HAROKOPIO UNIVERSITY OF ATHENS

SCHOOL OF HEALTH SCIENCE & EDUCATION

DEPARTMENT OF NUTRITION AND DIETETICS

A phase-II clinical trial on the effects of a nutritional supplement on Inflammatory Bowel Disease Patients

Doctoral Dissertation

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Athens, 2018

ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ ΚΑΙ ΑΓΩΓΗΣ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΔΙΑΙΤΟΛΟΓΙΑΣ-ΔΙΑΤΡΟΦΗΣ

Κλινική μελέτη φάσης ΙΙ διατροφικού συμπληρώματος σε ασθενείς με Ιδιοπαθή Φλεγμονώδη Νόσο του Εντέρου

Διδακτορική Διατριβή

Ευσταθία Παπαδά

Διαιτολόγος-Διατροφολόγος, MSc

Αθήνα, 2018

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Mrs Efstathia Papada was supported by the Leventis Scholarship Foundation (Academic Year 2017-2018).

I, Efstathia Papada

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Στους γονείς μου και

τον αδερφό μου

To my parents and

my brother

ACKNOWLEDGEMENTS

The present Doctoral Dissertation was conducted in the Laboratory of Chemistry-Biochemistry and Food Chemistry of the Department of Nutrition and Dietetics in Harokopio University of Athens under the supervision of Assistant Professor, Andriana Kaliora.

First of all, I would like to express my sincere gratitude to my supervisor, Assistant Professor, Andriana Kaliora, for giving me the opportunity to work on such an interesting project. I would like to thank her for the continuous support, encouragement and guidance with her valuable knowledge throughout every step of this Doctoral Dissertation. Successful study design, data analysis, interpretation of results, full papers publication and writing of the present Thesis would not be possible without her contribution.

I would also like to thank Professor Alastair Forbes for his valuable contribution during protocol design, interpretation of the results and editing of the scientific papers. I am also grateful for his advice, help and recommendations.

In addition, I would like to thank Professor Alexios-Leandros Skaltsounis, for his essential contribution in the completion of the bioavailability study. I would also like to thank him for his guidance and comments on the text.

Furthermore, I am grateful to Professor Nikolaos Kalogeropoulos for his guidance, advice and help in the GC-MS analysis in the Laboratory and his continuous support throughout the whole period of the completion of this Doctoral Dissertation.

I am also thankful to Professor Georgios Dedoussis, Professor Maria Kapsokefalou, and Associate Professor Georgios Bamias, for their guidance and comments on the text and their participation in the Examination Board of this Dissertation.

I would like to express my gratitude to Dr Ilias Smyrnioudis and Chios Mastic Growers Association for providing the supplements for the clinical trial and for the financial support of the experiments.

I am thankful to Associate Professor, Ljilja Torović, University of Novi Sad for her guidance during

the GC-MS experiments and Mrs Antigoni Tsiafitsa (HUA) for her support during the clinical trial and bioavailability study.

I would also like to thank the members of the Laboratory of Chemistry-Biochemistry and Food Chemistry (HUA) and the Department of Pharmacognosy and Natural Product Chemistry (NKUA).

I am grateful to Dr Aristea Gioxari, Postdoctoral Researcher, for her support, advice and guidance. Additionally, I am thankful to Mrs Charalampia Amerikanou for her help, support and true friendship throughout this period.

I owe my sincere gratitude to all the volunteers that took part in the bioavailability study and the clinical trial.

Last but not least, I am truly grateful to my parents, *Ioannis and Soultana*, my brother, *Evangelos*, and my best friend, *Georgia*. Without their support, love and patience throughout my academic journey, the completion of this Doctoral Dissertation would be impossible.

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Περίληψη

Η Ιδιοπαθής Φλεγμονώδης Νόσος του Εντέρου (ΙΦΝΕ) επηρεάζει 100/100.000 άτομα παγκοσμίως. Δεδομένου ότι οι φαρμακευτικές θεραπείες έχουν σοβαρές παρενέργειες, υπάρχει έντονο ερευνητικό ενδιαφέρον για φυσικά συμπληρώματα που θα μπορούσαν να χρησιμεύσουν ως πιθανές μη φαρμακολογικές θεραπείες. Η Μαστίχα Χίου (ΜΧ), ένα φυσικό προϊόν, με υψηλή περιεκτικότητα σε αντιοξειδωτικά και αντιφλεγμονώδη φυτοχημικά, κυρίως τερπένια, βρίσκεται υπό διερεύνηση.

Σκοπός της παρούσας Διδακτορικής Διατριβής ήταν: α) η διερεύνηση της απορρόφησης και βιοδιαθεσιμότητας των τερπενίων της ΜΧ σε υγιείς εθελοντές, β) η διερεύνηση της επίδρασης της χορήγησης ΜΧ στην κλινική πορεία της ΙΦΝΕ.

Για να αξιολογηθεί η βιοδιαθεσιμότητα των τερπενίων της ΜΧ, δεκαεπτά υγιείς άνδρες εθελοντές ακολούθησαν μια δίαιτα χαμηλή σε φυτοχημικά για 5 ημέρες. Μετά από ολονύκτια νηστεία, οι εθελοντές κατανάλωσαν σκόνη ΜΧ και δείγματα αίματος συλλέχθηκαν στη χρονική στιγμή 0 (πριν την κατάποση) και 0,5h, 1h, 2h, 4h, 6h, 24h (μετά την κατάποση). Η μέθοδος UHPLC-HRMS εφαρμόστηκε για την ανάλυση υψηλής απόδοσης του πλάσματος. Μετρήθηκε η αντίσταση του ορού στην οξείδωση, τα επίπεδα οξειδωμένης LDL (oxLDL) και ουρικού οξέος. Τα ελεύθερα αμινοξέα πλάσματος ταυτοποιήθηκαν και ποσοτικοποιήθηκαν με ανάλυση GC-MS.

Η ανάλυση UHPLC-HRMS/MS έδειξε ότι τα κύρια τερπένια ήταν βιοδιαθέσιμα από τη χρονική στιγμή 0.5h, φθάνοντας το μέγιστο μεταξύ των 2h και 4h. Η αντίσταση ορού στην οξείδωση αυξήθηκε σημαντικά τη χρονική στιγμή 4h, κορυφώθηκε κατά τη χρονική στιγμή 6h και παρέμεινε σημαντική μέχρι τις 24h. Τα επίπεδα oxLDL μειώθηκαν σημαντικά από τη χρονική στιγμή 1h μέχρι τη χρονική στιγμή 6h. Επιπρόσθετα, φάνηκε ότι οι συγκεντρώσεις των αμινοξέων μεταβάλλονται σε απόκριση της πρόσληψης τερπενίων.

Για να αξιολογηθεί η επίδραση της ΜΧ σε ενεργό ΙΦΝΕ, σχεδιάστηκε μια τυχαιοποιημένη, διπλά-τυφλή, ελεγχόμενη με εικονικό σκεύασμα κλινική δοκιμή φάσης-ΙΙ. Συνολικά, 60 ασθενείς με ΙΦΝΕ διαχωρίστηκαν τυχαία στην ομάδα που έλαβε ΜΧ (2,8g/ημέρα) ή εικονικό σκεύασμα για 3 μήνες συμπληρωματικά της σταθερής φαρμακευτικής θεραπείας. Αξιολογήθηκαν το ιατρικό και

διαιτητικό ιστορικό, το ερωτηματολόγιο για την ποιότητα ζωής (IBDQ), η ενεργότητα της νόσου, βιοχημικοί δείκτες, το οξειδωτικό στρες, τα ελεύθερα αμινοξέα πλάσματος, φλεγμονώδεις δείκτες κοπράνων κι αίματος. Ως πρωτεύον καταληκτικό σημείο ορίστηκε μία κλινικά σημαντική διαφορά μεταξύ των ομάδων στο IBDQ. Τα αποτελέσματα έδειξαν σημαντική βελτίωση στο IBDQ στην ομάδα ΜΧ σε σύγκριση με την έναρξη της παρέμβασης. Υπήρξε σημαντική μείωση στη λυσοζύμη κοπράνων σε ασθενείς που έλαβαν ΜΧ, με τη μέση μεταβολή να είναι σημαντική μεταξύ των δύο ομάδων, ενώ φάνηκε σημαντική αύξηση της λακτοφερρίνης και της καλπροτεκτίνης κοπράνων στην ομάδα placebo. Επιπρόσθετα, η oxLDL μειώθηκε σημαντικά στην ομάδα ΜΧ και οι λόγοι των oxLDL/HDL και oxLDL/LDL μειώθηκαν σημαντικά στην ίδια ομάδα με τις μέσες μεταβολές να είναι σημαντικά διαφορετικές μεταξύ των ομάδων.

Παρομοίως, για να αξιολογηθεί η επίδραση της ΜΧ σε ανενεργό ΙΦΝΕ, συνολικά 68 ασθενείς διαχωρίστηκαν τυχαία στην ομάδα ΜΧ (2,8g/ημέρα) ή στην ομάδα εικονικού σκευάσματος συμπληρωματικά της σταθερής φαρμακευτικής αγωγής. Το πρωτεύον καταληκτικό σημείο ήταν ο ρυθμός υποτροπής στους 6 μήνες. Παρόλο που η ΜΧ δεν αποδείχθηκε ανώτερη από το εικονικό σκεύασμα στη διατήρηση της ύφεσης, φάνηκε αναστολή στην αύξηση των ελεύθερων αμινοξέων πλάσματος στην ομάδα ΜΧ. Η IL-6 ορού, η καλπροτεκτίνη και η λακτοφερρίνη κοπράνων αυξήθηκαν μόνο στην ομάδα του εικονικού σκευάσματος.

Συμπερασματικά, αυτή η διατριβή έδειξε ότι τα κύρια τερπένια της ΜΧ είναι βιοδιαθέσιμα και είναι δυνητικά μεσολαβητές της αντιοξειδωτικής άμυνας in vivo. Η ρύθμιση της λυσοζύμης κοπράνων σε ενεργό ΙΦΝΕ ενδεχομένως να προτείνει έναν πρεβιοτικό μηχανισμό δράσης της ΜΧ. Τέλος, δεδομένου του ότι η ΜΧ αναστέλλει την αύξηση των ελεύθερων αμινοξέων πλάσματος που παρατηρούνται στην ανενεργό ΙΦΝΕ κι εφόσον οι μεταβολές των αμινοξέων θεωρούνται πρώιμος προγνωστικός δείκτης της ενεργότητας της νόσου, αυτό υποδεικνύει έναν πιθανό ρόλο της ΜΧ στη διατήρηση της ύφεσης.

Λέξεις κλειδιά: Ιδιοπαθής Φλεγμονώδης Νόσος του Εντέρου, Μαστίχα Χίου, τερπένια, κλινική δοκιμή, φυσικό συμπλήρωμα

Abstract

Inflammatory Bowel Disease (IBD) affects 100/100,000 persons worldwide. Since current medical treatments have serious side effects, there is a research interest in natural supplements that could serve as potential non-pharmacological treatments. As such, Mastiha, a natural product, with high content in antioxidant and anti-inflammatory phytochemicals, mainly terpenes, is under investigation.

The aim of the present Doctoral Dissertation was: a) to investigate the absorption and bioavailability of Mastiha's terpenes in healthy humans, b) To evaluate the effects of Mastiha on the clinical course of IBD adjunct to medical treatment.

To evaluate the bioavailability of Mastiha's terpenes, seventeen healthy male volunteers followed a low-phytochemical diet for 5 days. After overnight fasting, volunteers consumed Mastiha powder and blood samples were collected on time-points 0h (before ingestion) and 0.5h, 1h, 2h, 4h, 6h, 24h (post- ingestion). UHPLC-HRMS was applied for high throughput analysis of plasma. Serum resistance to oxidation, oxidized LDL levels (oxLDL) and uric acid levels were measured. Plasma free amino acids (AAs) were identified and quantified with GC-MS analysis.

UHPLC-HRMS/MS analysis showed that major terpenes were bioavailable since 0.5h, reaching a peak between 2h and 4h. Serum resistance to oxidation reached statistical significance at 4h, peaked at 6h and remained significant until 24h. oxLDL levels were reduced significantly from time point-1h until time point-6h. Additionally, It was shown that plasma free AAs are modulated in response to terpenes intake.

To evaluate the effects of Mastiha administration on active IBD, a Phase-II randomised, double-blind, placebo-controlled clinical trial was designed. A total of 60 IBD patients were randomly allocated to Mastiha (2.8g/day) or placebo groups for 3 months adjunct to stable medical treatment. Medical and dietary history, Inflammatory Bowel Disease Questionnaire (IBDQ), disease activity, biochemical indices, oxidative stress, plasma free AAs, faecal and blood inflammatory markers were assessed. A clinically important difference between groups in IBDQ was the primary outcome. The results showed a significant improvement in IBDQ in verum compared with baseline.

There was a significant decrease in faecal lysozyme in Mastiha patients with the mean change being significant and significant increases of faecal lactoferrin and calprotectin in placebo group. Additionally, oxLDL was significantly decreased in Mastiha arm and the ratios of oxLDL/HDL and oxLDL/LDL were significantly decreased in the same group with the mean changes being significantly different.

Similarly, to evaluate the effects of Mastiha administration on inactive IBD, a total of 68 patients were randomly allocated to Mastiha (2.8g/day) or placebo adjunct to stable medication. Primary endpoint was clinical relapse rate at 6 months. Although Mastiha was not proven superior to placebo in remission rate, attenuation in increase of free AAs levels in verum group was reported. Serum IL-6, faecal calprotectin and faecal lactoferrin increased only in placebo group.

In conclusion, this Thesis showed that Mastiha's major terpenes are bioavailable and are potential mediators of antioxidant defence in vivo. Regulation of faecal lysozyme in active IBD was shown pointing towards a prebiotic effect. Last, since Mastiha inhibited an increase in plasma free AAs seen in quiescent IBD and change of AAs is considered an early prognostic marker of disease activity, this indicates a potential role of Mastiha in remission maintenance.

Keywords: Inflammatory Bowel Disease, Mastiha, terpenes, clinical trial, natural supplement

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ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AGPs	Arabino-Galactanes Proteins
ANOVA	Analysis of Variance
ASCA	Anti-Saccharomyces cerevisiae antibodies
ATG16L1	Autophagy Related 16 Like 1
ATP	Adenosine Triphosphate
AZA	Azathioprine
BIA	Bioelectrical Impedance
BMI	Body Mass Index
CAC	Colitis Associated Cancer
CBC	Complete Blood Count
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CRF	Corticotropin-releasing factor
CRP	C-reactive protein
CVD	Cardiovascular Disease
DC	Dendritic Cells
DSS	Dextran Sodium Sulfate
DXA	Dual-energy X-ray Absorptiometry
EDTA	Ethylenediaminetetraacetic
FL	Faecal Lactoferrin
FLys	Faecal Lysozyme
FODMAP	Fermentable Oligo- Di- Monosaccharides and Polyols
γ-GT	gamma-Glutamylo-Tranferase
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally Recognised As Safe
GWAS	Genome-Wide Association Study
HBI	Harvey-Bradshaw Index
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HLA	Human Leukocyte Antigens
HR	High Resolution
IBD	Inflammatory Bowel Disease
IBDQ	Inflammatory Bowel Disease Questionnaire
IBS	Irritable Bowel Syndrome
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon-γ

	Acid
EMA	European Medicines Agency
EN	Enteral Nutrition
ESR	Erythrocyte sedimentation rate
FC	Faecal Calprotectin
FD	Faecal Defensin
LC-MS	Liquid Chromatography-Mass Spectrometry
LCT	Long Chain Triglycerides
LDH	Lactate Dehydrogenase
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule
MCT	Medium Chain Triglycerides
MCV	Mean Corpuscular Volume
MDR1	Multidrug Resistance Protein 1
MUFA	Monounsaturated Fatty Acids
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer cells
NKT	Natural Killer T cells
NMR	Nuclear Magnetic Resonance
NOD2	Nucleotide-binding Oligomerization Domain-containing protein 2
IgG	Immunoglobulin G
IL-	Interleukin-
IRGM	Immunity Related GTPase M
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
PRO-2	Patient Reported Outcomes-2
PRRs	Pattern Recognition Receptors
PUFA	Polyunsaturated Fatty Acids
RBB+C	Repeated Bead Beating plus Column
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SCCAI	Simple Clinical Colitis Activity Index
SCD	Specific-Carbohydrate Diet
SDS	Sodium Dodecyl Sulfate
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic-Pyruvic Transaminase
SNPs	Single Nucleotide Polymorphisms
TGF-β	Tranforming Growth Factor-beta

NSAIDs	Non-Steroids Anti-Inflammatory Drugs	**TL1A**	TNF-like protein 1A
ONS	Oral Nutritional Supplements	**TNBS**	Trinitrobenzene Sulfonic Acid
pANCA	Perinuclear Antineutrophil Cytoplasmic Antibodies	**TNF-α**	Tumour Necrosis Factor-alpha
oxLDL	oxidized Low Density Lipoprotein	**UC**	Ulcerative Colitis
PIL	Patient Information Leaflet	**UCDAI**	Ulcerative Colitis Activity Index
PBMC	Peripheral Blood Mononuclear Cells	**VCAM-**	Vascular Cell Adhesion Molecule-1
PBS	Phosphate-buffer Saline	**VZV**	Varicella Zoster Virus
PCR	Polymerase Chain Reaction	**WBC**	White Blood Cells
PDAI	Perianal Disease Activity Index	**5-ASA**	5-Aminosalicylates
PN	Parenteral Nutrition	**6MP**	6-Mercaptopurine

Curriculum Vitae

Efstathia Papada is a Dietitian-Nutritionist with a Bachelor degree (Distinction) from the Department of Dietetics and Nutrition, Harokopio University of Athens and a Master's degree (Distinction) in Clinical and Public Health Nutrition from the School of Medicine of University College London (UK). During her academic studies, her research interest focused on the nutritional management and the impact of natural compounds of diet on Inflammatory Bowel Disease. Her Doctoral Dissertation entitled "A phase-II clinical trial on the effects of a nutritional supplement on Inflammatory Bowel Disease Patients" was conducted in the Department of Nutrition and Dietetics in Harokopio University of Athens. She has also been further specialized in the dietary management of patients with gastroenterological diseases in Norfolk and Norwich University Hospital (NHS Foundation Trust, UK) with an Erasmus Traineeship scholarship. She has offered educational work in undergraduate dietetics students. She has also worked as a Lecturer in Private Vocational Training Institute, teaching Principles of Nutrition, Nutrition in Health, Therapeutic Nutrition, Nutrition and Exercise, Obesity and Metabolism. She has also participated in European and National Congresses as an invited speaker.

The present Thesis is supported by the following articles submitted in international peer-reviewed journals.

