH, nowadays there is increasing evidence supporting the role of adaptive immunity in liver inflammation as well [Sutti et al., 2020]. In 60% of NASH patients, B and T lymphocytes are detected and their size and prevalence are higher in patients with more severe lobular inflammation and fibrosis [Bruzzi et al., 2018]. CD4+ T helper cells are recruited to the liver in response to inflammatory signals and differentiate to IFNγ-producing T helper 1 (TH1) cells and to type 17 T helper (TH17) cells which produce the IL-17 family cytokines with a complex role in NASH [Sutti et al., 2014, Tang et al., 2011]. Also, cytotoxic CD8+ T cells are accumulating in liver in response to IFN signals and promote insulin resistance and liver glucose metabolism as shown in mice receiving a high-fat diet [Ghazarian et al., 2017]. Finally, B lymphocytes are infiltrating NASH liver biopsy samples maturing to plasma cells and producing IgM natural antibodies or highly antigen-specific IgA, IgG or IgE [Tsiantoulas et al., 2015]. Moreover, they show a profibrogenic activity as mice lacking mature B cells exhibit reduced fibrosis, collagen deposition, immune cells infiltration, and α-smooth muscle actin (α-SMA) expression [Thapa et al., 2016].

1.2.3. Diagnosis and NAFLD treatment

Most patients with NAFLD are asymptomatic or exhibit non specific symptoms. Liver function tests (such as aspartate aminotransferase AST and alanine aminotransferase ALT) may

be mildly elevated but not in all patients and does not reflect the disease severity. Radiological imaging, like ultrasound and magnetic resonance imaging are mainly used for diagnosis.

Liver biopsy is considered the “gold standard” in diagnosis and prognosis of NASH as it provides a validated grading and staging [Hassan et al., 2014], but it is expensive, invasive, with variable results and procedural complications. NAFLD diagnosis can be relied on imaging techniques of which Magnetic Resonance Imaging (MRI) is the gold standard [EASL Guidelines 2016].

Non-invasive predictive algorithms have been suggested as non-invasive diagnostic tools. The most studied scoring system is NAFLD fibrosis score (NFS) which includes age, BMI, insulin sensitivity, aminotransferase levels, platelets and albumin (-1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m2) + 1.13 × Impaired Fasting Glucose/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio – 0.013 × platelet (×109/l) – 0.66 × albumin (g/dl)) and has two cutoff points dividing patients to three categories (< -1.455 low probability of advanced liver fibrosis, >0.675 high probability of advanced liver fibrosis and e -1.455 – 0.675 indeterminate score). Other validated scores are Fibrosis-4 (FIB-4) which uses only age, aminotransferase levels and platelets (Age x AST) / (Platelets x (sqr (ALT)). Finally a new NASH score is developed (NASH Score= -3,05 + 0,562 x PNPLA3 genotype (CC=1, GC=2, GG=3) – 0,0092 x insulin(mU/L) + 0,0023 x AST (IU/L) + 0,0019 x (insulin x AST)) from a Finish population based study by Hyysalo et al, which includes not biochemical parameters, but also PNPLA3 genotype. The cut-off point of this score is -1.054 with a sensitivity of 71,6% and a specificity of 73,5%, for detecting advanced fibrosis [Hyysalo et al., 2013, Angulo et al., 2007]. The above scoring systems are based on routine, inexpensive clinical and biochemical parameters [Stefan et al., 2019], but lack diagnostic accuracy.

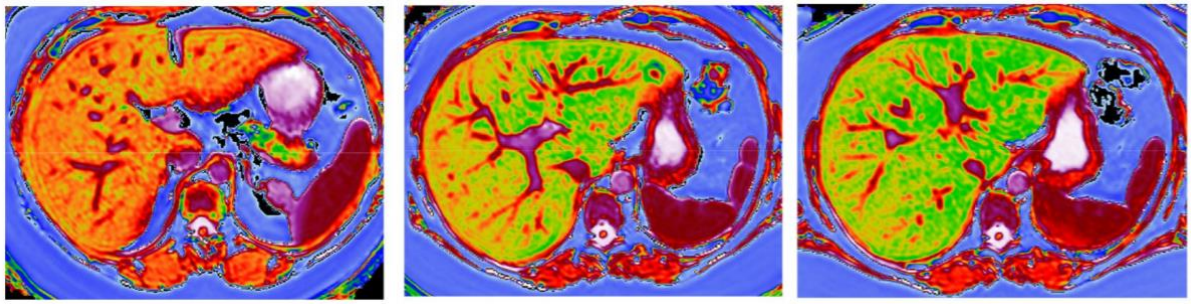


Figure 1.12. Representative LiverMultiScan images [Harrison et al., 2020]

On the contrary, MRI allows for high sensitivity and specificity in the detection of histologically confirmed steatosis, ranging from 76.7%-90.0% and 87.1%-91% respectively [Li et al., 2018]. Rajarshi et al have developed a novel scanning method with high accuracy for the assessment of liver fibrosis and steatosis using a software called LiverMultiscan (Perspectum Ltd, UK) [Pavlides et al., 2016]. LiverMultiScan is used for the quantification of fibrosis and inflammation using metrics such as changes in proton density fat fraction (PDFF), hepatic iron, iron-corrected (cT1) and Liver Inflammation Fibrosis score (LIF) [Fig. 1.12] [Harrison et al., 2020].

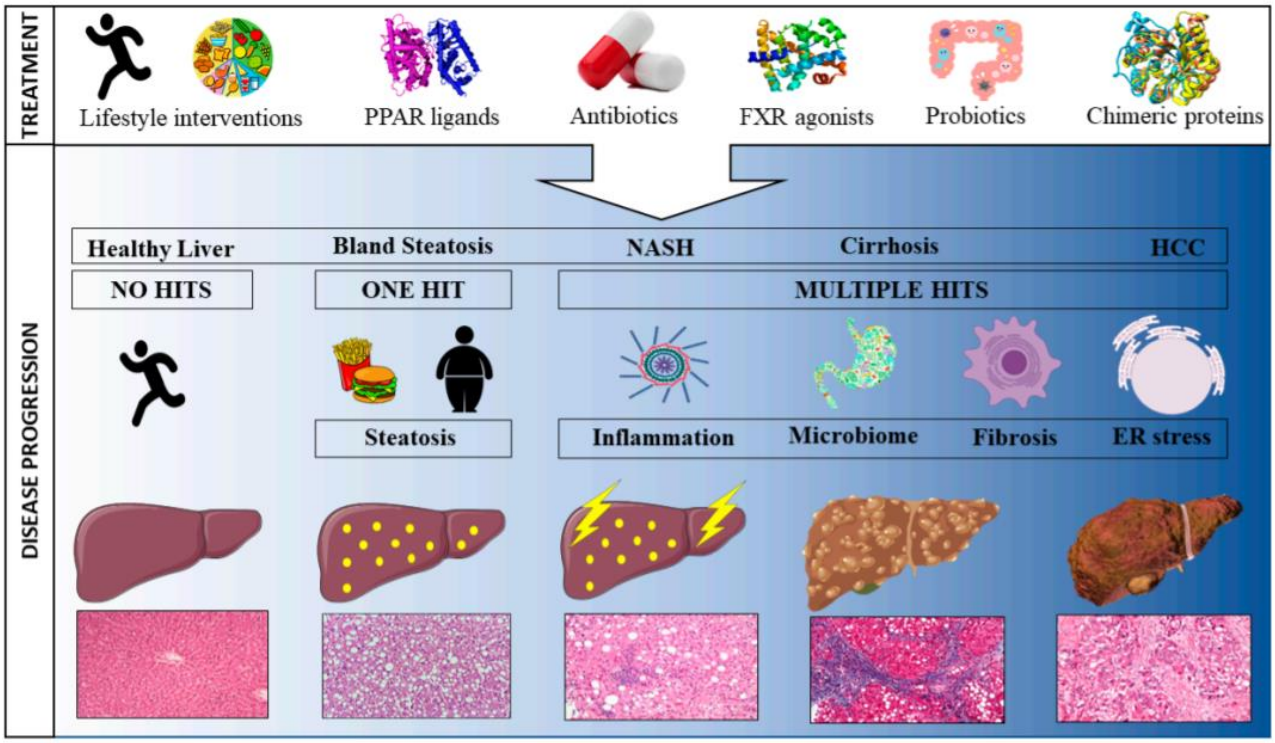


Figure 1.13. Potential treatments to attenuate disease progression [Smeuninx et al., 2020]

There is no specific medical treatment in NAFLD. The complex nature of the disease, where “multiple hits” cooperate, makes it difficult to handle the condition by targeting one risk factor. So, most treatments aim to multiple underlying mechanisms [Fig. 1.13]. Treatment against steatosis aims the two major mechanisms of elevated hepatic lipid synthesis, increased hepatic fatty acid delivery due to increased adipose TG lipolysis and de novo lipogenesis [Smeuninx et al., 2020]. For example, antagonists induce hepatic lipid clearance, as PPARα is a key regulator of fatty acid uptake, beta oxidation, ketogenesis, bile acid synthesis, and triglyceride turnover [Liss and Finck, 2017]. On the other hand, as fibrosis stage is predictive of

mortality, its reduction is a main objective of NASH-related therapeutics. Farnesoid X receptor (FXR) is a super family member of nuclear receptors whose activation regulate bile acid, lipids, cholesterol, and glucose homeostasis and can lead to fibrosis attenuation [Zhang et al., 2019].

Patients without fibrosis are at low risk of liver diseases within 10-15 years, but have a high risk of having or developing other metabolic conditions, such as T2DM, so it is important to manage their cardiovascular risk. Usually, insulin sensitizing agents (i.e metformin and pioglitazone), and lipid lowering drugs are used in this direction. On the other hand, body weight management is considered the keystone of treatment in all patients with NAFLD. Weight loss may reduce steatosis or biopsy score, whereas a considerable weight reduction, like the one occurring in bariatric surgery, may even result in resolution of NASH [Rinella et al., 2016]. Finally, as oxidative stress plays a crucial role in NAFLD, several therapeutic strategies with antioxidants have been proposed with vitamin E being the most studied. In the PIVENS study, there was a significant reduction in liver ballooning and inflammation, with no significant change in fibrosis. However, supplementation with vitamin E must be carried out with care, as it may be associated with increased risk of heart conditions and mortality [Hadi et al., 2018].

1.2.4. Metabolomics in NAFLD

The invasiveness and sampling variability of liver biopsy, the lack of a widely approved therapy, as well as the lack of evidence of the optimal diet and level of exercise requirement makes the discovery of new biomarkers of NAFLD for diagnosis, staging, and assessment of efficacy of new drugs an important scientific goal. Metabolomics may contribute towards this aim since they can be applied for the early diagnosis of the disease, the exploring of disease pathophysiology, the monitoring of NAFLD and the development of new drugs [Gitto et al., 2018].

Metabolic alterations are well described in NAFLD. First of all, lipid accumulation plays a dominant role in the pathogenesis of the disease, as lipid imbalance is often observed in blood and in liver tissue [Gitto et al., 2018]. Lipidomics is a subcategory of metabolomics oriented to the quantification of different lipids in cells, organs and body fluids using innovative techniques, therefore it is considered very important in the field of NAFLD [Buechler., 2014]. Mice with fatty liver have high hepatic concentrations of LPC, lysophosphatidylethanolamine (LPE), and

phospocholine (PC) species. [Van Ginneken et al., 2007]. In 2007, capillary gas chromatography was used to quantify and compare free fatty acids (diacylglycerol (DAG), triacylglycerol (TAG), free cholesterol (FC), cholesterol ester, and phospholipid contents) in normal and NAFLD livers. DAG and TAG increased significantly in NAFLD, but FFA remained unaltered between the two groups. Although the levels for linoleic acid and linolenic acid remained unaltered, there was a decrease in arachidonic acid, eicosapentanoic acid and docosahexanoic acid in NASH [Puri et al., 2007]. In a double-blinded study of patients with different stages of NAFLD a panel of 20 plasma metabolites that includes glycerophospholipids, sphingolipids, sterols, and various aqueous small molecular weight components involved in cellular metabolic pathways differentiated between NASH and steatosis [Gorden et al., 2015].

Metabolomics have also confirmed dysregulation in glucose metabolism, amino acids and bile acids levels in NAFLD. Global metabolomics analysis of the plasma profile of 35 NAFLD and NASH patients revealed higher levels of glycocholate, taurocholate, and glycochenodeoxycholate in subjects with NAFLD and higher glutamyl amino acids levels in both NAFLD and NASH compared with controls [Kalhan et al., 2011]. Urinary metabolomics in NAFLD and NASH patients revealed differences in 31 metabolites. Pathway analysis showed that the most strongly associated pathways are related to energy and amino acid metabolism, as well as to pentose phosphate pathway [Dong et al., 2017].

Other metabolite markers metabolic pathways altered in NAFLD or contributing in distinguishing different stages of NAFLD have been investigated as well. For example, betaine, a metabolite of choline, was found lower in NASH comparing to NAFLD patients and its levels were associated with disease severity, liver inflammation, ballooning degeneration and fibrosis [Sookoian et al., 2017]. Recently, a metabolite profiling using a combination of liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy created metabolite profile clusters for patients with NAFL, early NASH, and advanced NASH. This study was performed in 57 patients and 237 metabolites were targeted. The metabolites that differed significantly between the three patient categories are presented in Fig 1.14. Interestingly, the clustering between NAFL and NASH reveals different metabolites signature between these groups and some metabolites can identify the advanced NASH patients

[Ioannou et al., 2020]. The above emphasize the significance of the use of Metabolomics in discovering new NAFLD biomarkers and the need of more research in this field.

NASH vs. NAFL				Early NASH vs. NAFL			
Metabolite	***p* Value**	**Fold * Change**	**Method**	**Metabolite**	***p* Value**	**Fold * Change**	**Method**
Acetylglycine	0.03	0.57	MS	Hydroxyphenylpyruvate	0.002	0.83	MS
Cysteine	0.04	0.88	MS	Inositol	0.03	0.86	MS
Alanine	0.02	0.96	NMR	Cysteine	0.04	0.87	MS
Glucose	0.04	1.16	MS	Acetylcarnitine	0.04	0.90	MS
Erythrose	0.02	1.18	MS	Phenylalanine	0.03	1.12	NMR
Tyrosine	0.01	1.18	NMR	Tyrosine	0.02	1.18	NMR
Isovaleric acid	0.02	1.25	MS	Erythrose	0.04	1.18	MS
Leucic acid	0.04	1.28	MS	Alanine	0.03	1.18	NMR
Xanthine	0.02	1.49	MS	Tryptophan	0.04	1.19	NMR
Oxypurinol	0.01	1.54	MS				
Glycochenodeoxycholate	0.04	3.13	MS				
Advanced NASH vs. Early NASH				**Advanced NASH vs. NAFL**			
Metabolite	***p* Value**	**Fold * Change**	**Method**	**Metabolite**	***p* Value**	**Fold * Change**	**Method**
Spermidine	0.005	0.49	MS	Spermidine	0.005	0.33	MS
Oxaloacetate	0.01	0.85	MS	Acetylglycine	0.01	0.48	MS
Orotate	0.0009	0.85	MS	Glucose	0.04	1.20	MS
Linoleic acid	0.01	1.32	MS	Isovaleric acid	0.04	1.30	MS
Linolenic acid	0.01	1.33	MS	Leucic acid	0.02	1.30	MS
2-hydroxyglutarate	0.01	1.33	MS	2-hydroxyisovaleric acid	0.03	1.49	MS
				Xanthine	0.04	2.08	MS
				Oxypurinol	0.04	2.17	MS
				Glycocholate	0.02	2.22	MS
				Glycochenodeoxycholate	0.01	2.38	MS

* Fold changes shown are the ratios of NASH/ NAFL; Early NASH/ NAFL; Advanced NASH/ NAFL; Advanced NASH/ Early NASH. They are ordered from the lowest ratio (i.e., most "protective" against advanced disease) to the highest ratio (i.e., most highly associated with advanced disease).

Figure 1.14. Metabolites that differed significantly between patients with NAFL, early and advanced NASH. [Ioannou et al., 2020]

1.2.5. NAFLD nutrigenomics studies

As there are no specific drugs for the treatment of NAFLD, the lifestyle modification remains the most usual treatment approach. The investigation of a) the interplay between nutrition and cellular/genetic processes, b) the effect in potential modulation of clinical outcome by nutritional specific molecular pathways and c) gene and protein expression are crucial. Personalized dietary intervention which takes into account the genetic background of patients may assist in clinical translation, and provide an individualized therapeutic approach to the disease [Dongiovanni et al., 2017].

Diet impacts hepatic de novo lipogenesis by regulating the expression of genes involved in fatty acid synthesis. For example, PUFAs regulate the activity of several transcription factors (such as PPAR and sterol regulatory element binding proteins) which play a major role in hepatic carbohydrate, fatty acid, triglyceride, cholesterol and bile acid metabolism [Jump, 2002]. Fructose delivery in rat hepatocytes impairs the insulin signaling pathway by increasing c-jun N-terminal kinase activity and serine 307 phosphorylation of insulin receptor substrate-1 and reducing tyrosine phosphorylation of insulin receptor substrate-1 [Wei et al., 2005]. Rats fed with low Cu diet had increased hepatic expression of genes involved in inflammation and fibrogenesis, with decreased hepatic and serum Cu promoting lipid peroxidation and inducing NAFLD-like histopathology [Tallino et al., 2015].

Epigenetic manipulation by environmental stimuli, such as diet, may influence certain metabolic pathways such as one-carbon metabolism or NF-kB signaling. Several examples of dynamic changes in epigenetic marks by nutritional interventions have been reported [Lee et al., 2014]. Betaine supplementation in high fat diet mice ameliorated steatosis by down-regulating PPARα, apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTTP) mRNA expression, negatively correlated with DNA methylation of MTTP CpG sites [Wang et al., 2014]. Supplementation of a polyphenol extract derived from red wine in a high fat diet model reduced hepatic TG content with increased miR-122 levels accompanied by decreased Fas cell surface death receptor mRNA levels [Aoun et al., 2010]. High-fat diet increases the expression of miR-103 and miR-107 in mice and this effect is totally reversed by the continuous administration of polyphenols. Also, high fat diet does not alter the expression of miR-122, but dietary polyphenols significantly decrease its expression [Joven et al., 2012]. Lychee pulp phenolics improves liver lipid accumulation by reducing miR-33, which directly modulates ATP binding cassette transporters and carnitine palmitoyltransferase 1 [Su et al., 2017].

1.3.Inflammatory Bowel Diseases (IBD)

IBD is a heterogenous set of chronic, relapsing, and remitting inflammatory diseases affecting the gastrointestinal tract that cause enteric inflammation and often result in debilitating gastrointestinal symptoms [Gu et al., 2019]. Some of the symptoms are abdominal pain with, or without a change in bowel habit, diarrhea, rectal bleeding, and weight loss

[Soubières et al., 2016]. The two main manifestations of IBD are ulcerative colitis (UC) and Crohn's disease (CD) [Mentella et al., 2020]. There is no permanent drug cure and may result in long-term morbidity. Although, UC and CD possess similar characteristics, they are considered distinct conditions as they have different clinical characteristics. UC affects only the colon and is primarily expressed to the mucosal and less to the submucosal compartments. CD may involve any compartment of the gastrointestinal tract from the oral cavity to the anus and may involve all layers of the gut [Mentella et al., 2020, Rubin et al., 2012].

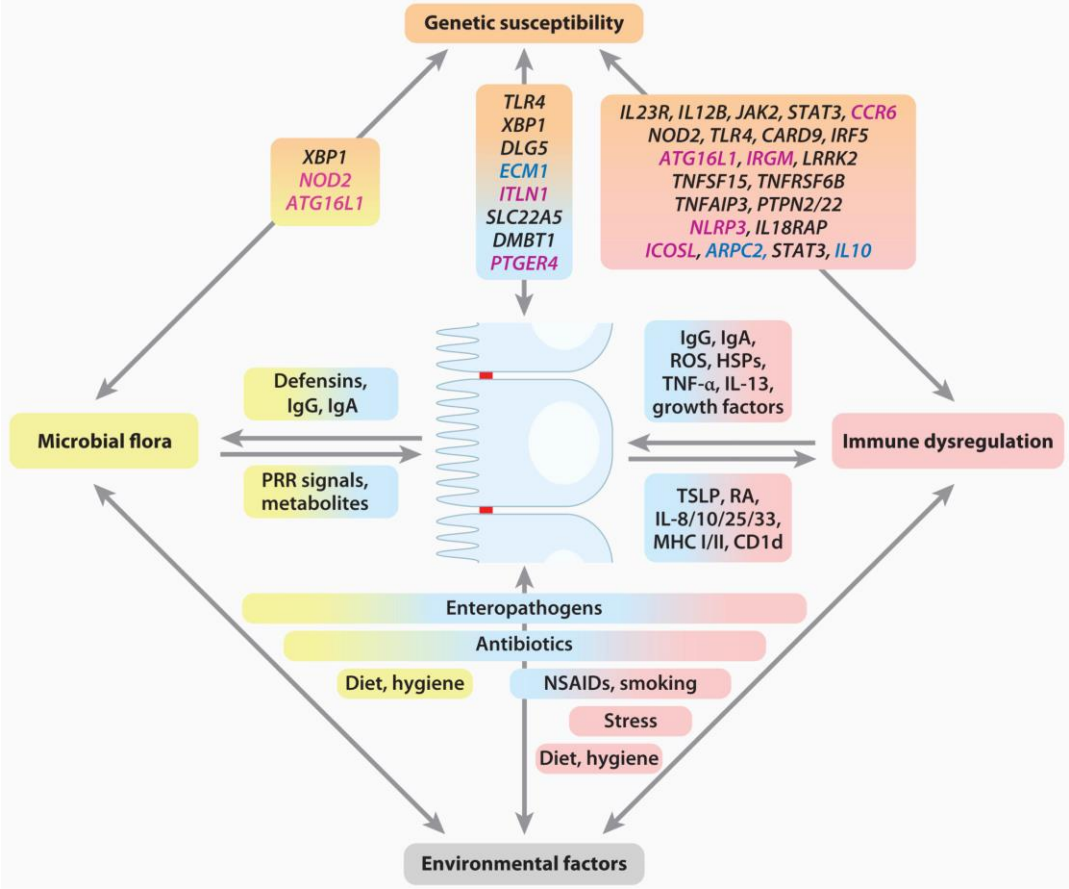


Figure 1.15. Factors affecting the development of IBD [Kaser et al., 2010]

IBD is a multifactorial disorder with several factors contributing to its development. The interaction of a genetically-susceptible host with other factors, such as the intestinal microbiota, the host immune system and several environmental factors affects the development and course of the disease [Kaiser et al., 2010] [Fig 1.15]. IBD is mainly considered a problem of Western societies, with their incidence and prevalence increasing worldwide [M'Koma, 2013].

1.3.1. Epidemiology

The incidence of UC is 0–19.2 per 100,000 in North America and 0.6–24.3 per 100,000 in Europe, and of CD 0–20.2 per 100,000 in North America and 0.3–12.7 per 100,000 in Europe. In Asia and Pacific UC's incidence is 0.76 whereas CD's is 0.54 per 100,000 [Ananthakrishnan, 2015]. Given the chronic nature, early onset and relatively low mortality of the disease, its prevalence increases over time due to the aging population and the increase of IBD incidence [Mak et al., 2020].

The prevalence of IBD is higher in the Western world, affecting up to 0.5% of the general population. The same stands for the incidence of IBD in the Western world, which ranges from 10 to 30 per 100,000 with a higher prevalence of UC than CD [Kaplan, 2015].

In Europe, the incidence has a continent-wide increase and is characterized by a north–south and an east–west gradient. More specifically, CD's incidence is 6.3 and 3.6 per 100,000 in Northern and Southern Europe respectively and UC's incidence is 11.4 and 8.0 per 100,000 in Northern and Southern Europe, respectively [Mak et al., 2020] [Fig 1.16a and 1.16b].

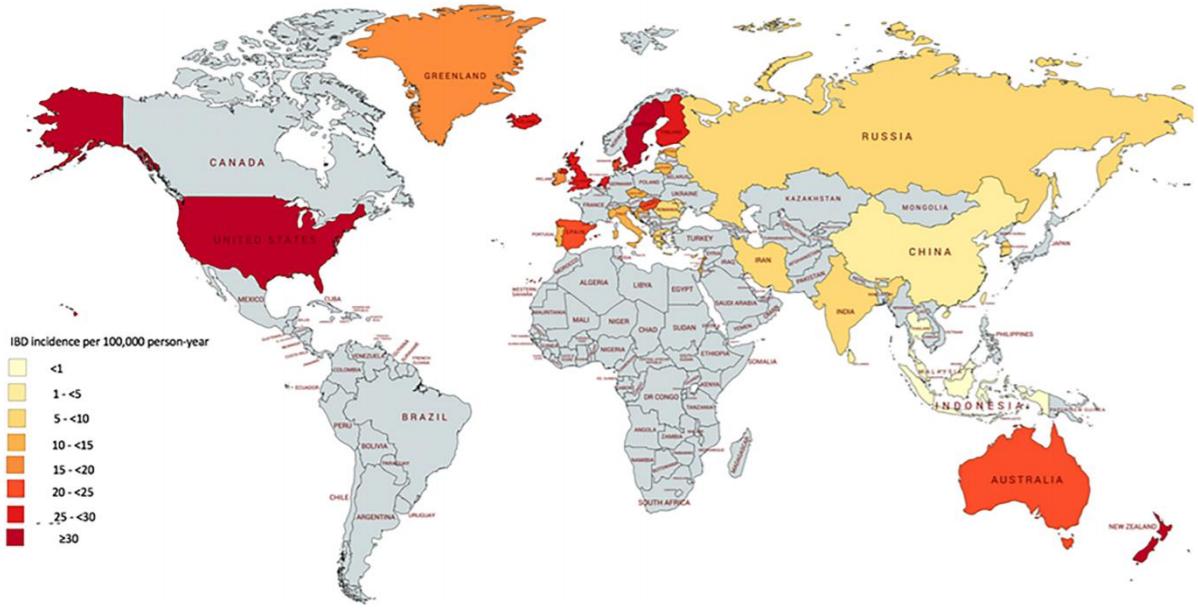


Figure 1.16a. Summary of the latest reported incidence of IBD according to population-based studies from 2010 to 2019 [Mak et al., 2020]

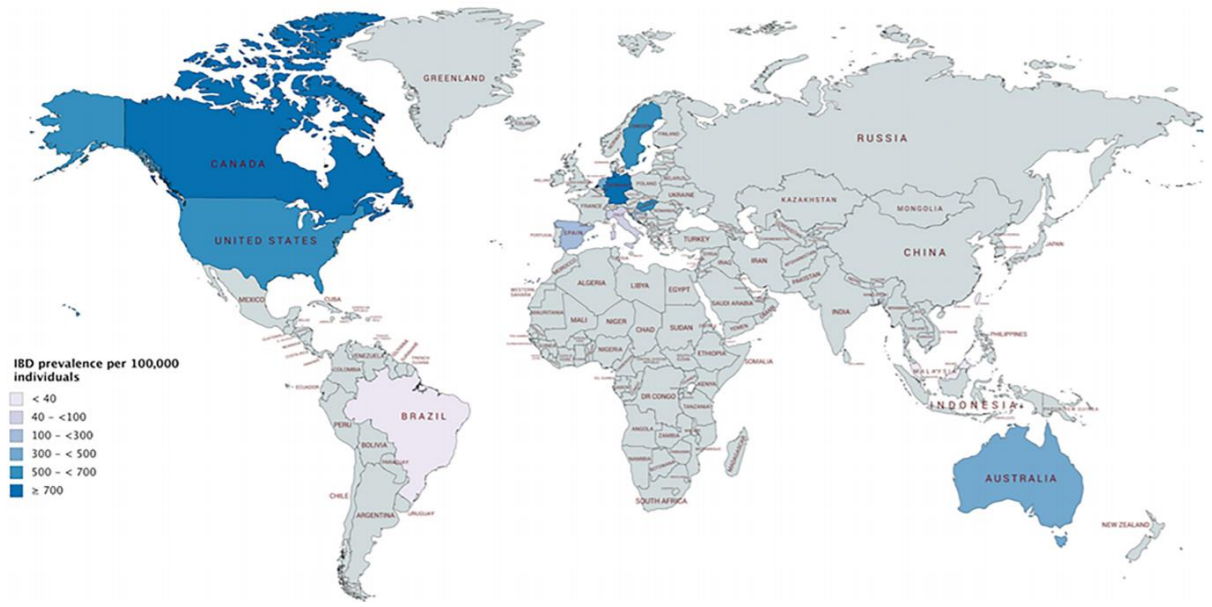


Figure 1.16b. Summary of the estimated prevalence of inflammatory bowel diseases (IBD) based on population-based studies from the past ten years [Mak et al., 2020]

1.3.2. Pathogenesis

The etiology of IBD is not totally clear, but it is believed that genetic susceptibility and environmental factors, along with dysfunction of intestinal epithelium which leads to imbalanced interactions with microorganisms and abnormal immune system lead to chronic inflammation [Kim et al., 2017].

1.3.2.1. Role of environmental factors

Several environmental risk factors have been described in IBD, including diet, smoking, appendectomy, breastfeeding, nonsteroidal anti-inflammatory drugs (NSAIDs) and microorganisms, however, none of the above completely explain the pathogenesis of IBD [Molodecky et al., 2010]. Fat intake is positively associated with IBD, whereas fruit, vegetables, and dietary fibers decrease the risk for IBD [Danese et al., 2004]. Increased consumption of animal proteins, sweets, sugar and low levels of vitamin D are positively associated with both UC and CD [Cosnes, 2010]. Smoking has a distinct effect on UC and CD, with tobacco use being associated with greater risk for CD and decreased incidence and risk of disease exacerbations in UC. Finally, oral contraceptives and NSAIDs have been associated with a higher risk for IBD

[Burke et al., 2017]. Epidemiological studies show that IBD is more common in urban centres most possibly due to the implication of the changes that accompany urbanisation, such as changes in lifestyle, exposure to environmental pollution, and dietary habits [Ananthakrishnan et al., 2017]. A meta-analysis of antibiotic exposure on the development of IBD including 7,208 IBD patients showed that all antibiotics except for penicillin were associated with CD, and more specifically metronidazole and fluoroquinolones were most strongly associated with new-onset CD in children [Ungaro et al., 2014]. Finally, the effect of mode of delivery, as well as duration of breastfeeding on intestinal microbiome and therefore the risk of developing IBD is well described. Infants born by caesarian section lack contact with the maternal gut or vaginal microbiota and have less microbial diversity than those born vaginally and the risk of IBD decreases along with the duration of breastfeeding [Ananthakrishnan et al., 2017].

1.3.2.2. Genetic and epigenetic factors in IBD

The interest in genetic risk for IBD appeared after observations of familial clustering in Ashkenazi Jews. The first linkage study indicated a locus on CARD15/NOD2 gene [Ye et al., 2016]. NOD2 is a cytoplasmic pattern recognition receptor which senses the muramyl dipeptide (MDP), a bacterial wall component, and through phosphorylations and activations leads to nuclear translocation of NFkB and upregulation of innate and adaptive immunity genes [Abreu et al., 2005, Strober et al., 2006]. The presence of NOD2 variants modifies the structure of LRR, the recognition of MDP and therefore the activation of NFkB in monocytes. Three SNPs, R702W (rs2066844), G908R (rs2066845), and the frameshift mutant L1007fsinsC, in or near the LRR domain represent 81% of disease-causing alterations within CARD15/NOD2 being independently associated with CD. These polymorphisms are more common in Caucasian CD patients (30% to 40% carriers) than in healthy controls (10% to 20%) [Ye et al., 2016]. In a Greek cohort R702W had allele frequency of 10%, 7.15% and 1% and G908R had allele frequency of 14.2%, 13.5% and 3.5% in CD, UC and healthy controls respectively [Gazouli et al., 2010].

GWAS identified several new SNPs associated with IBD. The strongest one was a protective variant in IL23R gene [Ye et al., 2016]. IL-23R is a receptor of cytokine IL-23, highly expressed on the cell membrane of immune cells which activates JAK2/STAT3 pathway [Eken et al., 2014]. IL23R gene was originally discovered as a CD susceptibility gene, but later it was also

confirmed in UC. The functional allele is more frequent in healthy controls, suggesting a protective effect from inflammation. R381Q encodes an amino acid change which may impair the IL-23R signaling pathway and reduce cellular response to IL-23 explaining its protective effect [Ferguson et al. 2010]. The allele frequency is 2.8% in CD patients, 5% in UC patients and 7.1% in Caucasian healthy controls [Coterill et al., 2010]. Of the 163 genetic loci that have been described in IBD, only 21 are CD-specific, and 20 are UC-specific. The rest are common in both CD and UC. Other IBD genes are related with innate (TLR4, STAT3, CARD9) and adaptive (TNFSF15, PTPN2, IL-12B) immune response pathways and highlight the role of autophagy and intracellular bacterial handing (CARD15/NOD2, ATG16L1) [Ye et al., 2016].

Epigenetic factors have been widely studied in IBD in the last decade. The first study reported a global DNA hypomethylation in UC patients compared to healthy controls and in active patients compared to inactive [Gloria et al., 1996]. In a GWAS metaanalysis Franke et al. [2010] showed an association of CD with a SNP in the DNMT3A gene whereas, DNMT1 and DNMT3B were shown to have higher expression levels in the mucosa of active UC [Sato et al., 2012]. Posttranslational histone modifications are the less studied epigenetic factors in IBD. Inhibition of HDACs in rat models of colitis, increases FoxP3 gene expression and improves the function of regulatory T cells indicating a potential role of HDACs in the pathogenesis of IBD [de Zoeten et al., 2010]. An increase in histone 4 (H4) acetylation in both experimental rats and inflamed mucosa of IBD patients and a downregulation in lysine HAT 2B lead to decreased H4J5 acetylation and IL-10 expression [Tsaprouni et al., 2011, Bai et al., 2016].

A growing number of studies shows distinct miRNA expression profiles in mucosal tissue (sigmoid and colon biopsies) and in peripheral blood of IBD patients compared to healthy individuals. Most studies are based in microarray and qRT-PCR methodology. The first study was in 2008, when Wu and colleagues examined sigmoid and colon biopsies from patients with active and inactive UC and CD and compared their microRNA profile with healthy subjects. 11 miRNAs showed significantly different expression in active UC versus control [Wu et al., 2008]. In the next years, further research confirmed some of those miRNA dysregulations and added some new. In 2010 Takagi et al. and Fasseu et al. confirmed the upregulation of miR-21 in patients with active UC. Fasseu et al., introduced miR-31 as a candidate miRNA that has altered expression in both UC and CD [Fasseau et al., 2010, Fischer and Lin, 2015, Takagi et al., 2010].

MiRNA-21 is one of the most highly expressed miRNAs associated with active IBD. MiR-21 induces the degradation of RhoB mRNA. RhoB is a member of the Rho GTP-binding protein family which display important role in the organization of cytoskeleton. Overexpression of miR-21 activates degradation of RhoB mRNA and subsequently increases intestinal epithelial permeability [Yang et al., 2013]. MiRNA-126 is upregulated in both CD (biopsy and blood) and UC (biopsy). A potential pathway regulated by miRNA-126 in UC is the IkBα pathway. IkBa is reduced in active UC and is negatively correlated with miRNA-126 expression. When mutating miR-126 binding site in the IKBA 39-UTR, gene expression is restored. So, upregulation of miRNA-126 could activate NFkB through inhabitation of IkBα [Feng et al., 2012]. MiR-31 increases with disease progression, targeting factor inhibiting hypoxia inducible factor 1, a hydroxylase that catalyzes HIF modification, a tumor angiogenesis mediator [Olaru et al., 2012] and miR-26b is shown upregulated with disease progression in tissues and serum of UC patients [Benderska et al., 2015]. Regarding Greek population, there is a study by Paraskevi et al. [2012] which examined the expression of 20 microRNAs in blood samples of 128 patients with CD, 88 patients with primary UC and 162 healthy controls. Eleven microRNAs (miR-16, miR-23a, miR-29a, miR-106a, miR-107, miR-126, miR-191, miR-199a-5p, miR-200c, miR-362-3p and miR-532-3p) were significantly higher in CD and five miRNAs (miR-16, miR-21, miR-28-5p, miR-151-5p, miR-155 and miR-199a-5p) in UC when compared to healthy controls.

Changes in miRNA levels are associated with disease development and can be quantified in both the inflamed tissue and the circulation making them promising biomarkers in differential diagnosis and prognosis of disease severity and drug resistance [James et al., 2020]. For example, fecal miR-16 and miR-223 correlate with clinical parameters, such as CRP and calprotectin [Schönauen et al., 2018], ileal mucosal miRNAs are altered in UC pouch patients and the alterated levels correlate with the degree of inflammation [Ben-Shachar et al., 2016]. Treatment for UC patients with JAK inhibitors, is used for moderate-to-severely active UC patients not responding to conventional therapies [Magro et al., 2020]. Pathak et al. [2015] identified SOCS1, a potent molecular switch that tunes the JAK pathway downregulated by miR-155. It was suggested that miR-155 expression in UC patients enhances inflammation through SOCS1.