PAPER 1

Papada E, Gioxari A, Brieudes V, Amerikanou C, Halabalaki M, Skaltsounis AL, Smyrnioudis I, Kaliora AC. **Bioavailability of terpenes and postprandial effect on human antioxidant potential. An open-label study in healthy subjects**. Mol Nutr Food Res. 2018; 62 (3) doi: 10.1002/mnfr.201700751 (IF 5.2)

PAPER 2

Papada E, Torović L, Amerikanou C, Kalogeropoulos N, Smyrnioudis I, Kaliora AC. **Modulation of free amino acid profile in healthy humans administered with mastiha terpenes. An open-label trial.** Nutrients. 2018; 10(6). pii: E715. doi: 10.3390/nu10060715 (IF 4.2)

PAPER 3

Papada E, Gioxari A, Amerikanou C, Forbes A, Tzavara C, Smyrnioudis I, Kaliora AC. **Regulation of faecal biomarkers in Inflammatory Bowel Disease patients treated with oral Mastiha supplement: A randomized controlled clinical trial.** Phytotherapy Research (in press) (IF 3.3)

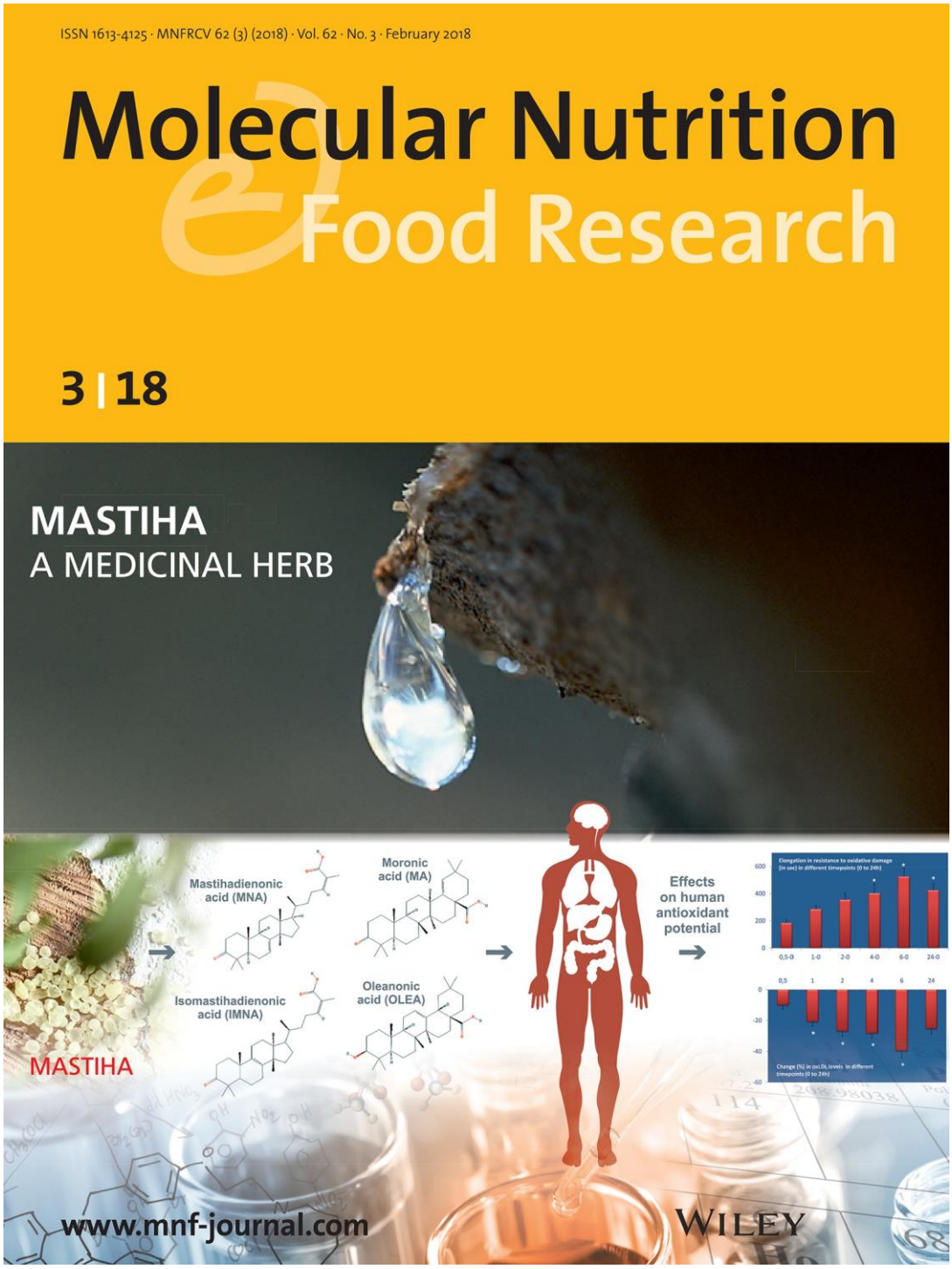
PAPER 4

Papada E, Amerikanou C, Torovic L, Tzavara C, Kalogeropoulos N, Forbes A, Kaliora AC. **Plasma free amino acid profile in quiescent Inflammatory Bowel Disease patients orally supplemented with Mastiha; a randomized clinical trial.** Phytomedicine (in press, https://doi.org/10.1016/j.phymed.2018.08.008) (IF 3.6)

PAPER 5

Papada E, Forbes A, Amerikanou A, Torović L, Kalogeropoulos N, Tzavara C, Triantafillidis JK, Kaliora AC. **Antioxidative efficacy of Mastiha supplement and effect on plasma amino acid profile in inflammatory bowel disease; a randomized, double blind, placebo-controlled trial.** Nutrients (revised version submitted) (IF 4.2)

Paper 1 was selected as the front cover of Molecular Nutrition and Food Research (Volume 62, Issue 3).



The present Thesis is supported by the following announcements in conferences.

1. Papada E, Amerikanou C, Torovic L, Kalogeropoulos N, Tzavara C, Smyrnioudis I, Forbes A, Kaliora AC. **Effects of Mastiha (Pistacia lentiscus) supplement on oxidative stress and on plasma free amino acid in Inflammatory Bowel Disease; a randomized, double blind, placebo-controlled trial.** 30th International Symposium on the Chemistry of Natural Products and the 10th International Congress on Biodiversity, 25-29.11.2018, Athens, Greece

2. Papada E, Torović L, Amerikanou C, Kalogeropoulos N, Kaliora AC. **A kinetic study on plasma free amino acids after supplementation with Mastiha; correlation with bioavailable terpenes and antioxidant status.** Bioavailability 2018. 10-13.09.2018, Norwich, United Kingdom

3. Papada E, Amerikanou C, Torovic L, Kalogeropoulos N, Tzavara C, Forbes A, Kaliora AC. **On the identification of plasma free amino acid profile in patients with quiescent IBD. A placebo-controlled randomised clinical trial with Mastiha supplement.** 40th ESPEN Congress. 01-04.09.2018, Madrid, Spain. *Mrs Papada was awarded with ESPEN 2018 Travel Fellowship for Young Investigators under 35 years old.*

4. Papada E, Torovic L, Amerikanou C, Gioxari A, Kalogeropoulos NK, Kaliora AC. **Metabolomic analysis of amino acids in Inflammatory Bowel Disease: The role in disease management.** 14th Hellenic Congress of Dietetics and Nutrition. 24-26.11.2017, Athens, Greece

5. Brieudes V, Kallergis E, Papada E, Gioxari A, Kaliora A, Mitakou S, Skaltsounis AL, Halabalaki M. **Bioavailability study and impact of Chios mastic supplementation on oxidative stress in healthy humans.** 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), 3-7.09.2017, Basel, Switzerland

6. Papada E, Gioxari A, Brieudes V, Lemonakis N, Stuppner H, Halabalaki M, Mikros E, Skaltsounis LA, Kaliora AC. **The effect of acute Mastiha supplementation on the metabolites profile and oxidative stress in healthy humans**. 8th Biologie Prospective Santorini Conference, Greece 3-5.10.2016

PART A

LITERATURE REVIEW

CHAPTER 1

INFLAMMATORY BOWEL DISEASE

1.1 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a chronic progressive immunologically mediated disease that includes Crohn's Disease (CD) and Ulcerative Colitis (UC). IBD onset happens more frequently in the second and third decade of life but there is also a peak onset between 55 and 65 years of life. IBD patients experience periods of relapses and remissions and they are on a greater risk for colorectal cancer (Ananthakrishnan, 2015).

CD and UC share some similar characteristics; however they are two distinct diseases. CD affects any part of the digestive tract, where normal segments interrupt inflamed parts forming "skip areas". Inflammation expands through the gastrointestinal wall layers resulting in formation of fistulas and abscesses. The most affected areas are the ileum and the colon. Formation of ulcerations and granulomas is quite common. On the other hand, UC is limited to the colon and the rectum. Inflammation is continuous and affects mainly the mucosa, while ulceration, oedema and haemorrhage may be present.

The prolonged nature of IBD affects health-related quality of life, economic productivity and social function of the patients. Furthermore, increased demand for physician needs, for medical therapy, for surgery due to complications and for hospitalizations raises the costs for public health. It is estimated that the direct and indirect costs ranged between $14.6 and $31.6 billion in 2014. It seems also that inappropriate treatments, lack of adherence to therapeutic regimens, as well as suboptimal treatment increase the cost burden. Therefore, this overwhelming economic burden of IBD makes early diagnosis and effective treatment at onset essential (Mehta, 2016).

1.2 Epidemiology

The incidence of IBD is increased amongst the populations of United Kingdom, Scandinavia, North America and Northern Europe. This could be partially attributed to the decreased exposure to UV. However, it seems that there is an increasing trend in other regions as well (e.g. Latin America, Asia, Africa, central and southern Europe). The prevalence of IBD is 100/100,000 persons in the general population. Jewish populations are predisposed to a three-fold greater risk for the disease, especially the Ashkenazi, the American and European Jewish populations. Men and women are equally affected but this depends on race and ethnicity (Ananthakrishnan, 2015).

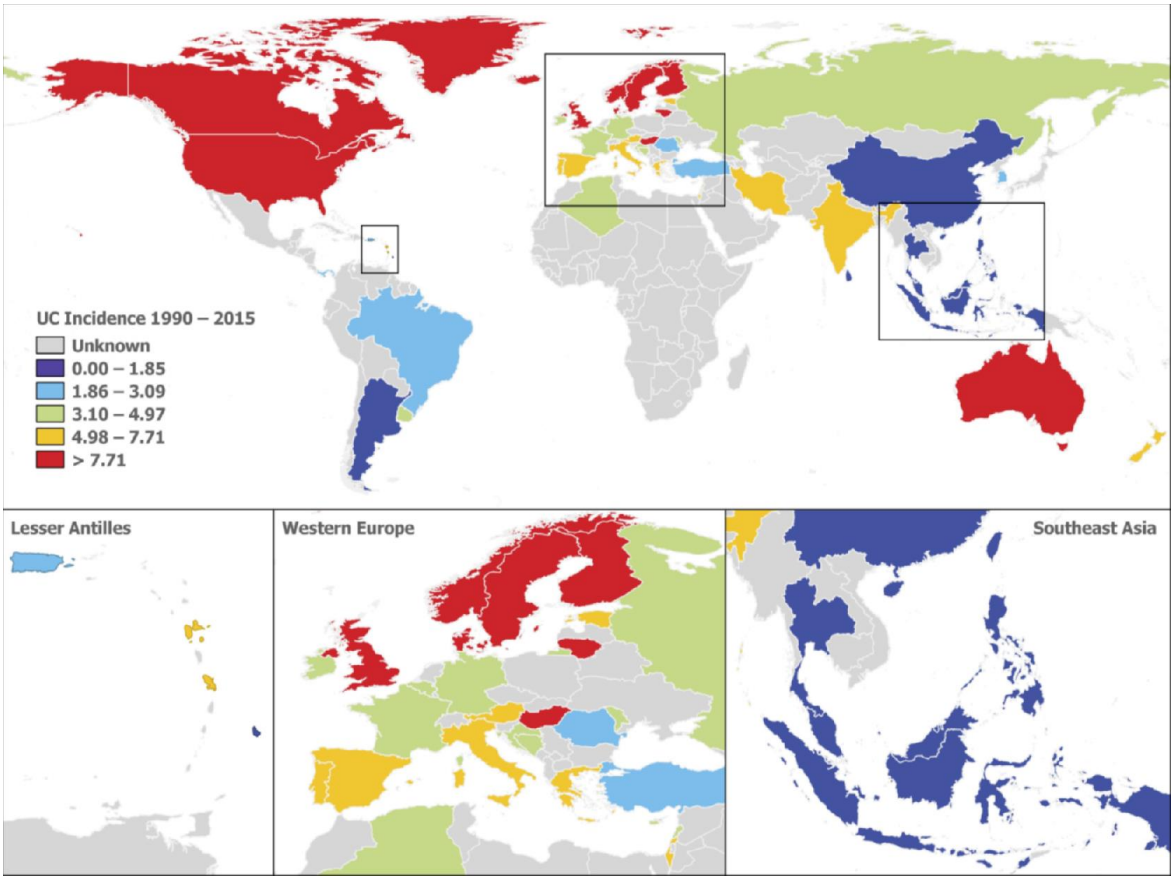


Figure 1. Worldwide map of incidence of UC stratified by quintile levels

(Adapted from Ng et al., 2018)

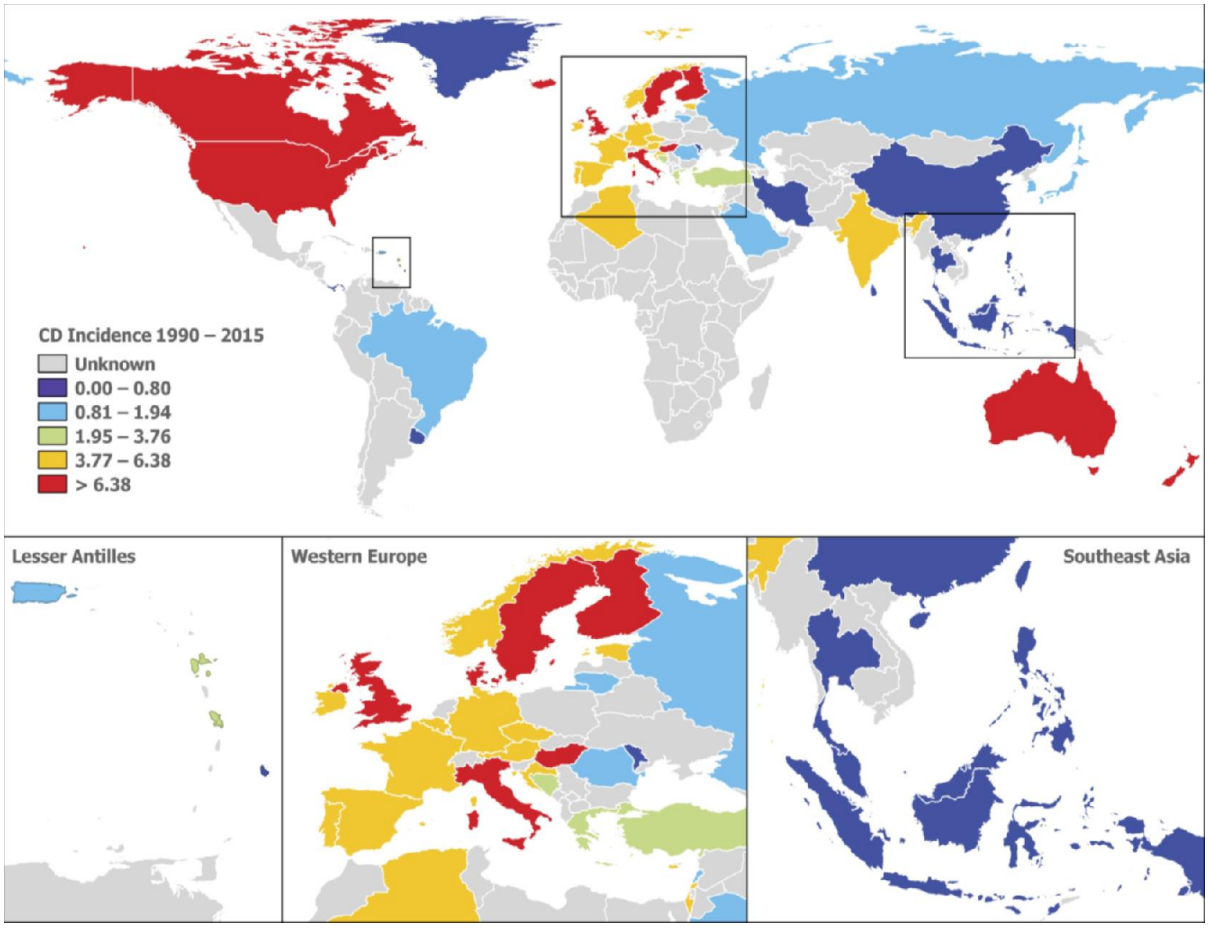


Figure 2. Worldwide map of incidence of CD stratified by quintile levels

(Adapted from Ng et al., 2018)

1.3. Pathogenesis

IBD pathogenesis has not been fully elucidated yet, although it seems to be a result of an abnormal response to normal antigens of the gastrointestinal tract. More specifically, a combination of genetic, immunological and environmental factors initially contributes to destruction of the intestinal epithelial barrier and increase of intestinal permeability leading to influx of immune cells in the intestinal lumen. This disregulation of the immunomodulation of intestinal mucosa leads into abnormal function of activated T cells, mononuclear cells and macrophages that causes lesions in epithelial cells and chronic inflammation (Novak & Molen, 2015).

1.4. Risk factors

As far as IBD aetiology is concerned, it seems that this disease develops at the intersection of genetic predisposition, immunological dysfunction, environmental factors and dysbiosis of gut microbiota (Ananthakrishnan, 2015).

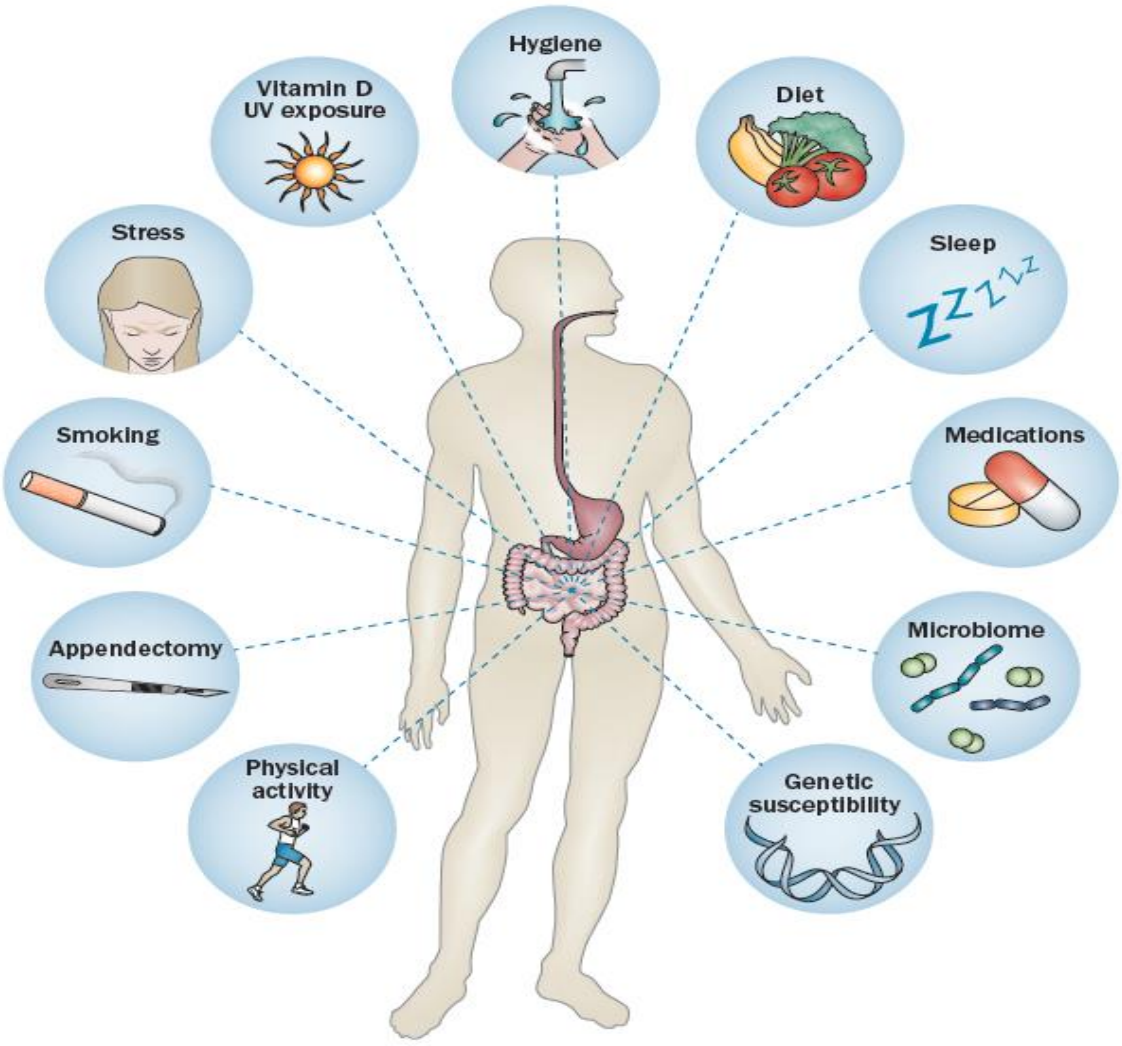


Figure 3. The interaction between genetics, environment, immunology and microbiome in the development of IBD (Ananthakrishnan, 2015).

1.4.1 Genetic factors

Approximately, 15% of patients with CD have a family member with IBD, and twin studies for CD have shown 50% concordance in monozygotic twins compared to <10% in dizygotics.