1.3.2.3. Intestinal epithelium and microbiota in IBD

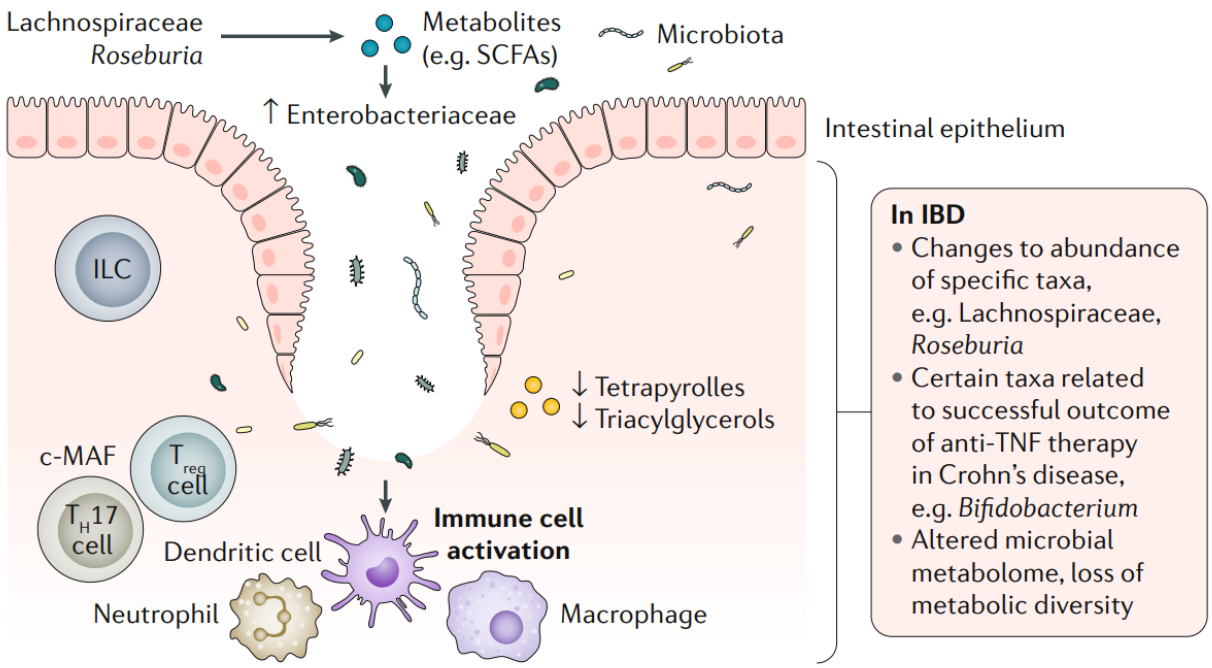


Figure 1.17. Intestinal barrier alterations in IBD [Neurath et al., 2019]

Intestinal epithelium consists of several different cells (enterocytes, goblet cells, Paneth cells etc) which form crypts and villi in a single column and a tight junction which protects lamina propria from pathogens and secrete mucus with antimicrobial action (i.e. defensins from Paneth cells) [Kim et al., 2017]. IBD is associated with various abnormalities in the above system [Fig 1.17]. For example, deletion of the gene encoding for mucine leads to colitis in mice. Genetic abnormalities or ER stress create Paneth cell dysfunction and susceptibility to IBD. Furthermore, the destruction of intestinal layer integrity, due to genetic (like N-cadherin mutations) or environmental factors facilitate external pathogens access into the lamina propria and activate immune responses which are leading to IBD [Kim et al., 2017]. Interestingly, a correlation between microbiome composition (for example Roseburia) and differentially methylation regions along with the presence of microorganisms tha tproduce butyrate (known as HDAC inhibitor) reveal that the interplay of gut microbiota with epigenetic mechanisms underlies the pathogenesis of IBD could be attributed partly to its association [Berni Canani et al., 2012].

On the other hand, microorganisms of the intestinal epithelium play a crucial role in the development of IBD. Normally, there are about 1000 different species (≈1012 cells) which

provide the host with important functions for the development of his metabolic and immune system. During the last years research has shown that gut microbiota, including bacteria, fungi, viruses, and other microorganisms are implicated in the pathogenesis of CD and UC [Zuo et al., 2018]. Reduced microbial diversity and other changes in several taxa reflect changes secondary to inflammation [Clooney et al., 2020]. In IBD there is a significant reduction in some species where at the same time a significant increase in others, called dysbiosis. IBD patients exhibit increased titers against commensal bacteria and antibiotics may help in some cases. Finally, the fact that genetic polymorphisms in genes implicated in bacterial recognition (such as NOD) and T cell immunity (such as IL-23R) are associated with IBD enhances the importance of gut microbiota in the development and progression of IBD [Becker et al., 2015, Kim et al., 2017].

Metagenomic studies explore changes in microbiota whereas metabolomic studies identify those microbial metabolites that are expressed differently in IBD compared to healthy controls. Some of the impaired species and their metabolites possess anti-inflammatory properties explaining why their depletion is harmful. On the other hand, pro-inflammatory bacterial species and metabolites are enriched in IBD and can be associated with its pathogenesis [Liu S et al., 2020]. Several studies have revealed that Proteobacteria (especially adherent invasive *Escherichia coli* (AIEC), Pasteurellaceae, Veillonellaceae, *Fusobacterium* species, and *Ruminococcus gnavus* are increased, whereas *Clostridium* groups IV and XIVa, *Bacteroides, Suterella, Roseburia, Bifidobacterium* species and *Faecalibacterium prausnitzii* are decreased in IBD. However, it is not always known whether these changes are causative or rather a result of inflammation or treatment [Glassner et al., 2019]

F. prausnitzii, a gram positive species, one of the main butyrate producers of the intestine [Lopez-Siles et al., 2017] is decreased in IBD patients compared with controls [Lloyd-Price et al., 2019]. In vitro cell stimulation by *F. prausnitzii* decreases IL-12 and IFN-γ production and increases IL-10 secretion. Also, *F. prausnitzii* can stimulate IL-10 secretion in dendritic cells. All the above suggest an anti-inflammatory role of *F. prausnitzii* in IBD [Rossi et al., 2016, Sokol et al., 2008]. Another anti-inflammatory genus which is found decreased in IBD is *Roseburia* which converts acetate that resides in the intestinal mucus layer to butyrate and therefore exhibits anti-inflammatory action [Vich-Vila et al., 2018].

AIEC crosses the mucous layer and resists antimicrobial peptides, adheres to intestinal epithelial cells, colonises the gut mucosa, promotes inflammation, and escapes autophagy inside macrophages [Liu S et al., 2020]. *Fusobacterium nucleatum* is increased in IBD patients' stools and correlates with disease activity. It has been shown that it can damage epithelial integrity and increase gut permeability. Also, it promotes cytokine secretion and STAT3 signaling pathways leading to T cells differentiation [Liu H et al., 2020]. Finally, dysbiosis in mycobiome and viroma has been reported with Candida species being associated with the inflamed gut and phages from IBD patients inducing IFN-γ via a pathway linked to aggravated intestinal inflammation [Gogokhia et al., 2019].

1.3.2.4. The role of immunity and inflammation in IBD

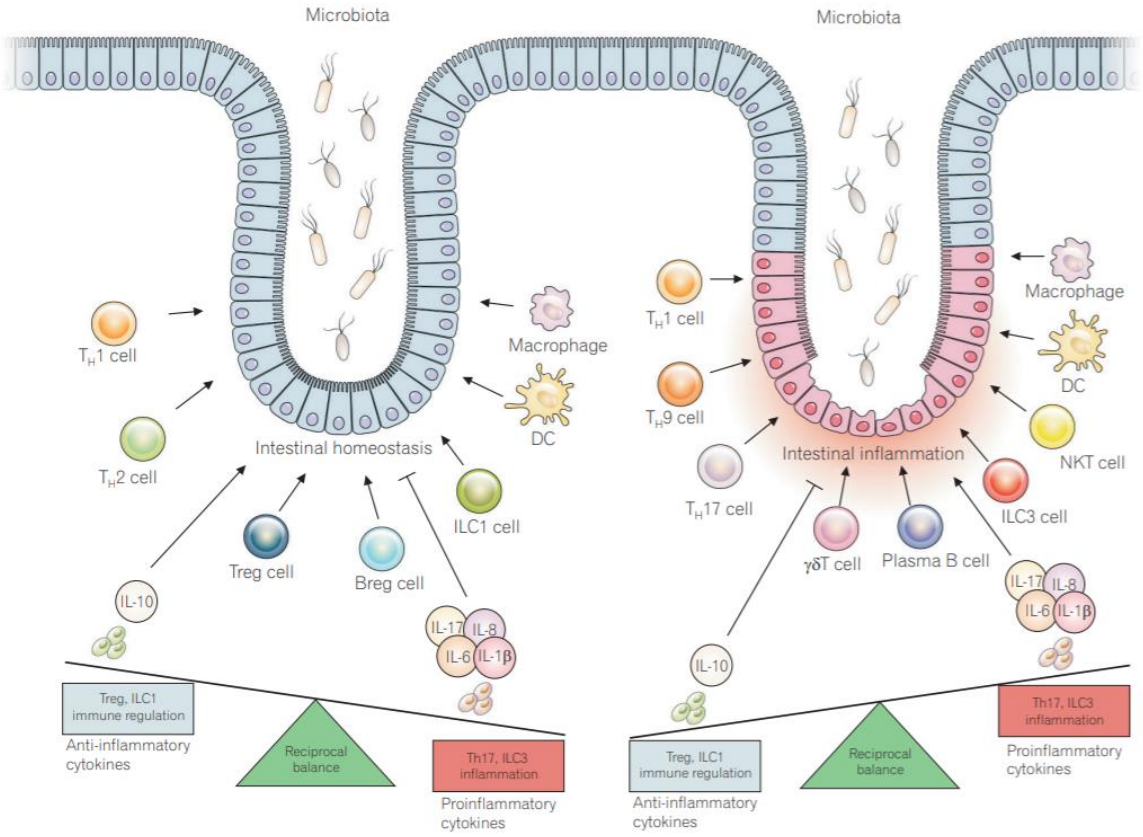


Figure 1.18. Reciprocal balance for intestinal immune homeostasis and inflammation [Lee et al., 2018]

A vicious cycle where abnormal innate immune system leads to adaptive immune disorders (Th1/Th2 regulation and Th17/Treg transformation imbalance) and cytokines increase

the innate immune damages (apoptosis, reduction of connection protein expression, decrease of antibacterial peptides), disturb the intestinal barrier function, and increase inflammation is observed in IBD [Huang and Chen, 2016]. Gut immune homeostasis is mediated by a balance between immune cells (Treg and Breg vs. Th17 and ILC1) and cytokines that maintain this environment. However, in IBD there is an imbalance in immune cells leading to the destruction of intestinal epithelial cells and the invasion of gut microbiota resulting to an uncontrolled release of cytokines [Fig. 1.18] [Lee et al., 2018].

Innate immune response is the first line of defense against PAMPs of microorganisms crossing the disrupted intestinal barrier. The main PRRs are TLRs and NLRs that identify recognition molecules of PAMPs and activates the production of IL, TNF and IFN and the maturation of APC cells in order to activate adaptive immunity. In IBD, macrophages increase dramatically and express costimulatory molecules such as CD40, CD80, and CD86, and inducers of intestinal inflammation, such as triggering receptor expressed on myeloid cells-1 (TREM-1) [Genua et al., 2014, Natale et al., 2019]. DCs and NK cells express more immune active molecules, such as IL-6, IL-12 and CD25, CD28, CD69, respectively than normal intestinal mucosa [Hart et al., 2005, Liu et al., 2008]. It has been proposed that the compromised mucus production (due to depletion of goblet cells) in UC and the defective intestinal barrier associated with complex defensin deficiencies in CD, are the key events in the pathogenesis of IBD [Koeninger et al., 2020].

Adaptive immunity and T cells regulation play an important role on IBD pathogenesis, as alterations in T cell subsets proliferation and differentiation cause an excessive increase of chemokines and cytokines, worsening the mucosal inflammatory process. Humoral immunity is also deregulated, as B cells produce an abnormal amount of antibodies, especially IgG, IgM, and IgA and several autoantibodies, such as ASCA and ANCA [Silva et al., 2016]. While normally, T-cells display mainly a regulatory phenotype, in IBD, there is increased Th1, Th2 and Th17 and reduced Treg and Tr1 responses [Tindemans et al., 2020]. Th1 cells are important for elimination of intracellular pathogens and mainly produce interferon IFN-γ and TNF-a, which activate macrophages and cytotoxic $CD8^+$ T cell responses against viruses and bacteria. Th1 cells accumulate in the intestinal tract of CD patients. Additionally, Th1 cells are more strongly associated with CD than UC. Th2 cells control parasite infection and IgE responses. IL-4 signals

their activation through STAT6 signaling and GATA-3 transcription factor. Th2-like cells are more associated with UC [Imam et al., 2018].

Th17 cells are induced by IL-6, IL-23, TGF-β and protect against extracellular pathogens at mucosal surfaces. Their differentiation is determined by the composition of the gut microbiota and various cytokines related to TH17, such as IL-17A and IL-17F are overexpressed in IBD patients comparing to healthy controls [Lee et al., 2018]. They induce neutrophil-specific chemokines (CXCLs), granulopoiesis and mediators of the acute phase response (such as IL-6). The role of IL-17 family members in IBD pathogenesis remains controversial as both protective and pathogenic functions have been reported. As such, while the Th17-associated IL-23-IL-17 axis is thought to play a role in IBD pathogenesis, Th17 cells cooperate with Treg to repair damages in the epithelial barrier in colitis [Tindemans et al., 2020]. Blocking of Th17 effector cytokines has had limited success and in some cases has even worsen the disease suggesting that targeting IL-17 in the intestine also blocks its barrier-promoting effects [Friedrich et al., 2019].

1.3.3. Diagnosis and IBD treatment

The diagnosis of IBD depends on the clinical findings after radiological, endoscopic and histological examinations along with a careful physical examination and a review of the patient's history. Though endoscopy is the gold standard for IBD diagnosis, it is invasive and burdensome to patients and has a high risk of perforation. That is why the need for finding alternative noninvasive tests has gained increasing attention [Fu et al., 2004]. Although there is not yet a blood or fecal biomarker sensitive or specific enough to be used alone in diagnosing and monitoring of IBD, some of the most commonly used are CRP, Erythrocyte sedimentation rate, Antineutrophil cytoplasmic antibodies (ANCAs) and anti-Saccharomyces cerevisiae antibodies (ASCA) in blood and calprotectin and lactoferin in stools [Soubières et al., 2016].

CD diagnosis is based on a combination of findings with the main one being the degree of focal, asymmetric, and transmural granulomatous inflammation of the luminal gastrointestinal tract [Lichtenstein et al., 2018]. Stool testing for fecal pathogens should be performed along with other fecal biomarkers that identify gut inflammation, such as a fecal calprotectin. Calprotectin has greater diagnostic accuracy than other biomarkers, correlates

with endoscopic scores, and helps to discriminate patients in remission from those with mild or moderate to severe activity [Penna et al., 2020]. Anemia, elevated platelets, serum CRP and erythrocyte sedimentation rate can also be used to detect and monitor inflammation but should be used with caution as 40% of patients with mild inflammation may have a normal CRP and erythrocyte sedimentation rate limits [Lichtenstein et al., 2018].

Although a complete colonoscopy helps in the differentiation with CD, UC diagnosis is usually based on a sigmoidoscopy with biopsy, especially in severe patients in order to reduce the risk of perforation. Endoscopic features of UC include loss of vascular markings, granularity of the mucosa, erosions, and in severe cases deep ulcerations and bleeding [Rubin et al., 2019]. CRP and erythrocyte sedimentation rate are also used but are nonspecific and fecal calprotectin has been proven more sensitive in UC than in CD [Takashima et al., 2015]. In UC fecal calprotectin correlates with clinical and endoscopic findings as well as the severity of the disease and can be used as a noninvasive marker of disease activity and to assess response to therapy [Kawashima et al., 2016, Sandborne et al., 2015].

Considering that IBD is an inflammatory disease most therapeutics approaches focus on anti-inflammatory agents and therapies that modulate the immune system. The most common anti-inflammatory and immunomodulating treatments include 5-Aminosalicylates (5-ASAs), corticosteroids, methotrexate, and anti-TNF agents [Neurath., 2017]. 5-ASAs are more frequently used in UC patients as they have little efficacy in CD. Their mechanism includes inhibition of cyclooxygenase, lipoxygenase, proinflammatory cytokines and NFkB [Allgayer, 2003]. Corticosteroids bind to a cytosolic receptor and translocate to the nucleus to regulate gene transcription [Oakley and Cidlowski, 2013]. Classic immunosuppressive drugs, such as azathioprine and methotrexate inhibit activation of immune cells, or block cell proliferation and induce apoptosis respectively. Finally, anti-TNF antibodies suppress intestinal inflammation as TNF is a pleiotropic cytokine which activates macrophages, implicates in Paneth cell death via necroptosis and regulates Tcell apoptosis [Neurath., 2017].

Nutritional management and manipulation of intestinal gut microbiota as means of controlling the disease remain the key focus of developing therapies. Regarding microbiome regulation, antibiotics, pre and probiotics are extensively used, with enteral nutrition being

considered to have an impact on gut microbiome as well [Neurath., 2017]. Nutrition during active phase of IBD is very important, with low fibre diet being recommended in moderate and severe disease activity and reintroduction of high fibre foods in periods with no symptoms or mild disease activity [Brown et al., 2011, Forbes et al., 2017]. Finally, there is an emerging interest in handling stress and psychological health of IBD patients in order to reduce symptoms and treatment needs [Berstein., 2014].

1.3.4. Metabolomics in IBD

Metabolomic and lipidomics analysis have been widely used to study IBD, aiming at the exploration of disease pathogenesis, the identification of disease biomarkers and the differentiation of disease subtypes. Also, it offers potential applications to therapeutic response assessment, as well as to revealing potential disease mechanisms in this direction [Gallagher et al., 2020]. 1H NMR spectroscopy is the most widely applied technique, with several studies in different kind of biofluids, such as serum, plasma, urine and fecal extracts as well as on biopsies [Storr et al., 2013].

As mentioned previously, microbial-derived metabolites are altered in IBD and influence immune maturation and homeostasis, host energy metabolism and maintenance of mucosal integrity. SCFAs are considered as metabolites of beneficial bacteria derived from microbiota-accessible carbohydrates. SCFAs regulate intestinal macrophages, modulate intestinal motility, support B cell development, influence energy metabolism and have protective effects in animal models of colitis [Lavelle et al., 2020]. Marchesi et al. [2007] and Bjerrum et al. [2015], have presented lower levels of short-chain fatty acids (SCFAs) in stool of IBD patients compared to healthy volunteers.

Bile acids and microbiota exert a bidirectional interaction in the gut. Bile acids are converted from primary to secondary bile acids by microbes in the colon and microbes are strongly affected by them either directly due to their antimicrobial effects or indirectly by stimulation of host antimicrobial peptides production [Ridlon et al., 2006]. In IBD faecal conjugated primary bile acids were found increased, whereas serum and faecal secondary bile acids were found decreased [Duboc et al., 2013].

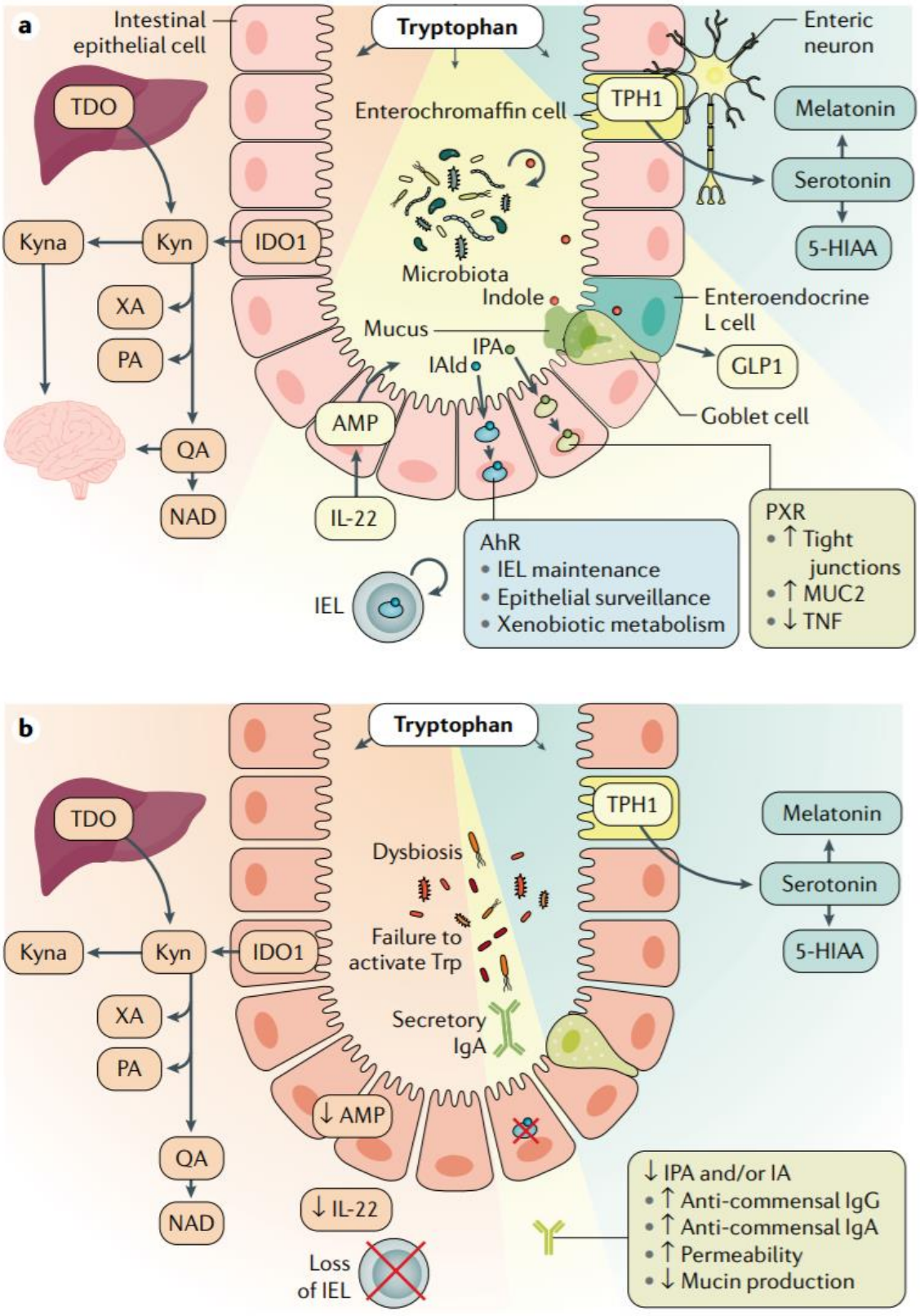


Figure 1.19. Tryptophan metabolism is dysregulated in IBD a. in healthy intestine b. in IBD [Lavelle et al., 2020]

Amino acids levels are also impaired in IBD. Phenylalanine, an essential aromatic amino acid that modulates intestinal inflammation, is decreased in serum of CD patients [Lai et al., 2019]. Fecal IBD samples usually contain higher levels of amino acids, such as the branched chain amino acids, taurine, glycine, lysine and alanine, whereas tissue BCAA are lower in IBD groups compared to controls [Gallagher et al., 2020]. Serum tryptophan, whose metabolism is essential in IBD, was significantly lower in patients with IBD than in controls and had a negative correlation with disease activity and CRP [Nikolaus et al., 2017]. Tryptophan is metabolized by microbiota to indoles and aryl hydrocarbon receptor (AhR) with diverse effects on mucosal immunity and homeostasis [Fig. 1.19] [Romani et al., 2007]. AhR activation in T cells regulates Th1/Th2 cell–associated immunity and IFN-γ, IL-17, IL-22 production, which are cytokines with either inflammatory or protective effects. Depending on the cell context, AhR signals differentially modulate Th response and act as initiators or attenuators of tissue-damaging T cell– dependent inflammatory processes [Monteoleone et al., 2011].

Metabolomics have also revealed differential lipid profiling in IBD. In TNF-impaired ileitis mouse model that mimics CD, 63 differentially expressed inflammatory markers were found in healthy and inflamed tissue, with most of them being implicated in metabolism of cholesterols, triglycerides, and phospholipids [Baur et al., 2011]. Thirty three lipid species in CD and 5 lipid species in UC were found negatively correlated with the disease in a study of 40 IBD patients using electrospray ionization–tandem mass spectrometry. Among them, plasmalogens, a class of membrane glycerophospholipids exhibited the most profound negative correlation, which is very interesting, as they are susceptible to oxidation and lower levels may be due to increased oxidative stress in CD [Fan et al., 2015]. Lloyd-Price et al [2019] reported higher concentrations of arachidonate (a precursor to immune and inflammatory-related molecules such as eicosanoids and prostaglandins) in dysbiotic IBD.

1.3.5. IBD nutrigenomics studies

Although pharmacogenomics have an increasing contribution to IBD prediction and therapy, nutrigenomics may have an even greater potential, due to the contribution of diet and gut microbiota to the pathogenesis of the disease. Prescribing an elemental diet, which acts through modulating inflammatory response may be of great benefit. Probiotics influence the

gut microbiome and immunonutrition, including omega- 3 fatty acids and certain phenolic compounds, may attenuate intestinal inflammation [Ferguson et al., 2007]. Alterations in NOD2 and TLR signaling are the most described molecular compounds targeted by nutrients, such as phytochemicals, fish oil, fatty acids and others [Fig. 1.20].

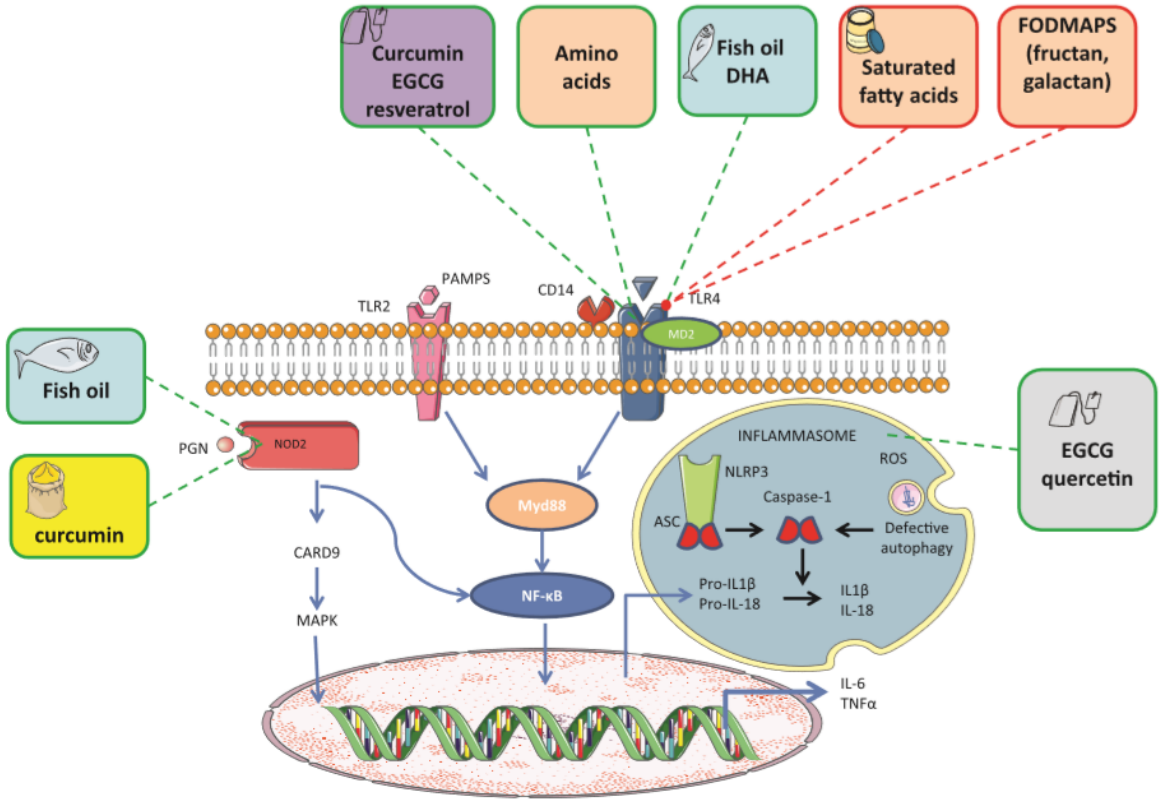


Figure 1.20. Dietary targets in IBD [Marion-Letellier et al., 2016]

Knockout mice are useful in nutrigenomics research and provide invaluable clues about diet effect in gene expression. IL-10−/− mice fed with arachidonic and eicosapentaenoic acid enriched diet, showed at least 40% lower colonic histological scores than mice fed on control diets. The expression of some inflammation genes was affected (i.e. down-regulation of TNF, IL-6 and up-regulation of PPAR) [Roy et al., 2007]. Treatment of Trinitrobenzene Sulfonic Acid (TNBS)-induced colitis mice with curcumin improved histopathologic signs of colonic inflammation and suppressed CD4+ T-cell infiltration and NFkB activation in colonic mucosa [Sugimoto et al., 2002]. In a similar model, resveratrol attenuated colonic injury, significantly reduced TNF-a, restored PGE2 values to normal and reduced COX-2 and the NFkB p65 protein expression [Ramon Mrtin et al., 2006]. In an in vitro study, lymphocytes from IBD patients were

treated with H_2O_2 or 2-amino-3-methylimidazo [4,5-f]quinoline in the presence of quercetin or epicatechin. Flavonoid supplementation decreased DNA damage resulting in a 48.6% reduction of H_2O_2-induced and a 43% reduction of induced DNA damage [Najafzadeh et al., 2009].

Yet, only a few nutrigenomics studies have been applied in IBD. A 6 week treatment with anthocyanin-rich bilberry extract was tested in 13 patients with mild to moderate UC. This treatment inhibited the expression of IFN-γ-receptor 2 in THP-1 monocytic cells and reduced IFN-γ, TNF-α and p65-NFkB in colon biopsies. Serum levels of TNF-α and MCP-1 were reduced and levels of IL-17A, IL-22 and IL-10 were higher in patients with successful treatment. The above suggested a modulation of T-cell cytokine signalling and inhibition of IFN-γ signal transduction as the molecular mechanism [Roth et al., 2016]. In a transcriptomics study of Marlow et al. on 2013, a Mediterranean-inspired anti-inflammatory diet was applied in a 6-week intervention in CD. Diet reduced established biomarkers of inflammation. As no single gene stood out, it was suggested that the cumulative effect of small changes in many genes and a trend of normalizing the microbiota was responsible for the beneficial effect [Marlow et al., 2013]. In 2005, Furrie et al tested the immune response of UC patients in a synbiotic therapy combining a probiotic (Bifidobacterium longum) and a prebiotic (an inulinoligofructose growth substrate). Sigmoidoscopy scores and mRNA levels of defensins, TNF-a and IL-1a, proinflammatory cytokines that induce defensin expression, were reduced [Furrie et al., 2005].

1.4. Connecting dots between NAFLD and IBD

Throughout the years, a possible association between NAFLD and IBD has been proposed. Thomas first described a link between “ulceration of the colon” and “a much enlarged fatty liver” early back in 1873 [Thomas, 1873]. Since then, several studies examined the prevalence of hepatic steatosis in IBD patients indicating a higher prevalence of NAFLD among IBD patients compared to the general population [Saroli Palumbo et al., 2019]. In fact, more severe IBD promotes the development of liver fat accumulation and severe liver steatosis further impaires IBD [Sartini et al., 2018]. A great variability among studies reporting the prevalence of NAFLD in IBD was observed, ranging from 1.5% to 39.5% and from 1.5% to 55% in CD and UC patients, respectively [Gizard et al., 2014]. The above results are quite interesting, as IBD is mainly considered a wasting disease with malabsorption, malnutrition, and severe weight

loss in some cases [Magri et al., 2019]. This variability, 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 related to underlying chronic inflammation.

***Inflammation**:* As both NAFLD and IBD are considered chronic inflammatory conditions, they share common features in their pathophysiology. Oxidative stress, chronic relapsing inflammation and immune activation, may be the common pathogenic factor contributing to the consistence of NAFLD and IBD [Chao et al., 2016]. Inflammatory mediators (adipokines, cytokines) triggered by oxidative hepatic environment and bacterial overgrowth lead to hepatic stress and hepatic disease progression in NAFLD [Delli Bovi et al., 2021]. Similarly, an exacerbated inflammatory response, caused by several causes, such as increased permeability, dysregulation of the innate and adaptive immune system and oxidative stress plays an important role in IBD [Guan, 2019].

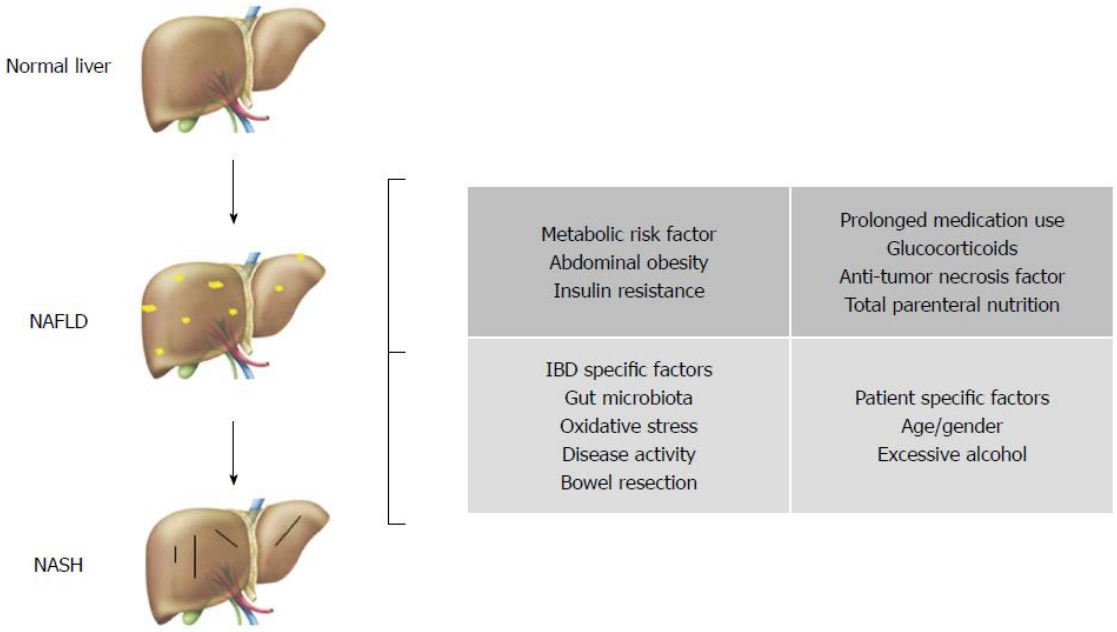


Figure 1.21. Potential pathogenic factors contributing to the coexistence of non-alcoholic fatty liver disease and inflammatory bowel disease [Chao et al., 2016]

Specific characteristics of each disease may contribute to the high prevalence of NAFLD in IBD patients. For example, NAFLD is considered the hepatic manifestation of metabolic syndrome. Contrary to what was believed in the past, although IBD patients are malnourished, they exhibit a high prevalence of central obesity, dyslipidemia and metabolic syndrome

[Dragasevic et al., 2019]. During the last years it is well established that metabolically driven pathologies, such as obesity and insulin resistance share common features with immunologically mediated disorders, such as IBD. Metabolic disorders cause strong inflammatory responses and inflammation is associated with metabolic alterations. There is an interplay between inflammatory pathways and the enteroendocrine system which regulates metabolic adaptation and the immune response in the luminal environment [Zietek et al., 2016].

Regarding IBD characteristics, disease activity and disease duration play an essential role in the link with NAFLD. It has been shown that more severe IBD is associated with the presence of more severe hepatic steatosis [Sartini et al., 2018]. Also, longer disease duration exposes patients to multiple NAFLD risk factors including chronic relapsing inflammation, metabolic comorbidities and hepatotoxic drugs, such as steroids, immunosuppressants and biological factors [Bessissow et al., 2016, Magri et al., 2019]. Methotrexate and thiopurines are well-known for their hepatotoxic activity, including hepatocellular injury and cholestatic disease. On the other side, TNF-alpha inhibitors, that are used in IBD treatment have been proved as a promising tool in the treatment of NASH, as they affect the circulating levels of leptin, a key peptide in appetite control and insulin resistance [McGowan et al., 2012].

Dysbiosis, a common feature in IBD and NAFLD: Alterations in intestinal microbiota have been associated with disease severity in both IBD and NAFLD, therefore they may act as a potential pathogenic link between the two conditions [Saroli Palumbo et al., 2019]. Intestinal dysbiosis, increased intestinal permeability and alterations of mucosal barrier play a critical role in both diseases. In NAFLD, it is well established that gut–liver axis dysfunction plays a key role in promoting the molecular mechanism of NAFLD and the progression to NASH. Gut microbiota and their bioactive metabolites, such as endotoxins, lipopolysaccharides, SCFAs, bile acids, cholic acids and other interact with a range of inflammatory factors including IL-6, IL-12, IL-1β and TNF-α that may drive fatty infiltration of the liver [Pan et al., 2020]. Similarly, in IBD the reduction of microbial diversity and the impairment of mucosal barrier which leads to unlimited passages of microbes to lamina propria and systemic bloodstream, result to immune tolerance to hyperactivation in the body [Yu, 2018].

1.5.Mastiha

According to the European Pharmacopoeia Monograph (01/2008:1876), Mastic or Mastiha (mastic gum) is the dried resinous exudate obtained from stems and branches of *Pistacia lentiscus (Pistacia lentiscus* L. *var latifolius Coss.),* an evergreen shrub of the Anacardiaceae family, common in the eastern Mediterranean area. Mastiha is an oleoresin obtained from a cultivated variety of cultivated clone of the mastic tree. The variety Chia, is uniquely cultivated in the southern part of a Greek island, Chios, due to climate characteristics. The name *Pistacia lentiscus* var. *chia* is mainly used in publications. For more than 2500 years mastic has been used in Greek medicine for several disorders like peptic ulcer and gastralgia, in the Mediterranenan cuisine as a seasoning, in preparation of beverages, in perfumery and in dentistry [Paraschos et al., 2012].

Mastiha was first time reported from Herodotes in the 5th century B.C where he described its use by the Egyptians as a “cover of the dead instead of glue”. The Romans used Mastiha as a wine flavoring agent [Pachi et al., 2020]. Dioscurides and Galinus considered Mastiha as a medical and culinary agent and reported its positive influences in health [Triantafyllou et al., 2007]. Mastiha has been playing an important role in the economy of Chios throughout history, from the ancient years, during the Ottoman occupation, as well as at present. In 1997 the European Union characterized Mastiha as a product of Protected Designation of Origin [Dimas et al., 2012]. Mastiha is Generally Recognised As Safe (GRAS) from the U.S. Food & Drug Administration and since 2015 the European Medicines Agency (EMA) has classified it to the category of traditional herbal medicines for two therapeutic indications, mild dyspeptic disorders and minor inflammations of the skin.

1.5.1. Chemical constituents

Mastiha resin is a complex natural resin with approximately 120 chemical compounds being reported. It consists of large amounts of triterpenoid molecules, which constitute the major chemical group and comprise approximately the 65–70% of the total resins' weight [Pachi et al., 2020]. Some of them are tetra- or pentacyclic triterpenoids [Fig. 1.21], such as mastic acid, isomastic acid, oleanolic acid, tirucallol, dipterocarpol, lupeol, fl-amyrin, fl-amyrone, oleanonic aldehyde and germanicol [Marner et al., 1991], but there are still many unidentified triterpenoids. Recently, 13 unidentified tirucallane triterpenoids have been isolated and

identified, with some of them exhibiting anti-inflammatory and nitric oxide (NO) inhibitory activities [Liu et al., 2021].

The resin also contains a considerable amount of polymeric material 1,4-poly-β-myrcene which has the *cis*- conformation for about 75% and is relatively unstable after isolation [Van den Berg et al., 1998]. This polymer constitutes about 25–30% of the dry weight of Mastiha and molds the above-mentioned chemical compounds into a resin [Pachi et al., 2020].

Also, Mastiha consists of the essential oil (Mastic oil, 3%), polysaccharides (polysaccharide-protein complexes such as arabino-galactan proteins) [Kottakis et al., 2007], phytosterols (such as tirucallol) [Paraschos et al., 2007], phenolics and other secondary metabolites [Fig. 1.2]. The major constituents of the essential oil of *Pistachia lentiscus* var. *chia* are α-pinene (63%), β-pinene (3.3%), β-myrcene (25%), limonene (1.5%), and β-caryophyllene (1%) [Koutsoudaki et al., 2005]. Several phenolic compounds have been identified in Mastiha resin, such as tyrosol, p-hydroxy-benzoic acid, vanillic acid and gallic acid [Kaliora et al., 2004]. Finally, *Pistacia lentiscus L*. leaves mainly contain flavonoids and phenolic acids such as myricetin glycoside, catechin, β-glucogallin and quercitrin gallate [Rodriguez-Perez et al., 2013]. The combination of more than 90 ingredients justifies the multiple uses of Mastiha oil in food and health care area.

Chemical composition of *Chios mastic* gum.

	Percentage of weight
1. Natural polymer poly-β-myrcene	25%
2. Masticadienonic acid	12%
3. Iso-masticadienonic acid	12%
4. Oleanonic acid	6%
5. Moronic Acid	4%
6. Masticadienolic acid	1%
7. Iso-masticadienolic acid	1%
8. Other acids	3%
9. Mastic oil	3%
10. Butyspermol	2%
11. Tirucallol	2%
12. Oleanolic aldehyde	2%
13. Oleanonic aldehyde	2%
14. Betulonal	2%
15. Caryophyllene oxide	2%
16. Masticadienolal	2%
17. Iso-masticadienolal	2%
18. Other alcooles	4%
19. Other aldehydes	8%

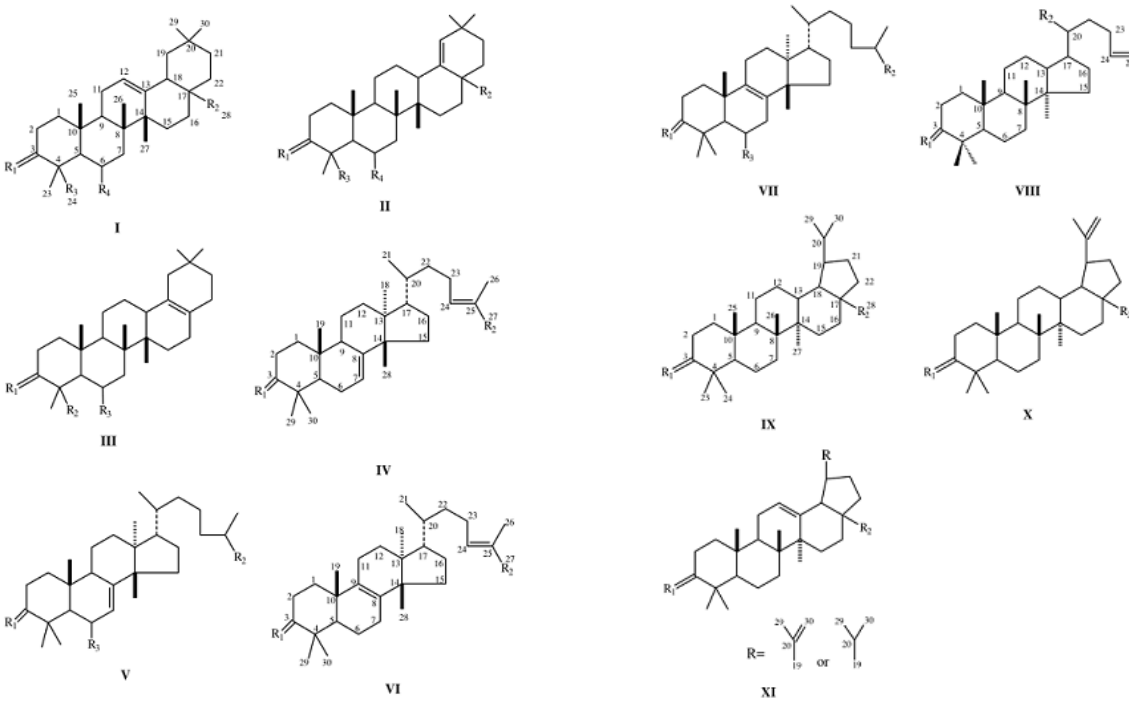


Figure 1.22. Chemical composition of Mastiha and skeletons of penta- and tetra-cyclic triterpene derivatives identified in Pistacia species [Assimopoulou et al., 2005, Dabos et al., 2009].

1.5.2. Biological activities

Mastiha is considered a wide-range therapeutic agent with several biological activities than can be attributed either to the gum itself or to its constituents [Paraschos et al., 2012]. Despite the great number of studies exploring the use of the gum itself as a herbal drug, the absorption and action of its main constituents has only been studied the recent years. Lemonakis et al. first examined the absorption of Mastiha's main triterpenic acids in mouse plasma [2011] and Papada et al [2017] and Brieudes et al. [2021] in human plasma. The above studies showed that 24Z-isomasticadienonic acid (IMNA) and 24Z-isomasticadienolic acid (IMLA) in mice, whereas IMNA and mastihadienonic (MNA) in humans are bioavailable. Also, Zachariadis and Langioli [2011] detected traces of several Mastiha constituents (such as α-pinene, β-myrcene, limonene, and other) in urine samples collected after dietetic use of various mastic products.

The elucidation of Mastiha's chemical composition is difficult due to its complex nature. Its main chemical group is triterpenes (aproximetelly the 65–70% of the total resins' weight). Also it comprises of volatile compounds (essential oil and water) that can be obtained after hydrodistillation, leaving a residue that is called "colophonio". Other chemical compounds appear in a low percentage of approximately 5%. Mastiha's compounds are molded into a resin due to the polymer (approximately 25–30% of the dry weight) [Pachi et al., 2020]. The crude resin remaining after steam distillation can be fractionated into the polymer, the acidic and the neutral fraction [Sharifi and Hazel, 2009].

Several studies have confirmed Mastiha's beneficial properties with a few trying to address the action of some of its constituents separately and are presented below.

1.5.2.1. Antimicrobial properties

Several studies have proven the antimicrobial activity of Mastiha. Early in 1980s several clinical trials showed that Mastiha reduces the intensity of ulcerations in duodenal ulcer caused by *Helicobacter Pylori* (HP). In a double-blind clinical trial with 38 patients with duodenal ulcer, endoscopically-proven healing was presented in 70% of patients on verum whereas in only 22% of patients on placebo [Al-Habbal et al., 1984]. These findings are of great importance as HP is considered one of the main etiological agents of gastritis, ulcer disease, gastric cancer and mucosa associated lymphoma [Aebischer et al., 2010]. Kottakis et al. proved that under the presence of HP neutrophil-activating proteins, the Mastiha extracted arabinogalactan proteins inhibit neutrophil activation verifying the crucial role in HP associated pathologies [Kottakis et al., 2009]. Mastiha's effect in HP has also been demonstrated via electron microscopy showing that Mastiha induced morphological abnormalities in HP cells [Marone et al., 2001]. Miyamoto et al., [2014] tested ten standard Mastiha constituents and identified that α-terpineol and (E)-methyl isoeugenol exhibit the most potent anti-pyloritic activity against both drug sensitive and drug resistant strains. Finally, when investigating anti-pyloritic activity of Mastiha's different fractions, it was shown that the polymer fraction had the greatest activity, probably because of the *in situ* oxidation that happens during mastication [Sharifi and Hazel, 2009]. This may also explain why the the absence of polymer seems to reduce Mastiha's activity [Pachi et al., 2020].

Apart from HP, Mastiha exhibits antimicrobial activity for other bacteria as well. As such, it shows selective hydroxyl scavenging activity against oral bacteria (*Porphyromonas gingivalis* and *Prevotella melaninogenica*) which is really useful for the oral health [Sakagami et al., 2009]. Mastiha oil (verbenone, α-terpineol and linalool) show antimicrobial activity against *Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis* with different sensitivity in individual bacteria suggesting that the efficacy of Mastiha is due to a synergistically action of its constituents [Dimas et al., 2012]. Essential oil's activity against several bacteria and fungi was proven higher than Mastiha leaves and resin (both acidic and neutral fraction) suggesting that its main constituent (α-pinene) is mainly responsible for its antimicrobial activity [Magiatis et al., 1999]. Finally, most recently Mastiha was tested as an antiviral drug against influenza A with promising results as it reduced morbidity of mice infected with the virus. It was suggested that Mastiha's antiviral activity is expressed through blocking of the early stage of viral replication and by reducing the cytopathogenic effect, RNAs, proteins and infectious particles of the virus [Kim et al., 2021].

1.5.2.2. Antioxidant and cardioprotective properties

The antioxidant activity of Mastiha has been first showed by an in vitro study of Andrikopoulos and colleagues. Mastiha was the most effective resin against copper-induced LDL oxidation in vitro among others and the methanol/water extract was the most effective compared with other solvent combinations [Andrikopoulos et al., 2003]. Mastiha can be used as an antioxidant agent as it is proven to inhibit protein kinase C, which attenuates H_2O_2 production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) [Triantafyllou et al, 2011]. Mastiha's polar extract has been shown to inhibit the cytotoxic effect of oxidized LDL (oxLDL) in PBMCs via regulating glutathione (GSH) levels and CD36 expression with the triterpenoid fraction exhibiting the most significant increase in GSH [Dedoussis et al., 2004]. Our group has recently shown that in both Mastiha resin and Mastiha oil, major terpenes' bioavailability is accompanied by a serum resistance to oxidation indicating that Mastiha's antioxidant activity may be attributed to its terpenes [Papada et al., 2017, Papada et al., 2020].

Given that Mastiha protects PBMCs from oxLDL's effect, the benefits in the cardiovascular system have been also examined. Mastiha, and more specifically isolated phytosterol tirucallol, significantly inhibits the expression of vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) in human aortic endothelial cells (HAECs) [Loizou et al., 2009]. Also, it decreases serum total cholesterol (TC), LDL, APOB, AST and ALT, and γ-glutamyltransferase (γ-GT) levels in the low-dose group, suggesting its hepatoprotective and cardioprotective action [Triantafyllou et al., 2007]. Nowadays, Mastiha's lipid lowering properties are well established as it has been shown to reduce total cholesterol [Kartalis et al., 2015] and this hypolipidemic activity is probably associated with camphene, one of its monoterpene constituents [Vallianou et al., 2011].

1.5.2.3. Chemopreventive properties

Mastiha's chemopreventive properties have been examined in *in vitro* studies and experimental animals. In an androgen-responsive prostate cancer cell line, Mastiha have been shown to inhibit the function of the androgen receptor (AR) and androgen-regulated genes (prostate-specific antigen (PSA) and NKX3) [He et al., 2006]. Furthermore, treatment of lewis lung adenocarcinomas (LLC) cells with Mastiha reduces the expression of vascular endothelial growth factor and NFkB activation. These two inhibitions are associated with tumor neovascularization and reduction in tumor size [Magkouta et al., 2009]. Furthermore, treatment of LLC cells with mastic oil inhibits the metastatic potential of those cells, as it reduces levels of secreted matrix metalloproteinase-2 (MMP-2), prevents actin remodeling and reduces the expression of adhesion molecules [Loutrari et al., 2011]. In a human colon cancer/immunodeficient mouse model, injection with hexane extract of Mastiha results in tumor growth suppression of about 35% [Dimas et al., 2009]. Finally, in a transcriptomics analysis of mastic oil-treated Lewis lung carcinomas, an altered expression of 925 genes has been observed. Among them, genes implicated in cell cycle, survival and NFkB cascade along with those encoding for phosphatase and tensin homolog (PTEN), E2F7, Heme oxygenase 1 (HMOX 1) and NOD-1 revealed interesting mechanistic links underlying the anti-proliferative effect of mastic oil [Moulos et al., 2009].

1.5.2.4. Effects on gastrointestinal system (GI)

Apart from the use as a potential agent for the treatment of duodenal ulcers, Mastiha has been proven effective in digestive and GI disorders. One hundred and forty eight patients with functional dyspepsia who received Mastiha exhibited a significant reduction in the severity of their symptoms, comparing to the placebo group [Dabos et al., 2009].

Mastiha's effect on inflammatory bowel disease (IBD) has been investigated in several studies. The first study ever reported to evaluate Mastiha for possible clinical effectiveness in patients with CD was by Kaliora et al in 2007. More specifically, Mastiha significally decreased the disease activity index, plasma IL-6 and CRP in patients with mildly to moderately active CD. In PBMCs obtained from patients, Mastiha reduced TNF-α secretion, whereas macrophage migration inhibitory factor (MIF) release was significantly increased, suggesting an immunomodulative activity of Mastiha on PBMCs [Kaliora et al., 2007, Kaliora et al., 2007b]. Oral ingestion of Mastiha decreased histological damage in TNBS induced colitic rats, an animal model of IBD, after a 3-day treatment [Gioxari et al., 2011]. Recently, Mastiha's favourable effects on IBD has been shown in a randomised, double-blind, placebo-controlled clinical trial with 128 IBD patients enrolled and randomly allocated to Mastiha or placebo groups. Mastiha supplementation decreased serum oxLDL and oxLDL/LDL or oxLDL/HDL [Papada et al., 2018a] and regulated faecal lysozyme [Papada et al., 2018b] in IBD patients in relapse. Also, it inhibited an increase in plasma free amino acids in IBD patients in remission [Papada et al., 2019].

Finally, there are only a few studies supporting the hepatoprotective activity of Mastiha [Pachi et al., 2020]. The only constituent of Mastiha that has been reported to have hepatoprotective activity, even though it is not one of its major constituents (approximately 1% in Mastiha oil) is camphene, which seems to prevent hepatic steatosis in mice [Kim et al., 2013]. Also, Mastiha treatment of diabetic rats showed improvement in the liver function by reducing ALT and AST [Ur Rehman et al., 2015]. There is only one study on the effect of Mastiha on animal models of NASH. This study revealed a significant reduction in plasma ALT, an improvement in hepatic steatosis and collagen content, and a reduction in NAFLD activity score on diet-induced obesity, NASH, and fibrosis (DIO-NASH model) mice [Kannt et al., 2019].

1.5.2.5. Antiinflammatory properties

Mastiha is a natural product with established anti-inflammatory properties in both preclinical and clinical studies [Papada and Kaliora, 2019]. Zhou et al. showed that Mastiha inhibits the production of NO and PGE2 by LPS-activated mouse macrophage-like RAW264.7 cells [Zhou et al., 2009]. In an asthma mouse model, Mastiha inhibited eosinophilia and the production of inflammatory cytokines (IL-5 and IL-13) as well as chemokines (such as eotaxin and eotaxin2) in bronchoalveolar lavage fluid [Qiao et al., 2011]. Loizou et al studied the anti-inflammatory activity of Mastic Neutral Fraction (MNF) in HAEC. Mastiha extract and tirucallol inhibited VCAM-1 and ICAM -1 expression in TNF-a-stimulated HAEC and attenuated the phosphorylation of NFkB-p65 [Loizou et al., 2009]. Oral ingestion of *Pistachia lentiscus* powder decreased cytokine (IL-6, IL-8, TNF-α and ICAM-1) levels in the intestinal epithelium of TNBS – Induced Colitis rats [Gioxari et al., 2011]. Also the NFkB-p65 protein level decreased in cell lysates of co-cultured human colon epithelial HT29 colorectal adenocarcinoma cell line and monocytes/macrophages treated with Mastiha, suggesting inhibition of NFkB activation and down-regulation of genes encoding for IL-8 and ICAM-1 as the mechanism underlying anti-inflammatory activity [Papalois et al. 2012].

2. Methodology

2.1. MAST4HEALTH

Mastiha Treatment for Obese with NAFLD Diagnosis (MAST4HEALTH) is a multicenter randomized and double blind placebo controlled (parallel arm) clinical trial aiming at exploring the effectiveness of Mastiha as a non-pharmacological intervention in NAFLD. MAST4HEALTH project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 691042. It consists of the following 13 partners (7 academic and 6 non academic partners) from 8 European countries (Bosnia Herzegovina, France, Germany, Greece, Italy, Serbia, Spain, United Kingdom):

<u>Academic partners</u>

- HAROKOPIO UNIVERSITY- (referred as The Coordinator or as HUA) Greece
- University of Novi Sad (referred as UNS) Serbia
- CONSIGLIO NAZIONALE DELLE RICERCHE (referred as CNR) Italy
- University of East Sarajevo, (referred as UES) Bosnia and Herzegovina.
- QUEEN MARY UNIVERSITY OF LONDON (referred as QMUL) United Kingdom
- INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (referred as INSERM) France
- FUNDACION PARA EL FOMENTO DE LA INVESTIGACION SANITARIA Y BIOMEDICA DE LA COMUNITAT VALENCIANA (referred as FISABIO) Spain

<u>Non academic partners</u>

- BIOTECH VANA SL (referred as BV) Spain
- RANDOX LABORATORIES Ltd (referred as RANDOX) United Kingdom
- MARASCIO FERNANDO Intervideo (referred as IIWS) Italy
- SANOFI-AVENTIS DEUTSCHLAND GMBH (referred as SANOFI) Germany
- Chios Mastiha Growers Association (referred as CMGA) Greece
- PERSPECTUM DIAGNOSTICS LTD (referred as PRSP) United Kingdom

2.1.1. Ethics and trial registration

The trial was conducted in three clinical trial centers (Harokopio University of Athens Greece, University of Novi Sad, Serbia and Consiglio Nationale de Ricerche, Pisa, Italy). Ethics Committees approvals were obtained from the three centres, HUA (Bioethics Committee 49/29-10-2015), CNR (Ethical Clearance by Commissione per l'Etica e l'Integrità nella Ricerca, February 2016) and Niguarda Hospital Ethics Committee 230-052017 (Comitato Etico Milano Area 3-11.05.2017), UNS (Faculty of Medicine Novi Sad, The Human Research Ethics Commission No. 01-39/58/1-27.06.2016). The trial was conducted following the Helsinki declaration and the Data Protection Act 1998, and was registered with ClinicalTrials.gov (Identifier: NCT03135873). All volunteers gave their informed consent after given a detailed information leaflet. "Ample time" was given for consideration and participants were free to leave the study at any time. A case report form was created for reporting adverse events.

2.1.2. Study design and study population

Recruitment took place from 2017 to 2019 following specific inclusion and exclusion criteria (Table 2.1).

After completing the baseline assessment, patients were randomly allocated to either the Mastiha or the Placebo group. To avoid allocation bias, randomisation was carried out by a computer-generated random number list. The randomization algorithm was designed to balance the size of each group per country and per sex, by picking a pseudo-random number from 0 to 1 (using Javascript's "random" method). The trial was blinded to treatment allocation for both researchers and patients.

Mastiha (100% natural) or matching Placebo (corn starch) capsules weighing 0.35 g each were given in 3 equal doses daily (total of 2.1 g). The dose was chosen based on its effectiveness towards inflammation in a pilot study while exhibiting no side effects [Kaliora et al., 2007]. Placebo was identical in physical form, sensory perception, packaging and labeling, with no pharmaceutical activity. Both groups received nutritional counseling to allow for body weight regulation up to 5%. The un-blinding took place on the completion of the study, once all statistical analyses were completed. Compliance and side effects were monitored biweekly

through phone calls and use of compliance calendars. Also, in each visit the number of remaining capsules in the boxes provided to the volunteers were measured. No side effect or any discomfort was reported. Treatment discontinuation was defined as a minimum of 6 consecutive days without treatment intake.

Table 2.1. Inclusion and exclusion criteria of MASTHEALTH study.

Inclusion criteria	Exclusion criteria
Sex: Male and female	Hepatotoxic Medication, Concomitant Liver Disease
Confirmed NAFLD/NASH (moderate to severe fibrosis) LIF score>2 or LIF score >1 and ≥2 MRI slices with LIF:2-4> 30% based on the sensitive LiverMultiScan technique (Perspectum Ltd, UK)	Decompensated Diabetes Mellitus (diabetes mellitus type 1, uncontrolled diabetes mellitus type 2 (HbA1c ≥ 7,5%)
18< Age <67 years	Dysthyroidism, hypopituitarism, Cushing syndrome / disease
Obesity (BMI≥30 kg/m^2)	Alcohol abuse (>20 g/day (women), >30 g/day (men), EASL Guidelines) or drug addiction
	Clinically or biochemically recognized systemic diseases
	Pregnancy test, lactation
	Vegan or lacto- and ovo-lacto-vegetarianism
	Psychiatric or mental disorder
	Recent loss in body weight or current diet
	Any use of antioxidant-phytochemical rich supplement, anti-, pre- or pro-biotics within 3 months pre-intervention
	Changes in drug treatment for e.g. hypertension, diabetes mellitus, 3 months prior or during the 6month intervention

	Antibiotic treatment during and 2 months prior to screening

2.1.3. Primary and secondary outcomes

The primary endpoint of MAST4HEALTH was the improvement in LIF score assessed by MRI scanning and the sensitive LiverMultiScan. LIF is a continuous score (0-4) derived from T1 and T2∗ values. T1 reflects the amount of extracellular fluid and reflects inflammation and fibrosis and T2∗ reflects the amount of iron deposition, which has a confounding effect on T1. LIF is based on cT1 that correlates with liver fibro-inflammation and NAFLD activity score [Eddowes et al., 2018]. LIF has been superseded by cT1 to reflect improved algorithm correction, ensuring cross-scanner and field strength reproducibility, as well as repeatability for this metric [Bachtiar et al., 2019].

Secondary endpoints were alterations in:

- Other MRI parameters (PDFF, hepatic iron content)
- BMI, liver function enzymes (ALT, AST, γ-GT, AST/ALT), lipid profile (TC, LDL, HDL, TG) and insulin resistance (fasting glucose, insulin, total diabetes risk, 75-g, 2 hour Oral Glucose Tolerance Test (OGTT), Homeostasis model assessment (HOMA-IR)) and NAFLD predictive scores (NFS, NASH score)
- Metabolomic profile
- Inflammatory markers
- Gut microbiota composition
- Epigenetic profile (microRNAs plasma levels)

2.1.4. Medical, anthropometric and lifestyle assessment

Detailed medical history was obtained including personal/family medical history 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 $(m)^2$. Total diabetes risk was

assessed using the validated Finnish Diabetic Risk Score (FINDRISK) questionnaire [Lindstrom et al., 2003]. Dietary intake was assessed using a 24-h recall record (three random days) and data was analysed using Nutritionist Pro™ (Axxya Systems) software for the estimation of caloric intake. Physical activity level was evaluated via the International Physical Activity Questionnaire (IPAQ)[Craig et al., 2003] and Metabolic Equivalent Task minutes per week (MET-min/wk) were derived according to the IPAQ scoring protocol. The sum of all METs has been considered as a total physical activity score.

2.1.5. Laboratory analyses

2.1.5.1. Sample collection and genotyping

Blood (25 ml) was drawn after an overnight fast at baseline and post-treatment (for plasma isolation whole blood was collected in EDTA whole blood tubes and was kept on ice until further processing, 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 for 10 min at a speed of 3000 rpm in order to isolate serum and plasma. Fecal samples were collected with the Omnigen-Gut system, following manufacturer instructions (http://www.dnagenotek.com/US/support/ciOMR200.html) and were shipped to FISABIO for 16S rRNA amplicon sequencing.

DNA was also isolated and genotyping was performed with the Infinium Global Screening Array (Illumina). The PNPLA3 rs738409 variant was extracted from the genotypic data of all samples and was used for the calculation of the NASH score.

2.1.5.2. LC-HRMS based metabolomics

Plasma samples were treated as described by Lemonakis et al. [2011] using cold acetonitrile for protein precipitation. For the metabolomic analysis, high-purity water was provided by a Millipore Direct-Q® 3 UV purification system (Merck Millipore Sigma, Burlington, MA, USA), while Optima™ LC-MS grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific (Waltham, MA, USA). For the Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) based metabolomics, liquid chromatography analysis was performed

using an Acquity® UPLC System (Waters). Detection was carried out on an LTQ-Orbitrap® XL hybrid mass spectrometer equipped with an ESI source (Thermo Scientific). Separation was achieved on an Acquity® HSS T3 (Waters) column (100 x 2.1 mm, 1.8 µm) using a standard gradient of water containing 0.1% (v/v) formic acid (A) and acetonitrile (B). MS data were acquired in negative mode, in the full scan range of m/z 115–1000, with a resolution of 30000.

All samples were analysed in duplicate, in random order. QC pooled samples were used and mass accuracy was maintained ≤5ppm. Raw data file preprocessing was achieved using the MZMine 2.53 software [Pluskal et al., 2011]. A generic streamline was employed, including mass detection, chromatogram building, chromatogram deconvolution, isotopic peak grouping, spectral alignment and gap filling to generate the peak list. Metabolite annotation was performed comparing the recorded HRMS and HRMS/MS spectra with online databases such as METLIN [Smith et al., 2005] and the Human Metabolome Database (HMDB)[Wishart et al., 2018].

2.1.5.3. Inflammatory markers

Inflammation markers (EGF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFNγ, TNFα, and VEGF-A) were measured in serum using Randox high sensitivity cytokine I multiplex array, using a Evidence Investigator analyser (Randox Laboratories Ltd, Crumlin, UK) in Randox Clinical Laboratory Services (Antrim, Northern Ireland) facilities.

2.1.5.4. Sequencing and analysis of 16S rRNA amplicons

DNA from fecal samples was extracted using the MagNA Pure LC DNA isolation kit II (Roche Life Science, Basel, Switzerland). The V3-V4 region of the 16S rRNA gene was amplified to construct amplicon libraries that were sequenced using the Reagent Kit v3 (2x300 cycles) in a MiSeq platform (Illumina, San Diego, CA, USA). The DADA2 pipeline [Callahan et al., 2016] (R package) was used to generate amplicon sequence variants (ASV) from raw sequences. Chimeras and sequences matching the human genome were filtered out to generate the final ASV abundance tables. The taxonomic information of the ASVs was obtained by comparison with the SILVA reference database (v.132) [Quast et al., 2013]. The sequences have been deposited in the European Nucleotide Archive under accession number PRJEB40538.

The proportions of 11 bacterial taxa were selected to be included in the main analysis (Flavonifractor, Bacteroides, Faecalibacterium, Dorea, Enterobacteriaceae, Rikenellaceae, Prevotella 9, Coprobacter, Ruminococcaceae UCG-014, Veillonella and Fusobacterium), based on their significant association with liver MRI outcomes at baseline (Table 2.2) or their previously reported associations with dysbiosis in NAFLD [Aron-Wisnewsky et al., 2020, Quesada et al., 2020]. The relative abundances of these taxa were obtained by total-sum scaling of the taxon count contingency tables obtained with the DADA2 pipeline. We also computed three microbiota parameters, namely the Chao1 richness estimator [Chao, 1984], the Shannon diversity index [Shannon, 1948] and the Bray-Curtis dissimilarity index [Bray and Curtis, 1957] to evaluate the overall change in gut microbiota composition and diversity. The Chao1 estimator and Shannon index are measures of within sample diversity. The Bray-Curtis dissimilarity is a well-established non-Euclidian measure of the distance between two bacterial communities (beta-diversity), which takes into account the differences in abundance for all bacterial taxa that are shared between two samples.

Table 2.2: Significant associations between taxon balances and MRI variables, in both groups, at baseline *bacterial group decreases with the associated MRI variables. **bacterial group increases with the associated MRI variables.

Balance	**Bacterial genera or genus-level groups**		**LIF**		**cT1**		**PDFF**		**Hepatic Iron**	
			n	**Beta**	**Pvalue**	**Beta**	**Pvalue**	**Beta**	**Pvalue**	**Beta**
Balance 1	*Faecalibacterium**, *Fusobacterium***, *Prevotella 9***	89	-0.210	0.178	-0.002	0.098	-0.034	**6.4e-08**	-0.252	0.556
Balance 2	*Veillonella***	89	-0.466	**1.5e-02**	-0.006	**5.9e-05**	-0.029	**1.3e-02**	0.231	0.895
Balance 3	Ruminococcaceae UCG-014*	89	0.618	**1.4e-03**	0.004	**9.2e-03**	0.011	0.250	-0.160	0.996
Balance 4	*Coprobacter***	89	0.262	**1.7e-03**	0.002	**1.2e-02**	0.005	0.242	-0.002	0.993

2.1.5.5. MicroRNAs quantification

Quantification of microRNAs implicated in inflammation (miR-16-5p, mir-21-5p and mir-155-5p) was performed. Total RNA was isolated from plasma using MagMAX™ mirVana™ Total RNA Isolation Kit by Thermo Fischer Scientific. This kit is designed for isolation of total RNA, including microRNA and uses MagMAX™ magnetic-bead technology, ensuring reproducible recovery of high-quality RNA. IMPLEN P330 nanophotometer was used to assess the average concentrations of RNA samples, as well as their purity. Concentration was measured in ng/ml and the A260/A230 and A260/A280 ratios were used to detect contamination of proteins or other organic compounds respectively.

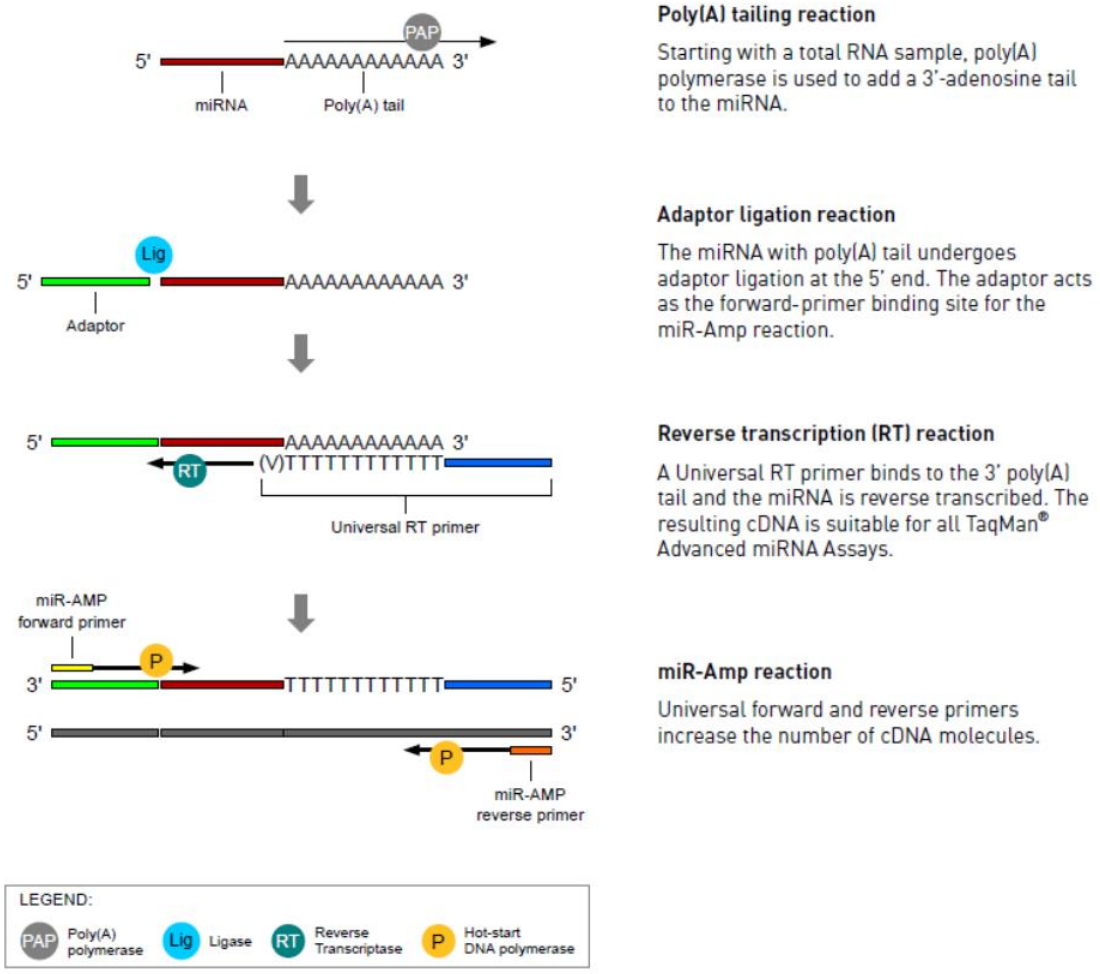


Figure 2.1. cDNA template preparation [Taqman Advanced miRNA assays-user quide, Thermo Fischer Scientific., 2016]

Preparing of cDNA templates was performed using Taqman Advanced miRNA assays (Thermo Fischer Scientific) as described in Fig 2.1. This assay uses universal primers that

uniformly amplify all targets even low-expressing miRNA targets that increase the sensitivity of the assay. Then, 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.) (**Fig. 2.2)**. 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 Cτ (ΔΔCτ) 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 are presented as fold change in expression using the 2ΔΔCt formula.

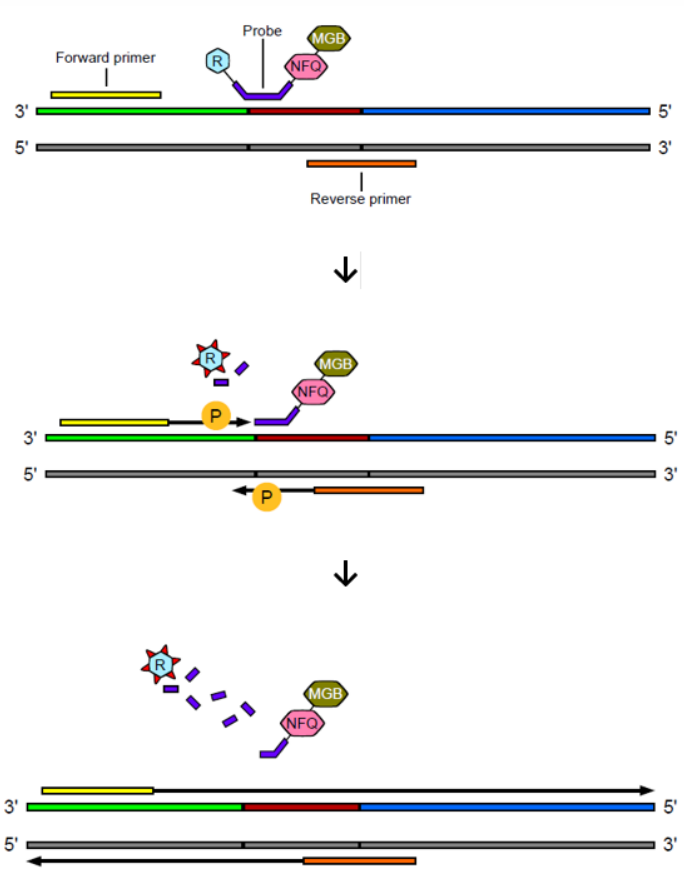


Figure 2.2. Real-time PCR steps [Adapted from Taqman Advanced miRNA assays-user quide [Thermo Fischer Scientific., 2016]

2.1.6. Sample size calculations and statistical analysis

A priori power calculations to calculate the optimal sample-size were based on the SD of the fold-change of liver fat content. A pooled SD of 0.68 was found for the fold-change in liver fat content measured by MR spectroscopy during a lifestyle intervention. With a sample size of 45 evaluable subjects per group and the given SD of 0.68 for the fold-change, the entire 95-% confidence interval for a treatment difference in the fold-change of liver fat content was thought to entirely be within a range from ± 0.35 of the true (but unknown) fold-change with a probability of at least 80% (= estimation power). Most recently this approach proved effective to find a 14% difference of the change of liver fat content in a multicenter, randomized, placebo-controlled clinical trial [Stefan et al., 2014]. Enrolled in each arm should be 50-55 patients (to allow for a 10% drop out). This was a conservative estimate as the sensitivity of the LiverMultiScan platform would allow to detect even small changes from the Mastiha treatment i.e. LIF score changes from 3.0 to 2.3 (cT1 950ms → 900ms), with 90% power in 26 patients per arm allowing for a potentially higher dropout rate.

For the current analysis, we estimated that we had 80% statistical power to detect a minimum effect size difference (effect size=difference in means/pooled SD), between the two treatment groups at the end of the trial, of 0.55 with a maximum of 52 samples per group.

In the primary analysis, 58 variables have been considered, including anthropometric, biochemical, liver MRI (20 variables), microbiota (14 variables) and metabolites (24 variables). Individuals on statin medication had their pre-medication levels approximated by dividing the LDL value by 0.7 and the TC value by 0.8, both at baseline and post-trial. Apart for the triglyceride levels (log transformed), the population normal distribution for the rest was assumed. To avoid overcorrection for multiple testing, the Pearson's coefficient for all pairwise correlations have been calculated and the multiple testing threshold of significance was set at 0.0015. Additionally, findings at nominal level of significance (P value≤0.05) were also reported.

To assess the effect of the Mastiha treatment, post-treatment mean levels between the Mastiha and the Placebo have been compared, via analysis of covariance (ANCOVA) models. The models have been adjusted for the corresponding baseline levels of the tested outcome (apart from the Bray Curtis index), age, sex and the centre of recruitment. Furthermore, a number of sensitivity analyses have been performed with a sequential adjustment for the baseline BMI

levels, the baseline physical activity levels, the difference in the caloric intake between post-treatment and baseline or the difference in the BMI level between post-treatment and baseline. Based on the latter sensitivity analyses, a further stratification of the study samples into two categories has been made, in order to carefully investigate the effect of Mastiha on the MRI outcomes (namely LIF, cT1, PDFF and hepatic iron): Class I obesity (BMI≤ 35 kg/m^2, N=65) and Class II or III obesity (BMI> 35 kg/m^2, N=33). In the stratified analysis, ANCOVA models (adjusted for age, sex and centre) have been applied to assess differences in the mean values of the MRI outcomes, separately for each BMI category. All ANCOVA models were implemented in R.

Regarding metabolites, we obtained levels for sixty five annotated metabolites and calculated the mean values per metabolite per sample per time point, based on duplicate measurements. Baseline and post-treatment metabolite levels have been transformed and normalized, via the "bestNormalise" package in R, prior to the analysis. As part of the exploratory analysis, a PCA including all 65 metabolites at baseline and post-treatment was performed, but we didn't observe any significant clustering between the intervention groups. In order to prioritize metabolites for further statistical analysis, all metabolites with a log2fold change >1.5 between the Mastiha and Placebo groups post-treatment have been selected.

The potential associations between gut microbiota composition and NAFLD-related variables were analyzed with the gneiss software [Morton et al., 2017] within the qiime2 platform [Bolyen et al., 2019]. Rather than focusing on individual taxa abundances, gneiss focuses on the ratios between taxa or groups of taxa, which facilitates the identification of the actual microbes that are changing. Multivariate response linear regression models were built to predict the matrix of abundance balances depending on the different MRI covariates measured at baseline.

Regarding inflammatory markers, 12 circulating biomarkers were natural log transformed prior to statistical analysis. We compared the post-treatment mean levels between the Mastiha and the placebo groups, via ANCOVA models and performed a number of sensitivity analyses and a stratification into two BMI categories as we previously desrcibed.