Recent and large genetic association studies, involving genome-wide association data for over 75,000 patients and controls, identified 163 susceptibility loci for IBD. However, these loci account only for 13% of CD and 7% for UC disease variance. More recently, a trans-ethnic analysis, with over 20,000 subjects, identified 38 new IBD loci as well. In most cases there is a polygenic contribution toward genetic susceptibility, but there is also a range of rare genetic disorders that can lead to early-onset IBD (before 5 years) or very early onset IBD (before 2 years). These kind of genetic variants show a wide effect on gene function and they are so rare in allele frequency that the genetic signals are not detected in genome-wide association studies. Due to progress in sequencing techniques, approximately 50 genetic disorders have been identified and associated with IBD pathology (Loddo & Romano, 2015).

For example, genetic susceptibility to CD is associated with a frameshift mutation of NOD2 gene, which is more common in CD patients and triggers inflammation and reduced response to bacterial cell products (Ogura et al., 2001). The correlation of CD with gene mutations NOD2 / CARD15 (in chromosomal region 16q12) and IL23R gene (in chromosomal region 1p31) has been confirmed, while there seems to be a strong correlation with genes in chromosomal regions 2q37, 3p21, 5p13, 5q33 , 10q21, 10q24 and 18p11 (Welcome Trust Case Control Consortium, 2007). The genes on chromosome 6 encoding the human leukocyte antigens (HLA) are also associated with IBD, but rather with the disease phenotype and extraintestinal manifestations (Cho & Brant, 2011). Additionally, genes involved in the autophagy mechanism like ATG16L1 (chromosome 2) and IRGM (chromosome 5) are also related with CD in GWAS (Parkes et al, 2007; Rioux et al, 2007). Furthermore, a series of missense mutations of the gene in the chromosomal region of 7q, which is responsible for the expression of a carrier membrane protein, the MDR1 ("multidrug resistance"), is associated with IBD (Brant et al, 2003).

However, the increasing incidence of IBD during the last decades cannot only be explained by

genetic disposition, since the incidence of genetic diseases is relatively stable. Thus, environmental factors may also play a crucial role.

1.4.2. Environmental factors

Diet*:* *Nutrition* seems to have a causal role in IBD development. Dietary patterns and specific components have been considered as either protective or aggravating. Western diets may increase the risk for IBD, while Mediterranean diet may exhibit a protective effect (Gentschew & Ferguson, 2012; Hou et al., 2011). Numerous studies have attempted to relate specific dietary components with the risk for IBD.

- ***Monosaccharides***: Although many studies have indicated a possible association of consumption of cola-type drinks, chocolate, sweets and artificial sweeteners with risk for IBD, a large prospective study in 2014 demonstrated no association between total intake of carbohydrates, sugar or starch and incidence of IBD (Chan et al., 2014).

- ***Proteins and fats***: Increased animal protein and fat consumption seem to increase the risk for IBD. More specifically, trans fatty acids and linoleic acid, a polyunsaturated fatty acid (PUFA), are associated with increased incidence of UC. Linoleic acid is a precursor of arachidonic acid, whose metabolites exhibit proinflammatory properties (Owczarek et al., 2016). A high ratio of n-6:n-3 polyunsaturated fatty acids (PUFA) is related with increased risk for CD (Forbes et al., 2011). On the contrary, monounsaturated fatty acids (MUFA) have a protective role.

- ***Fiber***: Dietary fiber holds a protective position against IBD. Ananthakrishnan *et al.* (2013) showed that a daily consumption of 24.3g (rather from fruit sources) decreases the risk for CD by 40%. However, this association is not confirmed in UC.

- ***Micronutrients***: *Vitamin D* deficiency is common in IBD patients (about 60%) and it is related with increased disease activity in CD patients (Jørgensen et al., 2013). Vitamin D is a pleiotropic hormone and its deficiency may lead to decreased colonic bacterial clearance, reduced expression of tight junctions in the intestinal epithelium, and elevated Th1-driven inflammation

at the gut level. However, it is not clear whether it is a result of malabsorption, or whether it contributes to disease onset and progression (Del Pinto et al., 2015). Other micronutrients that possibly exhibit a protective role against IBD include vitamin C, zinc and magnesium (Owczarek et al., 2016). Magnesium deficiency is frequent in IBD, being present in 13-88% of IBD patients, and it seems to contribute to IBD-related fatigue. Intracellular zinc is important for autophagy, bacterial clearance, as well as reduction of intestinal permeability. It is estimated that 20.5% of CD patients are zinc deficient and disease activity may affect zinc levels (Ananthakrishnan, 2015; Kruis & Nguyen, 2016). Vitamin C levels are lower in IBD patients compared with controls indicationg an altered antioxidant status in IBD (Hengstermann et al., 2008).

- ***Alcohol:*** A number of studies have shown that alcohol consumption may exhibit a protective effect on UC development. However, this is association is invalid when alcohol is combined with cigarette smoking. Regarding CD development, there are no significant differences between individuals consuming no alcohol and those consuming alcohol at least four times a week (Owczarek et al., 2016).

Breastfeeding: An interesting meta-analysis has shown that breastfeeding may be protective in IBD morbidity, with further studies being necessary in order to confirm this hypothesis. However, it is important for families with IBD history to be encouraged with breastfeeding (Klement et al., 2004).

Microbiome: The human gastrointestinal tract is colonized by a huge number of microorganisms, namely the microbiota. The microbiota includes mainly bacteria, but viruses and protozoans exist in a beneficial relationship with the host as well. Gut microbiota consists of mainly the Gram-negative Bacteroidetes (17%–60%) and Gram-positive Firmicutes (35%–80%). Other less widespread phyla include *Actinobacteria*, *Proteobacteria* and *Euryarchaeota.* This composition is dynamic but it is affected by host genetics, immunity, microbial species acquired at birth, antibiotic usage and diet.

Normally, the gut microbiota lives symbiotically with the host and contributes to epithelial injury protection, fat metabolism regulation, vitamin and essential amino acids, bile acid biotransformation, intestinal motility and angiogenesis, as well as immune system development.

Last but not least, gut microbiota resists colonisation of pathogenic bacteria through production of antimicrobial molecules. In this way, the gut epithelial barrier is protected from pathogens, bacterial overgrowth is prevented and susceptibility to enteric infections decreases (Ahmed et al., 2016).

Although a healthy host maintains immune homeostasis and has a tolerance towards microbiota, this homeostasis is dysregulated in IBD. There is an increase in pathogens and a decrease in beneficial microorganisms leading to *dysbiosis*. Generally, the microbial diversity and stability decreases and 25% fewer genes can be identified in faecal samples of IBD patients compared with healthy individuals. Additionally, IBD patients have more proinflammatory and less anti-inflammatory bacteria (Qin et al., 2010). Not only microbiome but also enteric virome is abnormal in CD and UC patients, with *Caudovirales* bacteriophages expanding significantly in the IBD affected intestine. It seems that changes in the virome may contribute to intestinal inflammation and dysbiosis (Norman et al., 2015).

Although it is possible that dysbiosis plays a central role in IBD pathogenesis, further research will clarify whether there is a causative relationship between IBD onset and dysbiosis or the later results from alterations in the gastrointestinal tract.

Infections: Infectious agents (e.g. *Listeria monocytogenes, Escherichia coli, Mycobacterium paratuberculosis, Salmonella, Campylobacter*) are involved in IBD pathogenesis, however their role has not been clarified yet (Danese et al., 2004). Infections may trigger the setting of genetic susceptibility, thus the molecular basis of this interaction should be further studied.

Drugs: Non-steroids anti-inflammatory drugs (NSAIDs), antibiotics, oral contraceptives and postmenopausal hormone therapy are positively associated with increased IBD risk (Ananthakrishnan, 2015).

Hygiene: Number of siblings, larger family size, drinking unpasteurized milk, living on a farm and exposures to pets (particularly early on in childhood) have been inversely associated with risk of IBD (Ananthakrishnan, 2015). According to the hygiene hypothesis, improvements in hygiene, such as clean water and non-contaminated food, decreased the exposure in common infectious agents,

leading in IBD later in life due to abnormal immune responses (Koloski et al., 2008). However, this association has not been confirmed (Castiglione et al., 2012).

Smoking: Smoking has a different impact on CD and UC, as it seems to be aggravating for CD pathogenesis, whereas it shows a protective role against UC. The risk for CD is twofold in smokers compared with nonsmokers (Mahid et al., 2006). On the contrary, smoking cessation increases the risk for UC within 2-5 years (Higuchi et al., 2012). Passive smoking has similar effects. Several hypotheses have been proposed to explain those effects. Alterations in smooth muscle tone, increase of oxidative stress, influence on endothelial function through nitric oxide production and gut mucous barrier integrity are some of the proposed mechanisms (Ananthakrishnan, 2015).

Appendectomy: Appendectomy has a similar divergent effect on CD and UC. More specifically, patients who underwent appendectomy for perforated on non-perforated appendicitis had a significantly lower risk for UC development compared with those underwent appendectomy with nonspecific abdominal pain (Andersson et al., 2001). In contrast, the risk for CD is greater for up to 20 years after appendectomy (Andersson et al., 2003).

Psychological profile: Chronic stress, depression, sleep disorders and unpleasant events may be associated with increased incidence of IBD, but most likely as a triggering mechanism leading to the first symptoms in predisposed subjects, worsening the symptoms or inducing relapses. Chronic stress is more associated with UC, whereas depression is related with CD (Ananthakrishnan, 2015; Maunder & Levenstein, 2008). Additionally, personality traits may contribute to IBD pathogenesis with neuroticism and perfectionism being quite common characteristics among IBD patients (Sajadinejad et al., 2012). Figure 4 presents the direct and indirect effects of stress possible mechanisms on IBD course.

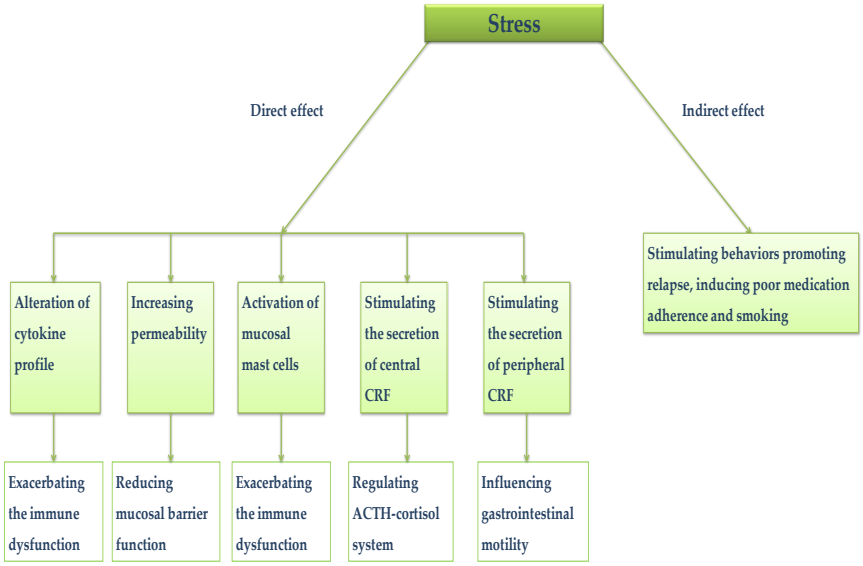


Figure 4. Stress influence on IBD course (Adapted from Sajadinejad et al., 2012).

1.4.3. Immunological factors

Normally the gut is in a state of controlled inflammation and there is a balance between pro-inflammatory and anti-inflammatory mechanisms that control the immune response. Innate immunity and adaptive immunity cooperate and complement each other in order to defend the host against pathogens. The "bridge" between adaptive and innate immune responses has not been fully elucidated yet. However, abnormal innate immune responses induce adaptive immunity imbalance, leading to inflammatory cytokines production that increase innate immune damages, abate intestinal barrier functions and aggravate inflammation, forming a vicious cycle (Huang & Chen, 2016). (See Chapter 2)

1.5. Clinical features and diagnosis of IBD

1.5.1. Clinical features

UC and CD share some similar clinical features, even if they are not specific for the disease and this is the reason for the delayed diagnosis in some cases. Additionally, there is heterogeneity of the symptoms between different patients and different periods of relapses or remission. Usually, UC is

more easily diagnosed than CD, since it gives more specific symptoms.

Intestinal manifestations of IBD

Bloody stools and diarrhoea with abdominal cramping are the most frequent features of UC. Position of the abdominal pain depends on the colonic involvement. On the contrary, intestinal CD symptoms are often delayed and depend on the severity and location of the disease. Postprandial abdominal pain is present with ileocolonic involvement. Nausea, vomiting and dysphagia accompany gastroduodenal CD. Colonic CD often resembles UC with bloody diarrhoeas. Fistulas are more common in CD. Last but not least, risk for Colitis Associated Cancer (CAC) is increased in both UC and CD when there is colonic involvement (Bernstein et al., 2015).

Extraintestinal manifestations of IBD

Extraintestinal features include rheumatic (e.g. peripheral arthritis), metabolic (e.g. osteoporosis, osteomalacia), dermatologic (e.g. erythema nodosum), ophthalmologic (e.g. episcleritis), hepatobiliary (e.g. cholelithiasis) and pancreatic (e.g. pancreatitis) complications (Larsen et al., 2010). Fever and weight loss are quite common general symptoms. Growth and sexual maturation delay in children is quite common due to malnutrition and corticosteroids administration (Bernstein et al., 2015).

1.5.2. Diagnosis

Diagnosis is based on the following examination according to the latest guidelines of the World Gastroenterology Organisation (Bernstein et al., 2015).

- ***Patient history*** (e.g. symptoms, family history, extraintestinal manifestations, other medical problems)
- ***Physical examination*** (general, abdominal region, perianal region, extraintestinal inspection)
- ***Stool examination***

- ✓ Faecal examinations to exclude bacterial, viral or parasitic causes of diarrhea.
- ✓ Testing for Clostridium difficile.
- ✓ Check for occult blood or faecal leukocytes.
- ✓ Lactoferrin and a_1 antitrypsin to rule out intestinal inflammation.
- ✓ Calprotectin to measure IBD activity.

- ***Blood examination***
  - ✓ Complete blood count (CBC).
  - ✓ Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and orosomucoid, which correlate imperfectly with inflammation and disease activity.
  - ✓ Electrolytes, albumin, ferritin, calcium, magnesium, vitamin B_{12}, serum cobalamin.
  - ✓ Liver enzymes and function testing.
  - ✓ Human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), varicella zoster virus (VZV), immunoglobulin G (IgG).
  - ✓ Perinuclear antineutrophil cytoplasmic antibody (p-ANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA) for cases of unclassified IBD. Positive p-ANCA and negative ASCA tests suggest UC. Negative p-ANCA and positive ASCA tests suggest CD.
  - ✓ Celiac antibody testing.

- ***Imaging and endoscopy*** (e.g. plain abdominal radiography, capsule endoscopy, sigmoidoscopy and colonoscopy with histopathological assessment)

Diagnosis of both CD and UC requires a combination of clinical, endoscopic, histopathologic, and radiologic features. However it remains challenging and differentiation between UC and CD can be difficult. New biomarkers for diagnosis and disease monitoring are necessary. Metabolomics has the potential to reveal disease mechanisms, identify biomarkers for diagnosis and monitoring

disease activity, as well as distinguish IBD from healthy controls, and CD from UC, with urine, serum or stool samples (Scoville et al., 2018).

1.6. Medical management

The medical management of CD and UC includes a wide range of pharmacological agents such as aminosalicylates (5-ASA), corticosteroids (CS), immunomodulators, i.e. azathioprine (AZA) and 6-mercaptopurine (6MP), biological therapy and antibiotics. In most cases, patients follow a stepwise therapy, starting from less aggressive medications for mild disease and continue to stronger and possibly more toxic therapies in more severe conditions. Surgical management is considered when patients do not respond to the medications described in Table 1 and it is still necessary in 30%-40% of patients with CD and 20%-30% of patients with UC. The risk for surgery in CD patients is associated with stenosing phenotype, perianal disease, smoking, younger age at diagnosis, and delay in biologic therapy. Surgery in CD is not curative but is required in cases medical therapy fails to control symptoms. Though, surgery in UC is curative and is suggested in cases of failure of medical management, acute complication including fulminant colitis, perforation, severe bleeding, toxic megacolon, and chronic conditions such as development of dysplasia or malignancy (Ferrari et al., 2016).

Table 1. Overview of disease status and drug therapy (Adapted from Bernstein at el., 2015)

	Distal UC	**Extensive UC**	**CD**
Mild	Rectal or oral 5-ASA Rectal CS	Topic and oral 5-ASA	Sulphasalazine or other 5-ASA for colonic disease only Metronidazole or ciprofloxacin for perianal disease Budesonide for ileal and/or right colon disease
Moderate	Rectal or oral 5-ASA Rectal CS	Oral CS Topical and oral 5-ASA AZA or 6MP Anti-TNF	Oral CS AZA or 6MP Methotrexate Anti-TNF
Severe	Rectal and oral 5- ASA Oral or intravenous CS Rectal CS	Intravenous CD Intravenous cyclosporine or Intravenous infliximab	Oral or intravenous CS Subcutaneous or intramuscular methotrexate Intravenous infliximab or subcutaneous adalimumab or subcutaneous certolizumab
Corticosteroid-resistant or dependent	AZA or 6-MP or preferably anti-TNF or combination AZA/6-MP + anti- TNF	AZA or 6-MP or anti-TNF or preferably combination AZA/6-MP + Anti-TNF Vedolizumab therapy is another alternative in moderate/severe disease	AZA or 6-MP or anti-TNF or preferably combination AZA/6-MP + Anti-TNF Vedolizumab therapy is another alternative in moderate/severe disease
Quiescent	Oral or rectal 5-ASA Oral AZA or 6-MP	Oral 5-ASA Oral AZA or 6-MP	AZA or 6-MP or methotrexate
Perianal			Oral antibiotics AZA or 6-MP Intravenous infliximab Subcutaneous adalimumab

1.7. Monitoring IBD activity

Several laboratory markers, from both blood and faecal samples, have been investigated in IBD, because clinical indices are subjective and there is a need to avoid repeative non-invasive tests. An ideal test should be simple, easy and rapid, low cost and reproducible. Additionally, it has to be sensitive and specific enough to help in differential diagnosis, objective measurement of disease activity and treatment efficacy, and predicting relapse and disease course. However, there is not such a biomarker that fulfills all the above criteria yet. Colonoscopy with histological analysis is considered the gold standard, however it is expensive, uncomfortable and poses patients in risk.

1.7.1. Laboratory tests in blood

C-Reactive Protein (CRP): CRP is a 224-residue protein synthesized by the liver in low concentrations. It remains the most used acute-phase reactant, even if it rises in response to many physiological processes. It is widely used in clinical practice, since it usually correlates with inflammation better in CD compared with UC.

Erythrocyte Sedimentation Rate (ESR): ESR expresses the rate at which erythrocytes migrate through the plasma, depends on plasma concentration and on the number and size of erythrocytes. ESR values gradually increase compared to CRP, and normalize in several days even after resolution of inflammation.

Both CRP and ESR are used in clinical practice in order to assess and monitor disease activity, to predict disease course and monitor response to therapy. They are inexpensive but have a low specificity (Cappello & Morreale, 2016).

Complete blood cell count (CBC): CBC including white blood cells (WBC), heamoglobin, mean corpuscular volume (MCV) and platelets, is used in routine evaluation of recurrence, diagnosis of intercurrent infections, diagnosis of anemia and monitoring of drug safety. An increase of WBC count is common in active IBD and in patients taking steroids. Anaemia is usual, either as anaemia of chronic disease or as iron-deficiency anemia. Elevated MCV is frequent in patients taking

azathioprine (AZA) or 6-mercaptopurine (6-MP) (Cappello & Morreale, 2016).

Serum iron, transferrin, ferritin, albumin, Vitamin B12, folate: These markers are used to evaluate malnutrition and selective deficiencies, as well as to diagnose iron-deficiency anaemia (Cappello & Morreale, 2016).

1.7.2. Laboratory tests in faeces

Until today, faecal samples were used as a routine test to evalute the presence of WBCs, pathogens and parasites. However, the lack of a reliable biomarker has led to the study of neutrophil-derived proteins in faeces to asess gut inflammation. Many proteins have been evaluated such as faecal calprotectin, lysozyme, lactoferrin, defensins, elastase and myeloperoxidase.

Faecal calprotectin (FC): Calprotectin is a 36-kDa inflammatory protein present in the cytosol of human neutrophils, macrophages, and monocytes. Calprotectin includes up to 60% of neutrophil cytosolic proteins. FC is directly proportional to neutrophil migration into the gastrointestinal tract during the inflammatory state (Vermeire et al., 2006). Stability is an advantage of FC, since it can be detected in faecal samples for more than a week. Even if it is nonspecific, because it can be elevated in case of colorectal carcinoma, infections, polyps and usage of NSAIDs, FC is used as a surrogate marker of intestinal inflammation in IBD and it is associated with endoscopic activity. Additionally, FC discriminates IBD from Irritable Bowel Syndrome (IBS), as well as it is useful for the assessment and monitoring disease activity, for the prediction of disease course and for monitoring response to therapy (Cappello & Morreale, 2016). In general, active IBD is indicated by very high levels of FC, and inactive IBD is usually indicated by FC levels less than 50 μg/g. However, the appropriate cut-off limits to define disease activity are still questionable by many clinicians and researchers. As such, FC cut-off levels are affected by the type of assay used, variations in method of extraction, heterogeneity in study design, time points of stool collection and disease phenotype (Chang et al., 2015).

Faecal Lactoferrin (FL): Lactoferrin is an additional stool neutrophil protein and it is frequently assessed in combination with FC in clinical trials. Lactoferrin is an 80-kDa, iron- binding protein present over most mucosal surfaces, where it is secreted. It has a comparable but slightly lower sensitivity and specificity compared with FC, as well as less stability at room temperature (up to 4 days) (Cappello & Morreale, 2016). FL could also be useful in differentiating diagnosis between IBD and IBS (Zhou et al., 2014).

Faecal Lysozyme (FLys): Lysozyme is an enzyme found naturally in human tears, saliva and other body fluids, able to destroy the cell walls of certain bacteria and to act as a mild antiseptic. It regulates innate immune response and it is expressed in both entities, CD and UC, mainly in small intestine, but markedly also in the colon, shown in both experimental animals (Coulombe et al. 2016) and in humans (Rubio, 2011; Fahlgren et al., 2013).

Faecal beta-defensins (FD): Beta-defensins are antimicrobial peptides that play a key role in the innate host defence against infections. They are mainly produced by epithelia at mucosal sites and they exhibit antimicrobial effects against Gram-positive and -negative bacteria, fungi and some viruses (Yamaguchi et al., 2009). Beta-defensin 2 may act as a marker of colonic inflammation and it is detectable in faecal samples. This molecule seems to cause adaptive immune responses through recruitment of dendritic cells and T cells during active inflammation (Kolho et al., 2014).

Novel biomarkers: They can be categorised in 3 groups: *serum cytokines* driving inflammation, (e.g interleukin (IL)-17 and IL-33/ST2); *enzymes involved in inflammation at tissue level* (e.g. adenosine deaminase, polymorphonuclear elastase, and matrix metalloproteinase-9) and *break- down products of the inflammatory process* (e.g. neopterin, serum M30, and faecal hemoglobin) (Vrabie & Kane, 2014).

1.7.3. Disease activity indices

Disease activity indices of UC and CD assess several parameters (e.g. soft stool frequency, abdominal pain) and result in a scoring system that categorises disease activity in remission, mild, moderate and severe. However, the assessment of these indices is largely subjective. Table 2 depicts the thresholds for disease activity according to symptom-based scoring systems in CD and UC.

Table 2. Thresholds for Disease Activity according to Symptom-Based Scoring Systems in CD and UC (Adapted from Peyrin-Biroulet et al., 2016).

Tool	Parameters assessed	Scoring system	Activity score thresholds			
			Remission	Mild	Moderate	Severe
Crohn's Disease						
CDAI	Soft stool frequency over past week Abdominal pain over past week General well-being over past week Taking drugs for diarrhea over past week Presence of complications Heamatocrit levels Percentage deviation from standard weight	Cumulative score with components given different weightings	<150	150-219	220-450	>450
Short CDAI	Soft stool frequency over past week Abdominal pain over past week General well-being over past week	Cumulative score with components given different weightings (+ constant to yield a mean value as close as possible to full CDAI)	<150	150–219	220–450	>450

Tool	Parameters assessed	Scoring system	Activity score thresholds			
			Remission	Mild	Moderate	Severe
PRO-2	Soft stool frequency over past week Abdominal pain over past week	Cumulative score with components given different weightings	<8	8–13	14–34	>35
HBI	General well-being on previous day Abdominal pain on previous day Liquid stool frequency on previous day Abdominal mass EIMs	Cumulative score	<5	5–7	8–16	>16
van Hess index	Albumin ESR Body mass index Abdominal mass Sex Temperature Stool consistency Resection Extraintestinal lesions	Cumulative score with components given different weightings	<100	100–149	150–210	>210
PDAI	Patient report of perianal discharge Pain with restriction of daily activities Restriction of sexual activity Type of perianal disease Degree of induration	Cumulative score with each component score on a scale from 0 (no symptoms) to 4 (severe symptoms)	0	<4 (inactive disease not requiring therapy)	≥4 (active disease requiring medical or surgical therapy)	≥4 (active disease requiring medical or surgical therapy)
Ulcerative Colitis						
Mayo score	Stool frequency Rectal bleeding Physician's global assessment Sigmoidoscopy	Cumulative score	0–2	3–5	6–10	11–12
UCDAI	Stool frequency Rectal bleeding Physician's global assessment Sigmoidoscopy	Cumulative score	0–2	3–8		9–12

Tool	Parameters assessed	Scoring system	Activity score thresholds			
			Remission	Mild	Moderate	Severe
Powell–Tuck index	Well-being Abdominal pain Bowel movement frequency Stool consistency Bleeding Anorexia Nausea/vomiting Abdominal tenderness Eye, joint, mouth, or skin complications Temperature Sigmoidoscopy	Cumulative score	≤3	4–10	11–14	>14
Partial Mayo Score	Stool frequency Rectal bleeding Physician's global assessment	Cumulative score	0-1	2-4	5-6	7-9
Truelove & Witts score	Evacuation no/day Blood in stools Fever Pulse Anaemia ESR	Qualitative score				
Rachmilewitz score	Bowel movement frequency Blood in stools Physician's global assessment Abdominal pain/cramps Temperature EIMs Laboratory findings (ESR, hemoglobin)	Cumulative score	0–4	5–10	11–17	>17
SCCAI (Walmsley)	Bowel movements (day) Bowel movements (night) Urgency of defecation Blood in stool Well-being Extracolonic features	Cumulative score	≤2 <2.5	3–20		

Tool	Parameters assessed	Scoring system	Activity score thresholds			
			Remission	Mild	Moderate	Severe
Lichtiger index	Diarrhea frequency Nocturnal diarrhea Visible blood (% of movements) Faecal incontinence Abdominal pain/cramping Well-being Abdominal tenderness Need for antidiarrheal medications	Cumulative score	≤3	4–8	9-14	>14
Seo index	Bowel movement frequency Blood in stool ESR Hemoglobin Albumin	Cumulative score with components given different weightings (+ constant to yield a mean value as close as possible to Truelove–Witts criteria)	<108 <120	<150	150-220	>220

1.8. Nutrition and IBD

Malnutrition and IBD

Malnutrition is a common feature in IBD, however it is more common in CD than UC patients. Protein-energy malnutrition is detected in 20-85% of CD patients, affecting 70-80% of hospitalised IBD patients and 20-40% CD patients of outclinics (Forbes et al., 2011; Hartman et al., 2009). The major mechanisms contributing to malnutrition in IBD include:

- decreased food intake due to anorexia, nausea, emesis and drugs interactions
- nutrients malabsorption due to intestinal inflammation and fistulas
- increased intestinal losses through diarrhoea, steatorrhoea and blood losses
- hypermetabolism due to increased production of proinflammatory cytokines and increased lipid oxidation rate. (Hartman et al., 2009)

Malnutrition is quite common in paediatric populations of IBD as well. Weight loss is a frequent feature of new cases presenting with IBD. Defective bone mineralisation, growth failure and delayed puberty problems (especially for boys) are some additional complications in paediatric IBD patients. Malnutrition, increased production of proinflammatory molecules and long-term steroid administration seem to contribute to these complications (Gerasimidis et al., 2011).

Changes in body composition

Compromised nutritional status reflects upon alterations in body composition of IBD patients. Body Mass Index (BMI) is lower in 37% of CD patients and 20% of UC patients. However, BMI is a superficial factor for the assessment of nutritional status, therefore, body composition should be assessed via Bioelectrical Impedance (BIA) or Dual-energy X-ray Absorptiometry (DXA), which is considered the gold standard for the determination of bone density, fat and lean body mass. Lean body mass is typically lower in IBD compared with the healthy population. More specifically, lean

body mass is decreased in 28% of CD and 13% of UC cases compared with healthy controls. Furthermore, fat mass is lower 31% of CD and 13% of UC patients. Fat distribution, especially fat localized around the intestine lumen, is suspected to be pathognomonic as well as a pathogenic factor for IBD. This phenomenon is referred as "fat wrapping" and it seems that the proinflammatory and immunomodulating adipokines secreted affect IBD pathogenesis (Bryant et al., 2013). Last but not least, bone mass is also compromised in IBD patient, since osteopenia and osteoporosis are common complications affecting almost every second patient. Early onset of the disease before 30 years of age or age over 50 is a risk factor for osteopenia and osteoporosis (Lim et al., 2014).

Micronutrient deficiencies

Iron deficiency is quite common due to either malabsorption or increased intestinal losses through bloody stools. It is the principal cause of anemia in IBD patients (approximately 57%). Intravenous preparations are preferable due to poor tolerance of oral iron supplements and risk of exacerbations (Nemeş et al., 2016). However, in cases of inactive disease oral iron supplements are the first-line treatment (Forbes et al., 2017). Calcium deficiency may be a result of malnutrition, malabsoption or longterm glucocorticosteroids treatment. Additionally, avoidance of dairy because of the fear of lactose intolerance contributes to calcium deficiency. Supplementation is crucial in case of reduced dietary intake to protect bone health (Lim et al., 2014; Owczarek et al., 2016). As far as vitamin D is concerned, IBD is significantly associated with having higher odds of vitamin deficiency, although it is not clear whether it results from IBD-related malabsorption due to intestinal mucosal damage, or it is a possible contributor to disease onset and progression (Del Pinto et al., 2015). Vitamin B12 deficiency is present in 28-48% of CD patients due to resection of the distal part of the intestine or with particularly intensified disease involving the distal GI tract. It should be monitored and vitamin B12 preparations should be administered parenterally when necessary. Other micronutrients deficiencies include folic acid, zinc, vitamin A and magnesium (Owczarek et al., 2016).

Objectives of nutritional care

The aims of nutritional care are to:

- Achieve and maintain remission
- Prevent disease progression
- Correct nutritional deficiencies
- Improve quality of life

Patients with IBD should be screened for malnutrition at the time of diagnosis and thereafter on a regular basis since they are at risk for malnutrition. In case of malnutrition, patients should be treated in order to improve prognosis and quality of life and decrease complication rates and mortality (Forbes et al., 2017). It is important to follow the routine described in Figure 5.

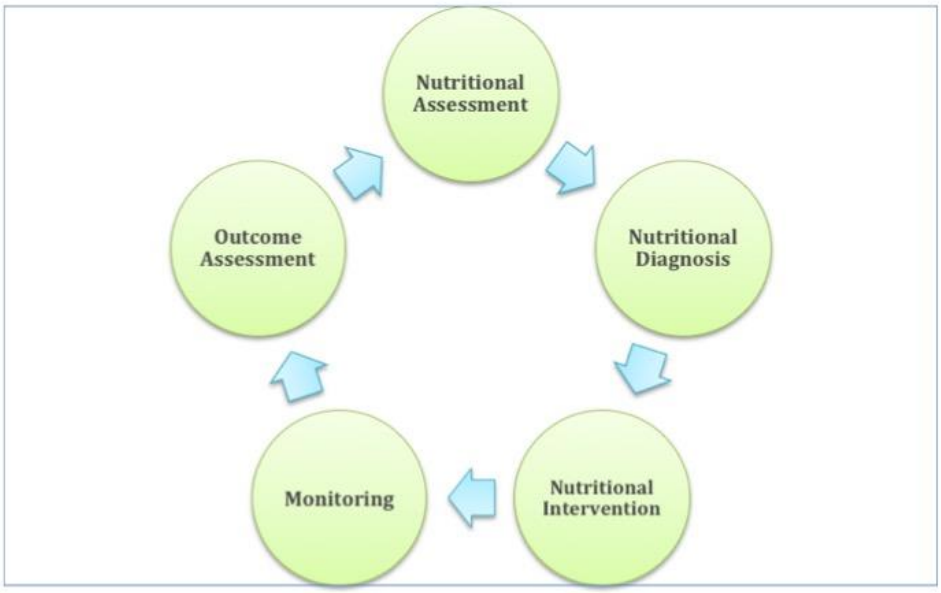


Figure 5. Nutritional care plan

Nutritional care during active phases of IBD

During periods of relapses low fiber intake is recommended for patients with diarrhoea and abdominal pain, except from patients with UC and rectal involvement that may suffer from constipation. Importantly, adequate energy intake must be ensured to avoid protein-energy malnutrition. Energy requirements are similar to those of the healthy population, but protein requirements are higher in active phases of IBD and protein intake should increase at 1.2-1.5 g/kg/d in adults (Forbes et al., 2017). Small and frequent meals are usually better tolerated. Patients should be monitored on a regular basis for micronutrient deficiencies, in case of which vitamin supplements are necessary.

A recent study assessing the dietary habits of patients with CD and UC showed that foods that intensify symptoms include raw fruits and vegetables, whole grains, fatty red meat, dairy, legumes, nuts and alcohol, but cooked fruits and vegetables, white rice and pasta, lean meat are considered "safer" by the patients (Cohen et al., 2013). However, there is no evidence for an IBD-appropriate diet in cases of relapse to promote remission, even if there is a strong interest upon low-FODMAP diet, specific-carbohydrate diet (SCD). Exclusion diets based on patient's individual intolerances cannot be recommended to achieve remission in active CD (Forbes et al., 2017).

Since IBD morbidity is linked with gastrointestinal bacterial flora abnormalities and an abnormal immune response to physiological flora, several studies have tried to clarify the effect of probiotic supplementation on IBD. There is a controversy among their results depending on the strain of the bacteria and the type of disease (Jonkers et al., 2012). There is no sufficient evidence to support probiotic supplementation in CD, but probiotic therapy using E. coli Nissle 1917 or VSL#3 can be considered in patients with mild to moderate UC for the induction of remission (Forbes et al., 2017).

Artificial nutrition

Artificial nutrition in the form of enteral nutrition (EN) or parenteral nutrition (PN) should be considered when there is a necessity. Oral Nutrition Supplements (ONS) are the first line used to support nutrition in addition to normal food. If oral intake is not possible tube feeding is the next step. PN should be considered only in cases of bowel obstruction, short bowel syndrome, enterocutaneous fistula and toxic megacolon. The following algorithm should be followed.

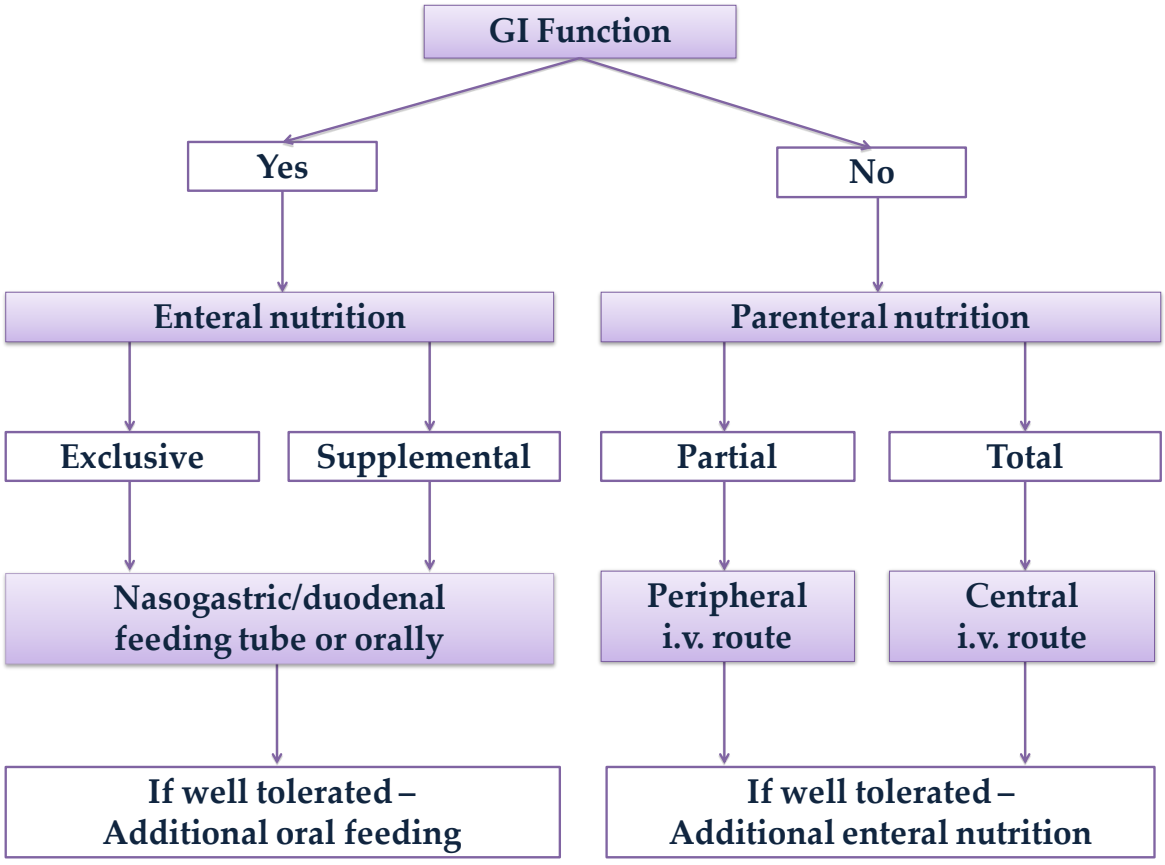


Figure 6. Rules of nutritional therapy depend on normal function of gastrointestinal system

(Adapted from Wędrychowicz et al, 2016)

Enteral nutrition

EN is recommended for the management of undernutrition, prevention of growth failure in children and improvement in the quality of life. Furthermore, it may prove effective in the treatment of active CD in cases of steroid intolerance in adults; nevertheless, steroids continue to be more effective than EN (Zachos et al., 2007). However, it should be noted that exclusive EN remains as a first line treatment for children and adolescents with active IBD to induce remission and to avoid side effects of steroids. EN seems to be safe and can be considered as supportive therapy according to standard nutritional practice in patients with severe UC. Last but not least, EN should be considered to enhance nutritional status during the perioperative period (Forbes et al., 2017).

Possible mechanisms that have been proposed regarding the mechanisms of efficacy of EN include sequestration of intraluminal antigens, modulation of the immune response of the bowel, downregulation of proinflammatory cytokines, restoration of the antioxidant status, regulation of the intestinal microflora and the intestinal motility, as well as promotion of epithelial healing (Triantafillidis et al., 2015).

The composition of the EN formula does not seem to influence its therapeutic potential. There is not a significant difference between **elemental** (amino acids, glucose, low in fat), s**emi-elementa**l (peptides, glucose polymers or starch, MCT), or **polymeric** (proteins, complex carbohydrates, LCT) formulae. However, the meta-analysis of Zachos et al (2007) showed a non-significant difference comparing elemental versus non-elemental formulas as well as a non-significant trend favouring low-fat formulas. The use of special substrates such as glutamine, arginine, n-3 fatty acids is not adequately supported by research data (Forbes et al., 2017).

Parenteral nutrition

PN is not suitable as a first-line treatment neither for CD nor for UC, since bowel rest does not seem to have a place in IBD nutritional management. PN should be considered only in cases of

malnourished patients, who are intolerant to food and EN or have an inaccessible gut (Forbes et al., 2017).

Nutritional care during inactive phases of IBD

The protein requirements in remission are generally not increased and it should be similar (about 1g/kg/d in adults) to that recommended for the general population (Forbes et al., 2017). There is no specific diet recommended during remission, but the patients are encouraged to follow a balanced diet, according to that recommended to the general population, avoiding alcohol consumption. However, patients tend to exclude "dangerous" foods from their diet fearing exacerbation of symptoms, thus compromising their nutritional status. Enteral nutrition may be useful during maintenance periods following two alternative strategies; either as nocturnal infusions of enteral diet during one of every 4 months, or as supplementary enteral nutrition with unrestricted daytime diet (Wędrychowicz et al, 2016).

Phytochemicals and IBD

Phytochemicals include a large group of compounds naturally occuring in fruits, vegetables, herbs, spices and botanical extracts. They are divided into subtypes such as polyphenols, carotenoids, anthocyanins, alkaloids, glycosides, saponins and terpenes. A plethora of studies have revealed that phytochemicals exhibit anti-inflammatory, antioxidant and antimicrobial properties (Islam et al., 2016).