2.2.IBD-GR

IBD-GR is a randomised, double-blind, placebo controlled (parallel arm) clinical trial aiming at testing the effectiveness of a herbal supplement with Mastiha in IBD patients.

2.2.1. Ethics and trial registration

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 the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines for Good Clinical Practice and was registered with ClinicalTrials.gov (Identifier: NCT02796339). All data were handled in accordance with the Data Protection Act 1998. All subjects gave their informed consent after being provided with a detailed information leaflet describing the study. “Ample time” was given for consideration and participants were free to leave the study at any time. A case report form was created for reporting adverse events.

2.2.2. Study design and study population

Recruitment lasted between May 2016 and September 2017 in Athens. The enrolment was based on certain inclusion and exclusion criteria presented in Table 2.3. After obtaining informed consent and the baseline assessment, patients were randomly allocated to either verum or placebo. In order to avoid allocation bias, randomisation was carried out by an independent bioinformatician, using a computer randomisation software 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. 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 2.3. Inclusion and exclusion criteria of IBD-GR study.

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

Compliance and side effects were monitored biweekly through phone calls and use of compliance calendars. Also, in each visit the number of remaining tablets in the boxes provided to the volunteers were measured. No side effect or any discomfort was reported. Treatment discontinuation was defined as a minimum of 6 consecutive days without treatment intake. All adverse events are being recorded in the Case Report Forms, even if they are not related to the

treatment. Recording of adverse events included the following: clinical symptoms, severity, relationship to treatment, expectedness.

2.2.3. Primary and secondary outcomes

The primary endpoint of IBD-GR was the improvement in quality of life depicted by Inflammatory Bowel Disease Questionnaire (IBDQ). IBDQ is a questionnaire for the assessment of quality of life of IBD patients and consists of 32 questions about bowel, social, systemic and emotional performance (score from 32 to 224 points).

Secondary endpoints included alterations in

- Metabolomic profile
- Inflammatory markers (IL-6, IL-10, IL-11, IL-17, IL-22)
- Gut microbiota composition
- Epigenetic profile (microRNAs)

2.2.4. Medical, anthropometric and lifestyle assessment

Medical history was obtained by experienced gastroenterologist and quality of life was assessed using the validated Greek version of IBDQ [Guyatt et al., 1989, Pallis et al., 2001], Harvey-Bradshaw Index (HBI) and Partial Mayo Score (PMS). Body weight was measured to the nearest 0.1 kg and height was measured to the nearest millimeter twice. Body Mass Index was calculated.

2.2.5. Laboratory analyses

2.2.5.1. Sample collection

Standard Blood sampling (20mL) was performed and samples were centrifuged at 3000rpm for 10 minutes at 4◦C for serum and plasma 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 stool extracts were kept for a maximum of 9 days at -20◦C until further analysis.

2.2.5.2. Metabolomic analysis

300uL aliquots of stool extracts were evaporated to dryness using a LABCONCO CentriVap Concentrator και CentriVap Cold Trap 800-522-7658. Samples were reconstituted to final volume of 600uL using phosphate buffer (pH=7.2) in D2O 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 12000 rpm at 4^{o}C. Supernatants were transferred to NOREL UP 5 mm NMR tubes for further NMR analysis.

All 1H-NMR spectra were acquired using a Varian 600 MHz spectrometer equipped with a triple resonance probe (HCN), at room temperature (25^{o}C). 1D NOE-PRESAT pulse sequence was used with the following parameters: 128 transients with 32K 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. All 1H-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 D2O region (4.68 5.00 ppm) was excluded. 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 180o X-nuclei pulses and gradient coherence selection) and gHSQCad (heteronuclear single-quantum 1-bond J-correlation spectroscopy with adiabatic 180o 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) [Filntisi et al., 2017] and the online NMR database HMDB [23].

2.2.5.3. Inflammatory markers

IL-6 (R&D Systems, Inc., Minneapolis, USA), IL-10 (OriGene Technologies, Inc., Maryland, USA), IL-17 (IL-17A), IL-11, IL-22 (Boster Biological Technology, Pleasanton, CA, USA), were

measured applying sandwich Enzyme-linked immunosorbent assay (ELISA) [Fig. 2.3] at baseline and at follow-up.

ELISA is a heterogenous colorimetric immunoassay which allows for accurate and sensitive detection of antigens. The main principle of ELISA is based on the physical properties of antigens and antibodies, which are linked with high affinity and specificity, as well as on the use of a specific complex, consisting of a homologous antibody and an enzyme, to detect the antigen-antibody conjugate. There are four types of ELISA: direct, indirect, sandwich and competitive.

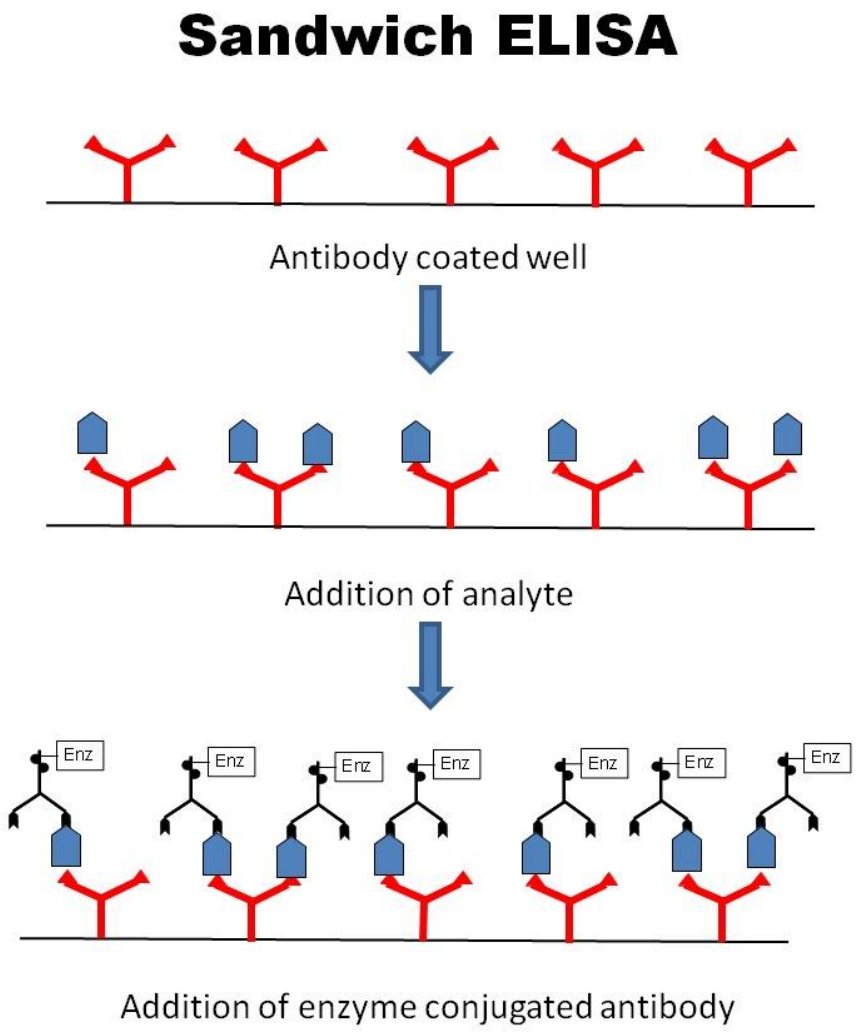


Figure 2.3. Diagram of a sandwich ELISA [Cox et al., 2012]

In sandwich ELISA [Fig 2.3], we used 96-well plates precoated with a monoclonal antibody from mouse specific for the cytokine under investigation. Standards and samples were

added to the wells, a biotinylated detection monoclonal antibody from mouse specific for the cytokine under investigation was added subsequently and then followed by washing with wash buffer. AvidinBiotin-Peroxidase Complex was added and unbound conjugates were washed away with wash buffer. Horseradish peroxidase (HRP) substrate, 3,3', 5,5”-tetramethylbenzidine (TMB) was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The optical density of the coloured product was read at 450nm using an ELISA reader (Biotek PowerWave XS2) and was proportional to the cytokines captured in plate.

2.2.5.4. Sequencing and analysis of 16S rRNA amplicons

DNA extraction from fecal samples was performed using a repeated bead beating plus column (RBB+C) method [Yu and Morrison 2004]. DNA was used at a concentration of 5 ng/µl in 10 mMTris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation. PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al. [2013]. Primers contained adapter overhanging sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit. After 16S rRNA gene amplification, amplicons were multiplexed and 1 ml of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer’s instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001). Quality assessment of sequencing reads was performed with the prinseq-lite program and sequences were analyzed using the qiime2 platform. Sequence denoising, paired-ends joining, and chimera depletion was performed with the DADA2 software. The taxonomic affiliations of the sequences were assigned by means of the Naive Bayesian classifier integrated in quiime2 using the SILVA_release_132 database (Quast et al, 2013).

One hundred percent-similarity sequence clusters were then obtained and considered as Operational Taxonomic Units (OTUs) for further analyses. Rarefaction curves and diversity metrics were obtained within qiime2. We visualized the variation in microbiota composition among treatments by means of clustering and PCA using phylogenetic and non-phylogenetic measures including the Jaccard, Bray-Curtis and UniFrac distances.

2.2.5.5. MicroRNAs quantification

MicroRNAs quantification was performed as described in section 2.1.5.5.

2.2.6. Sample size calculations and statistical analysis

Sample size calculation for patients in relapse was based on the findings of Irvine et al [2000]. A sample size of 58 subjects, 29 per arm, is sufficient to detect a clinically important difference of 28.3 between groups in IBDQ score assuming a standard deviation of 37.3 using a two-tailed t-test of difference between means with 80% power and a 5% level of significance. Considering a dropout rate of 10% the sample size required is 64 (32 per group) [Papada et al., 2019a]. Sample size calculation for patients in remission was based on the ground of the assumption that 10% in verum versus 40% in placebo will experience clinical relapse (in CD scoring HBI ≥5 and in UC scoring PMS ≥2). As such, a sample size of 64 patients are required to have a 80% chance of detecting (power 80%), as significant at the 5% level (α=0.05) [Pocock et al, 1983]. To allow for approximately 10% drop-out rate due to any reason, the number is increased to 70 patients [Papada et al., 2019b].

Data were expressed as mean ± standard deviation (SD) or with the median and the interquartile range (IQR). Quantitative variables were 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. 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).

In metabolomics, multivariate statistical analysis was employed to aligned 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 Parscaling allows any metabolites of low/medium intensity to affect the analysis only if they represent systematic variation. At first, 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 R2Y (goodness of fit) and Q2 (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 biomarker discovery, classification, and 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. Pairwise Wilcoxon and Kruskal-Wallis tests were performed to compare diversity metrics and abundance of specific bacterial taxa between different groups of samples.

3. Results and Discussion

3.1. MAST4HEALTH

3.1.1. Descriptives of the study population

Ninety-eight patients were randomised to Mastiha (N=41) or Placebo (N=57) for 6 months (CONSORT Flow diagram in **Figure. 3.1**). Out of the 98 volunteers who participated in the trial, 87 completed the intervention.

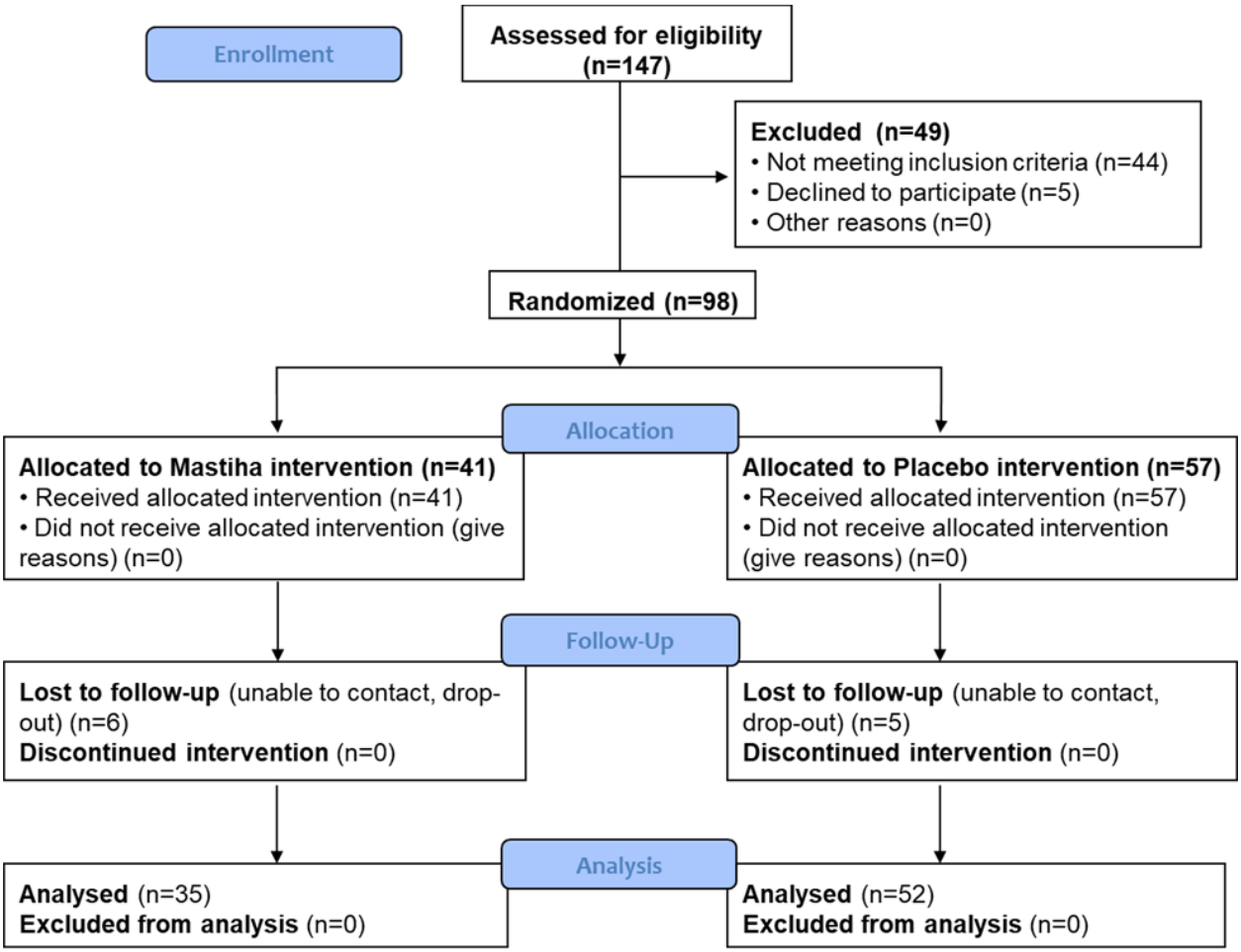


Figure 3.1. MAST4HEALTH CONSORT 2010 Flow diagram

Baseline characteristics are presented in **Table 3.1 a, b, c and d**. Overall, there were no significant differences between Mastiha and Placebo. Patients had moderate liver disease at baseline: mean LIF of 2.3 (SD=0.6), cT1 of 878.4 (79.5) ms, PDFF of 16.5 (12.00) % and hepatic iron 1.2 (0.2) mg/g. Mean baseline and end-of-trial values for body weight in the Mastiha group

were 100.80 kg (±15.84) and 100.23 kg (±15.00) respectively, (paired t-test P value=0.02). In Placebo, body weight was 105.13 kg (±19.75) and 104.05 kg (±20.62) respectively, (paired t-test P value=0.79) **(Table 3.1, 3.1a, 3.1b, 3.1c)**. Significant differences were observed between the two groups at baseline for circulating IL-1α, IL-2, IL-4, and TNF-α **(Table 3.2)**.

Table 3.1a: Baseline characteristics of the MAST4HEALTH participants (anthropometrics and biochemical parameters)

		All		Placebo		Mastiha	
Baseline Characteristics	**n**	**Mean (SD) or n**	**n**	**Mean (SD) or n**	**n**	**Mean (SD) or n**	**Pvalue***
Age (years)	98	48.83 (9.36)	57	48.95 (9.04)	41	48.66 (9.89)	0.929$
Sex (M/F)	98	68/30	57	42/15	41	26/15	0.386
Centre (GR/IT/SR)	98	38/30/30	57	23/17/17	41	15/13/13	0.931
Statin (Y/N)	98	12/86	57	8/49	41	4/37	0.636
T2D (Y/N)	98	4/94	57	2/55	41	2/39	1.000
Total Physical Activity Score	91	3622.17 (5128.18)	52	3536.78 (5345.85)	39	3736.04 (4889.48)	0.921
Smoking (Never/Ex/Current)	97	51/25/21	56	28/14/14	41	23/11/7	0,642
BMI (kg/m^2)	98	34.44 (4.41)	57	34.66 (5.05)	41	34.14 (3.38)	0.513
Glucose (mg/dL)	93	102.44 (15.64)	53	102.89 (14.38)	40	101.84 (17.33)	0.893
120 min-OGTT glucose (mg/dL)	87	131.57 (47.47)	47	126.88 (41.86)	40	137.08 (53.33)	0.260
Insulin (μU/mL)	94	18.94 (9.79)	54	18.63 (10.46)	40	19.38 (8.92)	0.586
HOMA_IR	90	4.88 (2.6)	51	4.83 (2.69)	39	4.95 (2.52)	0.654
FINDRISK_score	96	13.67 (3.77)	56	13.32 (3.69)	40	14.15 (3.89)	0.217
TC (mg/dL)	98	201.75 (37.43)	57	202.91 (37.61)	41	200.14 (37.59)	0.528
LDL (mg/dL)	97	127.14 (34.64)	56	129.69 (37.31)	41	123.65 (30.75)	0.276
Triglycerides (mg/dl)	98	148.21 (65.08)	57	141.96 59.35)	41	156.91 (72.15)	0.331
HDL(mg/dL)	98	44.49 (10.35)	57	44.31 (9.91)	41	44.75 (11.04)	0.980
ALT (IU/L)	95	37.78 (20.45)	56	36.7 (21.67)	39	39.33 (18.71)	0.253
AST (IU/L)	95	25.29 (11.12)	56	24.39 (11.72)	39	26.59 (10.19)	0.198
AST/ALT	95	0.74 (0.24)	56	0.74 (0.25)	39	0.74 (0.22)	0.477
γ-GT (U/L)	97	55.12 (60.22)	57	49.63 (54.97)	40	62.95 (66.94)	0.305
NFS_score	97	-1.97 (1.39)	57	-2.01 (1.53)	40	-1.91 (1.2)	0.713
NASH_score	88	-1.24 (0.94)	50	-1.32 (0.94)	38	-1.14 (0.94)	0.218
LIF	95	2.26 (0.62)	55	2.25 (0.68)	40	2.29 (0.55)	0.667
Hepatic_Iron (mg/g)	98	1.25 (0.22)	57	1.24 (0.24)	41	1.26 (0.2)	0.451
cT1 (ms)	95	878.36 (79.49)	55	879.88 (92.12)	40	876.26 (58.93)	0.876
PDFF (%)	96	16.47 (11.98)	57	16.09 (13.31)	39	17.02 (9.87)	0.547

Results are presented as mean (SD) for continuous variables and counts for categorical ones
*P value for the difference between Placebo and Mastiha groups was assessed with ANCOVA (adjusted for age, sex and centre) for the continuous variables and with Chi-square for the categorical ones
$ Adjusted only for sex and centre.

Table 3.1b: Baseline characteristics of serum inflammatory biomarkers for Mastiha and Placebo groups

	All		Placebo		Mastiha		
Baseline Characteristics	**n**	**Mean (SD) or n**	**n**	**Mean (SD) or n**	**n**	**Mean (SD) or n**	**Pvalue**
IL.1a (pg/ml)	95	0.193 (0.185)	55	0.155 (0.143)	40	0.246 (0.223)	**0,024**
IL.1b (pg/ml)	92	0.964 (0.521)	54	0.883 (0.388)	38	1.079 (0.654)	0,128
IL.2 (pg/ml)	87	1.97 (1.712)	49	1.636 (1.55)	38	2.402 (1.832)	**0,011**
IL.4 (pg/ml)	97	1.624 (0.539)	56	1.531 (0.377)	41	1.75 (0.688)	**0,043**
IL.6 (pg/ml)	97	1.851 (1.324)	56	1.915 (1.4)	41	1.764 (1.224)	0,472
IL.8 (pg/ml)	96	9.095 (5.934)	55	8.39 (5.128)	41	10.04 (6.822)	0,092
IL.10 (pg/ml)	96	0.771 (0.471)	55	0.732 (0.459)	41	0.825 (0.488)	0,166
IFNg (pg/ml)	93	0.373 (0.425)	53	0.321 (0.322)	40	0.443 (0.528)	0,055
TNF-a (pg/ml)	95	2.701 (1.127)	55	2.461 (0.685)	40	3.031 (1.49)	**0,027**
VEGF (pg/ml)	97	151.531 (113.965)	56	157.223 (113.504)	41	143.756 (115.541)	0,304

Pvalues for the Mastiha vs. Placebo group adjusted for age, gender and centre using ANOVA for continuous variables. Betas and p-values for the comparisons refer to log-transformed and scaled values, while raw mean±SD values are presented. Categorical variables were assessed with Chi-square.

Table 3.1c. Baseline characteristics of microbiota parameters per trial group.

Baseline Characteristics	**All**		**Placebo**		**Mastiha**		
Shannon microbiota diversity index	89	3.9 (0.45)	51	3.87 (0.43)	38	3.96 (0.47)	0,292
Chao1 microbiota richness index	89	223.92 (102.35)	51	212.2 (89.53)	38	239.65 (116.79)	0,107
Prevotella 9	89	0.01903 (0.03987)	51	0.01587 (0.03763)	38	0.02327 (0.04284)	0,392
Flavonifractor	89	0.00152 (0.00255)	51	0.00196 (0.0031)	38	0.00092 (0.00136)	0,022
Bacteroides	89	0.16462 (0.10801)	51	0.16018 (0.09685)	38	0.17058 (0.12251)	0,732
Dorea	89	0.00312 (0.0029)	51	0.00316 (0.00321)	38	0.00306 (0.00247)	0,857
Faecalibacterium	89	0.1363 (0.07717)	51	0.14211 (0.08023)	38	0.12849 (0.07319)	0,471
Enterobacteraceae	89	0.00664 (0.01942)	51	0.005 (0.01606)	38	0.00884 (0.02323)	0,272
Rikenellaceae	89	0.01161 (0.00922)	51	0.01063 (0.00939)	38	0.01292 (0.00895)	0,304
Coprobacter	89	0.0004 (0.00071)	51	0.00045 (0.00078)	38	0.00034 (0.00061)	0,419
Ruminococcaceae UCG-014	89	0.01368 (0.02933)	51	0.00852 (0.02045)	38	0.02061 (0.03734)	0,036
Veillonella	89	0.00192 (0.00864)	51	0.0028 (0.0113)	38	0.00073 (0.00153)	0,329
Fusobacterium	89	0.00028 (0.00123)	51	0.00048 (0.0016)	38	0.00002 (0.00006)	0,054

Pvalues for the Mastiha vs. Placebo group adjusted for age, gender and centre using ANOVA for continuous variables.

Table 3.1d. Baseline characteristics of plasma metabolites per trial group.

Baseline Characteristics	All		Placebo		Mastiha		
	n	Mean (SD) or n	n		n	Mean (SD) or n	Pvalue
Bilirubin	88	192074.36 (247931.69)	52	206672.58 (293913.2)	36	170988.05 (161703.31)	0,950
C10H15O	88	486394.44 (1521151.33)	52	602560.78 (1863991.12)	36	318598.61 (799937.41)	0,688
C11H12O5	88	18905.89 (64148.66)	52	22136.45 (80053.64)	36	14239.53 (29201.26)	0,999
C9H10O	88	159380.24 (255183.09)	52	146411.02 (227981.11)	36	178113.56 (292436.9)	0,931
Phenylpropane	88	1502212.47 (4316970.11)	52	1779871.66 (5153529.5)	36	1101149.19 (2710035.98)	0,662
Steroid_mz427.2152	88	617904.94 (688237.83)	52	519543.66 (419588.39)	36	759982.36 (941136.64)	0,321
Styrene	88	246974.33 (467998.59)	52	197966.4 (264617.26)	36	317763.56 (658538.29)	0,861
Cholic_acid	88	213881.15 (693274.33)	52	122826.78 (706402.36)	36	345404.12 (661419.79)	0,018
Deoxycholic_acid	88	308991.36 (367277.72)	52	229039.39 (185502.19)	36	424477.55 (511808.71)	0,095
Dihydroxyandrosterone_sulfate	88	1459520.57 (2438767.71)	52	1655427.07 (2959682.02)	36	1176544.52 (1371298.72)	0,879
Dopamine_quinone	88	71915.4 (371463.4)	52	89264.31 (471004.76)	36	46855.87 (136520.23)	0,898
Triterpenic_acid_sulfate_metabolite	88	8394.48 (33462.86)	52	5581.61 (22895.2)	36	12457.53 (44619)	0,212
LysoPC_16.1	88	753394.94 (343580.71)	52	732603.96 (338020.63)	36	783426.36 (354080.27)	0,537
LysoPC_18.1	88	3656793.49 (1206078.44)	52	3692520.09 (1301857.14)	36	3605188.39 (1068426.3)	0,837
LysoPE_16.0	88	9331712 (3053926.62)	52	9195439.89 (3344008.26)	36	9528549.49 (2611733.3)	0,527
LysoPE_18.1	88	16981807.71 (5729375.31)	52	16404241.11 (4830075.41)	36	17816070.59 (6810970.82)	0,360
LysoPE_20.0	88	28552960.96 (10553176.65)	52	27984881.42 (10496833.27)	36	29373520.3 (10728826.62)	0,588
LysoPE_22.7	88	4237998.54 (1549242.15)	52	4316075.13 (1530816.39)	36	4125221.25 (1590372.41)	0,436
Phenol_sulfate	88	1370588.18 (1937642.12)	52	1301249.23 (1634780.15)	36	1470744.43 (2328266.11)	0,709
Phenylsulfate	88	73846.03 (190383.34)	52	44374.66 (78709.76)	36	116415.78 (279132.61)	0,180
Pregnenolone_sulfate	88	824906.36 (695282.17)	52	753941.91 (603982.66)	36	927410.56 (807334.79)	0,100
Acid	88	600706.28 (549476.38)	52	563810.83 (430137.96)	36	653999.7 (689894.99)	0,601
Sulfoglycolithocholate	88	533994.96 (1051759.15)	52	369654.12 (403232.61)	36	771376.16 (1553923.48)	0,201
Testosterone_sulfate	88	72925284.09 (41858245.68)	52	71411057.69 (41615692.76)	36	75112500 (42700684.8)	0,335

Pvalues for the Mastiha vs. Placebo group adjusted for age, gender and centre using ANOVA for continuous variables.

3.1.2. Effect of Mastiha on liver inflammation and fibrosis through MRI, biochemical and multi-omic analyses **(The results are presented in Paper 1)**

Post-treatment levels between Mastiha and Placebo for 58 outcomes have been compared. The findings are summarised in **Figure 3.2** and **Table 3.2a, 3.2b. 3.2c**.

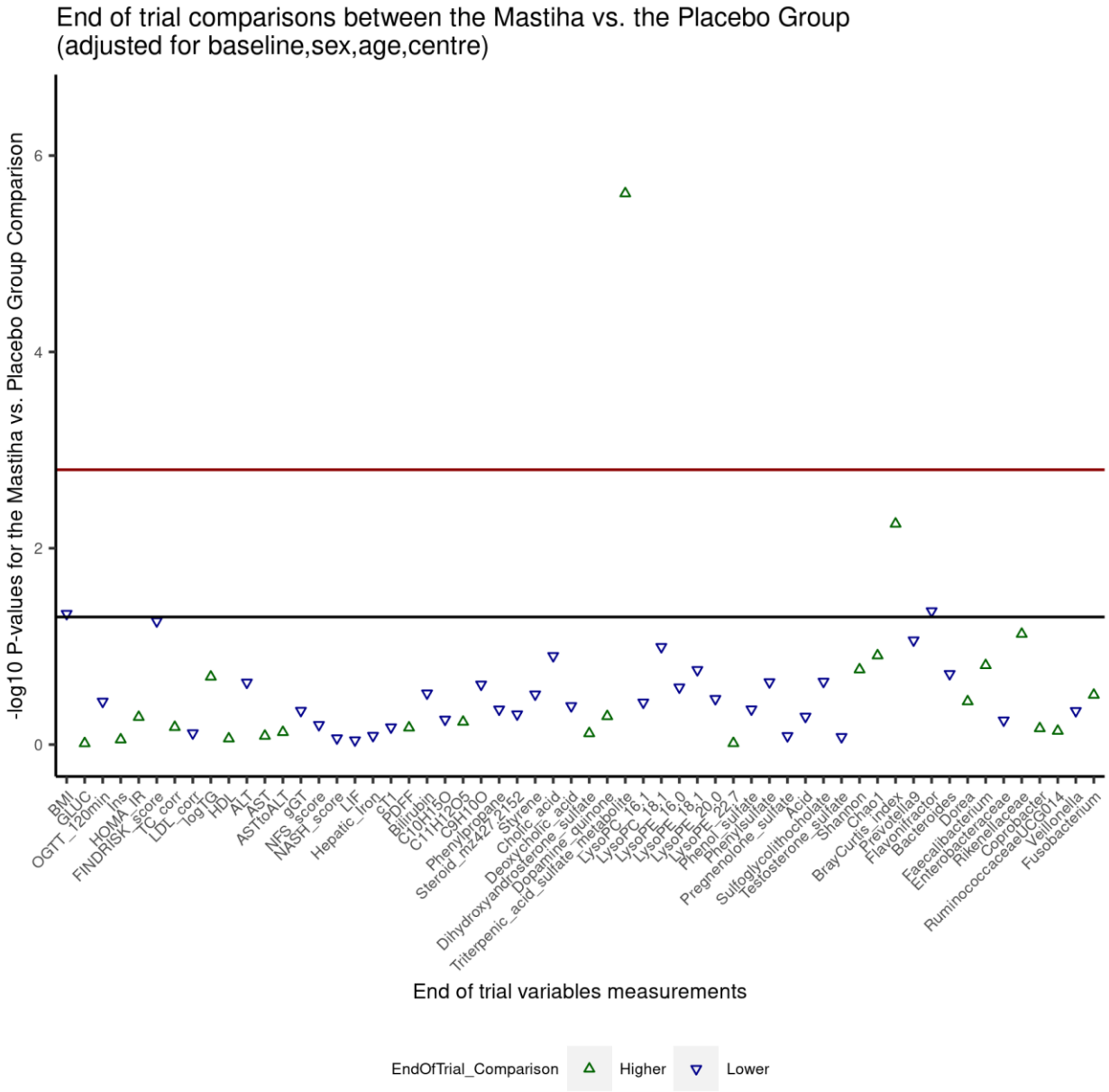


Figure 3.2. Differences in all outcomes assessed post treatment between the Mastiha and Placebo groups. Comparisons were performed using ANCOVA and adjusted for the corresponding baseline levels for each outcome, age, sex and centre (post-treatment outcome ~ mastiha vs placebo group + baseline outcome + age + sex + centre). Triangles indicate the P value (-log10 transformed) for the comparison. Blue descending triangles indicate lower mean values in the Mastiha group compared to the Placebo; while green ascending triangles indicate the opposite. The black horizontal line is marking nominal significance level (P value=0.05) and the red line the multiple testing significance level (P value=0.0015).

Table 3.2a ANOVA post-treatment comparisons between the Mastiha and Placebo groups for biochemical and MRI parameters

	Placebo		Mastiha		Post-treatment differences in relation to the Mastiha group	
Post-treatment outcome	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	**Pvalue**
BMI (kg/m^2)	52	34.43 (5.81)	35	33.54 (3.54)	-0.662 (0.328)	**0,047**
Glucose (mg/dL)	47	101.24 (18.65)	33	100.84 (15.88)	0.106 (3.011)	0,972
120 min-OGTT glucose (mg/dL)	40	131.97 (45.77)	31	130.67 (45.92)	-6.758 (7.395)	0,364
Insulin (μU/mL)	51	17.99 (10.59)	33	19.56 (9.43)	0.232 (1.671)	0,890
HOMA_IR	46	4.41 (2.92)	31	5.03 (2.66)	0.303 (0.474)	0,525
FINDRISK_score	52	14.02 (4.09)	34	13.32 (3.79)	-1.304 (0.67)	0,055
TC (mg/dL)	52	206.15 (44.33)	35	205.54 (47.05)	2.638 (6.069)	0,665
LDL (mg/dL)	48	138.72 (44.75)	34	130.89 (45.36)	-2.387 (7.958)	0,765
Triglycerides (mg/dl)	51	152.21 (73.80)	35	183.62 (73.26)	0.094 (0.073)	0,204
HDL(mg/dL)	52	43.16 (9.81)	35	41.86 (6.54)	0.186 (1.135)	0,870
ALT (IU/L)	51	37.82 (21.79)	33	36.88 (18.52)	-3.99 (3.32)	0,233
AST (IU/L)	51	25.43 (9.42)	33	26.61 (9.78)	0.449 (1.935)	0,817
AST/ALT	51	0.79 (0.34)	33	0.8 (0.27)	0.021 (0.064)	0,748
γ-GT (U/L)	52	45.75 (41.66)	35	47.74 (41.06)	-3.567 (4.715)	0,452
NFS_score	50	-1.72 (1.21)	35	-1.74 (0.99)	-0.065 (0.134)	0,630
NASH_score	47	-1.31 (0.83)	31	-1.23 (0.78)	-0.022 (0.127)	0,861
LIF	45	2.2 (0.61)	34	2.31 (0.56)	-0.014 (0.114)	0,902
Hepatic_Iron (mg/g)	48	1.2 (0.26)	35	1.23 (0.16)	-0.007 (0.031)	0,813
cT1 (ms)	43	873.52 (62.37)	32	872.7 (62.98)	-5.405 (12.459)	0,666
PDFF (%)	48	14.33 (9.53)	34	16.41 (10.02)	0.779 (1.836)	0,673

adjusted for the corresponding baseline levels for each outcome, age, gender and centre. Betas for the metabolites comparisons refer to transformed and normalized values, while raw mean±SD values are presented.

Table 3.2b ANOVA post-treatment comparisons between the Mastiha and Placebo groups for plasma metabolites, adjusted for the corresponding baseline levels for each outcome, age, gender and centre. Betas for the metabolites comparisons refer to transformed and normalized values, while raw mean±SD values are presented.

	Placebo		Mastiha		Post-treatment differences in relation to the Mastiha group	
Post-treatment outcome	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	**Pvalue**
Bilirubin	37	172367.93 (216845.59)	24	139479.85 (148553.67)	-0.245 (0.235)	0,300
C10H15O	37	239464.19 (486023.88)	24	121913.6 (258144.76)	-0.114 (0.192)	0,555
C11H12O5	37	6692.05 (15267.68)	24	13430.12 (33804.27)	0.109 (0.2)	0,588
C9H10O	37	279978.27 (558099.76)	24	131507.07 (162339.27)	-0.29 (0.246)	0,244
Phenylpropane	37	963199.06 (2034793.27)	24	506475.45 (1164195.85)	-0.155 (0.198)	0,438
Steroid_mz427.2152	37	634907.82 (590367.27)	24	612995.5 (702080.65)	-0.156 (0.224)	0,491
Styrene	37	404929.3 (867988.04)	24	212195.85 (280292.12)	-0.271 (0.263)	0,308
Cholic_acid	37	517027.54 (1055521.53)	24	166786.96 (351630.22)	-0.452 (0.29)	0,125
Deoxycholic_acid	37	378527.64 (522131.91)	24	266620.31 (237199.8)	-0.228 (0.271)	0,405
Dihydroxyandrosterone_sulfate	37	1413494.25 (1750436.32)	24	1462879.37 (1284466.41)	0.063 (0.214)	0,769
Dopamine_quinone	37	20548.07 (103651.96)	24	53389.94 (130745.52)	0.134 (0.205)	0,515
Triterpenic_acid_sulfate_metabolite	37	14232.7 (38763.01)	24	357785.41 (580523.51)	1.001 (0.189)	**2,43E-06**
LysoPC_16.1	37	838215 (413565.99)	24	767017.78 (368144.61)	-0.244 (0.271)	0,372
LysoPC_18.1	37	4138216.41 (1673159.41)	24	3642709.81 (1150525.37)	-0.459 (0.275)	0,101
LysoPE_16.0	37	10112823.62 (3706442.6)	24	9633559.37 (4088981.61)	-0.315 (0.277)	0,261
LysoPE_18.1	37	19110054.58 (8910722.67)	24	17275036.76 (6984279.83)	-0.381 (0.276)	0,174
LysoPE_20.0	37	29305634.59 (11081467.44)	24	28719657.42 (11328723.02)	-0.238 (0.248)	0,342
LysoPE_22.7	37	4422301.54 (1805941.12)	24	4259963.53 (1520210.01)	0.009 (0.239)	0,969
Phenol_sulfate	37	1490426.77 (1737212.46)	24	1522271.39 (2232458.55)	-0.19 (0.243)	0,437
Phenylsulfate	37	75119.34 (109633.91)	24	74470.18 (194778.31)	-0.283 (0.233)	0,231
Pregnenolone_sulfate	37	804033.63 (785050.1)	24	1083143.94 (1104183.3)	-0.05 (0.217)	0,817
Acid	37	578290.69 (307102.83)	24	558231.25 (350214.97)	-0.123 (0.189)	0,519
Sulfoglycolithocholate	37	420518.57 (495248.04)	24	382919.15 (428751.61)	-0.299 (0.245)	0,228
Testosterone_sulfate	37	71678378.38 (37571593.77)	24	78050000 (37098376.85)	-0.035 (0.169)	0,835

Table 3.2c ANOVA post-treatment comparisons between the Mastiha and Placebo groups for microbiota parameters, adjusted for the corresponding baseline levels for each outcome, age, gender and centre. Betas for the metabolites comparisons refer to transformed and normalized values, while raw mean±SD values are presented.