Many natural products have been tested in IBD and experimental animal models of colitis. Starting from *Curcuma longa* (Zingiberaceae) commonly known as Turmeric, it is an Indian spice taken from the rhizomes of the plant. Curcumin is the main active component of turmeric with established antioxidant, antimicrobial, anticancer, and anti-inflammatory properties. A few clinical studies have shown that parallel administration of curcumin and standard medical therapy improved its efficacy without side effects in the longterm and may be considered as a safe practice for maintaining remission and preventing relapse (Algieri et al., 2015). A recent study on 50 patients with mild to

moderate UC, who did not respond to the maximum dose of mesalamine, investigated curcumin's effect on inducing and maintaining remission. Patients were randomly divided into two groups given either curcumin capsules (3 g/day), or an identical placebo for 1 month, and continued mesalamine as well. Response to treatment and induction of clinical and endoscopic remission was significantly increased in the curcumin compared with the placebo group (Lang et al., 2015).

Another natural product that has been investigated in IBD treatment is the oleo-gum resin from *Boswellia serrata* (Burseraceae), which is a traditional Ayurvedic remedy used to treat inflammatory diseases, including UC. A survey performed in Germany regarding the use of complementary and alternative medicine showed that approximately 36% of IBD patients receiving Boswellia serrata extract reported satfisfaction with this treatment (Joos et al., 2016). Clinical trials have pointed out safety but lack of efficacy, although experimental animal models show more promising results, attributing the beneficial effects mainly on triterpenes, which are the most abundant. More specifically, boswellic acids are the major constituents and they are considered to principally contribute to the anti-inflammatory pharmacological properties of this product.

There are many other natural products that have been investigated in IBD treatment together with the conventional medical therapy, but there is lack of sufficient data to support their efficacy and recommend their usage (Algieri et al., 2015).

CHAPTER 2

IMMUNITY AND INFLAMMATORY BOWEL DISEASE

2.1. Immunity and IBD

Immunity is divided into innate and adaptive immunity. Innate immunity uses a large set of different pattern recognition receptors (PRRs) and a system for nonselective generation of antigen specific receptors. Adaptive immunity cooperates with innate immunity and contributes to pathogen recognition. Immunity function disorder is associated with the pathogenesis of IBD.

Table 3. Differences between innate and adaptive immunity (Adapted from Huang & Chen, 2016)

Item	**Innate immunity**	**Adaptive immunity**
Acquired form	Inherent (or congenital) Do not need to contact the antigens	Acquired Need to contact the antigens
Time to play roles	Early, rapid (minutes-4 days)	4-5 days
Immune recognition receptors	Pattern recognition receptor	Specific antigen recognition receptors
Immune memory	None	Generation of memory cells
Examples	Antibacterial substances, bactericidal substances, inflammatory cytokines, phagocytic cells, NK cells, NK T cells	T cells (cell immunity) B cells (humoral immunity)

Innate immunity

The intestinal innate immunity includes mainly the intestinal mucosal epithelial barrier, natural immune cells (e.g. macrophages, dendritic cells natural killer cells) and innate immune molecules (e.g. defensins, PRRs). This system is nonspecific and nonmemorial. However, it is closely related with the adaptive immune system, since it presents antigens to the original T cells and contributes to differentiation and maturity of immune cells in adaptive immunity.

Immune cells in the intestinal mucosa of IBD patients produce inflammation factors, such as TNF-α and IFN-γ, resulting in *epithelial cell apoptosis*, deteriorating function of epithelial cells resisting pathogens and increased permeability of intestinal mucosa (Bouma & Strober, 2003). Furthermore, the *tight junction protein* of the intestinal epithelium and its corresponding mRNA are significantly decreased in IBD patients, indicating that intestinal epithelial permeability increased especially during active phases of the disease (Gassler et al., 2001).

Regarding innate immune cells, *macrophages* that decompose engulfed pathogens into specific antigens determinants in order to present them to T cells, are dramatically increased in acute phases of IBD leading in proinflammatory factors secretion (Liu & Liang, 2008). *Dendritic cells (DC)* in the IBD colon interact with T cells promoting inflammatory cytokines synthesis and causing inflammation (Drakes et al., 2005). *Natural killer cells (NK)* and *natural killer T cells (NKT)* exist more in the intestinal mucosa of IBD patients expressing more immune active molecules, such as CD25, CD28 and CD69 compared with the healthy intestinal mucosa (Liu & Jiu, 2008). *Defensins* exhibit antimicrobial and chemotactic function, promote T cells, monocytes and DC gathering during inflammatory response, and thus, they act like a linkage between innate and adaptive immunity. CD has been characterised like a defensin deficiency syndrome, since its expression is significantly less in IBD patients, although its mRNA expression is significantly higher in UC patients (Wehkamp et al., 2003; Wehkamp et al. 2005).

Adaptive immunity

Adaptive immunity is characterised by specificity and immune memory. After stimulation of antigens, the original T cells differentiate into subsets Th1, Th2, Th17 and T reg cells. Th1 cells eliminate pathogens into the cells, Th2 cells provide protection from harmful parasites and regulate allergic reactions, Th17 cells remove extracellular bacteria and fungi and Treg cells promote tissue repair. Inflammation initiates when disorders of T cell responses and imbalance of T cell subsets stimulate excessive synthesis of cytokines and chemokines (Huang & Chen, 2016).

Th1 cells secrete different proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukins IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, TNF-α and interferon-γ (IFN-γ). Th1 cells play an important role in the pathogenesis of CD, since a lot of Th1 cytokines (e.g. IL-12, IL-18, TNF-α) are present in the intestinal mucosa of CD patients (Allez et al., 2004). Th2 cells secrete IL-4, IL-5, IL-13 and IL-10. IL-5 and IL-13 are increased in UC patients, so it seems that Th2 cells are related with UC pathogenesis (Strober & Fuss, 2011). Th1 and Th2 cells are in a dynamic equilibrium under normal situations, though, an imbalance between them usually determines an imbalance between proinflammatory and anti-inflammatory cytokines leading in uncontrolled immune reaction (Huang & Chen, 2015). Th17 cells secrete IL-17, Il-21 and IL-22 and are present in the bowel mucosa of IBD patients in higher levels (Fujino et al., 2003). Treg cells maintain immune tolerance by inhibiting other Th cells (e.g. Th1, Th2, Th17) via direct contacts with cells and release of cytokines (e.g. IL-10 and TGF-β). Treg cells are decreased in peripheral blood and intestinal mucosa of IBD patients, indicating a decreasing capacity of the immune system to maintain tolerance (Boden & Snapper, 2008). TNF-α holds a central position in the pathogenesis of IBD and this is demonstrated by its increased levels in both healthy and inflamed mucosa of CD patients. The release of TNF-α into the intestinal mucosa triggers the activation of Nuclear Factor Kappa B (NF-κB), which is a transcription factor that induces the expression of cytokines (IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α) and adhesion molecules (ICAM-1, VCAM-1) and further stimulates the inflammatory process (Viscido et al., 2005). Figure 7 illustrates how the innate immune abnormalities in IBD patients result in adaptive immune disorders (Th1/Th2 regulation imbalance and Th17/Treg transformation imbalance) (Huang & Chen, 2016).

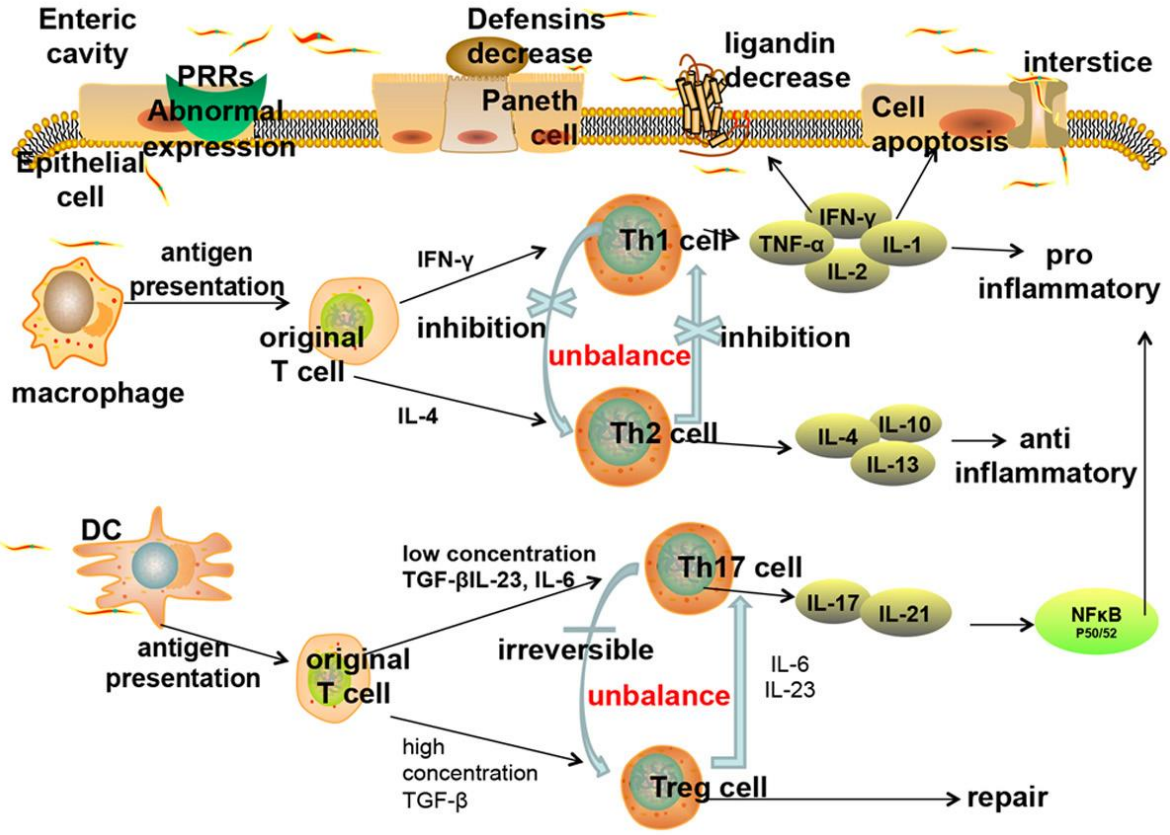


Figure 7. IBD-related immune disorders (Huang & Chen, 2016)

2.2. Mitochondrial dysfunction and oxidative stress in IBD

Mitochondrial function is indisputably essential to the maintenance of the intestinal epithelium. Since the epithelial barrier integrity is energy-dependent, mitochondrial dysfunction may play an important role in the onset and relapse of IBD. A plethora of studies have indicated that mitochondrial stress and alterations in mitochondrial function are present in the intestinal epithelium of IBD patients and mice with experimental colitis. Oxidative stress and impaired ATP production are characteristic of mitochondrial dysfunction, however it is not fully understood if this processes are a cause or a consequence of IBD (Novak & Molen, 2015).

Oxidative stress seems to have a critical role in the pathogenesis of intestinal inflammation. Even if Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are crucial for the homeostasis

of various molecular pathways, an imbalance between antioxidant (both endogenous and exogenous) and pro-oxidant mechanisms leads to oxidative stress, and thus, cellular damage and death through proteins, lipids, and DNA oxidation (Rezaie et al., 2007).

Several studies have shown that levels of ROS/RNS are increased within the intestinal epithelium of IBD patients. Additionally, elevated concentrations of oxidized molecules are also present in the plasma, serum, exhaled air, and saliva of patients with IBD. Antioxidant mechanisms are activated to defend oxidative stress, but since antioxidants (e.g. glutathione, ascorbate, α-tocopherol, β-carotene) are gradually depleted, more severe oxidative stress occurs (Rezaie et al., 2007). Serum antioxidant capacity seems to be significantly decreased in IBD patients compared with healthy controls (Koutroubakis et al., 2004). Impaired antioxidant mechanisms of the intestinal mucosa were demonstrated in IBD patients (Kruidenier et al., 2003). Increased oxidative DNA damage in peripheral leukocytes and decreased plasma antioxidants demonstrate that the oxidative damage is not limited to the intestinal mucosa (D'Odorico et al., 2001). Multiple studies have reported increased oxidative DNA damage both in blood and mucosa of patients with IBD (Novak & Molen, 2015).

Regarding mitochondria, they are the most abundant source of ROS in the cell (Beltrán et al., 2010). Under normal circumstances, low levels of ROS are produced and neutralized by the endogenous antioxidants. Nevertheless, damage or mutations destabilise mitochondria, and excessive oxidative stress may lead to a reduction of ATP, inhibition of the respiratory chain, and mtDNA damage. Persistent oxidative stress diminishes mitochondrial bioenergetics and homeostasis, resulting in cellular damage and eventually cell death (Novak & Molen, 2015).

Last but not least, the hypothesis of increased oxidative stress as an aetiological factor in IBD pathogenesis implicates also TNF-α, which seems to be increased in IBD. More specifically, it has been proposed that TNF-α produces ROS, which activate NF-κB. It is then that NF-κB increases the production of TNF-α leading into a vicious cycle (Head & Jurenka, 2003).

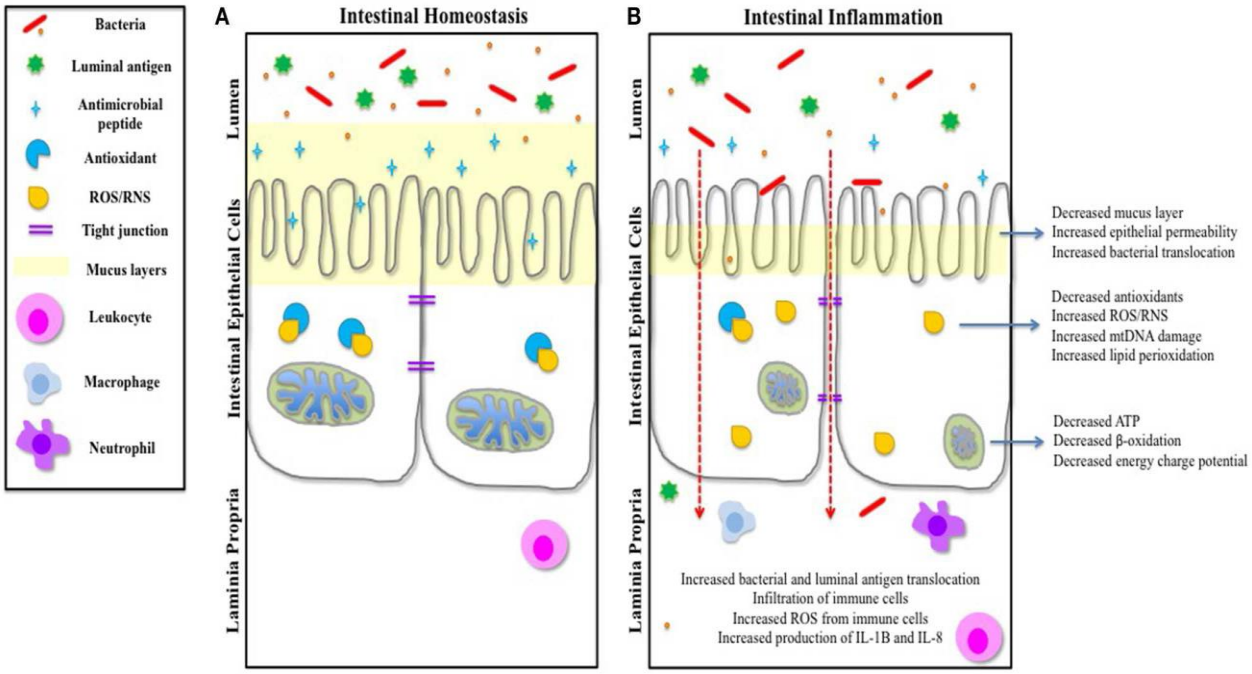


Figure 8. Intestinal epithelial cell function and intestinal homeostasis can be influenced by mitochondrial dysfunction (Novak & Molen, 2015).

CHAPTER 3

MASTIHA

3.1 Mastiha

Mastiha is the resinous secretion from the trunk of the shrub Pistacia Lentiscus (Anacardiaceae family), which grows exclusively in the southern part of Chios Island, Greece, due to particular soil and climate characteristics. Marking the trunk with a sharp tool causes the secretion and Mastiha flows as teardrops. Mastiha is well known since antiquity for its organoleptic characteristics and its beneficial properties. It is Generally Recognised As Safe (GRAS) from the U.S. Food & Drug Administration.

Ancient Greek physicians (Hippocrates, Dioscorides, Galenos) reported for first time the properties of Mastiha and recommended its use for its distinctive flavour and therapeutic properties. In ancient world, it was used to clean the teeth and to freshen the breath, in cosmetology for cleansing face and body, and also in a series of pharmaceutical formulas and nostrums, many of which have been recorded from time to time in international pharmacopeias. Dioscorides (1st century AD.) in *Materia Medica* referred to the therapeutic properties of Mastiha from the island of Chios mentioning that it helps in dyspepsia, in chronic coughing, while at the same time it acted as tranquilizer. He proposed the healing properties of Mastiha to support oral hygiene as well as to clean and fresh breath. He mentioned the use of the essential oil, to be applied in multiple ways for affections of the uterus, as well as for its styptic activities (Genandios 1914). From the 1st until the 7th century AD medical practitioners and botanists used Mastiha for more than 2500 years mainly for the treatment of stomach disorders like gastralgia, dyspepsia and peptic ulcer. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos and Galenos mentioned its properties and recommended its use (Paraschos et al., 2007). The effect of Mastiha in stomach inflammation, intestine and liver, is reported by Galen in «Simpliciun medicamentorum temperamentis ac faculatibus libri XI». The use of Mastiha continued to spread successfully during Byzantine. Additionally, it was Christopher Columbus who confessed «If spices worth their weight in silver, Mastiha worth its weight in gold».

3.2 Physical properties

During secretion Mastiha appears as a viscous, tacky and clear fluid, and after staying on the trunk or on the ground until the collection period (from June to September), it becomes solid in the form of teardrops (Savidis, 2000). This phenomenon happens due to partial evaporation of the essential oil (mastic oil) and the polymerization of the resin components. The density of Mastiha is 1.06, its acidity fluctuates between 50 and 75 and its colour is pale yellow or pale green. The hardness depends on the degree of polymerization, which is affected by temperature, the exposure time until collection and the size of teardrops. The more constant is the flow, the larger and softer the teardrops will be. In addition, Mastiha has initially a slight bitter taste, which disappears later. Mastiha has a melting point greater than 96 ºC.

3.3 Chemical composition

The resin consists of a variety of organic ingredients including a percentage of 30% of a natural polymer (poly-β-myrcene). Other constituents of Mastiha include volatile and aromatic ingredients that compose the essential oil, terpenic acids, phytosterols, phenolic compounds and a large number of other potentially active secondary metabolites, some of which have been isolated and determined in nature for the first time.

Overall, the main non-volatile natural products reported in the literature that have been isolated are: a) mastihadienonic acid, b) tirucallol, c) oleanolic acid, d) isomastihadienonic acid, e) 3-o-28-norolean-12-en, f) 20(S)-3β-acetoxy-20-hydroxydammar-24-en, g) 3-oxo-dammara-20(21),24-diene, h) 3β-hydroxymalabarica-14(26),17E,21-triene, i) 3-oxo-malabarica-14(26),17E,21-triene, j) 3-β-hydroxy-28-norolean-12-en, k) 3-oxo-28-norlup-20(29)-en, l) (8R)-3-Oxo-8-hydroxy-polypoda-13E,17E,21-triene, m) 1,4-poly-β-myrcene (Assimopoulou & Papageorgiou, 2005; Paraschos et al, 2007).

During the decades of the 90's and 2000's, the volatile components of Mastiha were the subject of several studies in the context of the analysis of the composition and activity of the essential oil. A

total of 90 components (50% monoterpene hydrocarbons, 20% oxygenated monoterpenes and sesquiterpenes 25%) with major components of the essential oil of resin, the α-pinene (79%) and the myrcene (3%) have been identified. A study of the three essential oils of Mastiha, leaves and branches, was published in 1999 by Magiatis and colleagues. In this study the major components identified were α-pinene (66.48%), myrcene (8.34%) and β- pinene (3.29%), the essential oil of leaves were myrcene (20,58%), germacrene D (13.30%), L-caryophyllene (8.33%), α-cadinol (7.33%) and δ-cadinene (7.00%), while the essential oil of the branches were myrcene (47.92%), germacrene D (15.46%) and E-caryophyllene (4.75%). In 2005, Koutsoudaki and colleagues indicated α-pinene, β-myrcene, β-pinene, limonene and trans-caryophyllene as the major components.

Additionally, Mastiha includes arabino-galactanes proteins (AGPs), which are a family of extensively glycosylated hydroxyproline-rich glycoproteins, analogous to animal proteoglycans that are thought to have important roles in various aspects of plant growth and development. AGPs in Mastiha have been isolated and partially characterized. More specifically, they consist of 3.1% protein, 78.5% neutral sugars (arabinose and galactose) and 18.4% uronic acids (Kottakis et al. 2008).

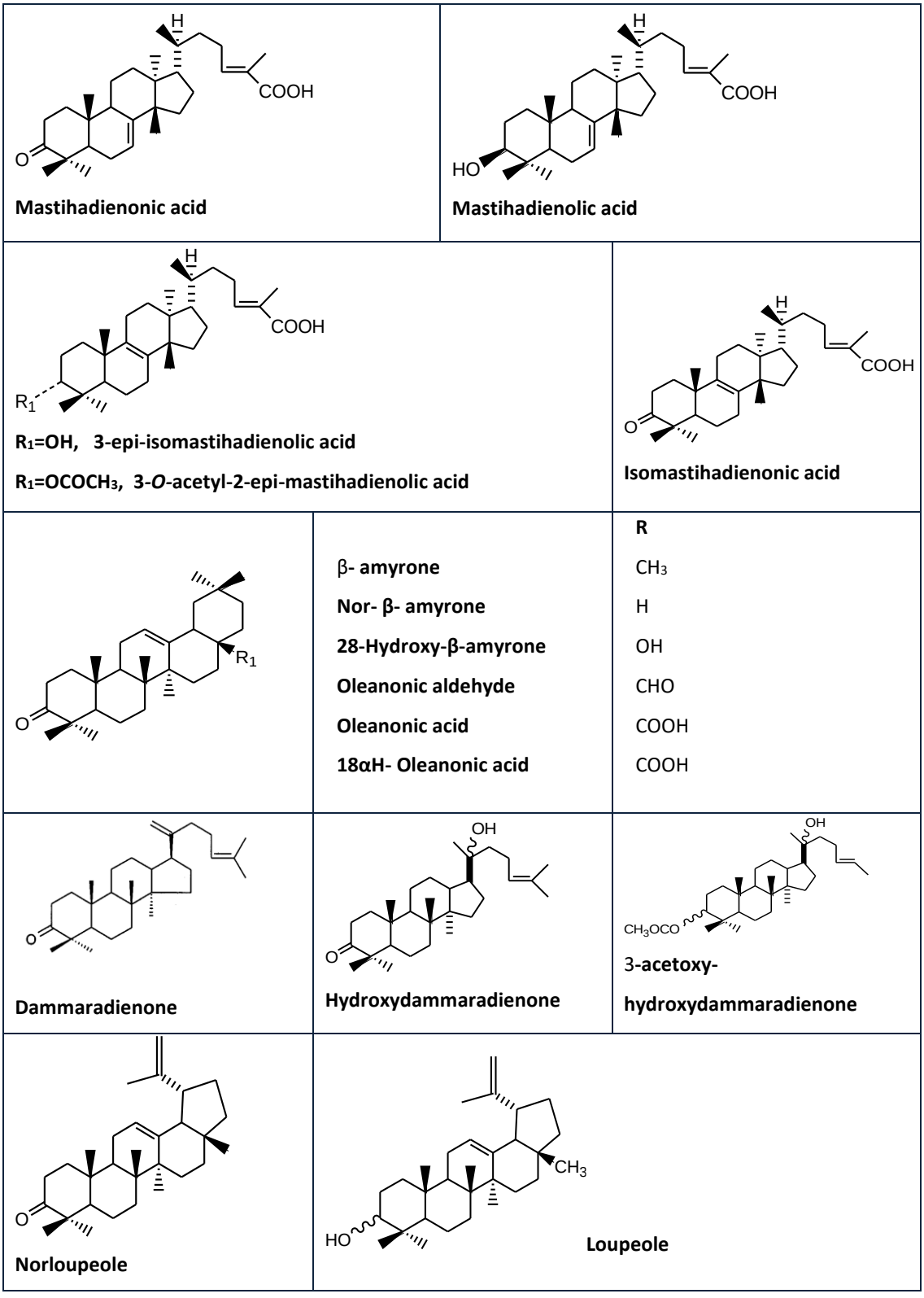


Figure 9. Triterpenes of Mastiha

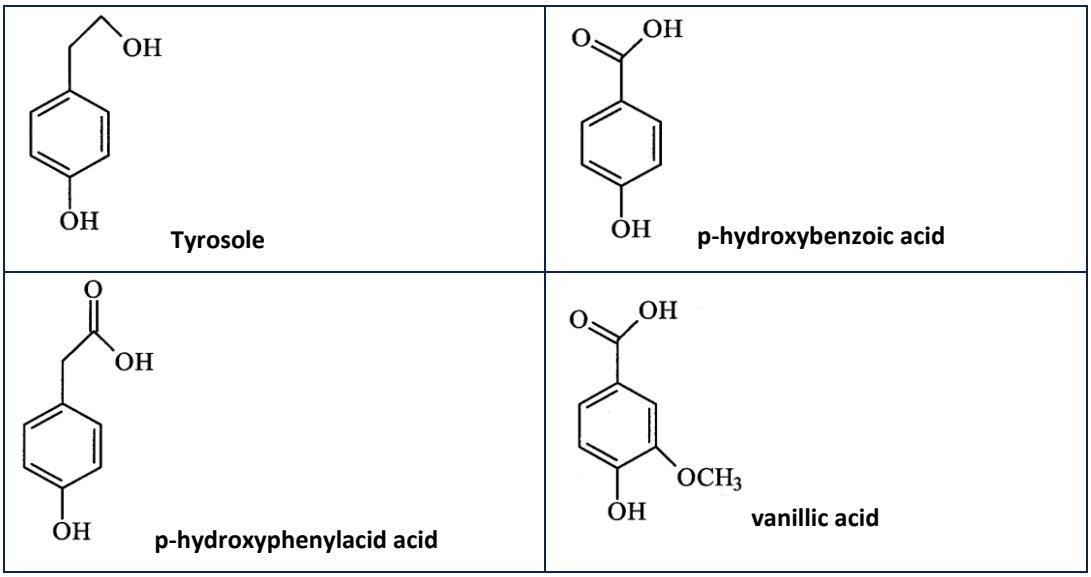


Figure 10. Phenolic compounds of Mastiha

3.4 Beneficial properties of Mastiha

The use of Mastiha as a therapeutic agent since antiquity led researchers from all around the world to investigate its beneficial properties. The first studies took place in the mid-80s and Mastiha attracts the scientific interest until nowadays due to its particular chemical composition.

Wound healing

The adhesive properties of Mastiha and its beneficial activity in healing wounds and post-operative incisions have been identified and studied by researchers for at least twenty years. Bandages, plasters, compresses and other healing means, applied in the protection and healing of wounds or postoperative incisions contain very often Mastiha as an ingredient. Relevant publications show, that when Mastiha is used in covering means and wound and incisions healing, it contributes to the effective skin regeneration and to wound healing, without any undesirable side-effect on the skin (irritation, itching, dermatitis, skin depigmentation, etc), as the conventional ingredients (Mikhail et al., 1986; 1989; Lesesne 1992; Yavuzer et al., 2005).

Antibacterial activity

A large body of evidence shows that Mastiha exhibits antibacterial activity. In the case of Streptococcus mutans the activity is comparable to vancomycin and significantly reduces plaque index and gingival index (Aksoy et al., 2006; Topitsoglou-Themeli et al. 1984). Furthermore, Mastiha shows selective antibacterial action against oral bacteria Porphyromonas gingivalis (Sakagami et al., 2009; Sterer 2006) and Prevotella melaninogenica (Sakagami et al., 2009).

In 1983 Mastiha total extract was shown to cause a 30-fold reduction in H. pylori colonization in the antrumand corpus of the stomach, in concurrence with the visible reduction in H. pylori colonization observed in the histopathology evaluations (Warren & Marshall, 1983). Finally, the researchers also suggested that habitual long term Mastiha consumption may be effective in moderating H. pylori colonization. Additionally, Kottakis et al. (2009), investigated the effect of Mastiha arabinogalactan proteins, in the presence of H. pylori neutrophil-activating protein, on the innate cellular immune effectors (neutrophils activations) comparing H. pylori-infected patients and healthy controls. Mastiha arabinogalactan proteins inhibited neutrophil activation, playing a crucial role in H. pylori-associated pathologies in gastric mucosa.

Sharifi and Hazell, (2009), showed that the most active fraction of Mastiha against H. pylori was its polymer, followed by the acidic fraction. Choli-Papadopoulou and colleagues, (2011) evidenced that arabinogalactan proteins derived from Mastiha, disrupt the process of neutrophil-endothelial cell attachment caused by H. pylori, an effect that points to a potential anti-inflammatory therapy for H. pylori patients. A more recent study (Miyamoto et al., 2014) examined which component of Mastiha is responsible for anti-H.pylori activity. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the essential oil of Mastiha led to the identification of 20 components, among which α-pinene (82.26 %) was the most abundant. Many components showed antibacterial activity against drug-resistant H. pylori growth in stomach.

Anti-inflammatory properties

Heo and colleagues, (2006), studied the in vivo effect of Mastiha in reducing the bowel damage induced by diclofenac and bacterial translocation in rats, a phenomenon caused by NSAIDs. Intestinal permeability, enteric aerobic bacterial counts in the distal ileum and cecum, intestinal adhesion, lipid peroxidation of distal ileum, and bacterial translocation to mesenteric lymph nodes, liver, spleen, kidney and heart, respectively increased by administration of diclofenac, but decreased after administration of Mastiha's essential oil at a dose of 1 ml/kg weight. The anti-inflammatory properties of Mastiha were investigated also by Kim and Neophytou in 2009 through the dextran-sulfate sodium (DSS) model of colitis. Their results indicated that supplementation with the essential oil delayed the onset and progression of the disease and prevented weight loss. Using Mastiha essential oil in combination with γ-tocopherol gave similar results to using Mastiha or γ-tocopherol alone.

Cytotoxic activity

Ethanol and hexane Mastiha extract induces apoptosis of colon cancer cells (Balan et al, 2005; Balan et al, 2007), and hexane extract may also decrease tumor size (Dimas et al, 2009). Similar results were found in studies on prostate cancer (He et al, 2006; He et al, 2007a, b). The cytotoxic activities of Mastiha and its major compounds have been reviewed by Giaginis and Theoharis (2011) and it is shown that Mastiha inhibited also cell proliferation of lung, and pancreatic cancer cells.

Hypolipidaemic properties

Mastiha administered in low dosages improves glucose and lipid disturbances in diabetic mice while alleviating hepatic damage (Georgiadis et al., 2014). The in vivo hypolipidaemic properties of the essential oil of Mastiha were also evaluated in hyperlipidaemic rats. In HepG2 cells the hypolipidemic action of the essential oil of Mastiha was attributed to camphene independently of HMG-CoA reductase activity. These results suggest that the hypocholesterolemic and hypotriglyceridemic effect of Mastiha are associated with a different mechanism of action than

that of statins (Vallianou et al, 2011).

Antioxidant activity

Dedoussis and colleagues, (2004), evaluated the effect of Mastiha polar extract, namely the terpenic and phenolic fraction, in the survival of peripheral blood mononuclear cells (PBMC). The extract was found to inhibit the oxidized low-density lipoprotein (oxLDL) triggered apoptosis of monocytes via downregulation of its receptor, CD36, expression and glutathione restoration. Moreover, Triantafyllou and colleagues, (2007), show that Mastiha decreases human serum total cholesterol, LDL, total cholesterol/HDL ratio, lipoprotein (a), apolipoprotein A-1, apolipoprotein B, SGOT, SGPT and gamma-GT (γ-GT) levels when supplemented at a dose of 5g daily.

Gastrointestinal disorders

A study on rats revealed that Mastiha decreased the expansion and intensity of experimentally induced gastric and duodenal ulcer (Al-Said et al, 1986). Clinical data about duodenal ulcer showed that Mastiha, supplemented at a dose 1g/day, resulted in alleviation of the symptoms in 80% of the cases, while the endoscopic examination confirmed that duodenal ulcer was cured in 70% of the cases (Al-Habbal et al, 1984). In patients with gastric ulcers, who were supplemented only with Mastiha at a dose of 2 g/day, symptoms resolved, while the treatment was confirmed endoscopically (Huwez et al, 1986). Furthermore, Dabos and colleagues, (2010), showed that Mastiha is effective in improving symptoms of functional dyspepsia. More specifically, when 148 patients fulfilling Rome II criteria for functional dyspepsia were randomly assigned to receive either Mastiha (1g/day) or placebo for a total of 3 weeks, the symptom score after treatment was significantly lower in the Mastiha than in the placebo group.

Regarding IBD, a pilot trial in CD patients with moderate disease activity has previously shown that Mastiha daily supplemented adjunct to conservative therapy was safe, but also regulated CRP and IL-6 in plasma and improve disease activity index (Kaliora et al., 2007a). In addition, Mastiha was shown to modulate the immune response in peripheral blood mononuclear cells, inhibiting the TNF-α secretion and inducing macrophage migration inhibitory factor (MIF) secretion (Kaliora et

al., 2007b).

In an experimental model of TNBS-colitis, Mastiha protected the intestinal epithelium barrier (Figure 11) and regulated inflammation by decreasing IL-6, IL-8, TNF-α and ICAM-1 in rat intestinal epithelium, even at the mRNA level (Gioxari et al., 2011; Papalois et al., 2012). Mastiha proved to regulate immunologic dysfunction by downregulation of NF-κB (Papalois et al., 2012). As a consequence, downregulation of NF-κB resulted in expression of several anti-apoptotic genes, inhibition of tissue damage, and protection of the intestinal barrier. Mastiha also reduced lactate dehydrogenase (LDH) release -a marker of membrane permeability and integrity- from the epithelial cell monolayer, consequently affecting cell membrane permeability.

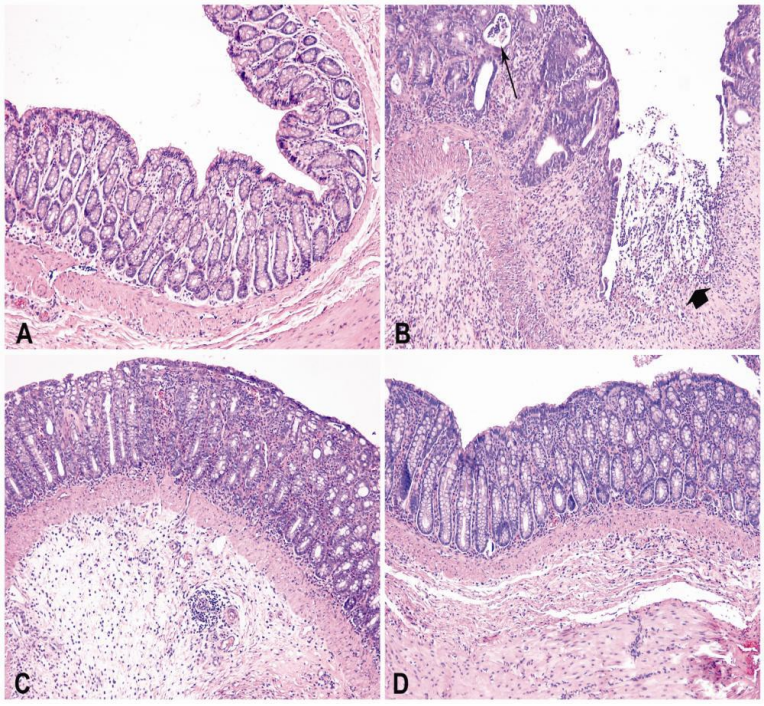


Figure 11: Colonic damage in TNBS-induced colitis rats treated with Mastiha (100 original magnification): (A) healthy, control group with normal mucosa; (B) TNBS model of colitis with severe crypt distortion, crypt abscess, inflammation, and ulceration; (C) TNBS-induced colitis rats treated with cortisone by elevated mucosal inflammatory cells and some crypt distortion still present; (D) animals daily treated with Mastiha characterized by sparse mucosal inflammatory cells and mild fibrosis (Gioxari et al, 2012).

Based on all data published, the European Medicines Agency (EMA) has recognised Mastiha as a herbal medicinal product with the following indications, a) mild dyspeptic disorders, and b) symptomatic treatment of minor inflammations of the skin and as an aid in healing of minor wounds (EMA, 2015).

3.5 Bioavailability of the phytochemicals of Mastiha

The bioavailability of Mastiha is still unexplored. However, there is an ongoing research upon the bioaccessibility and bioavailability of phytochemicals, such as phenolics and terpenes, compounds that are present in Mastiha as well. Terpenes, that are its major constituent of Mastiha, are characterised by low water solubility and poor bioavailability, but due to their importance as resources for pharmaceutical, food and cosmetic industries, there is a strong effort in the improvement of their clinical use (Lima et al., 2016).

The evaluation of phytochemicals bioavailability has brought into the spotlight analytical procedures for their detection and separation. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the fundamental tool in the determination of many types of phytochemicals, since it provides sensitivity, specificity and good separation in complex samples (Stylos et al., 2017).

Regarding phenolic compounds, plentiful interactions happen before and during digestion within the plant and/or the host's macromolecules. The *food matrix* is a well-known factor affecting phenolics bioavailability. Most studies on bioavailability of phenolic compounds have shown a peak in plasma antioxidant capacity 1-2h after intake. However, a postprandial study on 10 healthy volunteers consuming 15g of dietary fibre rich in phenolics demonstrated a significant increase in plasma antioxidant capacity 8h after intake (Pérez-Jiménez et al., 2009). These data demonstrate that dietary fibre (non-digestible cell wall components) interacts closely with phenolic compounds and delays their absorption in the small intestine, instead carrying them to the large intestine. Phenolics that are not bound to dietary fibre hinder digestion and absorption of proteins, carbohydrates, lipids, cholesterol, bile salts and micronutrients by inhibiting digestive enzymes and

enterocyte transporters as well as interfering with micelle formation. Some phenolic compounds are internalized by enterocytes, circulate through transcellular or paracellular transport, and then are effluxed back into the lumen or metabolised by phase I and phase II enzymes. Thus, the absorption and pharmacokinetics are affected by all the above-mentioned interactions in the gastrointestinal tract, as well as by the presence of other phenolic compounds (Domínguez-Avila et al., 2017).

Another factor that affects the bioavailability of phytochemicals is *genetic variability*. A recent postprandial study on 33 healthy males that consumed a test meal containing tomato puree with 9.7 mg of all-*trans* lycopene aimed at identifying a combination of Single Nucleotide Polymorphisms (SNPs) associated with the variability in lycopene bioavailability. The ability of to respond to lycopene was explained partially by a combination of 28 SNPs in 16 genes (Borel et al., 2015a). Further research upon the interindividual variability in dietary β-carotene bioavailability in healthy men showed that 69% of the variance in the postprandial chylomicron β-carotene response was explained by 25 SNPs in 12 genes (Borel et al., 2015b). The same research group investigated the bioavailability of lutein through tomato puree consumption or a supplement in 39 healthy men. Postprandial chylomicron lutein responses to meals were very variable between the subjects and have been attributed partially by 29 SNPs in 15 genes related to lutein and chylomicron metabolism (Borel et al., 2014).

Furthermore, the *type of formula* affects the bioavailability of the released phytochemicals. In case of berberine (an alkaloid) and betulinic acid (a triterpenoid), it seems that a patented dual channel spray gun technology enhances oral bioavailability of these promising anticancer agents (Godugu et al., 2014).

A postprandial study of Kanellos and colleagues (2013) aimed at investigating the impact of raisin supplementation in serum resistance to oxidation of 15 healthy volunteers and quantification of the content of phytochemicals. A total of 17 phytochemicals were identified and quantified in volunteers' plasma including oleanolic acid (a pentacyclic triterpenoid), vanillic acid, gallic acid, tyrosol and quercetin, which are present in Mastiha as well. Serum resistance to oxidation and

plasma total phenolics reached their peak 1h after raisin ingestion.

However, further research is necessary for the understanding of bioaccessibility and bioavailability of phytochemicals in Mastiha in order to explore the actual mechanisms of their beneficial properties and take the full advantage of them as a next step in the "battle" against chronic diseases.