	Placebo		Mastiha		Post-treatment differences in relation to the Mastiha group	
Post-treatment outcome	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	**Pvalue**
Shannon microbiota diversity index	48	3.9 (0.34)	32	4 (0.41)	0.1 (0.073)	0,172
Chao1 microbiota richness index	48	209.11 (63.78)	32	240.41 (80.48)	21.631 (13.888)	0,124
BrayCurtis microbiota dissimilarity index	45	0.49 (0.16)	29	0.59 (0.12)	0.102 (0.036)	**0,006**
Prevotella 9	48	0.02295 (0.05448)	32	0.01581 (0.0281)	-0.0141 (0.0081)	0,087
Flavonifractor	48	0.00321 (0.00526)	32	0.00091 (0.00096)	-0.0017 (0.0008)	**0,044**
Bacteroides	48	0.17591 (0.11498)	32	0.15366 (0.08651)	-0.0286 (0.0216)	0,191
Dorea	48	0.00309 (0.00264)	32	0.00396 (0.00367)	0.0007 (0.0007)	0,364
Faecalibacterium	48	0.13341 (0.07666)	32	0.15368 (0.09476)	0.0272 (0.0189)	0,156
Enterobacteraceae	48	0.02091 (0.05032)	32	0.01829 (0.04483)	-0.0061 (0.0106)	0,566
Rikenellaceae	48	0.01059 (0.00951)	32	0.01606 (0.0165)	0.0052 (0.0029)	0,075
Coprobacter	48	0.00054 (0.00167)	32	0.00067 (0.00151)	0.0002 (0.0004)	0,684
Ruminococcaceae UCG-014	48	0.00792 (0.02231)	32	0.01477 (0.0269)	0.002 (0.0057)	0,727
Veillonella	48	0.00273 (0.00999)	32	0.00313 (0.01173)	-0.0015 (0.002)	0,454
Fusobacterium	48	0.00001 (0.00005)	32	0.00065 (0.00356)	0.0006 (0.0006)	0,312

A very significant post treatment increase in a Mastiha derived metabolite (sulpho-conjugated) (**Figure 3.3**) within the Mastiha group compared to the Placebo (P value=2.43e-06) has been detected and remained robust across the sensitivity analysis, indicative of compliance to treatment protocol.

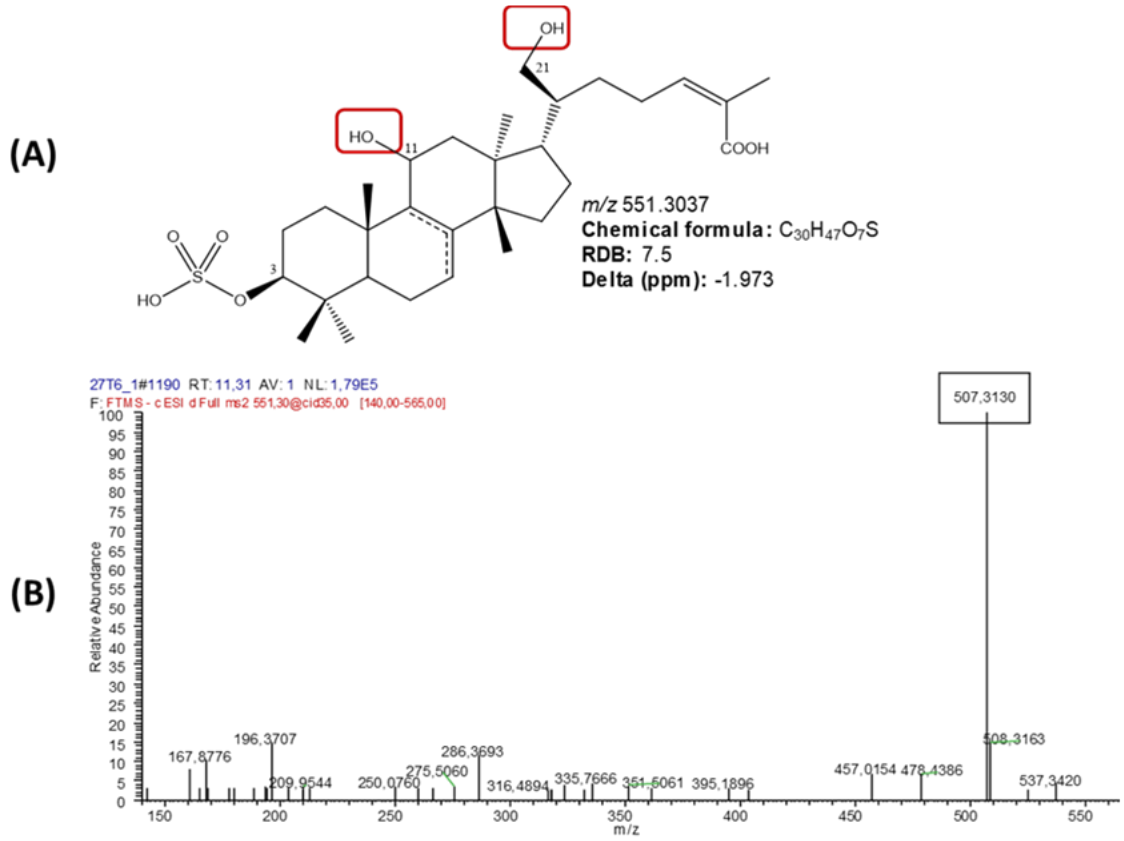


Figure 3.3. (A) Suggested structure of the tentatively identified triterpenic acid metabolite. (B) HRMS/MS spectrum of the detected triterpenic acids metabolite in negative mode.

None of the other outcomes met the multiple testing threshold of significance for an association with the Mastiha treatment. However, for the MRI parameters an increase in the signal magnitude and a change in the direction of effect have been found, after adjusting for the difference in BMI levels between post-treatment and baseline. Based on this, a further investigation of the MRI parameters has been assessed, after stratifying by BMI category (baseline characteristics after stratification are presented in **Table 3.3**). In BMI>35 kg/m^2 mean baseline values for LIF and cT1 in the Mastiha group were 2.52 (±0.46) and 890.46 ms (±36.79) respectively and in the Placebo group 2.5 (±0.68) and 915.65 ms (±112.89) respectively.

Table 3.3. Baseline characteristics of all samples and per trial group, stratified per BMI category. Pvalues for the Mastiha vs. Placebo group adjusted for age, gender and centre using ANOVA for continuous variables. Categorical variables were assessed with Chi-square.

	All (BMI<=35)		All (BMI>35)		Placebo (BMI<=35)		Mastiha (BMI<=35)			Placebo (BMI>35)		Mastiha (BMI>35)		
Baseline Characteristics	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Pvalue**	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Pvalue**
Age (years)	65	48.57 (9.47)	33	49.33 (9.25)	37	49.08 (9.08)	28	47.89 (10.09)	0,563	20	48.7 (9.21)	13	50.31 (9.6)	0,626
Gender (M/F)	65	47/18	33	21/12	37	29/8	28	18/10	0,328	20	13/7	13	8/5	1,000
Centre (GR/IT/SR)	65	17/27/21	33	21/3/9	37	9/16/12	28	8/11/9	0,918	20	14/1/5	13	7/2/4	0,508
NFS_score	64	-2.19 (1.22)	33	-1.54 (1.62)	37	-2.2 (1.35)	27	-2.18 (1.05)	0,661	20	-1.66 (1.8)	13	-1.35 (1.33)	0,816
NASH_score	59	-1.21 (0.88)	29	-1.32 (1.06)	33	-1.32 (0.99)	26	-1.07 (0.71)	0,115	17	-1.33 (0.87)	12	-1.31 (1.32)	0,286
LIF	63	2.14 (0.6)	32	2.51 (0.6)	35	2.1 (0.64)	28	2.19 (0.56)	0,497	20	2.5 (0.68)	12	2.52 (0.46)	0,421
Hepatic_Iron (mg/g)	65	1.27 (0.23)	33	1.21 (0.21)	37	1.27 (0.28)	28	1.27 (0.15)	0,530	20	1.19 (0.13)	13	1.26 (0.29)	0,717
cT1 (ms)	63	864.21 (68.95)	32	906.2 (91.89)	35	859.44 (71.92)	28	870.17 (65.85)	0,451	20	915.65 (112.89)	12	890.46 (36.79)	0,210
PDFF (%)	63	16.24 (11.22)	33	16.9 (13.5)	37	14.9 (11.74)	26	18.14 (10.35)	0,136	20	18.29 (15.91)	13	14.78 (8.78)	0,125

Post-treatment levels of both cT1 and LIF were lower (P value= 0.033 and 0.049, respectively) in Mastiha compared to the Placebo in BMI>35 kg/m^2 (**Figure 3.4, Table 3.4**). In BMI>35 kg/m^2 the mean post-treatment levels for cT1 and LIF were lower in Mastiha compared to Placebo. Finally, a pronounced reduction in both cT1 and LIF values was detected only in the Mastiha group with BMI>35 kg/m^2 (mean, SD: -29.61, 57.86 and -0.30, 0.54, for cT1 and LIF respectively).

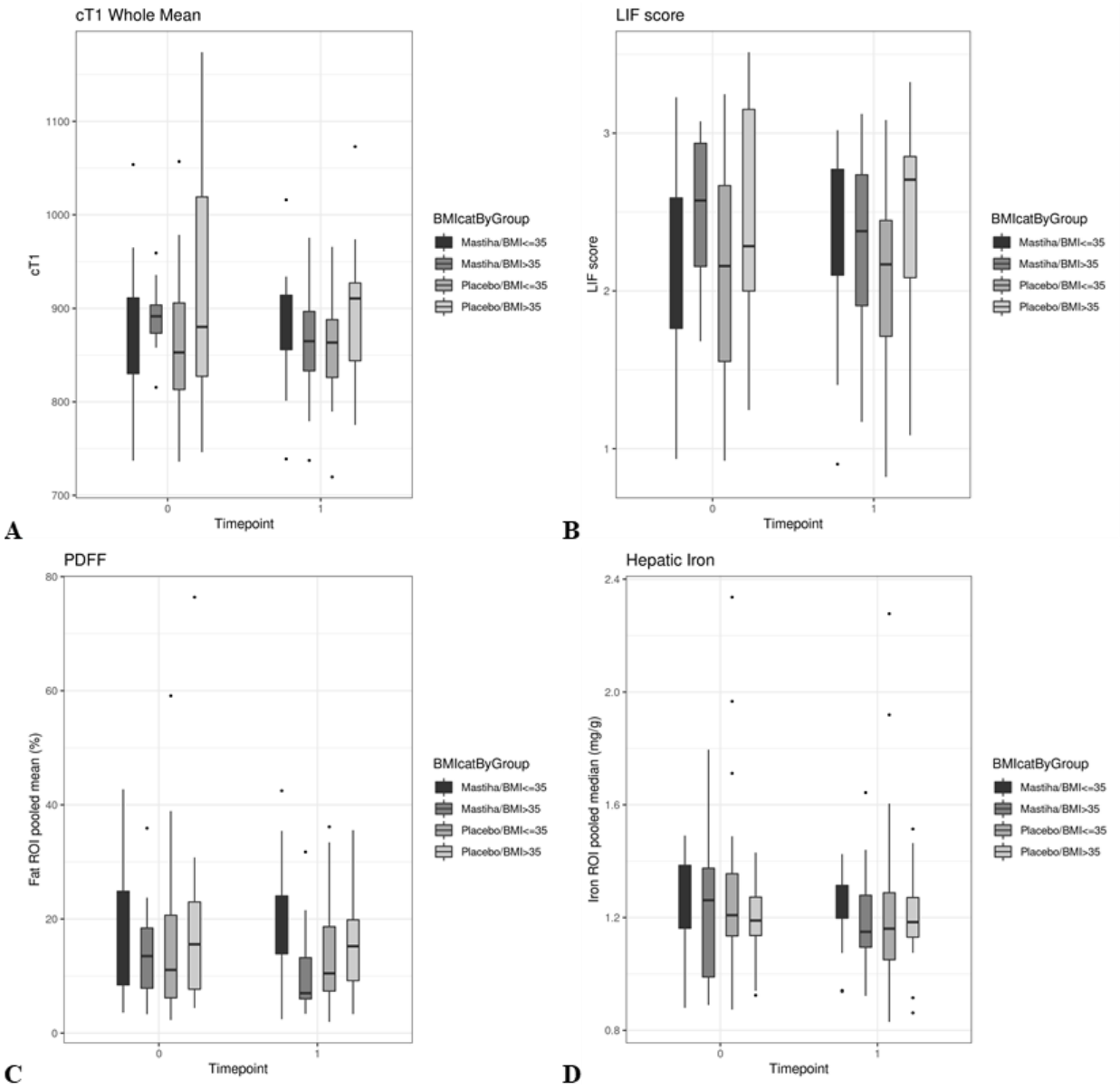
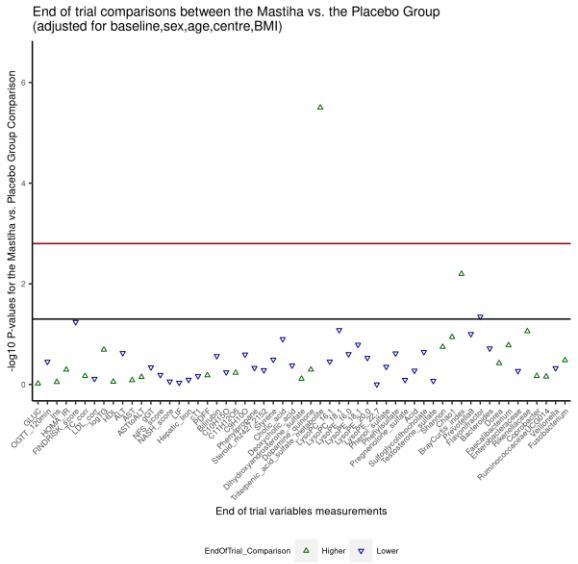


Figure 3.4. Box plots for the MRI parameters showing the values at baseline and post-treatment for the Mastiha and Placebo group, by BMI category. (A) cT1, (B) LIF, (C) PDFF, (D) Hepatic iron.

Table 3.4. Differences in cT1 and LIF between post-treatment and baseline for the Mastiha and placebo groups, stratified by BMI category. Pvalues are derived from ANOVA models comparing mean difference values between the treatment groups per BMI category.

	BMI<=35 kg/m^2						BMI>35 kg/m^2					
Differences in MRI outcome between post-treatment and baseline	**Placebo**		**Mastiha**		**Differences in relation to the Mastiha group**		**Placebo**		**Mastiha**		**Differences in relation to the Mastiha group**	
	n	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	**Pvalue**	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	**Pvalue**
dcT1	30	-1.79 (58.48)	20	10.19 (63.34)	12.696 (18.118)	0,487	13	22.02 (71.13)	11	-29.61 (57.86)	-58.488 (25.586)	**0,034**
dLIF	32	-0.04 (0.47)	22	0.07 (0.55)	0.117 (0.144)	0,423	13	0.24 (0.62)	11	-0.30 (0.54)	-0.623 (0.235)	**0,016**

In the un-stratified analysis, several associations at a nominal level of significance were identified. The Bray-Curtis dissimilarity index between baseline and post-treatment bacterial communities was larger in Mastiha vs. Placebo (P value=0.006, adjusted for age, sex and centre, **Table 3.2.c**). This difference was not attenuated when adjusted for baseline BMI levels (**Figure 3.5A**) or the difference in caloric intake between post-treatment and baseline (**Figure 3.5C**). The post-treatment relative abundance of Flavonifractor was lower in the Mastiha group compared to the Placebo (**Table 3.4**). The association was more significant after adjusting for the difference in caloric intake (P value=0.036).



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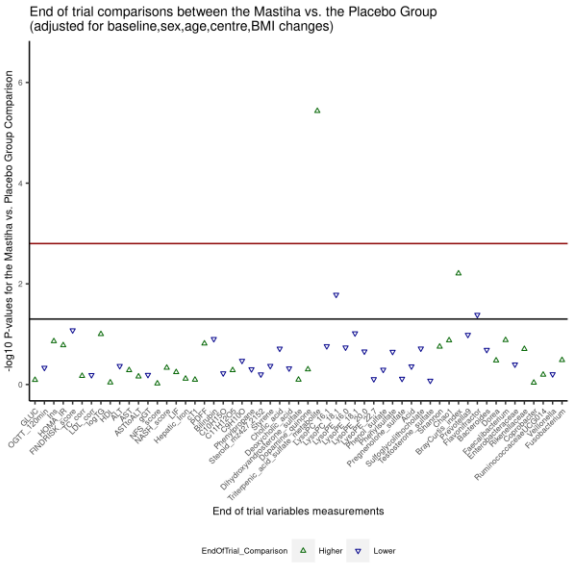

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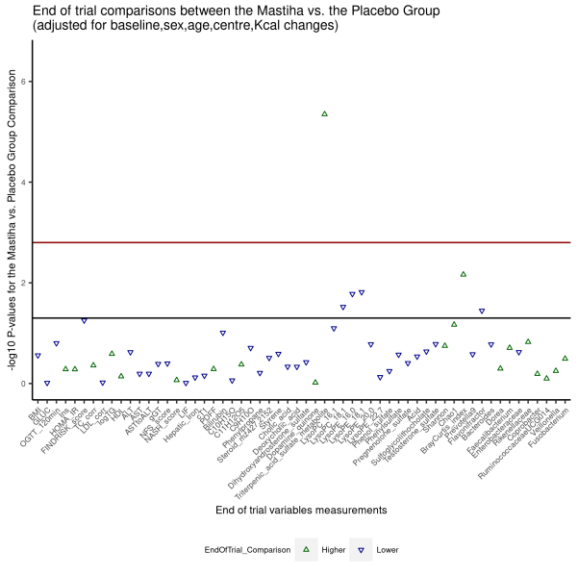

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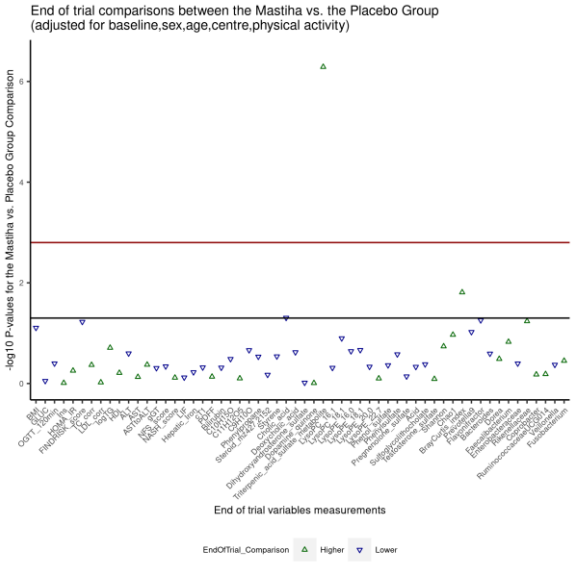

D

Figure 3.5. Sensitivity analysis for the differences in all outcomes assessed post treatment between the Mastiha and Placebo groups. Comparisons were performed using ANOVA and adjusted for (A) The corresponding baseline levels for each outcome, age, gender, centre and BMI at baseline, (B) The corresponding baseline levels for each outcome, age, gender, centre and the BMI difference between post-treatment and baseline, (C) The corresponding baseline levels for each outcome, age, gender, centre and the caloric intake difference between post-treatment and baseline, (D) The corresponding baseline levels for each outcome, age, gender, centre and the level of physical activity at baseline. Triangles indicate the P value (-log10 transformed) for the comparison. Blue descending triangles indicate lower mean values in the Mastiha group compared to the Placebo; while green ascending triangles indicate the opposite. The black horizontal line marks nominal significance level (P value=0.05) and the red line the multiple testing significance level (P value=0.0015).

A nominally significant decrease in post-treatment BMI in Mastiha group compared to Placebo (P value=0.047) (**Table 3.2.a**) was detected, but the effect was attenuated after adjustments for baseline levels of physical activity (**Figure 3.5D**) or the difference in caloric intake (**Figure 3.5C**).

Metabolites showed no differences post-treatment between the two groups in the main model (adjusted for the baseline metabolite level, age, sex and centre) apart from triterpenic acid sulphate (**Table 3.2.b**). However, several metabolite levels significantly decreased in Mastiha compared to Placebo (Lysophosphatidylcholines-(LysoPC) 18:1, P value=0.030, and Lysophosphatidylethanolamines-(LysoPE) 18:1, P value=0.015), when adjusting for the corresponding baseline metabolites, age, sex, centre and differences in caloric intake (**Figure 3.5C**). Similarly, in Mastiha group cholic acid decreased significantly compared to Placebo, after adjusting for baseline cholic acid, age, sex, centre and physical activity (**Figure 3.5D**).

3.1.3. Effect of Mastiha on serum inflammation biomarkers **(The results are presented in Paper 2)**

Table 3.5. ANCOVA post-treatment comparisons results between the Mastiha and placebo groups for all investigated outcomes, across different adjustment

Post-treatment outcome	Placebo		Mastiha		Post-treatment differences in relation to the Mastiha group adjusted for baseline levels, age, gender and centre		adjusted for baseline levels, age, gender, centre and baseline BMI		adjusted for baseline levels, age, gender, centre and baseline physical activity		adjusted for baseline levels, age, gender, centre and smoking status	
	n	Mean (SD)	n	Mean (SD)	Beta (SE)	*P*	Beta (SE)	*P*	Beta (SE)	*P*	Beta (SE)	*P*
EGF (pg/ml)	52	64.7692 (53.4996)	35	69.4571 (55.6378)	0.275 (0.192)	0,155	0.285 (0.192)	0,142	0.292 (0.199)	0,147	0.283 (0.189)	0,139
IL-1α (pg/ml)	51	0.1928 (0.1547)	34	0.2431 (0.2048)	-0.042 (0.229)	0,855	-0.045 (0.23)	0,845	-0.064 (0.237)	0,789	-0.043 (0.231)	0,852
IL-1β (pg/ml)	52	0.9054 (0.4705)	35	1.3567 (0.9956)	0.394 (0.205)	0,059	0.394 (0.206)	0,060	0.419 (0.218)	0,059	0.391 (0.206)	0,061
IL-2 (pg/ml)	45	1.7799 (1.2883)	33	2.3782 (1.7837)	0.076 (0.231)	0,744	0.083 (0.231)	0,720	0.146 (0.236)	0,539	0.065 (0.229)	0,779
IL-4 (pg/ml)	52	1.6498 (0.4884)	35	1.8203 (0.6783)	-0.038 (0.179)	0,831	-0.039 (0.18)	0,830	-0.016 (0.183)	0,933	-0.043 (0.179)	0,810
IL-6 (pg/ml)	52	1.525 (1.0448)	35	1.515 (0.863)	0.098 (0.199)	0,622	0.097 (0.198)	0,625	0.091 (0.21)	0,665	0.099 (0.2)	0,624
IL-8 (pg/ml)	52	8.9464 (5.1281)	34	10.271 (5.0639)	0.209 (0.206)	0,312	0.21 (0.207)	0,313	0.216 (0.21)	0,306	0.21 (0.207)	0,314
IL-10 (pg/ml)	51	0.8168 (0.75)	35	0.912 (0.9168)	0.031 (0.147)	0,835	0.031 (0.148)	0,837	0.051 (0.154)	0,741	0.03 (0.148)	0,838
IFNγ (pg/ml)	51	0.3614 (0.2977)	35	0.4 (0.3516)	0.008 (0.205)	0,970	0.014 (0.204)	0,944	0.074 (0.205)	0,717	0.008 (0.206)	0,969
TNF-α (pg/ml)	52	2.6282 (1.325)	34	2.955 (1.2304)	0.205 (0.19)	0,284	0.205 (0.191)	0,287	0.281 (0.192)	0,148	0.201 (0.19)	0,292
VEGF-A (pg/ml)	52	144.1058 (96.4747)	35	126.1714 (80.0079)	0.123 (0.114)	0,285	0.127 (0.11)	0,250	0.164 (0.114)	0,154	0.122 (0.114)	0,288

Table 3.6. Baseline characteristics of all participants stratified by BMI category and per trial group

Baseline Characteristics	All (BMI≤35kg/m²)		All (BMI>35kg/m²)		Placebo (BMI≤35kg/m²)		Mastiha (BMI≤35kg/m²)			Placebo (BMI>35kg/m²)		Mastiha (BMI>35kg/m²)		
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	*P*[1]	n	Mean (SD)	n	Mean (SD)	*P*[1]
EGF (pg/ml)	65	74.2077 (55.1764)	31	50.4516 (51.2224)	37	79.2432 (55.7292)	28	67.5536 (54.7246)	0,256	19	42.3158 (38.4698)	12	63.3333 (66.6229)	0,789
IL-1α (pg/ml)	64	0.1775 (0.1664)	31	0.225 (0.2184)	37	0.1478 (0.15)	27	0.2181 (0.1816)	**0,050**	18	0.1689 (0.1293)	13	0.3027 (0.2904)	0,374
IL-1β (pg/ml)	62	0.9654 (0.4904)	30	0.9603 (0.5874)	35	0.9 (0.3941)	27	1.0502 (0.5898)	0,212	19	0.8505 (0.3852)	11	1.15 (0.8192)	0,328
IL-2 (pg/ml)	60	2.1618 (1.8355)	27	1.545 (1.3331)	33	1.8261 (1.7162)	27	2.572 (1.9241)	**0,043**	16	1.2431 (1.077)	11	1.9841 (1.5884)	0,097
IL-4 (pg/ml)	65	1.6028 (0.4727)	32	1.6663 (0.6598)	37	1.5468 (0.4372)	28	1.6768 (0.5146)	0,200	19	1.5005 (0.2237)	13	1.9085 (0.9708)	0,095
IL-6 (pg/ml)	65	1.8071 (1.4854)	32	1.9413 (0.9265)	37	1.8114 (1.6047)	28	1.8014 (1.3404)	0,717	19	2.1168 (0.8773)	13	1.6846 (0.9711)	0,065
IL-8 (pg/ml)	64	9.0588 (5.6683)	32	9.1659 (6.5282)	36	8.296 (5.2103)	28	10.0396 (6.1648)	**0,043**	19	8.5684 (5.1039)	13	10.0392 (8.3443)	0,658
IL-10 (pg/ml)	64	0.8113 (0.5076)	32	0.6919 (0.3823)	36	0.7789 (0.5153)	28	0.8529 (0.5039)	0,203	19	0.6421 (0.3191)	13	0.7646 (0.4638)	0,598
IFNγ (pg/ml)	63	0.3826 (0.4366)	30	0.3533 (0.4063)	36	0.3167 (0.2156)	27	0.4706 (0.6147)	0,158	17	0.3294 (0.4853)	13	0.3846 (0.2881)	0,268
TNF-α (pg/ml)	63	2.8156 (1.206)	32	2.4748 (0.9286)	36	2.5357 (0.7288)	27	3.1887 (1.5812)	0,061	19	2.3195 (0.5845)	13	2.7019 (1.2732)	0,210
VEGF-A (pg/ml)	65	161.8077 (128.2488)	32	130.6563 (74.7874)	37	167.2568 (129.1862)	28	154.6071 (128.9989)	0,570	19	137.6842 (73.3281)	13	120.3846 (78.6827)	0,430

***P*[1]** for the trial groups comparison. Outcome comparisons are adjusted for age, sex and centre. Differences in categorical variables were assessed with Chi-square test

Table 3.7. ANCOVA post-treatment comparisons results between the Mastiha and Placebo groups for all investigated outcomes, stratified by BMI category. Effect sizes refer to log-transformed and scaled outcomes.

	BMI≤35kg/m^2						BMI>35kg/m^2					
Post-treatment outcome	**Placebo**		**Mastiha**		**Post-treatment differences in relation to the Mastiha group, adjusted for age, sex and centre**		**Placebo**		**Mastiha**		**Post-treatment differences in relation to the Mastiha group, adjusted for age, sex and centre**	
	n	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	***P***	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**Beta (SE)**	***P***
EGF (pg/ml)	36	65.8889 (52.1373)	23	78.9348 (58.4569)	0.38 (0.251)	0,137	16	62.25 (58.1295)	12	51.2917 (46.7534)	0.032 (0.308)	0,919
IL-1α (pg/ml)	36	0.1983 (0.1649)	22	0.2507 (0.1952)	0.031 (0.277)	0,913	15	0.1797 (0.1312)	12	0.2292 (0.2296)	-0.304 (0.408)	0,464
IL-1β (pg/ml)	36	0.925 (0.5008)	23	1.3259 (0.9886)	0.287 (0.264)	0,283	16	0.8613 (0.4057)	12	1.4158 (1.0506)	0.568 (0.349)	0,118
IL-2 (pg/ml)	30	1.8435 (1.287)	21	2.4695 (1.8832)	-0.032 (0.29)	0,912	15	1.6527 (1.3263)	12	2.2183 (1.6623)	0.148 (0.401)	0,716
IL-4 (pg/ml)	36	1.6642 (0.5257)	23	1.7961 (0.697)	-0.031 (0.213)	0,883	16	1.6175 (0.4056)	12	1.8667 (0.6687)	0.106 (0.348)	0,764
IL-6 (pg/ml)	36	1.2678 (0.9576)	23	1.615 (0.899)	0.481 (0.247)	0,057	16	2.1038 (1.0275)	12	1.3233 (0.7902)	-0.475 (0.294)	0,121
IL-8 (pg/ml)	36	8.5531 (4.8143)	23	10.2041 (5.3707)	0.305 (0.235)	0,199	16	9.8316 (5.8415)	11	10.4109 (4.5968)	0.215 (0.403)	0,600
IL-10 (pg/ml)	35	0.8743 (0.8803)	23	0.8652 (0.4396)	-0.014 (0.184)	0,938	16	0.6909 (0.3062)	12	1.0017 (1.4826)	0.18 (0.272)	0,514
IFNγ (pg/ml)	36	0.3869 (0.3226)	23	0.4261 (0.4077)	-0.042 (0.256)	0,869	15	0.3 (0.225)	12	0.35 (0.2135)	0.265 (0.343)	0,450
TNF-α (pg/ml)	36	2.7197 (1.5332)	22	3.127 (1.099)	0.395 (0.24)	0,105	16	2.4222 (0.6471)	12	2.6396 (1.438)	-0.057 (0.322)	0,862
VEGF-A (pg/ml)	36	141.0556 (105.224)	23	133.1957 (88.815)	0.244 (0.131)	0,068	16	150.9688 (75.748)	12	112.7083 (60.897)	-0.186 (0.2)	0,364

When comparing the post-treatment biomarkers between the two groups we didn't identify any significance difference **(Table 3.5)**. The results remained robust across all different sensitivity analysis. We further stratified our individuals by BMI category and the baseline characteristics are presented in **Table 3.6**. Significant differences were observed between the two groups at baseline in BMI≤35kg/m^2 for circulating IL-1α, IL-2 and IL-8. There were no significant differences between the two groups in the two BMI categories **(Table 3.7)**.

3.1.4. Discussion

NAFLD is characterized by excessive fat accumulation in the liver, not caused by alcohol consumption (<20 g ethanol per day for women and <30 g ethanol per day for men). It is one of the most common liver diseases in Western populations and its prevalence is constantly increasing and is currently estimated to be 24% [EASL Guidelines 2016]. It ranges from simple steatosis to NASH, which can potentially progress to advanced liver disease, cirrhosis and hepatocellular carcinoma. NAFLD is associated with obesity, dyslipidemia, insulin resistance and high cardiometabolic risk [Stefan et al., 2019, Younossi et al., 2017].

Liver biopsy is the gold standard for NASH and imaging techniques for NAFLD with MRI being the gold standard. Liver biopsy is expensive, invasive, with variable results and procedural complications. Also, its repetition for monitoring disease progression might be unfeasible and changes in liver fat alone are not predictive of histological changes. So there is an urgent need for reliable, accurate, and non- or minimally invasive methods like imaging [Piazzolla et al., 2020]. MRI offers good sensitivity and specificity in detecting histologically confirmed steatosis, ranging from 76.7%-90.0% and 87.1%-91% respectively. LiverMultiScan (Perspectum Ltd, UK) is a multiparametric MRI technique for the quantification of fibrosis and inflammation and has been successfully used to detect and stage liver disease in clinical trials [Harrison et al., 2020, Li et al., 2018]

As there is no definite treatment approved for NAFLD, lifestyle modification remains the main mode of therapy. A combination of dietary modifications and increased physical activity aims at decreasing body weight, and improving glycemic control, dyslipidemia, and cardiovascular risk [Nseir et al., 2014]. No medication can be considered as a standard therapy for NAFLD with anti-NAFLD agents targeting at insulin resistance and lipid accumulation,

displaying significant adverse side effects. Hence, there is an urgent demand for alternative treatment candidates with high efficacy and minimal side effects for the treatment of NAFLD and many studies focus on natural products and their potent effects against NAFLD [Yao et al., 2016].

In the last decade, clinical trials of non pharmacologic agents for the treatment of NASH are mounting. The effects of dietary natural products in the management of NAFLD have been investigated in several human studies. For example, n-3 PUFA from seal oils have been proven to improve ALT, serum lipid levels and normalize ultrasonographic evidence in patients with NAFLD [Zhu et al., 2008]. NAFLD patients administered with curcumin for 8 weeks exhibited a significant reduction in ultrasonography liver fat content, BMI, lipids, and hepatic enzymes compared with the placebo group [Rahmani et al., 2016]. Resveratrol significantly decreased AST, ALT, glucose, LDL and HOMA-IR in NAFLD patients compared with the placebo group and significantly reduced serum cytokines levels of TNF-α, cytokeratin 18 fragment, and fibroblast growth factor 21 [Chen et al., 2015].

Mastiha, is a natural nutritional supplement based on the dried resinous exudate from stems and branches of the tree Pistacia lentiscus. It consists of several bioactive compounds, such as terpenes, the poly-β-myrcene (approximately 20%), phytosterols, and phenolic compound and possesses anti-bacterial, antioxidant and anti-inflammatory activity [Papada et al., 2019]. Recently, the effect of Mastiha was investigated on mice with diet-induced obesity, NASH and fibrosis (DIO-NASH model). Mastiha supplementation significantly reduced plasma ALT activity, hepatic steatosis, and the histological NAFLD activity score. [Kannt et al., 2019].

We hereby report the results of the multicentre randomised double-blinded and placebo-controlled clinical trial where the effect of Mastiha on liver inflammation and fibrosis was investigated through MRI, biochemical and multi-omic analyses. To the best of our knowledge, this is the first study on the effect of a natural product in NAFLD applying a more integrated approach, including not only biochemical and inflammatory parameters of the disease, but also, MRI imaging, epigenetic and microbiome alterations.

No significant changes in primary outcomes were detected in the main analysis of the study. However, the efficacy of Mastiha in the primary outcome was clear when stratifying the

samples by BMI category. Specifically, lower post-treatment levels of cT1 and LIF in Mastiha compared to Placebo, only in Class II or III obesity (BMI>35 kg/m^2) have been detected. cT1 and LIF have been previously shown to strongly correlate with increasing liver fibrosis, as assessed by Ishak stage, and especially cT1 has been suggested as a useful tool in the monitoring of longitudinal changes in patients with NASH. When stratifying cT1 into groups (<840 ms, 840-990 ms, >990 ms), the risk of clinical events is increasing with increasing cT1 [Jayaswal et al., 2020]. Similarly, in the biopsy-confirmed mouse model of advanced NASH, hepatic pathology improved and NAFLD activity and expression of collagen genes (Col1a1 and Col4a1) were reduced upon Mastiha intake. Interestingly, the fact that these improvements occurred in the absence of weight loss, suggested a possible direct effffect of Mastiha on the liver rather than an indirect effffect via reducing adiposity [Kannt et al., 2019].

In our study, we showed that the Bray-Curtis dissimilarity index was significantly greater among patients with NAFLD that received the Mastiha, compared to the Placebo. Intestinal microbiota dysbiosis is well established in NAFLD and contributes to its pathogenesis as it involves altered composition and reduced diversity, it increases intestinal permeability to antigens and contributes to hepatic inflammation and fibrosis [Marra et al., 2018]. Several studies have identified bacterial genera, families and phyla that differ significantly in NAFLD patients and affect disease pathogenesis through several mechanisms, such as increased intestinal permeability (i.e. Lachnospiraceae), decrease in short chain fatty acids (SCFA) producing bacteria (i.e. Firmicutes) and elevated serum endotoxin production (i.e. *Bacteroides*, Enterobacteriaceae) [Quesada-Vázquez et al., 2020, Svegliati-Baroni et al., 2020]. As intestinal microbiota dysbiosis favours NAFLD progression, modification in microbiota composition is important in the resolution of the disease.

The bidirectional communication between gut microbiota and bile acid metabolism has a substantial role in NAFLD. The gut microbiota is involved in the conversion of primary bile acids into secondary bile acids in the intestine. Dysbiosis may lead to decreased secondary bile acids synthesis with consequent decreased activation of nuclear receptors important in lipid metabolism and energy regulation, leading to NAFLD [Chen et al., 2019]. On the other hand, any bile acid alterations due to high-fat diet-induced metabolic changes may affect the composition of the gut microbiota, which in turn may influence lipid and energy metabolism leading to

NAFLD [He et al., 2016]. We found decreased cholic acid levels only in the Mastiha group suggesting a potential effect of Mastiha on the interaction between gut microbiota and bile acid metabolism. The observed effect on microbiota composition may be associated with increased secondary bile acids synthesis, and decreased cholic acid levels, thus contributing to the regulation of lipid and energy metabolism.

Herein, the Mastiha group had lower proportion of Flavonifractor compared to the Placebo. Data on the Flavonifractor signature in NAFLD is contradictory, with either increased or decreased levels compared to healthy [Jiang et al., 2015]. Flavonifractor is involved in the catabolism of quercetin, a flavonoid with antioxidant and anti-inflammatory properties and is considered a potentially proinflammatory species [Moco et al., 2012, Mulders et al., 2018]. The downregulation of Flavonifractor and Prevotella (we detected a positive association between Prevotella and PDFF at baseline and a trend towards a lower abundance of Prevotella post-treatment in the Mastiha group) may be related to the anti-inflammatory activity of Mastiha. Weak trends of change in the relative abundance of other important bacterial taxa, previously associated with NAFLD, were also found in the Mastiha group, namely a decrease in Enterobacteriaceae and Bacteroides and an increase in Faecalibacterium. Overall, Mastiha's beneficial effect on patients' microbiota was likely through the decrease of inflammatory and endotoxin-producing bacteria and the increase of anti-inflammatory ones. In agreement with our findings, an alteration on fecal microbiome not paralled with an improvement in liver histopathology in patients under symbiotic treatment was reported by Scorletti et al.[2020]

According to our findings there is strong evidence that Mastiha administration might exhibit a beneficial effect in phospholipid homeostasis, as we detected a significant reduction of LysoPCs and LysoPEs only in the Mastiha group. Phospholipids have been placed at the center of NAFLD/NASH pathogenesis, since numerous reports associate their levels with increased risk of liver injury and oxidation and patients suffering from different grades of hepatic fat accumulation possess significantly higher concentrations of these metabolite groups [Beyoglu et al., 2013, Kahlan et al., 2011]. Mastiha's lipid lowering properties are nowadays well established. Recently, it has been shown that it reduces total cholesterol with a stronger effect on overweight and obese patients [Kartalis et al., 2015]. Also, it has been shown that the hypolipidemic activity of Mastiha is associated with one of its monoterpene constituents,

camphene [Vallianou et al., 2011]. Camphene seems to prevent hepatic steatosis in mice via the activation of AMPK signalling. Thus it contributes to the decreased expression of transcription factors involved in lipogenesis, such as sterol regulatory element binding protein 2 (SREBP2), a key regulator in phospholipids homeostasis [Kim et al., 2013]. The above suggests that changes in phospholipid levels can be attributed to Mastiha and that a potential mechanism of its action is through regulation of transcription factors related to lipogenesis.

While this study has some interesting results, it is subject to a number of limitations, such as the absence of confirmatory biopsies as part of the trial, and the relatively small sample size of the Mastiha and Placebo groups. Furthermore, the duration of the trial might not have been sufficient for significant changes in the investigated parameters.

In conclusion, after six months of Mastiha supplementation, we observed **a significant improvement on microbiota dysbiosis and lipid metabolite levels in patients with NAFLD**. Although no significant effect of the Mastiha on the primary outcomes was identified in the un-stratified analysis, **an improvement of the liver fibrosis as assessed via MRI has been observed in severely obese patients**. Mastiha **improved microbiota dysbiosis** mainly through decreasing the abundance of inflammatory taxa. The beneficial effect on the microbiota parallel with a **decrease in plasma cholic acid and phospholipids**, may be attributed to the bioavailable triterpenic acids of Mastiha. Overall, Mastiha could be considered an emerging non-pharmacological agent in NAFLD. More clinical trials are required to replicate and further investigate these initial findings.

3.2.IBD-GR

3.2.1. Descriptives of the study population

One hundred and twenty (n=129) nine patients met our criteria. A total of 62 patients were in relapse and 67 in remission. Eighty-six were diagnosed with CD and 43 with UC. Sixty-eight were randomised to the Mastiha group and 61 to the placebo group (CONSORT Flow diagram in **Figure 3.6**). Out of the 129 volunteers who participated in the trial, 91 completed the intervention.

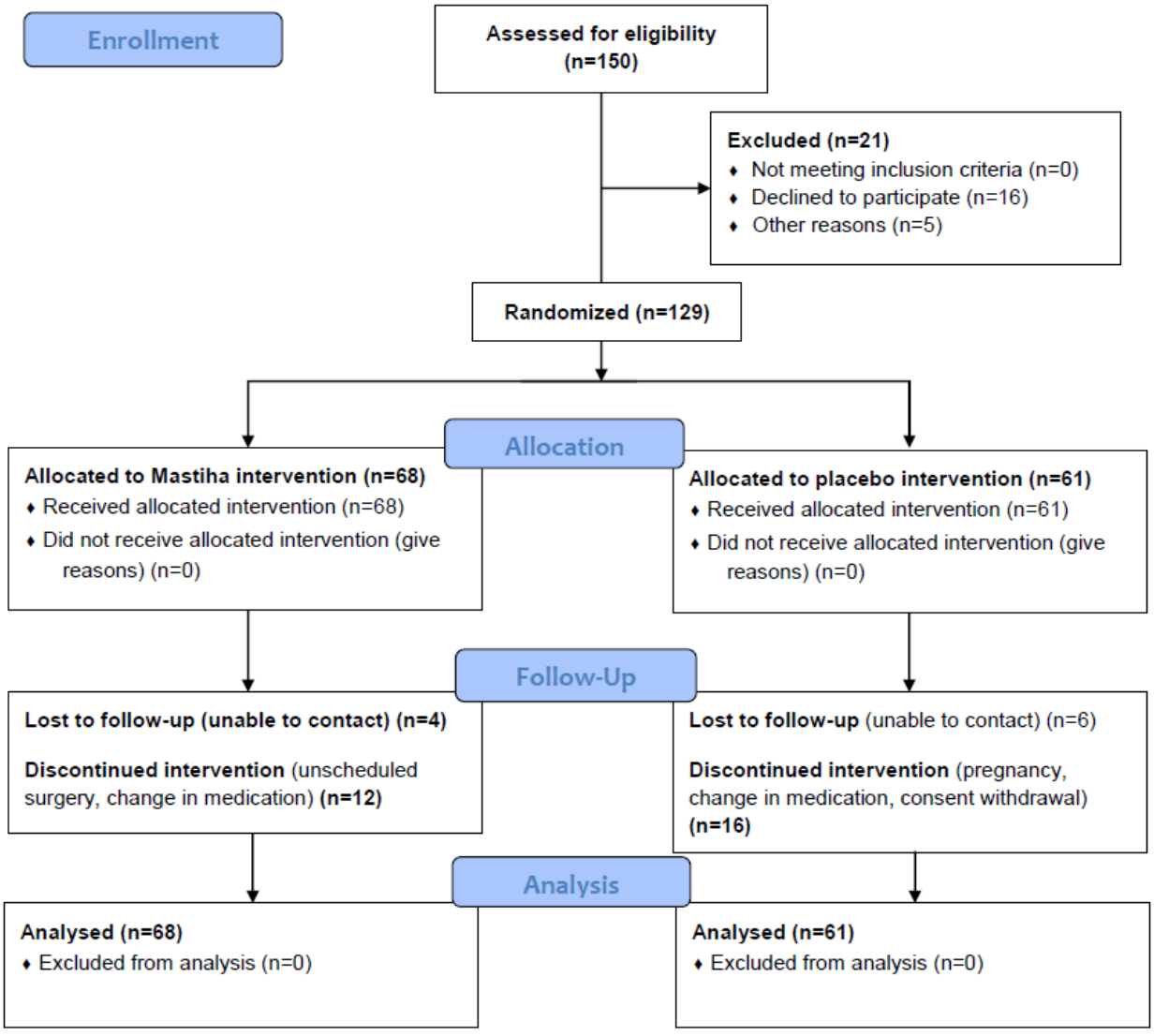

Figure 3.6. IBD-GR CONSORT 2010 Flow diagram

Baseline characteristics in IBD patients in relapse and in remission are given in **Table 3.8**. IBDQ was higher and HBI and PMS were lower in remission comparing to relapse, as expected (p<0.001). 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. Correlation analyses were conducted for serum IL-17A and other inflammatory markers. The only significant correlation was between IL-17A and IL-6 (r= 0.714, p= 0.006) and IL-17A and calprotectin (r=-0.553, p=0.050) in UC patients in relapse. No significant correlation was observed in IBD patients in total, in patients in remission and in CD patients in relapse.

Table 3.8. Baseline characteristics of the IBD-GR study participants

	IBD in relapse N=62	**IBD in remission N=67**	**Pvalue**
Sex (F/M)			
Females	34 (54.8)	29 (43.3)	0.190‡
Males	28 (45.2)	38 (56.7)	
Age (years) mean (SD)	41 (15.7)	37.5 (10.7)	0.136+
BMI (kg/m^2) mean (SD)	23.8 (5.8)	24.4 (3.9)	0.469+
Disease duration (years) mean (SD)	11.4 (9.1)	9.4 (6.7)	0.150+
Disease location			
Ileal	18 (29.0)	18 (26.9)	0.784‡
Ileocolonic	14 (22.6)	15 (22.4)	0.979‡
Colonic	3 (4.8)	7 (10.4)	0.328‡‡
Pancolitis	12 (19.4)	12 (17.9)	0.833‡
Left-sided	7 (11.3)	6 (9.0)	0.660‡
Other	7 (11.3)	14 (20.9)	0.140‡
Medication			
Mesalamine	24 (39.3)	34 (51.5)	0.169‡
Azathioprine	14 (23.0)	21 (31.8)	0.264‡
Corticosteroids	21 (34.4)	13 (19.7)	0.061‡
IBDQ mean (SD)	144.7 (25.5)	177.1 (27.1)	**<0.001+**
HBI median (IQR)	6 (5.5 – 8.5)	3 (1 – 3)	**<0.001++**

PMS median (IQR)	4 (2.5 – 5)	1 (0 – 1)	**<0.001++**
IL-6 (pg/mL) median (IQR)	5.9 (2.2 – 16.6)	2.1 (1.1 – 7.5)	**0.001++**
IL-10 (pg/mL) median (IQR)	4.6 (4.2 – 5.5)	5.6 (4.5 – 6.6)	**0.016++**
IL-11 (pg/mL) median (IQR)	235.3 (125.7 - 622.0)	189.9 (109.0 - 384.1)	0.630
IL-22 (pg/mL) median (IQR)	15.0 (6.4 – 33.1)	26.3 (5.2 – 38.7)	0.729
IL-17A (pg/mL) median (IQR)	17.8 (10.4 – 27.9)	25.6 (14.6 – 39.1)	**0.050++**
CRP (mg/L) median (IQR)	3.7 (1 – 9.6)	1.5 (0.3 – 4.2)	**0.023++**
Calprotectin (μg/g) median (IQR)	927.2 (372.9 – 2415.2)	422.1 (180.3 – 915.7)	**0.002++**
Defensin (ng/g) median (IQR)	12 (4 – 35.4)	6.8 (4 – 20.1)	0.310++
Lysozyme (μg/g) median (IQR)	8.7 (6.9 – 15.6)	7.1 (6 – 10.1)	**0.026++**
Lactoferrin (μg/g) median (IQR)	39.6 (15.8 – 192.5)	20.6 (7.1 – 63.5)	**0.024++**

+Student's t-test; ++Mann-Whitney test; ‡Pearson's chi-square test; ‡‡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.

3.2.2. Effect of Mastiha on inflammatory markers and faecal metabolic profile (**The results are presented in Paper 3)**

Table 3.9 (a.,b.,c.,d.,e.) describes serum cytokines levels at baseline and at follow up in all patients and in CD and UC. In active IBD, IL-6 increases significantly in both Mastiha and placebo groups, but the mean change was not different between groups. Similarly, IL-11 increases significantly in all patient groups, with the mean changes being indifferent as well. 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. No significant changes were observed in the other cytokines and patient groups.

Table 3.9a. Changes in IL-6 serum levels in IBD patients post intervention

		IL-6 baseline (pg/mL)	**IL-6 post-treatment* (pg/mL)**	**Comparison of serum levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	**Differences in the degree of changes between the groups after including age, sex and BMI as covariates**
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	**RELAPSE**					
	Placebo	14.4 (16.8)	24.3 (43.8)	**0.030**	0.955	0.734
	Mastiha	11.5 (12.3)	15.7 (13.3)	**0.021**		

	REMISSION					
	Placebo	3.6 (4.4)	8.0 (10.9)	0.139	0.708	0.667
	Mastiha	7.0 (10.3)	6.2 (7.6)	0.826		
CD	**RELAPSE**					
	Placebo	13.4 (11.7)	15.7 (16.9)	0.533	0.546	0.467
	Mastiha	12.5 (9.9)	16.9 (15.6)	0.194		
	REMISSION					
	Placebo	3.5 (4.7)	6.2 (12.2)	0.392	0.528	0.566
	Mastiha	5.6 (8.1)	6.8 (9.8)	0.701		
UC	**RELAPSE**					
	Placebo	5.7 (10.5)	8.5 (17.1)	0.687	0.568	0.416
	Mastiha	5.2 (8.1)	6.8 (9.8)	0.555		
	REMISSION					
	Placebo	3.6 (3.5)	10.9 (10.7)	0.060	0.830	0.857
	Mastiha	10.6 (10.1)	4.7 (5.1)	0.284		

Table 3.9b. Changes in IL-10 serum levels in IBD patients post intervention

		IL-10 baseline (pg/mL)	**IL-10 post-treatment* (pg/mL)**	**Comparison of serum levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	**Differences in the degree of changes between the groups after including age, sex and BMI as covariates**
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	**RELAPSE**					
	Placebo	8.8 (18.9)	9.5 (20.1)	0.454	0.607	0.449
	Mastiha	6.1 (2.7)	6.1 (2.7)	0.951		
	REMISSION					
	Placebo	6.7 (3.7)	6.1 (2.7)	0.254	0.053	0.064
	Mastiha	5.4 (1.8)	4.9 (1.6)	0.077		
CD	**RELAPSE**					
	Placebo	4.5 (0.9)	4.7 (0.9)	0.533	0.144	0.539
	Mastiha	5.3 (1.8)	5.5 (2.3)	0.194		
	REMISSION					
	Placebo	6.3 (1.7)	5.9 (1.2)	0.392	0.390	0.265
	Mastiha	5.0 (1.3)	4.9 (1.6)	0.701		
UC	**RELAPSE**					
	Placebo	5.2 (0.9)	5.0 (0.9)	0.687	0.477	0.139
	Mastiha	6.0 (2.9)	5.8 (2.8)	0.555		
	REMISSION					
	Placebo	7.4 (5.4)	6.3 (4.1)	0.060	0.471	0.673
	Mastiha	6.2 (2.7)	5.1 (1.3)	0.284		

Table 3.9c. Changes in IL-11 serum levels in IBD patients post intervention

		IL-11 baseline (pg/mL)	IL-11 post-treatment* (pg/mL)	Comparison of serum levels before and post-treatment in each group	Differences between the groups in the degree of changes	Differences in the degree of changes between the groups after including age, sex and BMI as covariates
		Mean (SD)	Mean (SD)	P[a]	P[b]	P[c]
IBD	**RELAPSE**					
	Placebo	268.8 (398.9)	819.5 (79.3)	**0.006**	0.620	0.440
	Mastiha	358.2 (279.4)	822.7 (242.8)	**0.009**		
	REMISSION					
	Placebo	198.4 (262.6)	579.6 (119.4)	**0.016**	0.185	0.202
	Mastiha	27.7 (274.4)	613.1 (130.2)	**0.001**		
CD	**RELAPSE**					
	Placebo	362.2 (497.6)	820.4 (97.9)	**0.023**	0.468	0.491
	Mastiha	244.7 (539.8)	896.3 (97.9)	**0.027**		
	REMISSION					
	Placebo	200.9 (140.2)	568.5 (150.5)	**0.005**	0.486	0.405
	Mastiha	253.8 (140.1)	618.7 (154.4)	**0.004**		
UC	**RELAPSE**					
	Placebo	311.5 (265.8)	682.8 (271.5)	**0.042**	0.362	0.284
	Mastiha	350.7 (248.5)	827.0 (17.8)	**0.025**		
	REMISSION					
	Placebo	200.9 (322.5)	568.5 (21.67)	0.075	0.462	0.248
	Mastiha	332.6 (277.9)	599.4 (20.3)	**0.017**		

Table 3.9d. Changes in IL-17 serum levels in IBD patients post intervention

		IL-17 baseline (pg/mL)	IL-17 post-treatment* (pg/mL)	Comparison of serum levels before and post-treatment in each group	Differences between the groups in the degree of changes	Differences in the degree of changes between the groups after including age, sex and BMI as covariates
		Mean (SD)	Mean (SD)	P[a]	P[b]	P[c]
IBD	**RELAPSE**					
	Placebo	33.6(37.7)	43.1(50.2)	0.374	0.444	0.597
	Mastiha	28.5(34.9)	38(42.3)	0.072		
	REMISSION					
	Placebo	40.8(31.3)	34.8(54.4)	0.083	**0.003**	**0.001**
	Mastiha	23.6(15.7)	47.4(45.5)	**0.006**		
CD	**RELAPSE**					
	Placebo	23.8(17.5)	40.3(44.7)	0.157	0.664	0.561
	Mastiha	24.6(23.3)	32.7(31.4)	0.136		

	REMISSION					
	Placebo	43.3(33.9)	42.6(62.4)	0.498	**0.019**	**0.010**
	Mastiha	24(16.6)	53.3(46.6)	**0.003**		
UC	**RELAPSE**					
	Placebo	53.2(57.6)	48.9(62.4)	0.767	0.462	0.371
	Mastiha	36(50.8)	48.3(58.2)	0.308		
	REMISSION					
	Placebo	36.8(27.6)	22.2(37)	**0.033**	0.117	0.104
	Mastiha	22.4(13.8)	31.7(40.6)	0.859		

Table 3.9e. Changes in IL-22 serum levels in IBD patients post intervention

		IL-22 baseline (pg/mL)	**IL-22 post-treatment* (pg/mL)**	**Comparison of serum levels before and post-treatment in each group**	**Differences between the groups in the degree of changes**	**Differences in the degree of changes between the groups after including age, sex and BMI as covariates**
		Mean (SD)	**Mean (SD)**	**P[a]**	**P[b]**	**P[c]**
IBD	**RELAPSE**					
	Placebo	32.0 (54.3)	60.3 (92.1)	0.485	0.462	0.926
	Mastiha	29.7 (29.4)	51.0 (145.0)	0.650		
	REMISSION					
	Placebo	27.0 (29.4)	51.0 (145.0)	0.734	0.273	0.602
	Mastiha	33.5 (26.0)	41.7 (102.1)	0.929		
CD	**RELAPSE**					
	Placebo	36.7 (61.4)	71.5 (142.3)	0.823	0.598	0.478
	Mastiha	25.9 (33.7)	40.7 (165.9)	0.740		
	REMISSION					
	Placebo	27.5 (26.8)	44.7 (108.7)	0.782	0.475	0.535
	Mastiha	31.4 (25.9)	53.5 (101.7)	0.872		
UC	**RELAPSE**					
	Placebo	23.6 (39.0)	40.4 (109.9)	0.484	0.545	0.430
	Mastiha	36.6 (55.5)	85.6 (110.2)	0.807		
	REMISSION					
	Placebo	26.2 (17.7)	61.1 (105.2)	0.750	0.686	0.673
	Mastiha	38.6 (27.0)	12.7 (136.5)	0.796		

[a] p-value for time effect (Wilcoxon signed rank tests),

[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.

Analyses were conducted on an ITT basis. *after 3 months for patients in relapse, after 6 months for patients in remission

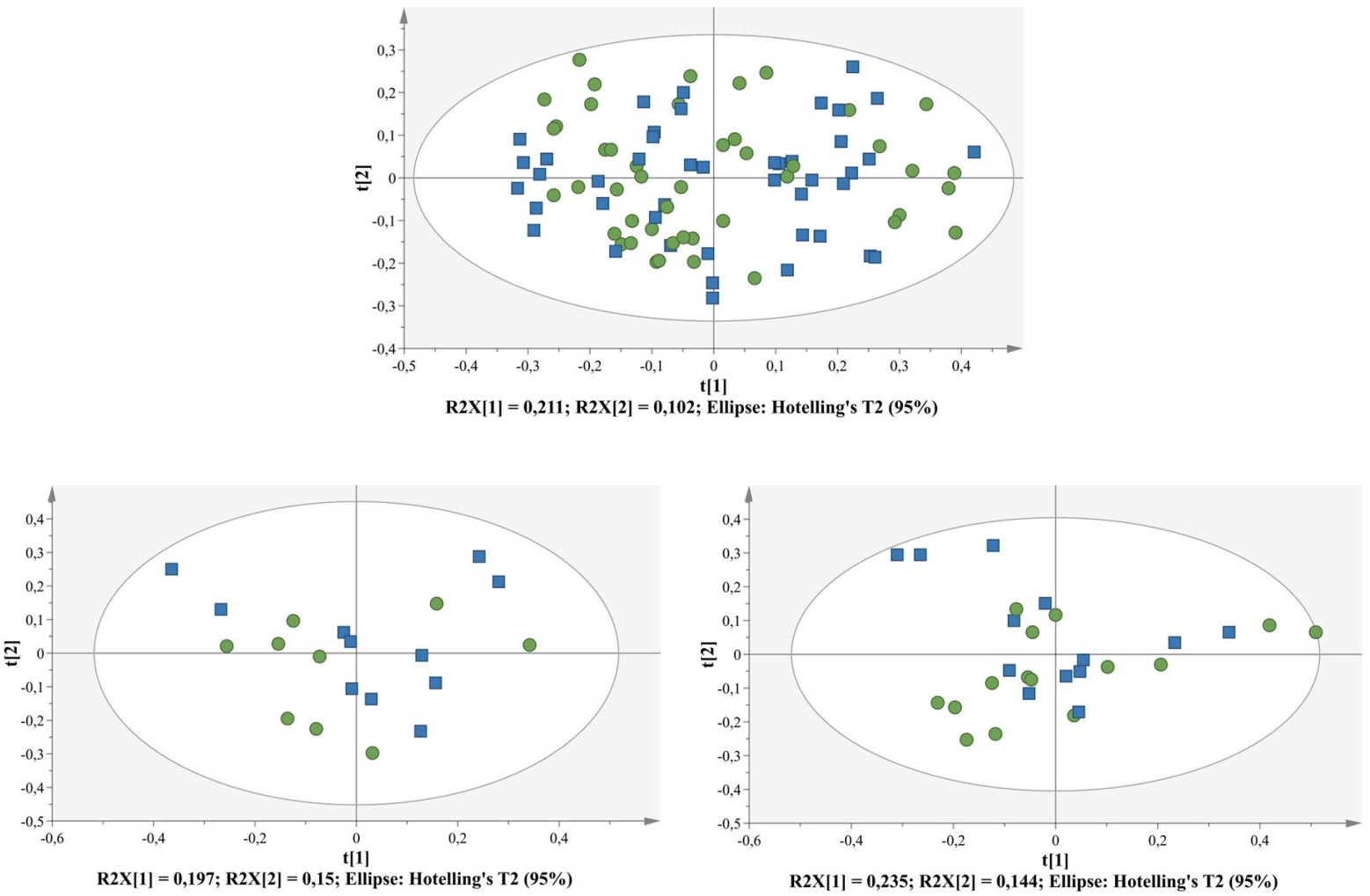

Figure 3.7. Up: PCA model in all samples, A=2, N=90, R2X(cum)=0,313, Q2(cum)=0,266. Green circles represent samples before the intervention and blue squares samples after the intervention. Down left: PCA of IBD samples in relapse in the Mastiha group. A=2, N=20, R2X(Cum)=0,35, Q2(cum) = 0,05. Down right: PCA of IBD samples in remission in the Mastiha group, A=2, N=28, R2X(Cum)=0.38, Q2(cum) = 0.23. Green circles and blue squares represent samples before and after Mastiha intervention respectively.

Regarding the faecal metabolic profiling, ninety samples before and after Mastiha intervention were included in a multivariate initial PCA model, as presented in **Figure 3.7**, without clear differentiation trends. Consequent models were generated for different sample subcategories. Specifically, for the case of IBD patients in remission, 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 (**Figure 3.8**).

Permutation testing further validated the generated model while ROC curves for samples before and after intervention provided 95.5% of true positives indicating high predictive ability (**Figure 3.9**). The color-coded S-line plot (**Figure 3.10**) 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 Mastiha 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. For comparison reasons, unsupervised and supervised statistical models were generated for IBD patients both in relapse and remission receiving placebo. Neither case provided validated models.

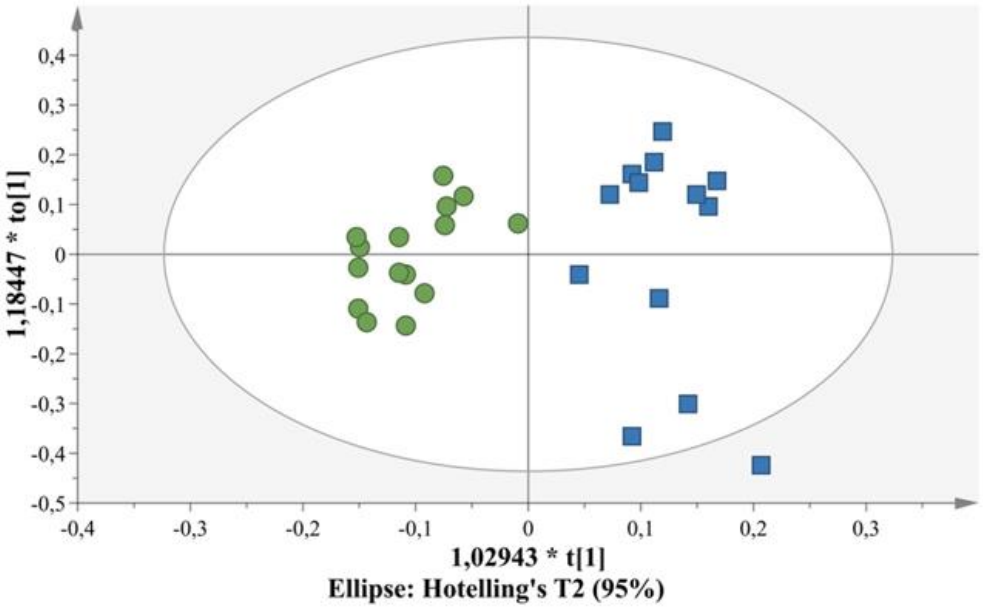

Figure 3.8. OPLS-DA model of IBD in remission, A=1+1, N=28 R2X (Cum)=0.88, Q2 (cum) =0.67. Green circles and blue squares represent samples before and after Mastiha intervention respectively.

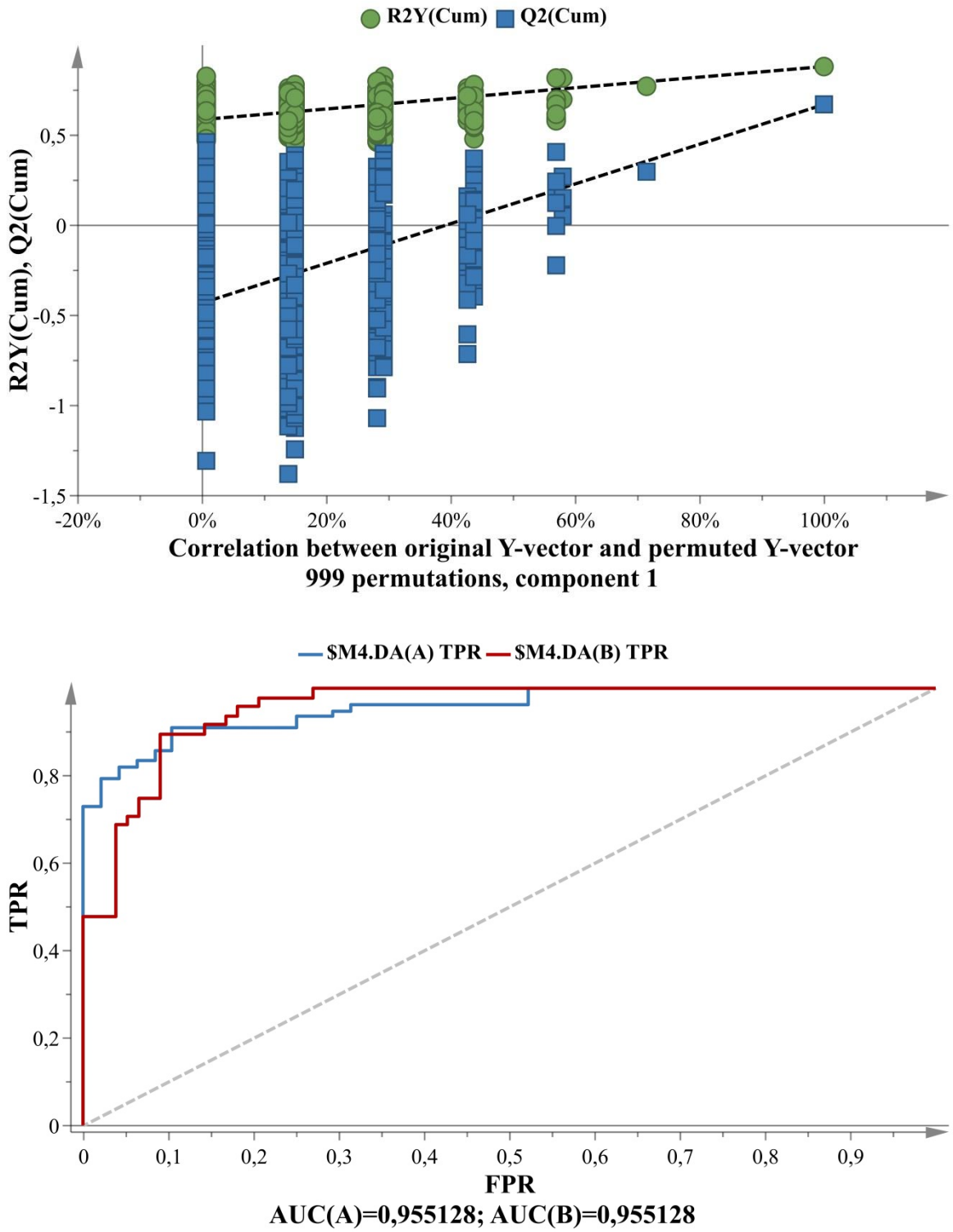

Figure 3.9. Permutation testing for validation (up) and ROC curve (bottom).

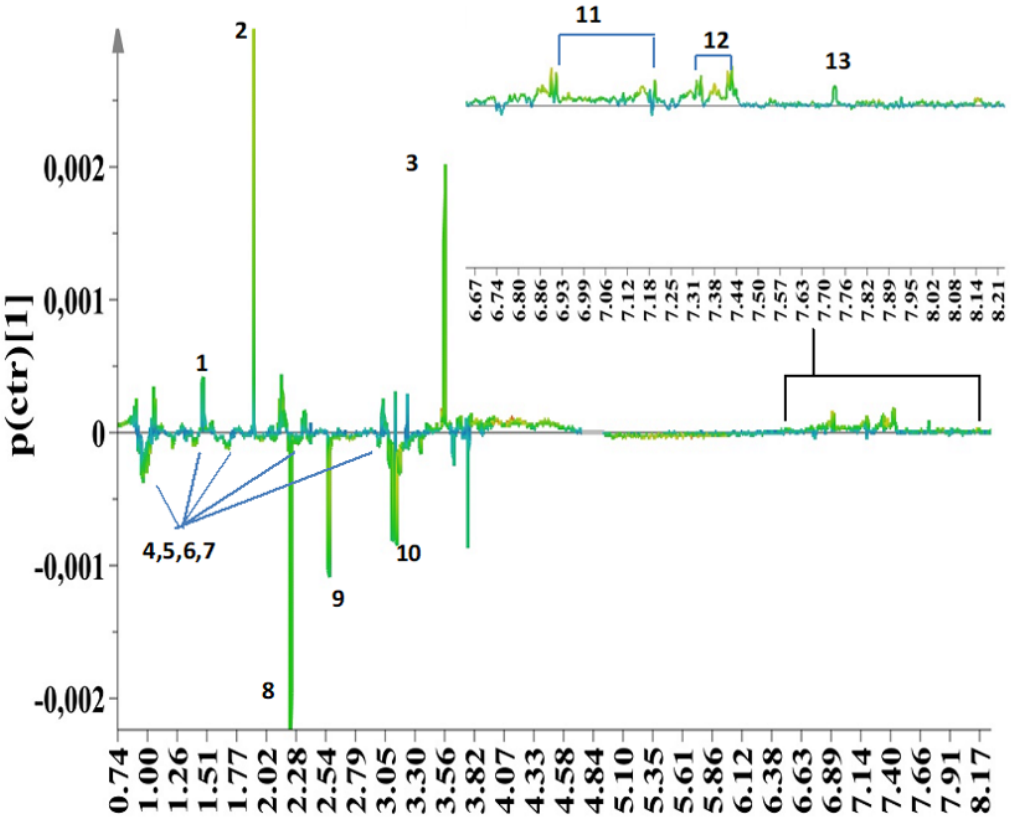

Figure 3.10. Color coded S-line plot for the IBD in remission model before and after Mastiha intervention with magnification of the aromatic region of the pseudo NMR spectra. 1. Alanine, 2. Acetic acid, 3. glycine, 4,5,6,7: valine, isoleucine, leucine, lysine, 8: acetone, 9: succinic, 10: unknown, 11: tyrosine, 12: phenylalanine, 13: tryptophan.

In general, 1H NMR spectra assignment revealed secondary metabolites (**Figure 3.11**) 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. MetaboAnalyst platform was utilized to explore the metabolic pathways related to the abovementioned metabolites (**Table 3.10**). 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 **Figure 3.12**.

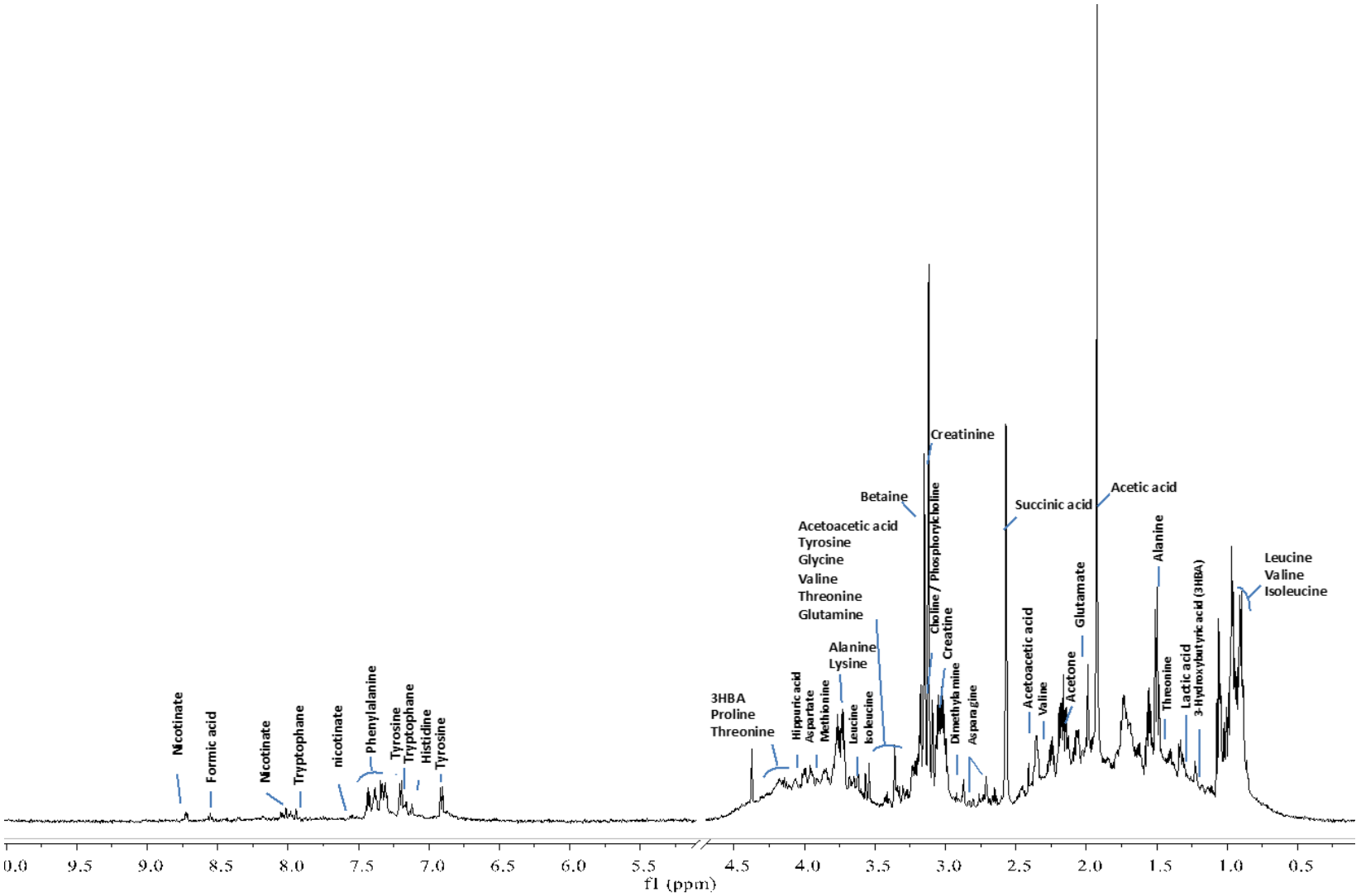

Figure 3.11 Representative 1D- NOE NMR spectrum with key identified metabolites.

Table 3.10. Metabolic pathway analysis results from MetaboAnalyst platform

Metabolic pathway	Total	Hits	Raw p	Impact
Glycine, serine and threonine metabolism	48	5	1.82E-06	0.12069
Phenylalanine metabolism	45	4	4.74E-05	0.15056
Aminoacyl-tRNA biosynthesis	75	4	0.0003563	0
Arginine and proline metabolism	77	3	0.0056244	0.13394
Phenylalanine, tyrosine and tryptophan	27	2	0.0074642	0.008
Valine, leucine and isoleucine biosynthesis	27	2	0.0074642	0.02173
Nitrogen metabolism	39	2	0.015242	0
Butanoate metabolism	40	2	0.016	0.08996
Synthesis and degradation of ketone bodies	6	1	0.029573	0
Tyrosine metabolism	76	2	0.052917	0.04724
Taurine and hypotaurine metabolism	20	1	0.095485	0.02158
Citrate cycle (TCA cycle)	20	1	0.095485	0.09024

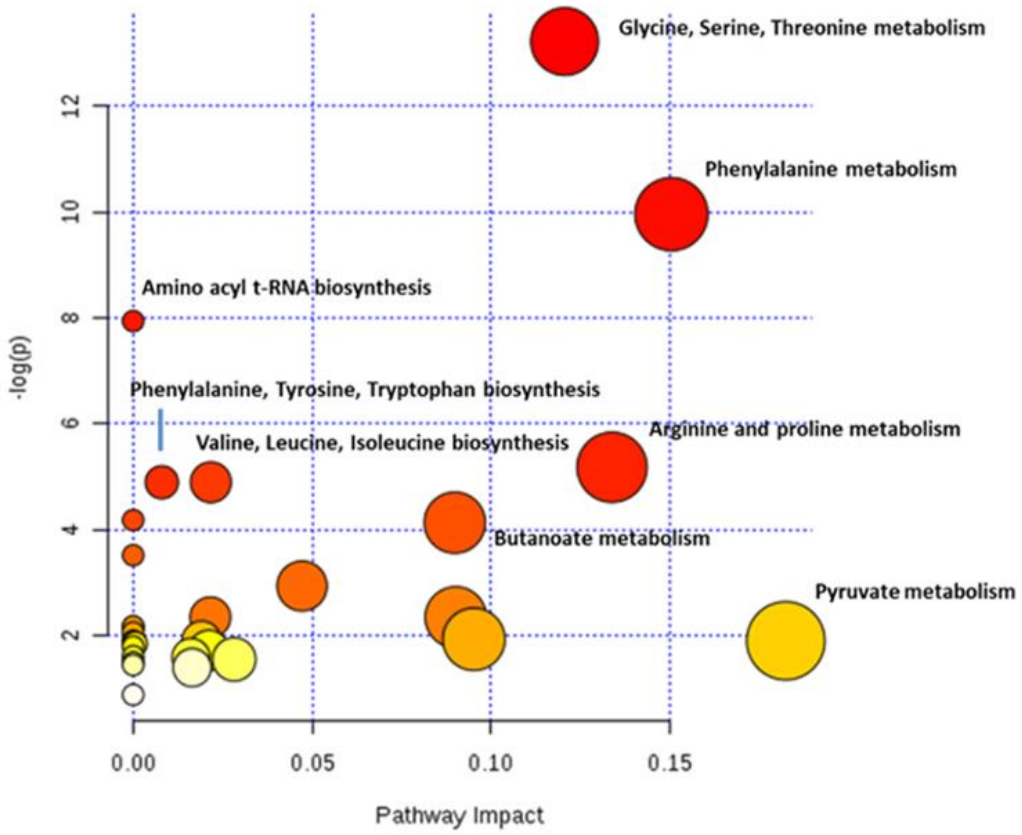

Figure 3.12. Schematic representation of perturbed pathways identified in MetaboAnalyst 3.0. x-axis represents the pathway impact and y-axis the pathway enrichment. Larger size and darker color of each node (pathway) represents higher pathway enrichment and higher pathway impact values.