Based on the preliminary study of Kaliora and collegues (2007a) that checked upon safety of Mastiha consumption in CD patients and their first encouraging data on efficacy of Mastiha on disease severity and inflammatory markers, this thesis aims at investigating the effects of Mastiha on the clinical course of IBD adjunct to medical treatment. However, since the bioavailability of its main bioactive compounds, namely terpenes, is unknown, a bioavailability study in healthy humans is also conducted in the context of this thesis.

PART B

METHODOLOGY

CHAPTER 1

BIOAVAILABILITY OF THE MICROCONSTITUENTS OF NATURAL MASTIHA IN HEALTHY ADULTS.

This study was registered at ClinicalTrials.gov (NCT02847117) and was approved by the Ethics Committee of Harokopio University of Athens (43/23-07-2014).

1.1 Aim and study design

Aim: The present study aimed at investigating whether Mastiha's compounds are absorbed and bioavailable in healthy adults.

Study design: This was an interventional, single group assignment, open label bioavailability study.

Twenty apparently healthy men, aged 20-40 years old, were enrolled based on certain inclusion and exclusion criteria (Table 4). The staff of the study provided detailed information regarding the aims, the methods, anticipated benefits and potential hazards of the study and all volunteers received the Patient Information Leaflet (PIL) (Appendix 1). Ample time (48 hours) was provided in order to decide whether they wanted to participate in the protocol. Each subject agreeing to participate signed an Informed Consent document and the staff explained to volunteers that they were under no obligation to enter the trial and that they could withdraw at any time during the trial, without having to give a reason. A copy of the signed Informed Consent was given to the participant (Appendix 1).

After enrollment, the volunteers underwent a medical and dietary assessment and their health status was evaluated through a complete blood count. Then, they were instructed to follow a low-phytochemical diet for five days, meaning that they excluded fruits, vegetables, legumes, coffee, tea, alcoholic beverages and chocolate (Appendix 1). On the day of the experiment and after overnight fasting, the volunteers consumed 10g of natural Mastiha and blood samples were obtained on timepoints 0h, 30min, 1h, 2h, 4h, 6h and 24h after Mastiha intake. Until timepoint 6h, they were allowed to consume only water (250mL in total). Serum and plasma were obtained after centrifugation of blood samples. All samples were kept at -80◦C until analysis.

Table 4. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Sex: Male	BMI > 25 kg/m^2
Age: 20-40 years old	Alcohol or drug abuse
BMI: 18.5-24.9 kg/m^2	Medication, vitamin or inorganic supplements
	Vegan or macrobiotic diet before and during the study
	Gastrointestinal diseases, such as atrophic gastritis, IBD, peptic ulcer or GI cancer

1.2 Sample size calculation

A 0.35 proportion of participants experiencing no increase in plasma terpenes at or before 6h post ingestion was assumed. The pre-specified 95% confidence limits were between 26,3 – 44,7%. Thus, the estimated sample size needed was at least 14 subjects.

1.3 Outcomes

Primary Outcome Measures

- The content of the main triterpenic acids identified in Mastiha, namely mastihadienonic and isomastihadienonic acids in plasma

Secondary Outcome Measures

- Regulation of oxidative stress biomarkers
- Amino acids profile identification (targeting at a profile of 50 amino acids)

1.4 Study flowchart

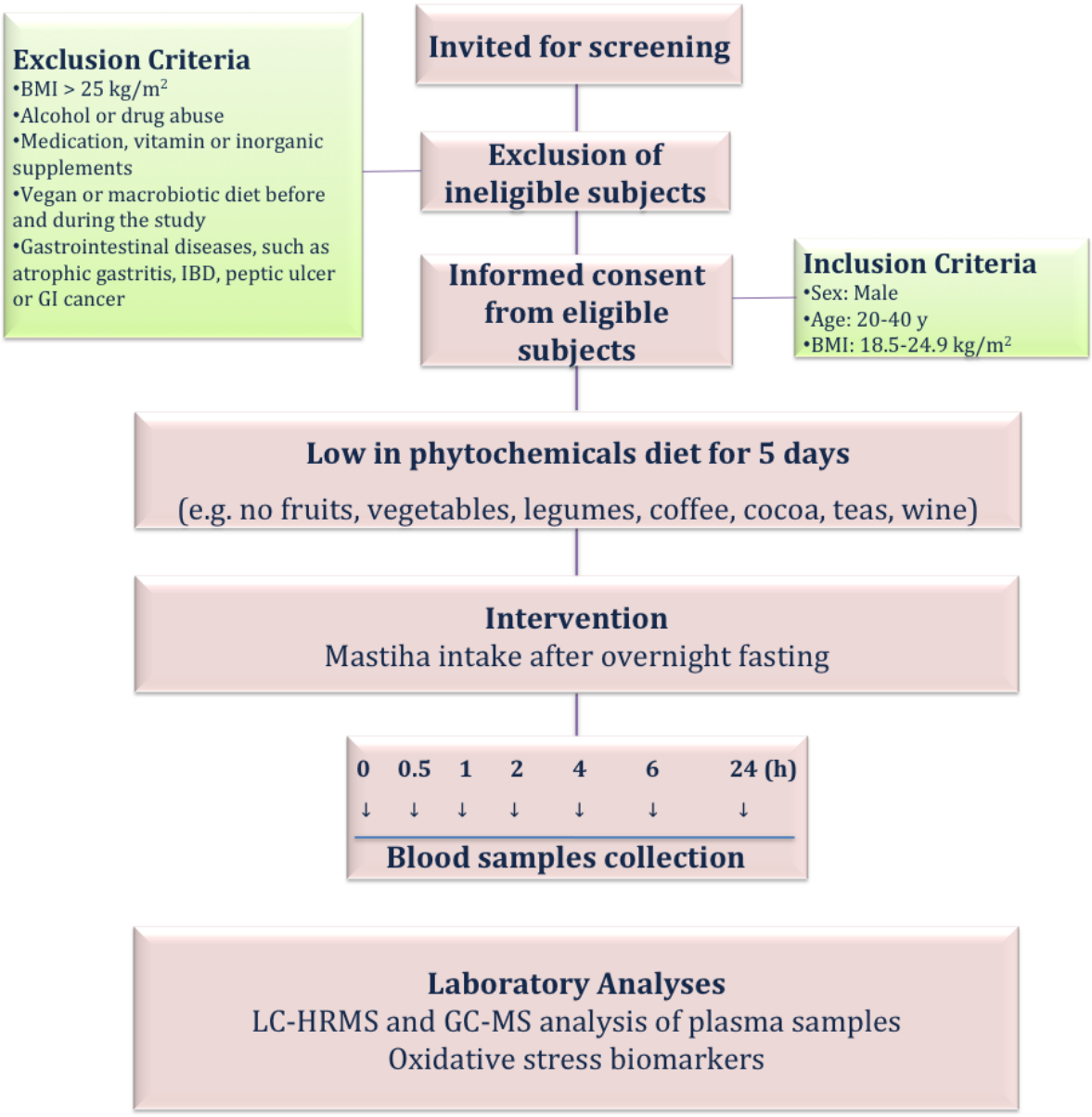


Figure 12. Bioavailability study flowchart

1.5 Methods

1.5.1 Quantification of Mastiha's metabolites with LC-HRMS

Chromatography separates the compounds of a mixture based on the different distribution coefficient between the mobile and tha stationery phases. LC uses a liquid mobile phase and it is used for qualitative and quantitative determination of a particular composition. It achieves qualitation through consistency between a sample and the target component in the peak time and quantitation through a standard curve that is created after different concentrations of standards are injected. LC is the most effective method to separate complex samples, but it cannot obtain any structural information (Pang et al., 2016).

MS completes qualitation by providing information on relative molecular mass and structural characteristics and quantitation by the relationship of the peak and compound content that the peak represented. MS is characterized by high selectivity and sensitivity (Pang et al., 2016). Tandem MS can also be employed offering structural information facilitating identification.

LC-MS takes the advantage of the physical separation potential of LC and the detection capacity of MS. Thus, LC-MS is widely used for the analysis of molecules in the presence of complex substances, for example microconstituents of natural products (Pang et al., 2016).

High resolution (HR) is necessary to separate peaks from one another and to confirm that particular ions of only one kind contribute to a measurement, and it is crucial for experiments involving complex mixtures, such as samples generated from a matrix (for example biological or environmental).

Herein, ultra-high-pressure liquid chromatography/tandem mass spectrometry coupled with high resolution/high accuracy mass spectrometry (UHPLC-HRMS/MS) was applied to identify the content of the isomers mastihadienonic and isomastihadienonic acids in plasma.

Standards preparation

Since Mastiha's constituents are not commercially available, fractionation of Mastiha and isolation

of pure substances from the acidic fraction was necessary to use them as standards. In summary, the isolation included a) the removal of the polymer content of Mastiha by selective precipitation, b) alkaline and acidic extraction for the separation of acidic and neutral compounds and c) application of HUPLC-HRMS/MS method for the initial profiling of total mastic extract without polymer.

Liquid chromatography analysis was performed on an Accela® High-Speed LC System (Thermo Scientific) and detection was carried out on a LTQ-Orbitrap® XL hybrid mass spectrometer equipped with an APCI source (Thermo Scientific). More specifically, separation was carried out on a Fortis® C18 (Fortis Technologies) column (150 mm × 2.1 mm, 1.7 μm) using a gradient of water supplemented with 0.1% (v/v) formic acid (A) and acetonitrile:methanol (1:1, v/v) (B). Column temperature was controlled at 40°C. Elution started with 95% A for 3min and decreased to 0% A in 21min. These conditions were kept for 2min before getting back to initial conditions for a 5min re-equilibration. 10μL of total Mastiha extract without polymer at a concentration of 100μg/mL (in H2O-MeOH, 1:1, v/v) were injected. MS data were acquired in negative mode, in the full scan m/z range of 100–1000, with a resolution of 30000. Data dependent acquisition was simultaneously performed using a CID value of 30% and a mass resolution of 7500. Capillary temperature and APCI Vaporizer temperature were set respectively at 275°C and 325°C whereas corona voltage was of 6 kV. Tube lens and capillary voltage were, respectively, tuned at -125 V and -10 V. Finally, nitrogen was used as the sheath gas (40 arbitrary units) and auxiliary gas (20 arbitrary units).

Plasma samples preparation

For the best extraction and isolation of Mastiha's compounds in plasma, samples protein precipitation with cold acetonitrile took place (cold AcN, 4°C, in the presence of formic acid). Then, in a centrifugal vacuum evaporator the supernatants were dried down and stored in -80°C until the analysis. After reconcentration in H_2O-MeOH samples were injected for analysis with the HUPLC-HRMS/MS method.

Liquid chromatography analysis was performed on the same instrument as described above except that the LTQ-Orbitrap® XL hybrid mass spectrometer was equipped with an ESI source (Thermo

Scientific). Separation was carried out on a Fortis® C18 (Fortis Technologies) column (150mm × 2.1mm, 1.7µm) using a gradient of water (A) and acetonitrile (B) both supplemented with 0.1% (v/v) formic acid. Column temperature was controlled at 40°C and the autosampler tray was maintained at 4°C. The gradient elution began at 100% A for 2min and decreased to 0% A in 15min. These conditions were kept for 5min before getting back to initial conditions for a 2min re-equilibration.

10µL plasma sample were injected. MS data were acquired both in positive and negative modes, in the full scan m/z range of 100–1000, with a resolution of 30000. Data dependent acquisition was simultaneously performed using a CID value of 30% and a mass resolution of 7500. Capillary temperature was set at 320°C in both polarities, whereas source voltage was 3.5 kV in ESI+ and 2.7 kV in ESI-. Tube lens and capillary voltage were, respectively, tuned at 120 V and 40 V in the positive mode and at -120 V and -40 V in the negative mode. Finally, nitrogen was used as the sheath gas (40 arbitrary units) and auxiliary gas (8 arbitrary units).

1.5.2 Plasma kinetic analysis

Maximum plasma concentrations (Cmax) and times to achieve maximum plasma concentrations (Tmax) were acquired through each individual's plasma concentration–time curve. The area under the plasma concentration–time curve (AUC) was calculated following the linear trapezoidal rule.

1.5.3 Amino acids profiling with GC-MS

Gas chromatography mass spectrometry (GC/MS) is a technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identfied and quantified.

After injection into the GC inlet, the sample solution is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The compounds of the sample are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at an ion source where compounds are converted to ions. A mass analyser separates the positively charged ions according to various mass related properties. Next, ions enter a detector whose output is amplified to boost the signal.

Herein, GC-MS was applied for the profiling of amino acids in plasma samples in an Agilent (Wallborn, Germany) series GC 6890 N gas chromatograph, coupled with an HP 5973 Mass Spectrometer detector (EI, 70 eV), split-splitess injector and an HP 7683 autosampler. An aliquot (2 μL) of the derivatized samples was injected into the GC at a split ratio of 1:15. Amino acids separation was achieved using a Phenomenex Zebron ZB-A amino acid analysis dedicated column (length = 10 m, internal diameter = 0.25 mm, film thickness = 25 μm). Carrier gas was helium at constant flow of 1.1 mL/min. The injector and transfer line temperatures were 250 and 340 °C, respectively. Initial oven temperature was 110 °C, increased to 320 °C at 30 °C/min and held at 320 °C for 3 min. A selective ion monitoring (SIM) GC/MS method was applied for the detection of 26 amino acids, based on the ±0.05 retention time (Rt) presence of target and qualifier ions at the predetermined ratios. The retention times, target and qualifier ions of the amino acids are shown in Table 5. Quantification was carried out employing norvaline as internal standard and constructing reference curves for every amino acid by means of standard solutions.

Table 5. Retention times, target and qualifier ions of the amino acids and internal standard.

Amino acid	Rt (min)	Target Ion (m/z)	Qualifier Ions (m/z)
Alanine	1.161	130	88
Allo-isoleucine	1.703	172	130
α-Aminoadipic acid	3.396	244	98
α-Aminobutyric acid	1.366	144	102
β-Aminoisobutyric acid	1.534	116	143, 172
Asparagine	2.150	155	69
Aspartic acid	2.723	216	130
Cysteine	5.897	248	216
Glutamic acid	3.081	230	170
Glutamine	3.720	187	84
Glycine	1.261	116	102
Histidine	4.585	282	168
Hydroxyproline	2.879	172	130
Isoleucine	1.731	172	130
Leucine	1.675	172	86
Lysine	4.407	170	128
Methionine	2.742	203	277
Ornithine	4.134	156	70
Phenylalanine	3.100	206	190
Proline	2.051	156	243
Serine	1.980	156	203
Thioproline	2.719	174	147
Threonine	1.943	160	101
Tryptophan	5.959	130	
Tyrosine	4.877	206	107
Valine	1.462	158	116
Norvaline (Internal Standard)	1.590	158	72

Plasma samples preparation

Plasma samples were prepared as follows: A solid phase extraction was followed by a derivatization and a liquid/liquid extraction. Derivatized samples were immediately analyzed by gas chromatography-mass spectrometry (EZfaast Amino Acid Analysis, Phenomenex Inc.).

1.5.4 Oxidative stress evaluation

Total serum oxidizability assay

Total serum oxidizability was applied to estimate oxidative stress and it is considered a method of indirect assessment of antioxidants bioavailability. More specifically, an increase in serum resistance to oxidation is usually associated with increase of antioxidants in serum, that themselves exhibit antioxidant activity or induced the synthesis of endogenous antioxidants.

The method is based on the formation of conjugated diene hydroperoxides that absorb at 245nm after oxidation of serum samples with copper sulfate. The final products of oxidation are aldehydes and ketones. Indicative lipid oxidation products are depicted in Figure 13.

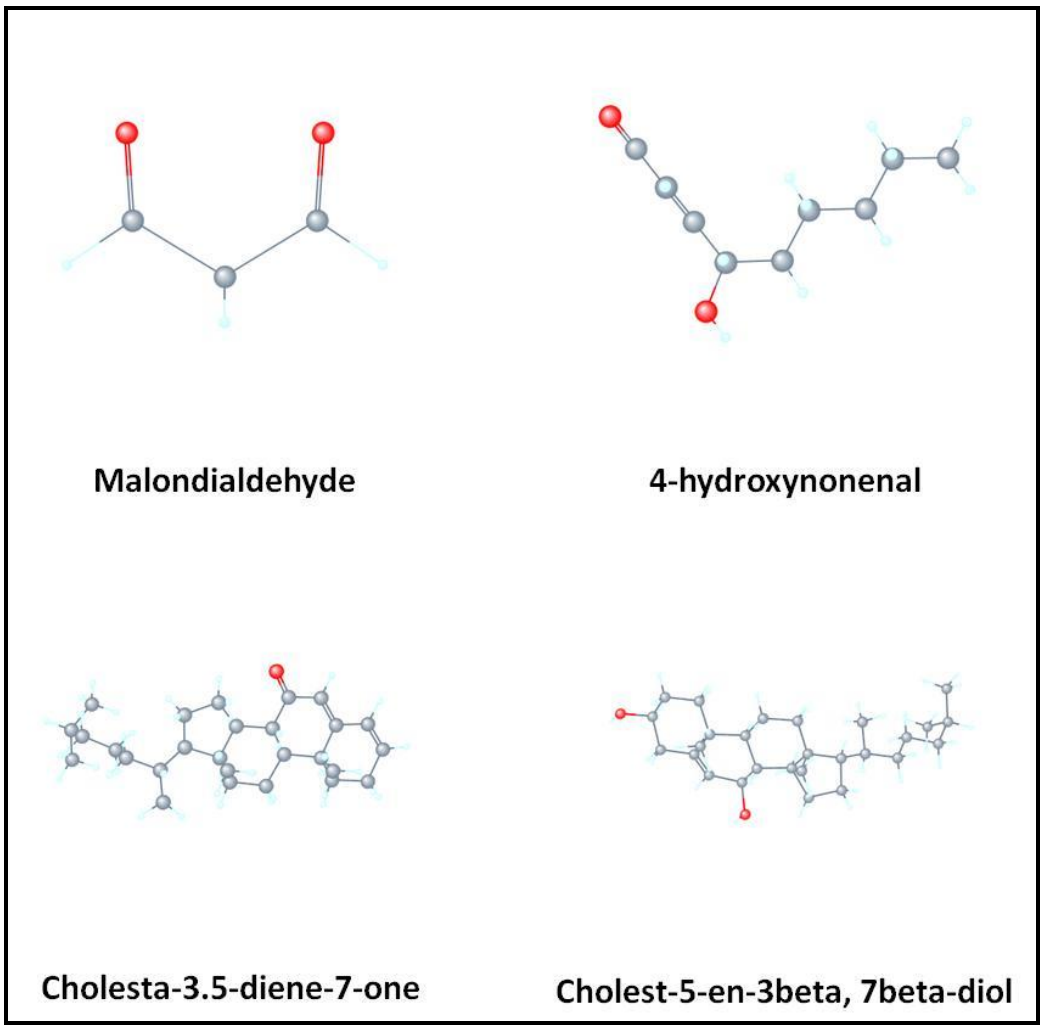


Figure 13. Indicative lipid oxidation products

A total of three phases—lag, propagation, and decomposition—were detected from each curve (Figure 14). During the lag phase, serum antioxidants are consumed and when serum is depleted of antioxidants, oxidizable lipids are oxidized to lipid hydroperoxides and 7-ketocholesterol (propagation phase), and after that they are converted to a variety of other products, including reactive aldehydes (decomposition phase). The kinetics of oxidation were analysed in terms of the lag-time preceding oxidation and expressed in seconds (t_{LAG}) according to Aurrekoetxea and colleagues (2010).