3.2.3. Effect of Mastiha on gut microbiota

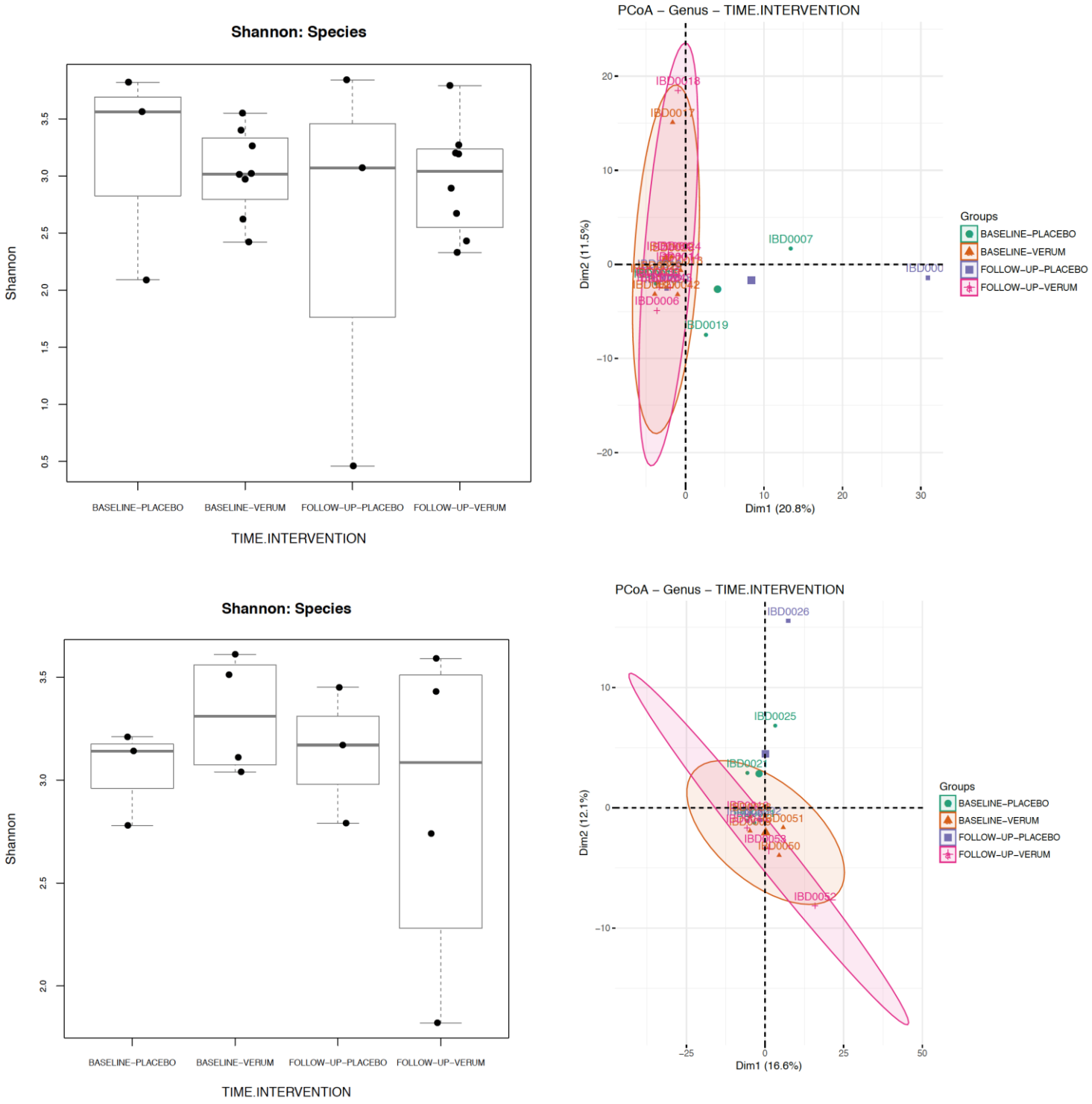

Figure 3.13. Up: Shannon diversity index at species level and global microbiota composition (PCoA plot) at genus level in CD patients Down: Shannon diversity index at species level and global microbiota composition (PCoA plot) at genus level in UC patients.

Microbial profiling using 16S ribosomal RNA (rRNA) sequencing was performed in 54 samples. A series of comparisons of gut microbiota diversity and composition using phylogenic and non-phylogenetic measures including the Jaccard, Bray-Curtis and UniFrac distances showed no significant differences in diversity or global microbiota composition. The variation in microbiota composition among treatments was visualized by means of clustering and PCA (**Figure 3.13**).

Although no significant differences in diversity were reported however some differences in relative abundances of bacterial taxa were observed. More specifically, In CD patients treated with Mastiha, relative abundance of *Tyzzerella* (Lachnospiraceae) decreased (p=0.05). Also, in CD patients relative abundance of *Terrisporobacter* (Peptostreptococcaceae) was higher (p=0.04), while Saccharimonadaceae (p=0.02) was lower in Mastiha vs placebo at follow-up. In UC patients, *Holdemania* (Erysipelotrichaceae) decreased in the Mastiha group (p= 0.03) and Enterobacteriaceae was higher (p= 0.05), whereas *Sellimonas* (Lachnospiraceae) (p= 0.04) and *Anaerostipes* (Lachnospiraceae) (p= 0.05) was lower in Mastiha vs placebo at follow-up.

3.2.4. Discussion

IBD is a complex immune condition with its exact etiology remaining unclear. Genetic heterogeneity, along with immune dysregulation, imbalance interaction with microbiome and several environmental factors, shape the development of the disease [Kaser et al., 2010]. Intestinal inflammation is controlled by both innate and adaptive immune signals with cytokines playing a key role determining T cell differentiation of T-helper 1 (Th1), Th2, T regulatory and Th17 cells [Sanchez-Munoz et al., 2008]. In recent years, biological therapies that target different molecular pathways, including cytokine networks have been developed, such as those targeting TNF-a, IL-6, and IL-17. 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 role of the microbiome in IBD is well established with several studies showing a decreased diversity and bacterial load in IBD patients comparing with healthy controls. This dysbiosis is observed especially in regions with active inflammation [Damman et al., 2012, Sommer et al., 2017]. Several disease-associated taxonomic and functional shifts have been

reported with an observed reduction in beneficial bacteria such as the phyla Firmicutes and an increase in harmful bacteria, such as the Enterobacteriaceae family [Kostic et al., 2014].

The signatures of microbiome and metabolome may be combined in order to characterize the fecal profile of IBD patients. Gut metabolites may reflect the complex crosstalk among hosts, microbes, and the transformations that the metabolites undergo [Dorrestein et al., 2014]. H-NMR and GC-MS studies have revealed alterations in bacterial products, suggesting an imbalance in bacterial ecology leading to dysbiosis, which is a major factor in IBD [Vernocchi et al., 2016]. Metabolomics can be used for identification of disease biomarkers in biological samples acquired via non-invasive processes and 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 [Storr et al., 2013].

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 [Larussa et al., 2017]. Our scientific group has previously shown Mastiha's effect on patients with IBD, with reduction in disease activity indices and cytokine levels in plasma and in blood mononuclear cells [Kaliora et al., 2017], as well as in faecal inflammatory markers [Papada et al., 2018] and in serum oxLDL [Papada et al., 2019] having been reported.

With interest to the immunoregulatory role of Mastiha, the aim of this study was to explore Mastiha's effect on cytokine serum levels in a Greek IBD cohort. Also, we aimed at investigating whether this effect is associated with alterations in stool metabolic profile. Finally, gut microbiota profiling would elucidate whether the above changes caused by Mastiha supplementation can be related with changes in gut microbiota composition.

Herein we observed 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 [Friedrich et al., 2019]. 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 [Andoh et al., 2002] and induce mucin production in epithelial cells of the airways [Kao et al., 2004]. 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 [Moschen et al., 2019]. 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 [Pickich et al., 2019]. 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 [Papalois et al., 2012, Qiao et al., 2011].

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 [Hovhannisyan et al., 2011]. 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 [McGeachy et al., 2007]. 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 [Kohlo et al., 2017]. 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 [Lever et al., 2010], while it has been correlated to Crohn's disease or ulcerative colitis in children and adolescents from 6 to 18 years old [Kohlo et al., 2017].

Marchesi et al. [2007] and Bjerrum et al. [2015], 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 [Joossens et al., 2011, Machiels et al., 2014]. SCFAs act beneficially as they constitute the main source of energy for intestinal epithelial cells. In particular, butyric acid, apart from being a source of energy, has proven anti-inflammatory properties which enhance the protection of epithelial barriers [Hamer et al., 2008].

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 [Liu et al., 2017]. Phenylalanine has been previously found at higher levels in IBD patients [Bjerrum et al., 2017] and has been positively correlated with tyrosine even during induction of remission. Hippuric acid has been previously associated with Clostridia populations in gut [Storr et al., 2013]. The relative abundance of Clostridia has been correlated with intestinal inflammation levels [Kohlo et al., 2017] and its populations have been found reduced in patients with UC [Rajilić-Stojanović et al., 2013]. 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 [Krupp et al., 2012].

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 [Hamer et al., 2008, Krupp et al., 2012]. 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 [Marchesi et al., 2007] 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 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 [Gálvez et al., 2014]. 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 [Romani et al., 2008]. 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 [Veldohen et al., 2008]. 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. Several natural products have been proposed as potent IDO inhibitors, in a similar way to our proposed action of Mastiha [Zulfiqar et al. 2017] and their mechanism of action is presented in **Figure 3.14**. For example, curcumin has been found to reverse IDO-mediated suppression of T-cell responses, by supressing STAT1 activation in bone marrow-derived DCs [Jeong et al., 2009]. Likewise, p-coumaric acid inhibits IDO expression at the

transcriptional level by inhibiting STAT1 activation in IFN-γ-stimulated murine DCs [Kim et al., 2007].

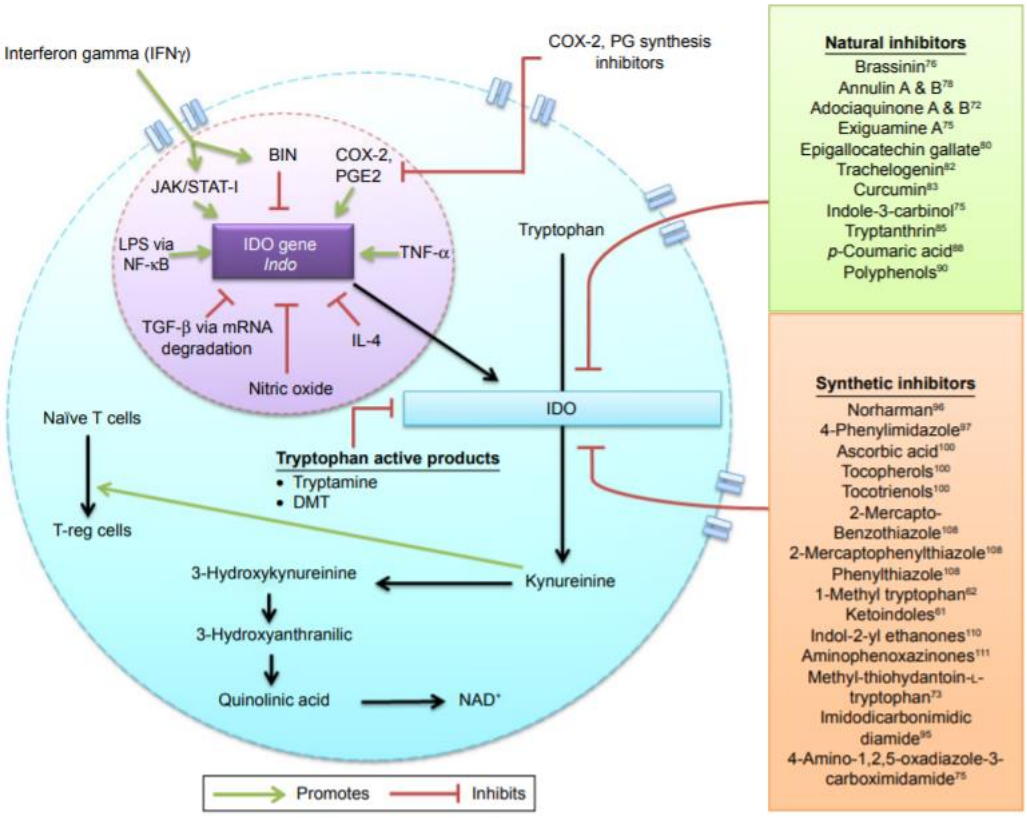

Figure 3.14. IDO pathway and inhbitors [Zulfiqar et al. 2017]

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 [Baker et al., 2018].

Regarding gut microbiota composition there were no alterations in diversity or global microbiota composition found in this intervention. However, we observed some differences in relative abundances of bacterial taxa. More specifically, in CD patients, relative abundance of *Tyzzerella* (Lachnospiraceae) decreased in the Mastiha group and relative abundance of

Terrisporobacter (Peptostreptococcaceae) was higher, while Saccharimonadaceae was lower in Mastiha vs placebo at follow-up. *Tyzzerella* has been found increased in CD patients in several studies and has been associated with increased risks of cardiovascular diseases suggesting that its decrease can be beneficial [Ascher and Reinhardt, 2018, Obermullet et al., 2020, Olaisen et al., 2020]. *Terrisporobacter* genus has been found increased in knock-out mice with colitis phenotype compared to the control group [Jangid et al., 2020]. Interestingly, Saccharimonadaceae is upregulated in AhR−/− mice after AhR ligand supplementation, suggesting that the activation of AhR pathways is associated with an increase in Saccharimonadaceae [Schanz et al., 2020]. In UC patients, *Holdemania* (Erysipelotrichaceae) decreased in the Mastiha group (p= 0.03) and Enterobacteriaceae was higher (p= 0.05), whereas *Sellimonas* (Lachnospiraceae) (p= 0.04) and *Anaerostipes* (Lachnospiraceae) (p= 0.05) was lower in Mastiha vs placebo at follow-up. *Holdemania* was reported increased in UC versus controls in a large longitudinal intercontinental study [Clooney et al., 2021]. Although Enterobacteriaceae are known for their implication in IBD and their increased levels in both CD and UC [Dalal et al., 2014], in a recent study, this family shows an imbalance in IBD with increased abundance level in CD and decreased in UC patients compared to the controls [Alam et al., 2020]. Finally, Lachnospiraceae are among the core of gut microbiota and the main producers of SCFAs, but have also been associated with several intestinal diseases [Vacca et al., 2020].

While this study has some interesting results it is subjected to some limitations, such as the absence of endoscopy at follow-up and the fact that cytokines were 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.

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-**

17Aa levels in serum that may be associated with **a possible shift of Th-17 cells to a regulatory role**, more protective for quiescent IBD. More studies are needed to further explore the role of Th-17 cells in IBD and confirm whether the above observations also exist in a mucosal level, as well as the possible effect of phytochemicals on Th-17 cells differentiation and consequent healing of the colonic ulcerations. Mastiha's proposed mechanism of action is presented in **Figure 3.15.**

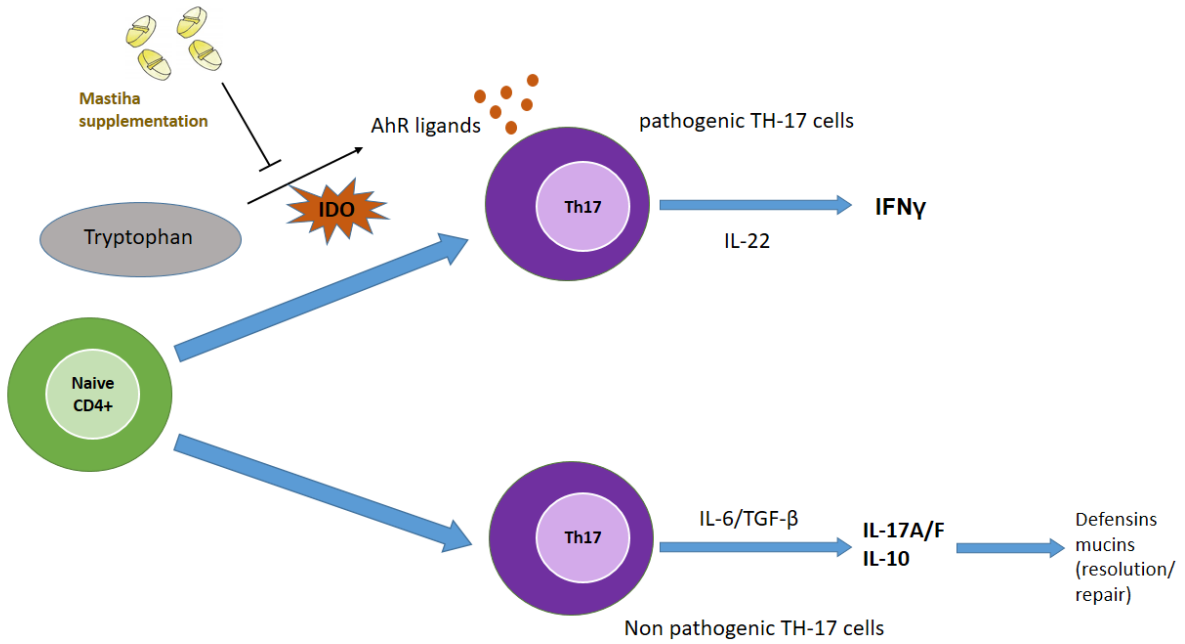

Figure 3.15. Mastiha's proposed mechanism of action in IBD

3.3.The regulatory mechanism of miRNA expression

3.3.1. Baseline characteristics of the patients that were included in the miRNA analysis

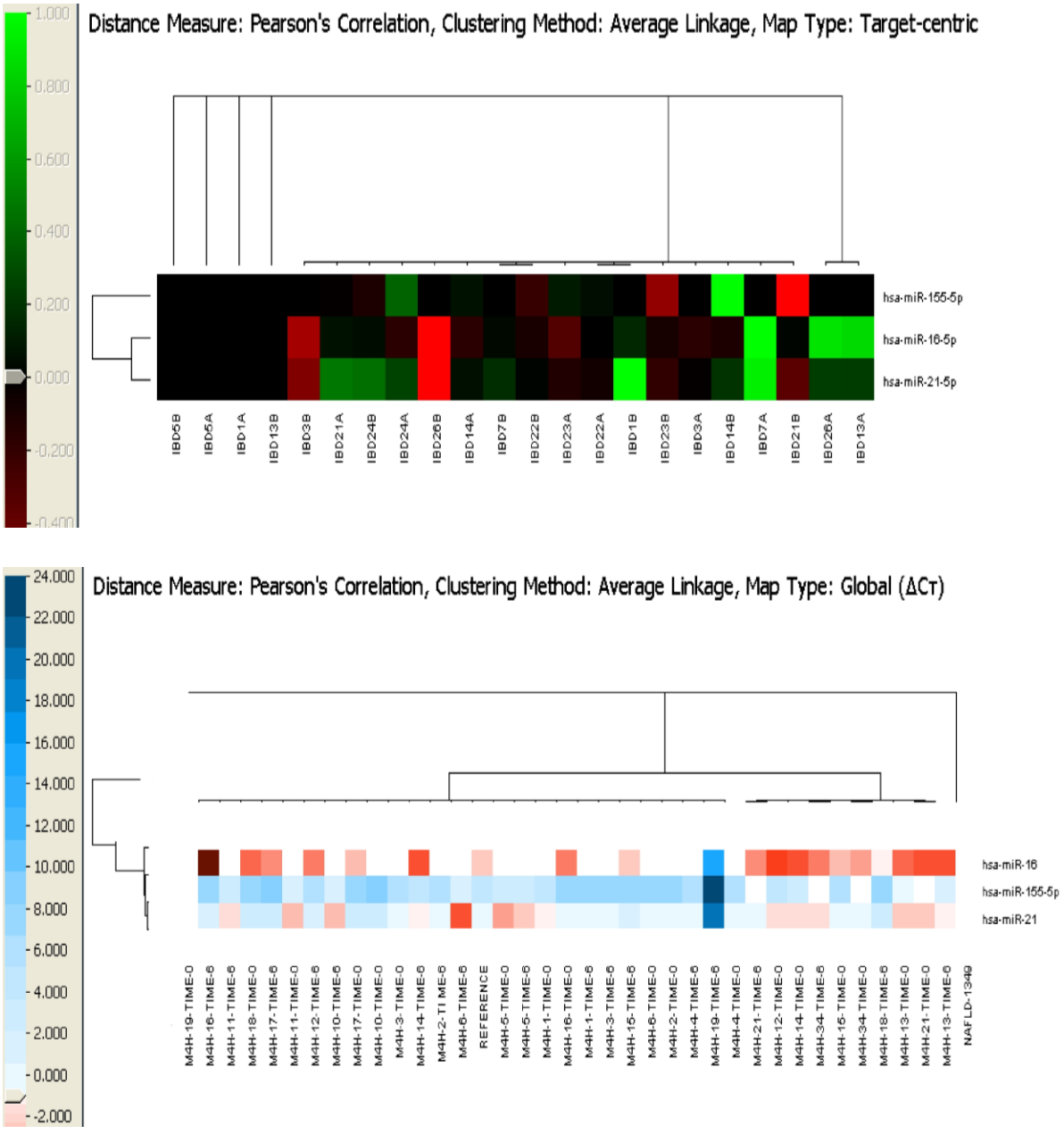

Figure 3.16 An example of a heat map plot of miRNA expression profiles produced with ExpressionSuite Software after quantification with the StepOnePlus™ Real-Time PCR System.

The above results from both interventions pointed towards an anti-inflammatory role of Mastiha. In order to further explore Mastiha's anti-inflammatory mechanism of action and

potential common molecular pathways of immunoregulation in NAFLD and IBD, we focused on epigenetic mechanisms and most specifically on microRNAs that are implicated in inflammation. 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 3.11**]. MicroRNA quantification was performed in miR-16-5p, miR-21-5p and miR-155-5p.

Table 3.11. Selected miRNAs for quantification and their functions

miRNA	Function	Inflammation	NAFLD	IBD
miR-16	inhibits cell proliferation, invasion, angiogenesis, cell cycle progression, promotes cell apoptosis, regulates tumorigenesis [Bonci et al., 2008, Cimmino et al., 2005, Yan et al., 2019]	regulates immune-mediated tissue repair and production of inflammatory mediators, such as TNF-a, suppresses the activation of inflammatory macrophages though MAPK and NF-κB signalling, improves inflammation-induced insulin sensitivity [Liang et al., 2016, Talari et al., 2015, Yan et al., 2019]	involvement in liver fibrosis through autophagy of activated stellate cells, increased circulating miR-16 levels in NAFLD patients, correlate with fibrosis stage, in NASH negatively correlate with AST and fibrosis prediction scores [Cermeli et al., 2011, Dongiovanni et al., 2018, Lopez-Riera et al., 2018].	activates NF-κB signaling pathway in human colonic mucosa of active UC patients, increased circulated levels in CD and UC patients compared to healthy controls [Paraskevi et al., 2012, Tian et al., 2016]
miR-21	oncogenic role, targets cancer related genes, regulates cell proliferation, invasion and migration, apoptosis [Meng et al., 2007, Si et al., 2015]	regulates chronic inflammatory processes and T cell effects, Controls TLR-signaling, PI3K/AKT/GSK3β, MyD88, MAPK pathways, induces DNA-hypomethylation , activates release of pro-inflammatory cytokines, [Fabbrei et al., 2012, Momen-Heravi et al., 2017, Shi et al., 2015]	involved in liver lipid metabolism through various targets, contributes to NASH, hepatocellular injury, inflammation and fibrosis via PPAR-α, upregulated in the serum of NAFLD patients, correlates positively with AST, ALT and fibrosis scores [Lopez-Riera et al., 2018, Wang et al., 2019].	differentiation, apoptosis, and activation of T cells, upregulated in both intestinal tissues and circulation and is upregulated and associated with disease activity in UC patients [Paraskevi et al., 2012, Yan et al., 2020]
miR-155	regulation and differentiation of cells of haematopoietic origin, regulation of type 1 Angiotensin II receptor, maintain the oxygen homeostasis [Faraoni et al., 2009, Yang et al., 2016]	controls B cell differentiation, antibody production, Th1, Th2 and Th17 differentiation, enhances AHR signaling, mitosis, reduces signaling for toll-like receptors, SOCS, ERK/MAPK, and B-cell receptors [Gracias et al., 2013, Higgs and Slack, 2013, Li et al., 2017]	up-regulated in hepatocytes and liver tissue of patients with NAFLD, regulates LXR α-dependent adipogenic signaling pathways [Blaya et al., 2019, Huang et al., 2019]	up-regulated in UC and CD, its deficiency protects mice from experimental colitis, key role in the differentiation of B and T cells. miR-155-/- mice express reduced Th17 cells [Archanioti et al., 2011, Lu et al., 2017, Singh et al., 2014]

In the microRNA analysis, we included 67 patients from the MAST4HEALTH study and 60 patients from the IBD-GR study [Flowchart in **Figure 3.17**]. The baseline characteristics of the patients that were included in our analysis are presented in **Tables 3.12** and **3.13**. No significant differences between the Mastiha and the placebo group were observed in neither of the inflammatory conditions.

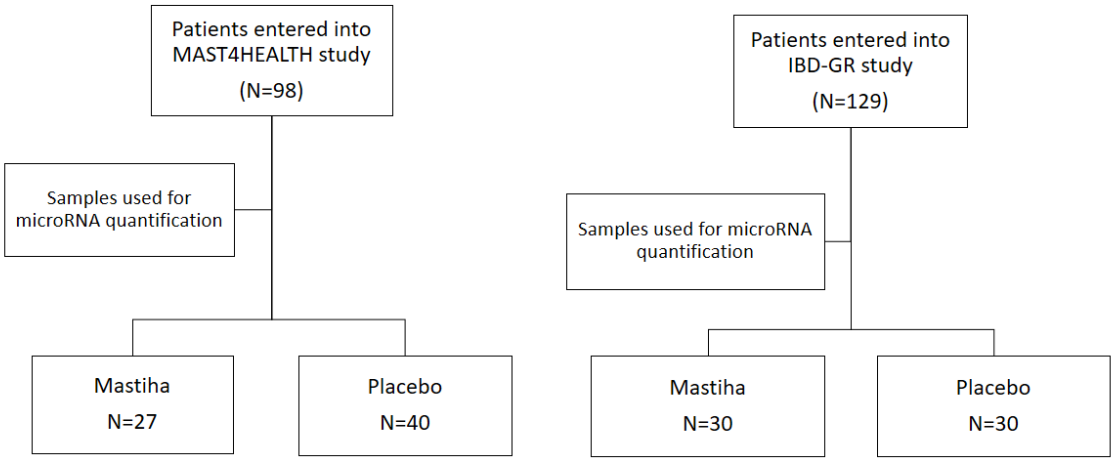

Figure 3.17. Flowchart of samples used for microRNA quantification

Table 3.12. 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

Table 3.13. Baseline characteristics of patients of the 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, HBI: Harvey & Bradshaw Activity Index, PMS: Partial Mayo Clinic Score

3.3.2. miRNA plasma levels post- intervention; unfolding the molecular pathway of Mastiha activiy

Next, we examined whether Mastiha supplementation can modulate the circulating levels of these three microRNAs in a way that explains its anti-inflammatory activity. **Tables 3.15** and **3.15 (a., b., c.)** present microRNA levels at baseline and at follow up in all patients and in different disease categories in NAFLD and in IBD cohorts respectively.

In NAFLD, 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 cTt1>868.6 and ct1<868,6 that correspond to higher and lower liver inflammation. 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..

In IBD, 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 statistically indifferent. 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.

Table 3.14. Changes in miRNA plasma levels in NAFLD patients post 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=14)	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=14)	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=14)	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 3.15a. Changes in miRNA-16 plasma levels in IBD patients post 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]
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=7)	2.321 (2.043)	1.866 (1.340)	0.266		
	REMISSION					
	Placebo (N=14)	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=8)	2.118 (1.739)	0.946 (0.883)	0.347		
	REMISSION					
	Placebo (N=6)	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 3.15b. Changes in miRNA-21 plasma levels in IBD patients post intervention

		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]
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=7)	0.183 (0.118)	0.298 (0.390)	0.206		
	REMISSION					
	Placebo (N=14)	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**		
	RELAPSE					
	Placebo (N=7)	0.216 (0.180)	0.464 (0.329)	0.244	0.187	0.303
	Mastiha (N=8)	0.153 (0.106)	0.204 (0.242)	0.659		
	REMISSION					
	Placebo (N=6)	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 3.15c. Changes in miRNA-155 plasma levels in IBD patients post 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=7)	0.055 (0.052)	0.075 (0.118)	0.652		
	REMISSION					
	Placebo (N=14)	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=8)	0.045 (0.038)	0.056 (0.065)	0.490		
	REMISSION					
	Placebo (N=6)	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		

[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

3.3.3. Discussion

Over the last few years, miRNAs have emerged as important regulators in various biological processes, including cell proliferation, differentiation, autophagy, metabolism and immune responses [Zhou et al., 2017]. It has been shown that they can influence several molecular signaling pathways associated with inflammatory responses [Chandan et al., 2019]. Their role has been investigated in both NAFLD and IBD. In NAFLD, there is increasing evidence that several miRNAs regulate molecular pathways associated with lipid metabolism, oxidative stress and liver inflammation [Lin et al., 2020]. In IBD, microRNAs implicate in regulation of intestinal epithelial barrier function, cell membrane trafficking, and interfere with inflammatory signaling pathways, such as the NF-κB and IL-6/STAT3 [James et al., 2020].

In our MAST4HEALTH study we observed an improvement in MRI liver parameters associated with liver inflammation and fibrosis accompanied with a decrease in the abundance of inflammatory taxa. In IBD-GR study an immunoregulatory role of Mastiha through the regulation of Th17 cells function and differentiation was proposed. To further explore this anti-inflammatory role of Mastiha we focused on three miRNAs with a critical role in inflammatory processes in both diseases.

No effect of Mastiha was detected in the levels of miR-16 and miR-21. MiR-16 acts as a regulator of immune-mediated tissue repair and the production of inflammatory mediators, such as TNF-a [Yan et al., 2019]. It has been shown to be increased in NAFLD patients and to positively correlate with fibrosis in early fibrosis, whereas negatively in NASH [Cermeli et al., 2011, Lopez-Riera et al., 2018]. In IBD, it promotes activation of NF-κB signaling pathway in human colonic mucosa of active UC patients [Tian et al., 2016] and its circulated levels are higher in CD and UC patients than healthy controls in a Greek IBD population [Paraskevi et al., 2012]. 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 [Momen-Heravi et al., 2018]. In NAFLD it is involved in liver lipid metabolism and contributes to inflammation and fibrosis via PPAR-α [Wang et al., 2019]. It is upregulated in the serum of NAFLD patients and correlates positively with AST, ALT and fibrosis scores [Lopez-Riera et al., 2018]. 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 [Yan et al., 2020].

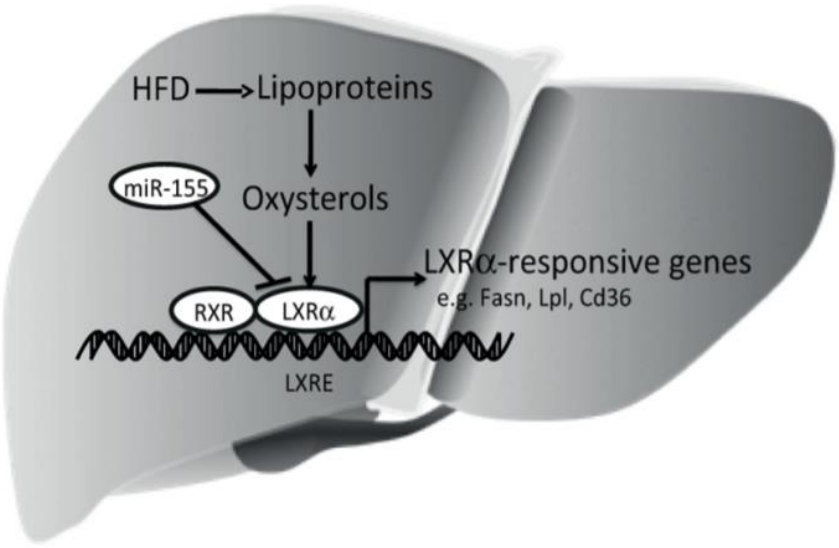

Figure 3.18. Schematic diagram indicating the pathway of miR-155 inhibition of LXRa-induced gene regulation of lipid accumulation during hepatic steatosis [Miller et al., 2013].

MiR-155 is a critical regulator of inflammation, with several activated immune cells overexpressing it responding to many inflammatory stimuli, such as TNF-a, interferons, PAMPs and DAMPs and TLR ligands [Mahesh and Biswas, 2019]. It controls inflammation in multiple levels, like B cell differentiation and antibody production, and controls Th1, Th2 and Th17 differentiation [Gracias et al., 2013]. MicroRNA-155 is considered one of the biologically most relevant miRNAs in liver diseases as it is implicated in pathways related to liver injury, steatosis, inflammation, fibrosis, and carcinogenesis [Hartmann and Take, 2016]. Although it has been reported to be upregulated in hepatocytes and liver tissue of patients with NAFLD [Blaya et al., 2019], its role may be protective or exacerbating, depending on the condition. Nevertheless, its implication in lipogenesis is through the regulation of liver X receptor (LXR) α-dependent adipogenic signaling pathways that lead to NAFLD [Huang et al., 2019]. Interestingly, HFD miR-155-/- mice developed increased hepatic steatosis compared to controls, associated with increased expression of hepatic genes involved in glucose regulation, fatty acid uptake and lipid metabolism [Miller et al., 2013] [Figure 3.18]. 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 only in the placebo group in patients with cT1<868,6, confirming that its role changes depending on the disease condition.

MiR-155 is up-regulated in both UC and CD and its deficiency protects mice from experimental colitis [Lu et al., 2017]. It has a key role in the differentiation of B and T cells and contributes to the development of regulatory T cells [Archanioti et al., 2011]. MiR-155-/- mice express reduced systemic and mucosal IFN-γ-expressing CD4+ T cells, and more specifically, Th17 cells [Singh et al., 2014]. 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 action has been proposed for cinnamaldehyde, an active compound from cinnamon that has been shown to reduce inflammation via miR-155 inhibition in colon tissues [Qu et al., 2019]. Furthermore, a study by Liu et al. [2018] 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 Th-17 regulatory role of Mastiha.

Our results suggest miR-155 as a key regulator in the mode of action of Mastiha as its levels were significantly changed in both RCTs. In NAFLD, mir-155 is implicated in the regulation of lipogenic genes, whereas in IBD, it regulates Th-17 differentation. These results parallel with the lipid regulatory action of Mastiha in the case of MAST4HEALTH [Amerikanou et al., 2021], and the Th-17 regulatory action in the case of IBD-GR [Amerikanou et al., 2021b] suggesting that Mastiha may exhibit different beneficial actions in these diseases, but under a common mechanism, in which regulators such as miRNA-155 play a pivotal role. The above suggested mechanism is presented in **Figure 3.19**.

Although miRNA quantification was performed in circulation and not in the inflamed tissues, this limitation is counterbalanced by the fact that miRNA expression in peripheral blood has been proven to reflect mucosal changes and alterations in circulating inflammatory cells [Link and Goel, 2013]. Finally, the methodologies applied for this analysis ensure the reproducible recovery of high-quality material.

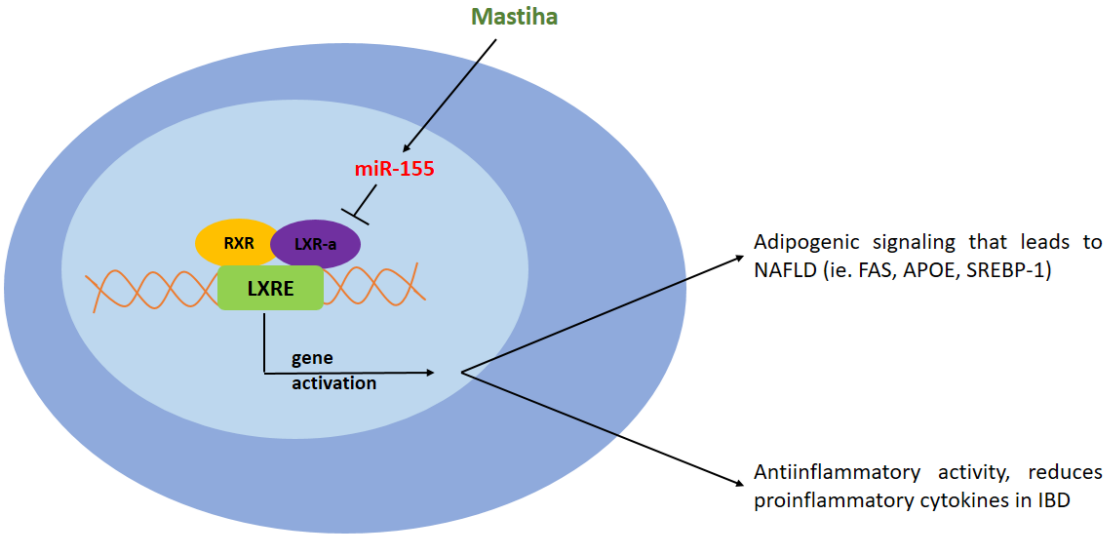

Figure 3.19. Mastiha's suggested mechanism of action in miRNA regulation. Mastiha may manipulate the miR155/LXR pathway through regulation of serum miR-155 levels. More specifically, in NAFLD patients it ameliorates a decrease of miR-155, which can be associated with disease progression. In patients with active UC, it ameliorates an increase of miR-155, which is associated with proinflammatory effects.

Conclusively, **miRNA quantification provided a new insight into Mastiha's anti-inflammatory properties**. MiR-155 may be one of the key mediators of Mastiha's action, probably through **regulation of lipogenesis in NAFLD** and **Th-17 differentiation in IBD**. The changes detected in the plasma levels of miR-155 observed in this RCT support a need to further evaluate Mastiha's molecular mechanisms focusing on miRNAs regulatory role. Nowadays there is an increasing interest in exploring phytochemicals activities through regulation of miRNAs. Nutrimiromics is a new discipline that focuses **on the influence of the diet on the modification of gene expression due to miRNAs**, and their implications in chronic diseases [Quintanilha et al., 2017].

4. Conclusive remarks

Inflammation is a critical biological response to harmful stimuli, such as pathogens, damaged cells and toxic compounds. It plays an important role in tissue repair and regeneration and is considered one of the most important mechanism of defence. Although, short-term and controlled inflammation is important for healing, however inflammatory dysregulation and chronic inflammatory responses lead to a variety of chronic inflammatory conditions. Inflammatory diseases include a great variety of disorders and are considered the most significant cause of death worldwide.

NAFLD and IBD are two inflammatory diseases with a high prevalence in Western societies, mainly due to the westernised lifestyle. Both conditions are the object of great scientific and clinical interest which will increase the next years as the burden of the health care impact is significant. NAFLD, on one hand, may have no or silent symptoms on early stages, but can lead to serious life threatening conditions and has no approved treatment, and IBD, on the other hand, has serious impact on the life quality of patients, with frequently hospitalisations and severe treatment side effects. The above, pinpoint the need for new promising options for treatment and management of these diseases with the least side effects. Also these two conditions, except from chronic inflammation, they share other common pathogenic features, such as increased intestinal permeability, gut dysbiosis, metabolic alterations and oxidative stress. In fact, there is increasing evidence about the co-existence of NAFLD and IBD with a great prevalence of NAFLD in IBD. The great variability on this prevalence 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 related to underlying chronic inflammatory condition. Therefore, IBD patients' increased risk for NAFLD may be related to intestinal disease-related factors, such as disease duration that exposes patients to multiple NAFLD risk factors including chronic relapsing inflammation, metabolic comorbidities and hepatotoxic drugs, such as steroids, immunosuppressants and biological factors.

The influence of natural products in disease management is quite interesting, with phytochemicals and derivatives from plants exhibiting promising options to improve treatment efficiency and decrease side effects. Mastiha is a resin excreted by the shrub *Pistacia lentiscus*,

cultivated on Chios Island, Greece. It consists of a plethora of bioactive constituents, including phenolic compounds and phytosterols, and is rich in terpenic acids. It has been known, as an effective ingredient in the treatment of gastrointestinal disorders since ancient times. Recently, EMA has officially granted two health claims to Mastiha, one for mild dyspeptic disorders and one for symptomatic treatment of minor inflammations of the skin.

A lot of research has been conducted on the beneficial effects of Mastiha in inflammatory diseases. Especially, in IBD, Mastiha supplementation adjunct to treatment was proven effective in regulation of several biomarkers associated to the disease, such as inflammatory cytokines, oxidative stress markers, faecal lysozyme, amino acid profile regulation and others. In NAFLD, there was only one study of Mastiha in a NASH mouse model that presented reduced plasma ALT, hepatic steatosis, and histological activity scores. This thesis aimed at investigating the molecular pathways under which Mastiha exhibits its beneficial properties in inflammation through two randomized double blind placebo controlled clinical trials in inflammatory diseases, NAFLD and IBD. Our aim was to investigate the effect of Mastiha supplementation in each disease applying a more integrative and multi-omics approach. Consequently, after exploring the similar patterns appearing in the two RCTs, we focused on common pathways that could explain the proven anti-inflammatory activity, through epigenetic modifications, and especially through regulation of miRNA levels.

The main findings derived from MAST4HEALTH trial:

- Mastiha **reduced parameters** of **liver inflammation/fibrosis** only in **severely obese** patients.
- Mastiha **increased dissimilarity of gut microbiota**, as shown by the Bray-Curtis index, and downregulated Flavonifractor, a known inflammatory taxon.
- Mastiha **decreased phospholipids and cholic acid levels** in plasma compared to Placebo.

The main outcomes from the IBD-GR trial:

- Mastiha **influenced the metabolic profile of IBD patients in remission** exhibiting, in between others, **increased levels of glycine and tryptophan**. Tryptophan derivatives are involved in immunoregalutory mechanisms, such as the Th17 cells differentiation.

- Mastiha **increased IL-17A levels in serum** that may be associated with a possible shift of Th-17 cells to a more **protective regulatory role**.

- Mastiha **regulated some gut microbiota** related to inflammatory processes.

The above coincide to an anti-inflammatory role of Mastiha expressed in the levels of various biomarkers, such as metabolites, gut microbiota and inflammatory markers. The above regulations should be a result of an implication in the regulation of the inflammasome and various molecular signaling pathways that result in the activation of inflammatory responses. MicroRNAs are small non-coding RNAs that have the ability to regulate gene expression by binding to target mRNAs and promoting or inhibiting their translation. They have emerged key gene regulators to control inflammation, as they target targeting key signalling molecules in inflammatory diseases, such as NAFLD and IBD. Also, it is known that phytochemicals exert their beneficial roles in diseases by directly controlling miRNA expression. Therefore, the final aim of this thesis was to explore whether microRNAs common to these inflammatory conditions with pivotal role in both NAFLD and IBD may facilitate the anti-inflammatory effect of Mastiha. Our results suggest miR-155 as a key regulator in the mode of action of Mastiha as its levels were significantly changed in both RCTs. In NAFLD, mir-155 is implicated in the regulation of lipogenic genes, whereas in IBD, it regulates Th-17 differentation. These results parallel with the lipid regulatory action of Mastiha in the case of MAST4HEALTH, and the Th-17 regulatory action in the case of IBD-GR suggesting that Mastiha may exhibit different beneficial actions in these diseases, but under a common mechanism, in which regulators such as miRNA-155 play a pivotal role.

Conclusively, the above results support the multiple health benefits by Mastiha use, underlying a possible common mechanism that lies under these beneficial properties. Certainly, there is need for further research on the unravelling of the molecular mechanisms associated with the regulatory role of Mastiha. Especially the key role of miRNA-155 in Mastiha's action has

to be confirmed in different samples as well, not only in a larger cohort but also in different tissues and in in vitro studies.

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APPENDIX A

MAST4HEALTH STUDY QUESTIONNAIRES

CONSENT FORM

Title of the study

Mastiha treatment for Obese Patients with NAFLD diagnosis "MAST4HEALTH"

Chief investigator

.....

Study Centre

.....

..... Ethics Committee has approved this study.

Please read carefully the Patient Information Leaflet (PIL) and ask anything you would like to know before you sign this form.

I ……..

1. Accept that I have read the PIL about the clinical trial and asked any possible questions.
2. Give permission to anyone that takes part in this clinical trial and is authorised by the Chief Investigator to have access to my personal medical notes.
3. Understand that I can withdraw the clinical trial whenever I want without giving any reason for my decision.
4. Agree to be a participant in this clinical trial voluntarily.

Signature of the participant ……. Date …….

Signature of the person taking the consent …….. Date …….

Signature of a witness …….. Date ……

Signature of the Chief Investigator …….. Date …….

PATIENT INFORMATION LEAFLET

Title of the study

Mastiha treatment for Obese Patients with NAFLD diagnosis "MAST4HEALTH"

We would like to inform you about a research study that you are being asked to take part in. This study has been approved by the Ethics Committee of the Institute. A description of the study and details about participation are included below. Please read the following information carefully, and then we will answer any questions that you may have. Additionally, talk to others about the study if you wish. If you decide to participate, please sign this form. By signing you are authorizing us to include you in the study. Your participation in this study is voluntary. You can refuse to participate and you may withdraw your consent to participate at any time without any disadvantage for your future medical care. Enroll in the study only if you really want to. Please read carefully this patient information sheet and consent form, and ask all the questions you may have. Do not sign this informed consent document if you have questions that have not been answered to your satisfaction.

If you consent to giving blood/stools as part of this study, these specimens will become the property of our Institution. Specimens could lead to discoveries that may be of value for Public Health. You do not have any right to money or other compensation stemming from products that may be developed from the specimens.

Purpose of the study

The purpose of this study is to investigate the possible efficacy of a natural nutritional supplement, called Mastiha, against Non Alcoholic Fatty Liver Disease/ Non Alcoholic Steatohepatitis (NAFLD/NASH).

Background

NAFLD/NASH affects approximately 50% of diabetics and 76% of obese patients. Since current NAFLD treatment therapies are limited, much attention has been focused on the identification of potential dietary substances or bioactive phytochemicals in fruits, vegetables, and plants or their products. Mastiha supplement, a natural product of Greece exerts antioxidant/anti-inflammatory and lipid lowering properties. Therefore, hereby a multicenter randomized double blind placebo controlled (parallel arm) clinical trial is designed.

Design of the study

If you decide to take part in this clinical trial after you sign the informed consent the following procedures will take place. You will need to visit the hospital two times for the whole study period (6 months).

Screening assessments

We will check if you are eventually eligible for this study (recording of demographic data, medical history and medication, physical examination, taking a blood sample, MR and liver Multiscan, Fibrotest, pregnancy test for women). If you are eligible, you will be allocated randomly to Group 1 (placebo) or to Group 2 (verum).

Baseline assessments

Before the initiation of the study, laboratory markers will be assessed including Gamma-glutamyl transferase, Alanine transaminase, and Aspartate transaminase, lipid profile (total cholesterol, high-density lipoprotein cholesterol, triglycerides, low-density lipoprotein cholesterol and fasting glucose, insulin, homeostasis model assessment (HOMA-IR)). Stool sample will also be collected for gut microbiota assessment. Genomic DNA will be isolated from peripheral blood samples. After that, randomization will take place and placebo or verum will be administered together with nutritional counseling for 6 months.

Follow-up assessment

At the end of the trial (6 months) all the initial assessment will take place.

Risks and benefits

No adverse effects have been referred after Mastiha consumption as a nutritional supplement and as a herbal remedy. During blood collection, every measure to prevent distress and discomfort will be put in place. Blood samplings will be performed with butterfly needles by specialized staff following all the standard hygiene rules. Blood sampling is a routine procedure without special risks and it is a part of the clinical procedure for the clinical diagnosis or the follow up herein. The sample collection for the study does not carry additional risk. Multiliver scan is a safe diagnostic tool approved by the European Union, which uses no radiation and no contrast. The scans are 15-20 minutes long. Metal implants (such as pacemakers or large metal objects/jewellery) should not be used during the scan. Usually a liver scan can be also considered

as a spleen scan because the spleen often is examined as well due to its proximity and close functional relationship to the liver. Moreover the liver scan can reveal information about various conditions, such as tumors, abscesses, hematomas, organ enlargement, or cysts, organ function and blood circulation. Due to its potent antioxidant, anti-inflammatory and lipid-lowering properties, and since it contains terpenes and polyphenols, associated with amelioration of hepatic steatosis, improvement of lipid metabolism, reduction of oxidative stress markers and hepatic inflammation, this supplement may have positive effects on NAFLD/NASH patients. We hope that the results of this study will allow us to a new approach in treatment, to choose the best individual treatment for each subject with this disease in future.

Confidentiality

It is important for you to know that any personal data will be available only to authorised personnel participating in this study.

In some circumstances Researcher may need to disclose identifying information (e.g., if the participant reveals the presence of certain communicable diseases, or imminent harm to self and others). If, as a result of this study, we obtain information that could significantly affect your health or well being (e.g. from the Multiliver scan), we will attempt to inform you of the existence of this information, provided you consent. You may then decide if you wish to know what we have learned. Results from genetic analysis for research purposes rather than clinical reasons will not be shared with you (or your family). However, if you consent, Researcher may share results of genetic analysis where early treatment of a disease that is genetically linked could improve disease prognosis. In this case, Researcher will need to provide ethical and scientific justification in Supervisory Board and Coordinator for passing on such information to you.

Thank you for your time. You can now decide whether you wish to take part or not. Please feel free to share any questions with our well-trained personnel.

CLINICAL RECORD OF PATIENT – QUESTIONNAIRES

VOLUNTEER ID:……..

VISIT DATE:____ / ____ / ____

DATE OF TAKING BLOOD SAMPLE:____ / ____ / ____

Fasting: YES ☐ NO☐

Are you on any medication? YES ☐ NO☐

Please indicate if you have taken a drug prior to giving blood (last 24h):

Drug	Aetiology
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	

<u>Personal details</u>
SURNAME: ……..……..……..
GIVEN NAME: ……..……..……
SEX: M☐ F☐

DATE OF BIRTH:____ / ____ / ____
PLACE OF ORIGIN: ……..……..……
ETHNICITY: ……..……..……
Father's origin: ……..……..……
Mother's origin: ……..……..……

VOLUNTEER'S CONTACT DETAILS
tel: ……..……..……
email:

ACCOMPANYING PERSON DETAILS

NAME .....
tel: ……..……..……

Files attached to the clinical record:

1. Clinical data
2. Anthropometric data
3. Lifestyle data
4. Food Frequency Questionnaire
5. Physical Activity Questionnaire
6. Type 2 Diabetes Risk Assessment Form

Comments:

1.
2.
3.

Adverse Events (AEs)/ Serious Advert Events (SAEs)	Date
1.	
2.	
3.	
4.	

CLINICAL DATA

1. **Blood group:…….**

- **Arterial pressure / Heart rate**
- Systolic blood pressure**:…….**
- Diastolic blood pressure**:……**
- Heart rate (beats per min):.....

2. **Menstrual cycle**
   - Are you still in menstrual cycle? ☐ **YES** ☐ **NO**
   - Duration of period ……..
   - Age of first period
   - Age of ending period
   - Did you ever have amenorrhea? ☐ **YES** ☐ **NO** (duration ..... age .....aetiology.....)
   - Have you ever used contraceptive pills? ☐ **YES** ☐ **NO**
   - Hysterectomy? ☐ **YES** ☐ **NO**
   - Hormones ☐ **YES** ☐ **NO**
   - Children.....

   Male .....
   Female .....

3. **Medication**
   Are you on any medication or following a medical intervention (e.g. chemotherapy, radiotherapy)
   ☐ **YES** ☐ **NO**
   If **YES**

Name of drug or medical intervention	**Aetiology**	**Frequency**

4. **Surgery**
   Have you ever had any surgery?
   ☐ **YES** ☐ **NO**
   If **YES**

Type of surgery	**Aetiology**	**Year of surgery**

5. **Has your doctor ever told you that you suffer/ed from:**

Non-alcoholic fatty liver disease	☐ YES	☐ NO

(NAFLD)		
Non-alcoholic steatohepatitis (NASH)	☐ YES	☐ NO
Coronary artery disease	☐ YES	☐ NO
Stable Angina	☐ YES	☐ NO
Unstable Angina	☐ YES	☐ NO
Myocardial Infarction	1.☐ YES AGE..... 2.☐.YES AGE:.....	☐ NO
Heart failure	☐ YES	☐ NO
Cardiac arrhythmia	☐ YES	☐ NO
Thrombosis	☐ YES	☐ NO
Stroke	☐ YES AGE:.....	☐ NO
Hyperlipidemia	☐ YES	☐ NO
Hypertension	☐ YES	☐ NO
Type I Diabetes	☐ YES AGE:.....	☐ NO
Type II Diabetes	☐ YES	☐ NO
Cervical syndrome	☐ YES	☐ NO
Liver failure	☐ YES	☐ NO
Renal failure	☐ YES	☐ NO
Asthma	☐ YES	☐ NO
Chronic Bronchitis	☐ YES	☐ NO
Chronic Anxiety, Depression	☐ YES	☐ NO
Psoriasis	☐ YES	☐ NO
Allergy	☐ YES	☐ NO
Osteoporosis	☐ YES	☐ NO
Lupus	☐ YES	☐ NO
Gastric ulcer	☐ YES	☐ NO
Gastroesophageal reflux	☐ YES	☐ NO
IBD	☐ YES	☐ NO
Hypothyroidism	☐ YES	☐ NO
Hyperthyroidism	☐ YES	☐ NO
Cancer **Type – specify:**	☐ YES	☐ NO
Other – please specify:		

6. Are your parents alive?

Father	☐YES ☐ NO
Mother	☐ YES ☐ NO

If NO

Age of death	Reason of death
Father	
Mother	

7. Family history:

Diseases	Siblings					Father	Mother
	1	2	3	4	5		
Non-alcoholic fatty liver disease (NAFLD)							
Non-alcoholic steatohepatitis (NASH)							
Coronary artery disease							
Stable Angina							
Unstable Angina							
Myocardial Infarction							
Heart failure							
Cardiac arrhythmia							
Thrombosis							
Stroke							
Hyperlipidemia							
Hypertension							
Type I Diabetes							
Type II Diabetes							
Gastric ulcer							
Gastroesophageal reflux							
IBD							
Hypothyroidism							
Hyperthyroidism							
Cancer **Type – specify:**							
Other – please specify:							

ANTHROPOMETRIC DATA

1. **Body Weight (kg)**

Current body weight:……..……..

Fasting: YES ☐ NO☐

Typical body weight: ……..……..

2. **History**

	Kg	Age	Period of time	Reason
Weight Loss				
Weight GAIN				

Birth weight:……..……..

3. **Height (m)……**

4. **Body circumferences (cm)**

Waist circumference (cm): ……..…

Hip circumference (cm):…….

5. **Body composition data**

Fat mass (Kg)……

Fat mass (%)……

Muscle mass (Kg)……..

Muscle mass (%)……..

Bone mass (Kg)……..…..

Bone mass (%BW)……..

LIFE STYLE QUESTIONNAIRE

1 MARITAL STATUS

- Married ☐
- Divorced ☐
- Separated ☐
- Widowed ☐
- Stable relation ☐
- No relation ☐

2 EDUCATION

- No education ☐
- Primary School ☐
- Secondary education ☐
- Higher Education ☐
- Other ☐

Total number of years in education:……

3 PROFESSION

- Full time – state employee ☐
- Full – private sector employee ☐
- Part time – state employee ☐
- Part time – private sector employee ☐
- Self employed ☐
- House work ☐
- Unemployed ☐
- Pensioner ☐

Please describe your profession……

SMOKING

4 Which of the following statements describes you best?

- I smoke daily ☐
- I smoke occasionally but not daily ☐
- I used to smoke daily but I no longer smoke ☐
- I used to smoke occasionally but I no longer smoke ☐
- I have never smoked ☐

5 At which age did you start smoking?……

6 If you used to smoke but now you do not smoke please answer the following questions:

How long ago did you quit smoking?……..……..

Duration of smoking (years).....……

7 If you smoke, do you smoke....

Cigarettes (with filter)	☐	
Cigarettes (without filter)	☐	
Cigars	☐	
Other	☐	**please specify: ……**

8 Number of cigarettes per day.....

24 Hour Recall form

Meal	Food (…../…../…….)	Food (…../…../…….)	Food (…../…../…….)
BREAKFAST			
SNACK			
LUNCH			
SNACK			
DINNER			
SNACK			

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

1. FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

2. Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure tim → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Finnish Diabetes Association

TYPE 2 DIABETES RISK ASSESSMENT FORM

Circle the right alternative and add up your points.

1. Age

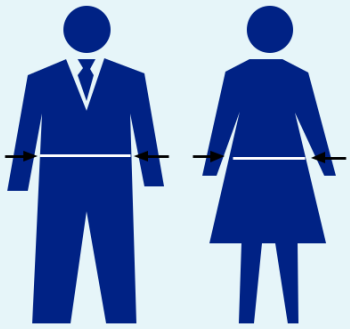
0 p. Under 45 years
2 p. 45–54 years
3 p. 55–64 years
4 p. Over 64 years

Body-mass index (See reverse of form)

1. p. Lower than 25 kg/m2
2. p. 25–30 kg/m2
5. p. Higher than 30 kg/m2

2. Waist circumference measured below the ribs (usually at the level of the navel)

	MEN	WOMEN
0 p.	Less than 94 cm	Less than 80 cm
3 p.	94–102 cm	80–88 cm
4 p.	More than 102 cm	More than 88 cm



4. Do you usually have daily at least 30 minutes of physical activity at work and/or during leisure time (including normal daily activity)?

0 p. Yes
2 p. No

5. How often do you eat vegetables, fruit or berries?

0 p. Every day
1 p. Not every day

6. Have you ever taken medication for high blood pressure on regular basis?

0 p. No
2 p. Yes

7. Have you ever been found to have high blood glucose (eg in a health examination, during an illness, during pregnancy)?

0 p. No
5 p. Yes

8. Have any of the members of your immediate family or other relatives been diagnosed with diabetes (type 1 or type 2)?

0 p. No
3 p. Yes: grandparent, aunt, uncle or first cousin (but no own parent, brother, sister or child)
5 p. Yes: parent, brother, sister or own child

Total Risk Score

☐ **The risk of developing type 2 diabetes within 10 years is**

Lower than 7	**Low:** estimated 1 in 100 will develop disease
7–11	**Slightly elevated:** estimated 1 in 25 will develop disease
12–14	**Moderate:** estimated 1 in 6 will develop disease
15–20	**High:** estimated 1 in 3 will develop disease
Higher than 20	**Very high:** estimated 1 in 2 will develop disease

Please turn over

Finnish Diabetes Association

WHAT CAN YOU DO

TO LOWER YOUR RISK OF DEVELOPING TYPE 2 DIABETES?

You can't do anything about your age or your genetic predisposition. On the other hand, the rest of the fac-tors predisposing to diabetes, such as overweightness, abdominal obesity, sedentary lifestyle, eating habits and smoking, are up to you. Your lifestyle choices can completely prevent type 2 diabetes or at least delay its onset until a much greater age.

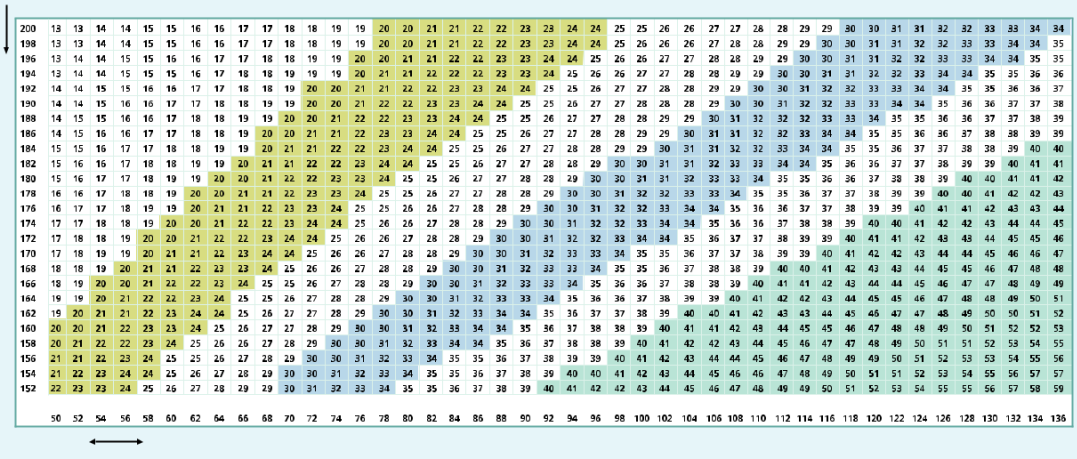
If there is diabetes in your family, you should be care-ful not to put on weight over the years. Growth of the waistline, in particular, increases the risk of diabetes, whereas regular moderate physical activity will lower the risk. You should also pay attention to your diet: take care to eat plenty of fibre-rich cereal products and vegetables every day. Omit excess hard fats from your diet and fa-vour soft vegetable fats.

Early stages of type 2 diabetes seldom cause any symptoms. If you scored 12–14 points in the Risk Test, you would be well advised to seriously consider your physical activity and eating habits and pay attention to your weight, to prevent yourself from developing diabetes. Please contact a public-health nurse or your own doctor for further guidance and tests.

If you scored 15 points or more in the Risk Test, you should have your blood glucose measured (both fast-ing value and value after a dose of glucose or a meal) to determine if you have diabetes without symptoms.

BODY-MASS INDEX

The body-mass index is used to assess whether a person is normal weight or not. The index is calculated by dividing body weight (kg) by the square of body height (m). For example, if your height is 165 cm and your weight 70 kg, your body-mass index will be 70/(1.65 x 1.65), or 25.7.



APPENDIX B

IBD-GR QUESTIONNAIRES

ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ
HAROKOPIO UNIVERSITY

INFORMED CONSENT

Within this document I consent to participate voluntarily in the trial «A CLINICAL TRIAL ON THE EFFECTIVENESS OF A NUTRITIONAL SUPPLEMENT IN INFLAMMATORY BOWEL DISEASE PATIENTS».
The study will be conducted at the facilities of Harokopio University of Athens. The aim of the trial is to investigate the effectiveness of intake of natural Mastiha in the form of tablet in patients with active or inactive Crohn's Disease or Ulcerative Colitis.
I assure that I have been orally informed about the experimental protocol in which I will participate if I sign this document. Specifically, I am aware that I will have to:

1. Undergo a clinical examination by the physician of the study.
2. Complete a questionnaire with the quidance of the study personell including:
    - demographics
    - health status (personal and family medical history)
    - disease activity
    - quality of life
    - smoking habits
    - dietary history
    - physical activity.
3. Undergo anthropometry measurements, namely height and weight.
4. Undergo a blood examination at baseline and at the end of the intervention.
5. Consume 3g of Mastiha daily in the form of tablets that will be supplied by the study personell adjunct to the medical therapy prescribed by the physician that treats me.
6. Participate in the trial for 3 months if I am in the active phase of IBD.
7. Participate in the trial for 6 months if I am in the inactive phase of IBD.
8. Collect and deliver a faecal sample at baseline and after the end of the intervention.

I am aware that all the above will be conducted under the supervision of a physician and a dietitian and there is no danger for my health status.

- **I agree** to follow steps 1-8.
- **I understand** that any question will be answered by the study personell.

- **I understand** that any personal information and test result will remain confidential and will not be published nominally.

I give my consent to voluntarily participate in this trial under the supervision of Assistant Professor, Andriana Kaliora.

Name_____Address_____

Tel.☎ Fax E-mail

Date ____/____/_____

Signature Witness's Signature

_____ _____

PATIENT INFORMATION LEAFLET

Title of the study

«A CLINICAL TRIAL ON THE EFFECTIVENESS OF A NUTRITIONAL SUPPLEMENT IN INFLAMMATORY BOWEL DISEASE (IBD) PATIENTS»

We would like to inform you about a research study that you are being asked to take part in. This study has been approved by the Ethics Committee of Harokopio University. Please read the following information carefully, and then we will answer any questions that you may have. Additionally, talk to others about the study if you wish. If you decide to participate, please sign this form. By signing you are authorizing us to include you in the study. Your participation in this study is voluntary. You can refuse to participate and you may withdraw your consent to participate at any time without any disadvantage for your future medical care. Enroll in the study only if you really want to. Please read carefully this patient information sheet and consent form. Do not sign this informed consent document if you have questions that have not been answered to your satisfaction.

If you consent to give blood/stools as part of this study, these specimens will become the property of our Institution. Specimens could lead to discoveries that may be of value for Public Health. You do not have any right to money or other compensation stemming from products that may be developed from the specimens.

Purpose of the clinical trial

The purpose of this trial is to investigate the efficacy of a natural nutritional supplement, namely Mastiha, on the main IBD entities, Ulcerative Colitis (UC) and Crohn's disease (CD).

IBD involves inflammation and oxidative stress. Currently, medical management aims at amelioration of inflammation and induction of remission. Additionally, there is an increasing research interest upon natural compounds of the diet. One of these includes Mastiha, a natural unprocessed nutritional supplement, which is secreted almost exclusively from the trunk of the shrub Pistacia Lentiscus. Several animal and human studies have shown that Mastiha exhibits anti-inflammatory, antioxidant, antibacterial and chemopreventive properties. Studies in patients with Crohn's disease and in experimental IBD demonstrated safety and promising results in reduction of inflammatory markers and Crohn's disease severity, attributed in regulation of immunologic dysfunction and restoration of intestinal integrity and permeability. Most recently, at a meeting of the Committee on herbal medicinal products to the European Medicines Agency (EMA/HMPC/46758/2015), Mastiha was recognized as a natural medicine and was classified to the category of traditional herbal medicines in two therapeutic indications: 1. mild dyspeptic disorders, diarrhea problems; 2. inflammation and wound healing.

Screening assessments

After signing the Consent Form, we will check if you are eligible for this study (Screening assessment will include medical history, physical examination, demographic data). If you are eligible, you will be allocated randomly to Group 1 (placebo) or to Group 2 (verum).

Design of the study

If you decide to take part in this clinical trial the following procedures will take place:

Visits: Being eligible and having given the consent of participation, you will need to visit Harokopio University two times for the whole study period.

Baseline assessments: Medical history, Dietary history, Disease Activity Indices, Body Weight (kg), height (cm), Body Mass Index (kg/m^2), Inflammatory Bowel Disease Questionnaire will be assessed. Blood collection (a total of 20cc) will be collected pre- and post- intervention. Serum and plasma will be isolated by centrifugation. Stools will be collected for gut microbiote alterations and measurement of inflammation. After that, randomization will take place and placebo or verum will be administered together for 3 or 6 months according to disease activity (3 months if you are in relapse or 6 months if you are in remission).

Follow-up assessment: At the end of the trial all the initial assessment will take place.

Risks and benefits

No adverse effects have been referred after Mastiha consumption as a nutritional supplement and as an herbal remedy. During blood collection, every measure to prevent distress and discomfort will be put in place. Blood samplings will be performed with butterfly needles by specialized staff following all the standard hygiene rules. Blood sampling is a routine procedure without special risks and it is a part of the clinical procedure for the clinical diagnosis or the follow up herein. The sample collection for the study does not carry additional risk.

Due to Mastiha potent antioxidant and anti-inflammatory properties, this may have positive effects on IBD patients. We hope that the results of this study will allow us to a new approach in treatment, to choose the best individual treatment for each subject with this disease in future.

Confidentiality

It is important for you to know that any personal data will be available only to authorised personnel participating in this study. In some circumstances Researcher may need to disclose identifying information (e.g., if the participant reveals the presence of certain communicable diseases, or imminent harm to self and others). If, as a result of this study, we obtain information that could significantly affect your health or well being, we will attempt to inform you of the existence of this information, provided you consent. You may then decide if you wish to know what we have learned. Results from genetic analysis for research purposes rather than clinical reasons will not be shared with you (or your family). However, if you consent, Researcher may share results of genetic analysis where early treatment of a disease that is genetically linked could improve disease prognosis. In this case, Researcher will need to provide ethical and scientific justification in Coordinator for passing on such information to you. Thank you for your time.

You can now decide whether you wish to take part or not. Please feel free to share any questions with our well-trained personnel.

MEDICAL HISTORY

DEMOGRAPHIC DATA

A.1. Patient code

A.2. Date of birth :

A.3. Marital status:

Married Single Divorced Widowed

Years of education:

1-9 years……..

10-12 years……..

>12 years……..

ANTHROPOMETRY

Height:

Weight:

Body Mass Index:

DISEASE

Ulcerative Colitis ….

Crohn's Disease ….

Allergic or atopic reactions	
History of smoking	*Current smoker YES …. NO …..* *If YES, years of smoking ……. No of Cigarettes/day ……* *Past smoker YES…. NO…..* *Years of smoking cessation ……*
Alcohol consumption	*Abstention (< 3 portions/year): ....* *Consumption: Light (<2 portions/day) .... Mild (2-4 portions/day) .... Heavy (>4 portions/day) ....* *Consumption in the past ....*

IBD HISTORY

Brief history of IBD	
Activity	
Location	
Duration	
Age of first symptoms	
Age of diagnosis	
Complications *Hepatic* *Skin* *Skeletal system* *Ocular problems* *Blood disorderd* *Embolic epeisoded* *Respiratory system* *Urinary system* *Enteral complications* *Perianal damage* *Massive bleeding* *Toxic megacolon* *Polyps* *Stenosis* *Fistula* *Abscess* *Cancer*	

MEDICAL THERAPY

TREATMENT	***BASELINE***	***FOLLOW-UP***
5-ASA		
Corticosteroids		
Azathioprine		
Metronidazole		
Ciprofloxacin		
EN formula		
T. P. N.		
Biological therapy		
Other		

SURGICAL THERAPY : YES …. NO ….

YEAR OF SURGERY:

KIND OF SURGERY:

DIETARY HISTORY

PATIENT CODE:

- AGE:
- CURRENT BODY WEIGHT (KG):
- USUAL BODY WEIGHT (KG):
- HEIGHT (CM):
- BMI :

- PHYSICAL ACTIVITY:
    - SEDENTARY LIFESTYLE
    - LIGHT PHYSICAL ACTIVITY
    - MODERATE PHYSICAL ACTIVITY
    - HEAVY PHYSICAL ACTIVITY

- FOLLOWING A SPECIFIC DIETARY PATTERN;

24-HOUR RECALL

	QUANTITY	TYPE OF FOOD AND DRINK
BREAKFAST (time……..)		
SNACK (time……..)		
LUNCH (time……..)		
SNACK (time……..)		
DINNER (time……..)		
SNACK (time……..)		

ASSESSMENT OF CD ACTIVITY

Harvey & Bradshaw Activity Index

1) **Number of loose stools per day**
2) **Abdominal pain** (0=absence, 1= mild, 2= moderate, 3=severe)
3) **General health condition** (0= very good, 1= good, 2= moderate, 3= bad, 4= very bad)
4) **Abdominal mass** (0= absence, 1= dubious, 2= definite, 3= definite + sensitive)
5) **Complications** (1 point for every complication): arthralgia, mouth ulcers, uveitis, erythema nodosum, puoderma nodosum, anal fistula, abscess)

General score	**< 5**	**remission**
	5-7	**mild disease**
	8-16	**moderate disease**
	>16	**severe disease**

ASSESSMENT OF UC ACTIVITY

PARTIAL MAYO ACTIVITY INDEX FOR UC

1. Stool Frequency (based on the past 3 days)

Normal number of stools =0
1-2 stools more than normal =1
3-4 stools more than normal =2
5 or more stools more than normal =3

2. Rectal Bleeding (based on the past 3 days)

No blood seen =0
Streaks of blood with stool less than half the time =1
Obvious blood with stool most of the time =2
Blood alone passed =3

3. Physician's Global Assessment (to be completed by Physician)

Normal (sub scores are mostly 0) =0
Mild disease (sub scores are mostly 1) =1
Moderate disease (sub scores are mostly 1 to 2) =2
Severe disease (sub scores are mostly 2 to 3) =3

The physician's Global Assessment acknowledges the Sub scores, the daily record of abdominal discomfort and functional assessment and other observations such as physical findings, and the patient's performance status.

Total Partial Mayo Index Score [sum of all above items]

Remission = 0-1 Mild Disease = 2-4 Moderate Disease = 5-6 Severe Disease =7-9

INFLAMMATORY BOWEL DISEASE QUESTIONNAIRE

This questionnaire is designed to find out how you have been feeling during the last two weeks. Please circle only one number for each question.

1. How frequent have your bowel movements been during the last 2 weeks?

a) Bowel movements as or more frequent than they have ever been
b) Extremely frequent
c) Very frequent
d) Moderate increase in frequency of bowel movements
e) Some increase in frequency of bowel movements
f) Slight increase in frequency of bowel movements
g) Normal, no increase in frequency of bowel movements

2. How often has the feeling of fatigue or being tired and worn out been a problem for you during the last 2 weeks?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

3. How often during the last 2 weeks have you felt frustrated, impatient, or restless?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

4. How often during the last 2 weeks have you been unable to attend school or work because of your bowel problem?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

5. How much time during the last 2 weeks have your bowel movements been loose?

a) All of the time

b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

6. How much energy have you had during the last 2 weeks?
a) No energy at all
b) Very little energy
c) A little energy
d) Some energy
e) A moderate amount of energy
f) A lot of energy
g) Full of energy

7. How often during the last 2 weeks did you feel worried about the possibility of needing surgery because of your bowel problem?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

8. How often during the last 2 weeks have you had to delay or cancel a social engagement because of your bowel problems?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

9. How often in the past 2 weeks have you been troubled by cramps in your abdomen?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

10. How often in the past 2 weeks have you felt generally unwell?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

11. How often during the last 2 weeks have you been troubled because of fear of not finding a bathroom?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

12. How much difficulty have you had, as a result of your bowel problems, doing leisure or sports activities you would liked to have done during the last 2 weeks?

a) A great deal of difficulty; activities made impossible
b) A lot of difficulty
c) A fair bit of difficulty
d) Some difficulty
e) A little difficulty
f) Hardly any difficulty
g) No difficulty; no limit sports or leisure activities

13. How often during the last 2 weeks have you been troubled by pain in the abdomen?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

14. How often during the past 2 weeks have you had problems getting a good night's sleep, or been troubled by waking up during the night?

a) All of the time
b) Most of the time
c) A good bit of the time

d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

15. How often during the past 2 weeks have you felt depressed or discouraged?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

16. How often during the past 2 weeks have you had to avoid attending events where there was no bathroom at hand?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

17. Overall, in the past 2 weeks, how much problem have you had with passing large amounts of gas?
a) A major problem
b) A big problem
c) A significant problem
d) Some trouble
e) A little trouble
f) Hardly any trouble
g) No trouble

18. Overall, in the last 2 weeks, how much of a problem have you had maintaining or getting to the weight you would like to be at?
a) A major problem
b) A big problem
c) A significant problem
d) Some trouble
e) A little trouble
f) Hardly any trouble
g) No trouble

19. Many patients with bowel problems often have worries and anxieties related to their illness. These include worries about getting cancer, worries about never feeling better, and worries about having a relapse. In general, how often during the last 2 weeks have you felt worried or anxious?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

20. How much of the time during the last 2 weeks have you been troubled by a feeling of abdominal bloating?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

21. How often during the last 2 weeks have you felt relaxed and free of tension?

a) None of the time
b) A little of the time
c) Some of the time
d) A good bit of the time
e) Most of the time
f) Almost all of the time
g) All of the time

22. How much time during the last 2 weeks have you had a problem with rectal bleeding with your bowel movements?

a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

23. How much time during the last 2 weeks have you felt embarrassed as the result of soiling, or because of an unpleasant odor caused by your bowel movement?

a) All of the time
b) Most of the time
c) A good bit of the time

d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

24. How much of the time during the past 2 weeks have you been troubled by a feeling of having to go to the bathroom even though your bowels are empty?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time
25. How much of the time during the last 2 weeks have you felt tearful of upset?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

26. How much of the time during the last 2 weeks have you been troubled by accidental soiling of your underpants?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

27. How much of the time in the 2 weeks have you felt angry as a result of your bowel problems?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

28. To what extent has your bowel problem limited sexual activity during the last 2 weeks?

a) No sex as a result of Crohn's disease
b) Major limitation as a result of Crohn's disease
c) Moderate limitation as a result of Crohn's disease
d) Some limitation as a result of Crohn's disease
e) A little limitation as a result of Crohn's disease
f) Hardly any limitation as a result of Crohn's disease
g) No limitation as a result of Crohn's disease

29. How much of the time during the last 2 weeks have you been troubled by feeling sick to your stomach?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

30. How much of the time during the past 2 weeks have you felt irritable?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

31. How often during the last 2 weeks have you felt a lack of understanding from others?
a) All of the time
b) Most of the time
c) A good bit of the time
d) Some of the time
e) A little of the time
f) Hardly any of the time
g) None of the time

32. How satisfied, happy, or pleased have you been with your personal life during the past 2 weeks?
a) Very dissatisfied, unhappy most of the time
b) Generally dissatisfied, unhappy
c) Somewhat dissatisfied, unhappy
d) Generally satisfied, pleased
e) Satisfied most of the time, happy
f) Very satisfied most of the time, happy
g) Extremely satisfied, could not have been more happy or pleased