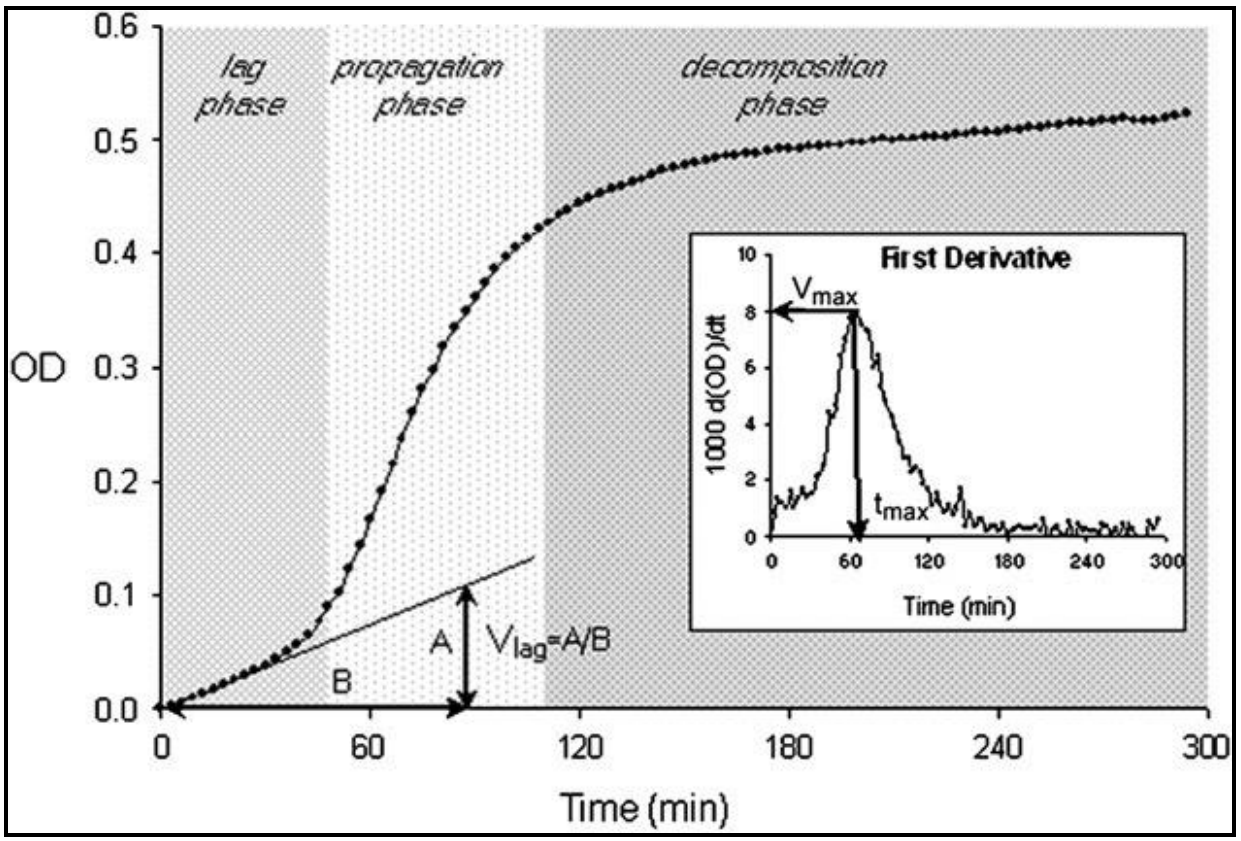


Figure 14. Representative curve illustrating the kinetics of Cu^{2+}-induced serum oxidation (Aurrekoetxea et al, 2010).

Samples preparation

Samples were diluted 1:12 in Phoshate Buffer Solution (PBS). Short rest of the serum sample before metal ion addition allowed the initiation of the oxidation (Figure 15). The reaction started after addition of a mixture of $CuSO_4$ with PBS. The increase in absorbance was plotted against time. Copper-induced oxidation was monitored every 2 minutes for a total of 4 hours (37°C) in a Biotek PowerWave XS2 Elisa Reader at 245nm.

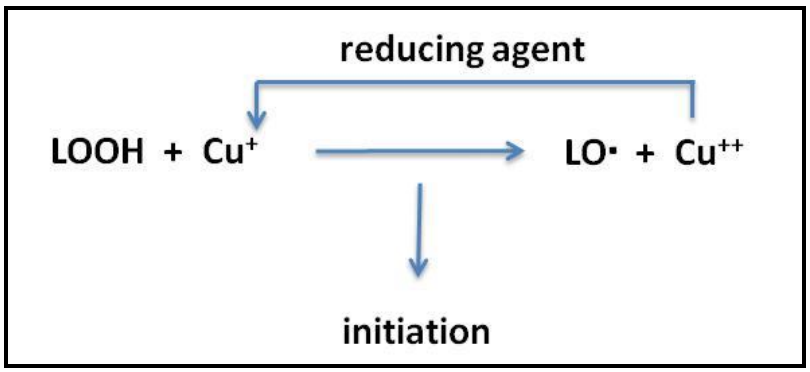


Figure 15. Initiation of lipids oxidation

Quantification of oxLDL in plasma

The measurement of oxidized low-density lipoprotein (oxLDL) as a biomarker of oxidative stress originates from the oxidative modification hypothesis of atherosclerosis. Oxidised LDL in blood is considered the most specific biomarker for oxidative stress evaluation in humans; most recently its measurement applying immunological methods (i.e. antibodies) has been recognized by European Food Safety Authority (EFSA) as a reliable method to assess oxidative damage with appropriate specificity (EFSA, 2011). Herein, the levels of oxLDL in plasma were measured in an ELISA reader (Biotek PowerWave XS2) at 450nm by applying sandwich Elisa (Mercodia, AB).

Sandwich ELISA is one of the most effective immunosorbent assays for detecting antigens with high sensitivity (Figure 16). In short, the wells of microtiter plates are coated with specific (capture) antibody and they are incubated with standards and samples containing antigen. After that, unbound antigen is washed out and an antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added. Another incubation follows, unbound conjugate is washed out and substrate is added. After final incubation, the hydrolysis of substrate is measured and this amount is proportional to the amount of antigen in the standard/sample.

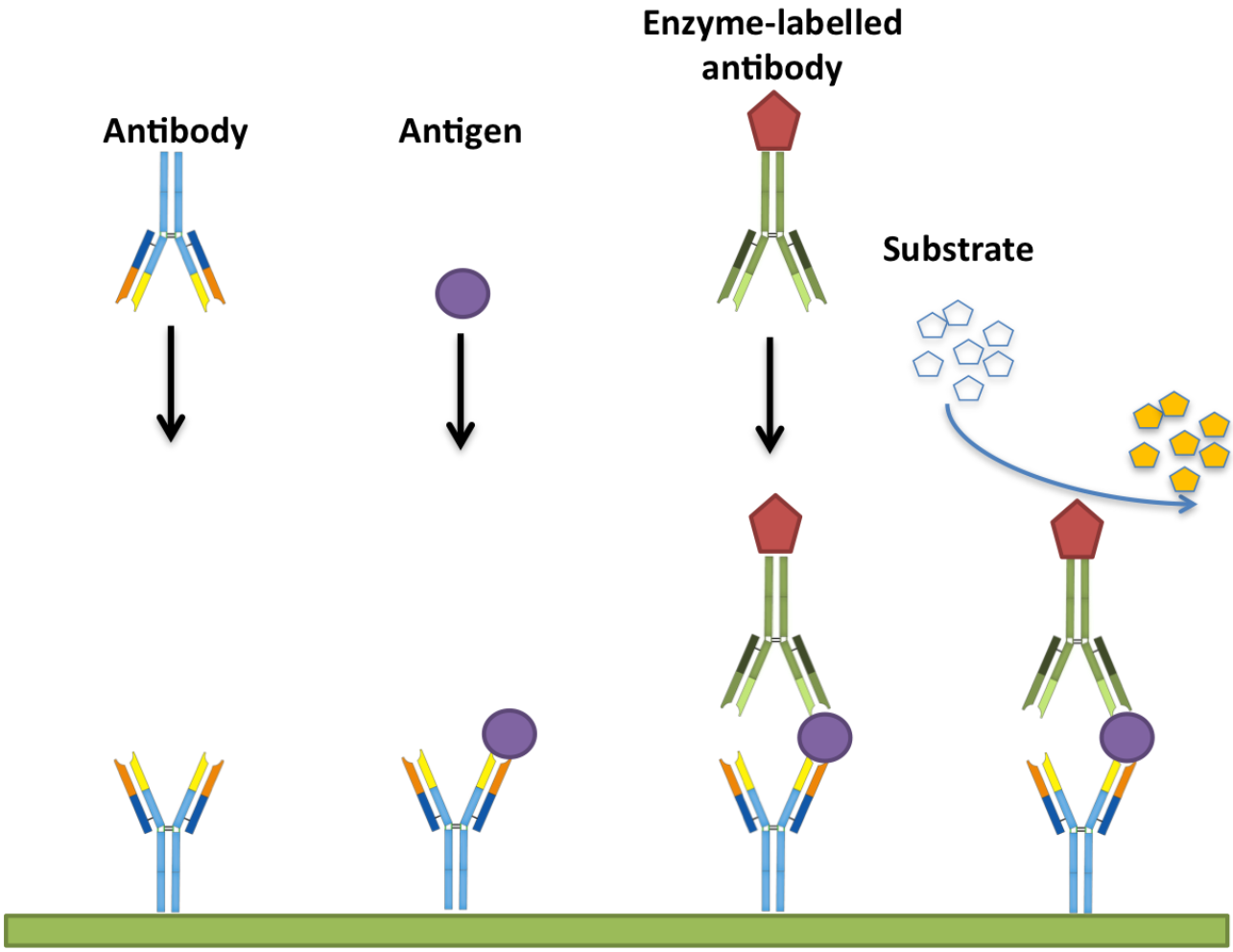


Figure 16. Sandwich ELISA principle.

Samples preparation

In order to prepare the plasma samples for the assay, LDL precipitation took place by mixing the samples with the LDL Precipitation Solution. After incubation at room temperature for 5 minutes and centrifugation for 20 minutes at 2000xg, the pellet had to be visible. Then, the pellet was collected for resuspension and dissolution in PBS. Further dilution of the samples in Assay Diluent was the final step before running the sandwich ELISA protocol.

Quantification of uric acid in serum

Uric acid in serum is considered as a marker of oxidative stress. In the presence of the enzyme uricase, uric acid is oxidized and produces H_2O_2. Next, the reaction of H_2O_2 with a phenolic derivative and 4-aminophenazone is catalyzed by the enzyme peroxidase (POD) and produces a red-coloured product (Figure 17). An increase in absorbance at 510 nm is proportional to the concentration of uric acid in the sample.

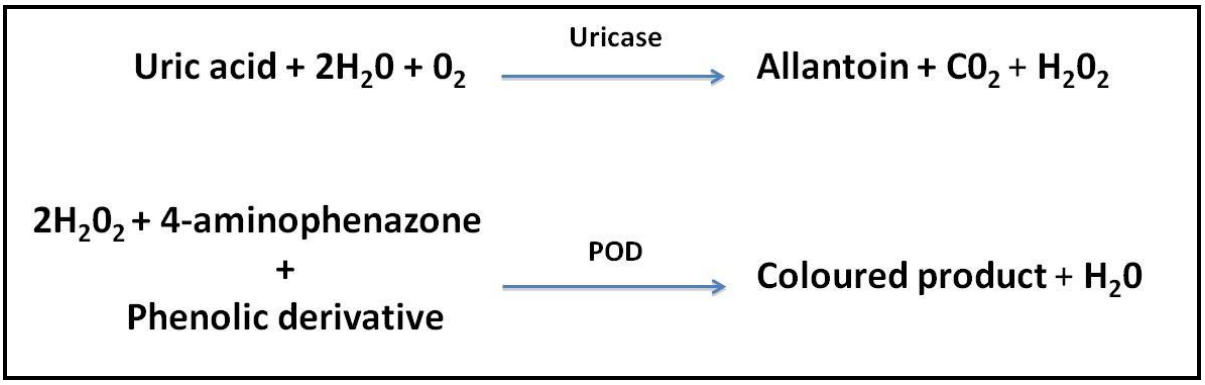


Figure 17. Uricase method for quantification of uric acid in serum

Samples preparation

Serum samples, standard and blank solutions were mixed with the working solution and incubated at 37°C for 5 min. according to the instructions of the manufacturer (Biosis, GR). After addition of distilled water, the absorbance was measured at 510nm in an ELISA reader (Biotek PowerWave XS2).

1.5.5 Statistical analysis

Statistical analysis was conducted with the SPSS software (SPSS for Windows, version 20.0, SPSS Inc., Chicago, IL, USA). Repeated measures ANOVA was performed to detect differences in terpenes concentrations, oxLDL levels, t_{LAG}, uric acid and amino acids levels. Pearson correlation coefficients were calculated to assess the relationship between amino acids concentrations and oxidative stress markers. Level of statistical significance is set at $p<0.05$.

CHAPTER 2

A STUDY ON THE EFFECTS OF A NUTRITIONAL SUPPLEMENT WITH NATURAL MASTIHA IN INFLAMMATORY BOWEL DISEASES.

This study was registered at ClinicalTrials.gov (NCT02796339) and was approved by the Ethics Committee of Harokopio University of Athens (49/29-10-2015) and IASO Hospital.

2.1 Aim and study design

Aim: The objective of this trial was to assess the effects of a supplement with Mastiha on IBD.

Study design: This was a randomised, double-blind, placebo controlled, parallel group clinical trial.

The enrolment was stimulated through the Inflammatory Bowel Disease Unit, IASO hospital, with the collaboration of the Hellenic Society of CD and UC patients. Confirmed IBD patients, with distinguished UC or CD were enrolled based on certain inclusion and exclusion criteria (Tables 6 & 7). The staff of the study provided detailed information regarding the aims, methods, anticipated benefits and potential hazards of the study and all patients received the PIL (Appendix 2). Ample time (48 hours) was provided in order to decide whether they wanted to participate in the protocol. Each patient agreeing to participate signed an Informed Consent document and the staff explained to patients that they were under no obligation to enter the trial and that they could withdraw at any time during the trial, without having to give a reason. A copy of the signed Informed Consent was given to the participant (Appendix 2).

Adult patients with biopsy proven IBD were randomly allocated to either Mastiha or placebo group according to a computer-generated scheme. Since access to the patients full list was not available at beginning of the trial due to privacy policy of the Hellenic Society of IBD patients, simple randomization was applied. After obtaining informed consent, the independent of the recruitment process contact would allocate participants to intervention. Randomization was applied by a computer generated random number list prepared by an independent investigator. Blinding of all other staff, analysts and participants was strictly maintained. The Mastiha group received Mastiha tablets at a dose of 2.8 g daily (4 x 700mg), while placebo group received respectively placebo

tablets. The intervention lasted 3 months for patients in relapse and 6 months for patients in remission. They received the tablets for the whole period of intervention at the start of the trial. Both groups continued their medical treatment, which should have been unaltered throughout the trial. Additionally, all patients received standard nutritional advice by dieticians and were encouraged to report any adverse effects during the intervention.

Tablets composition

Verum tablets: 70% Mastiha, 14% microcrystalline cellulose, 14% dibasic calcium phosphate anhydrous and 2% magnesium stearate.

Weight: 0.98 g/tablet

Placebo tablets: 49% microcrystalline cellulose, 49% dibasic calcium phosphate anhydrous, and 2% magnesium stearate.

Weight: 0.99 g/tablet

The resin was powdered prior to inclusion in tablet and the powder obtained was off-white to yellowish. The verum and placebo tablets shared identical appearance and organoleptic characteristics (Figure 18).

Figure 18. Verum and placebo tablets

Table 6. Eligibility criteria for patients in relapse

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	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
Active disease; CD: defined by Harvey & Bradshaw Activity Index ≥5 in CD, UC: defined by Partial Mayo Clinic Score ≥2 in UC	Enteral or Parenteral Nutrition; Alcohol or drug abuse, Vitamin or inorganic supplements, vegan or macrobiotic diet before and during the trial
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	Any malignancy in the year prior to screening; cardiovascular disease; peptic ulcer
Stable medication during the trial	Pregnancy, lactation

Table 7. Eligibility criteria for patients in remission

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
Inactive disease (>3 months); CD: defined by Harvey & Bradshaw Index ≤4, UC: defined by Partial Mayo Activity Index (0-1)	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
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	Any malignancy in the year prior to screening; cardiovascular disease; peptic ulcer
	Pregnancy, lactation

2.2 Sample size calculation

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).

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 was 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.

2.3 Outcomes

2.3.1. Patients in relapse

Primary Outcome

- A clinically significant improvement in quality of life as assessed by Inflammatory Bowel Disease Questionnaire (IBDQ) post-intervention

Secondary Outcomes

- Improvement in inflammatory markers in serum and stool post-intervention
- Reduction in disease severity as reflected by decrease in Harvey-Bradshaw Index (HBI) and Partial Mayo Score (PMS) post-intervention
- Improvement in biochemical markers post-intervention

- Improvement in oxidative stress markers post-intervention
- Identification of the effects on amino acids profile post-intervention

2.3.2. Patients in remission

Primary Outcome

- A reduction in clinical relapse rate in the period of 6 months

Secondary Outcomes

- Improvement in inflammatory markers in serum and stool post-intervention
- Improvement in biochemical markers post-intervention
- Improvement in quality of life as assessed by Inflammatory Bowel Disease Questionnaire (IBDQ) post-intervention.
- Identification of the effects on amino acids profile post-intervention

2.4 Study flowcharts

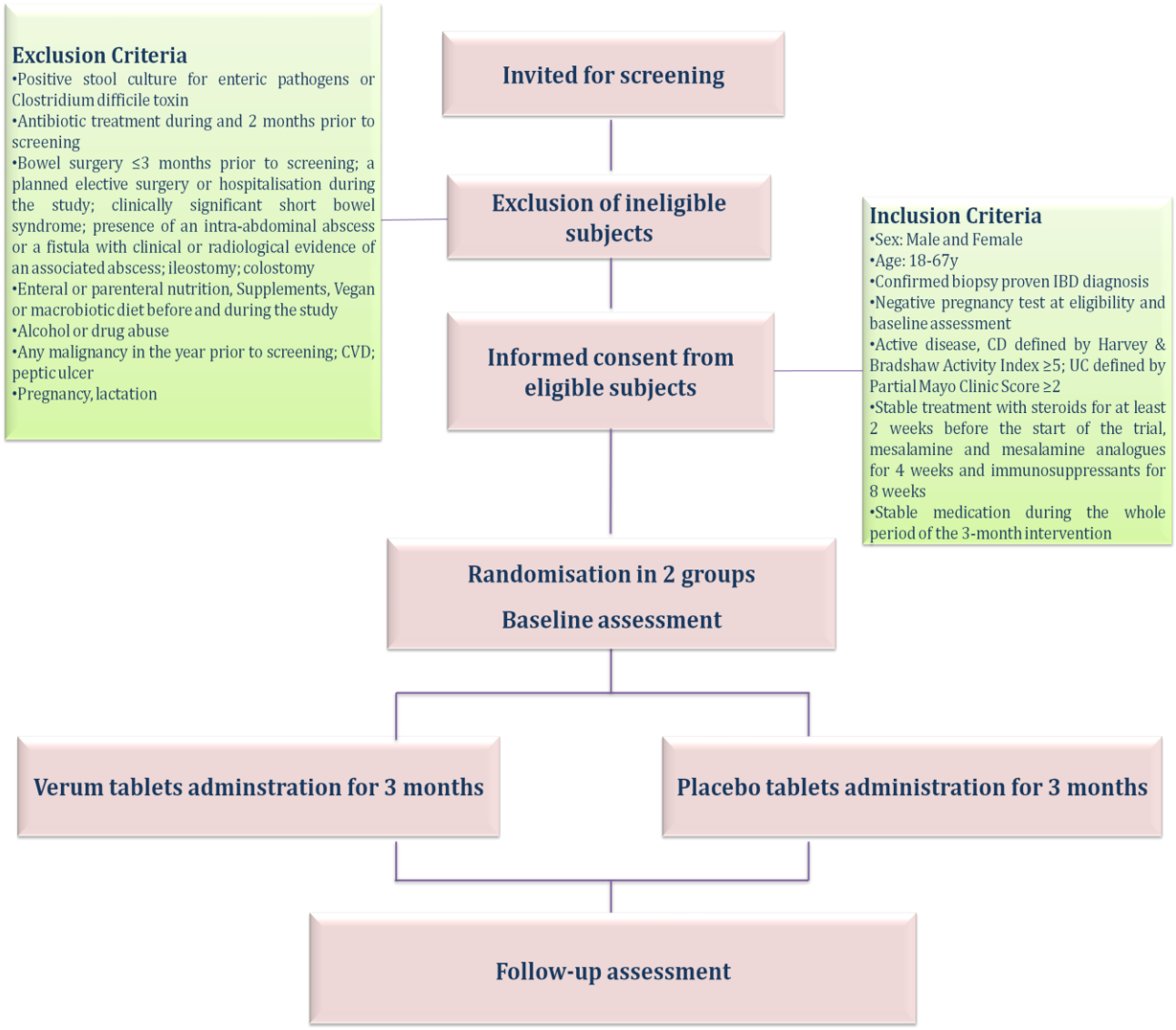


Figure 19. Study flowchart for patients in relapse

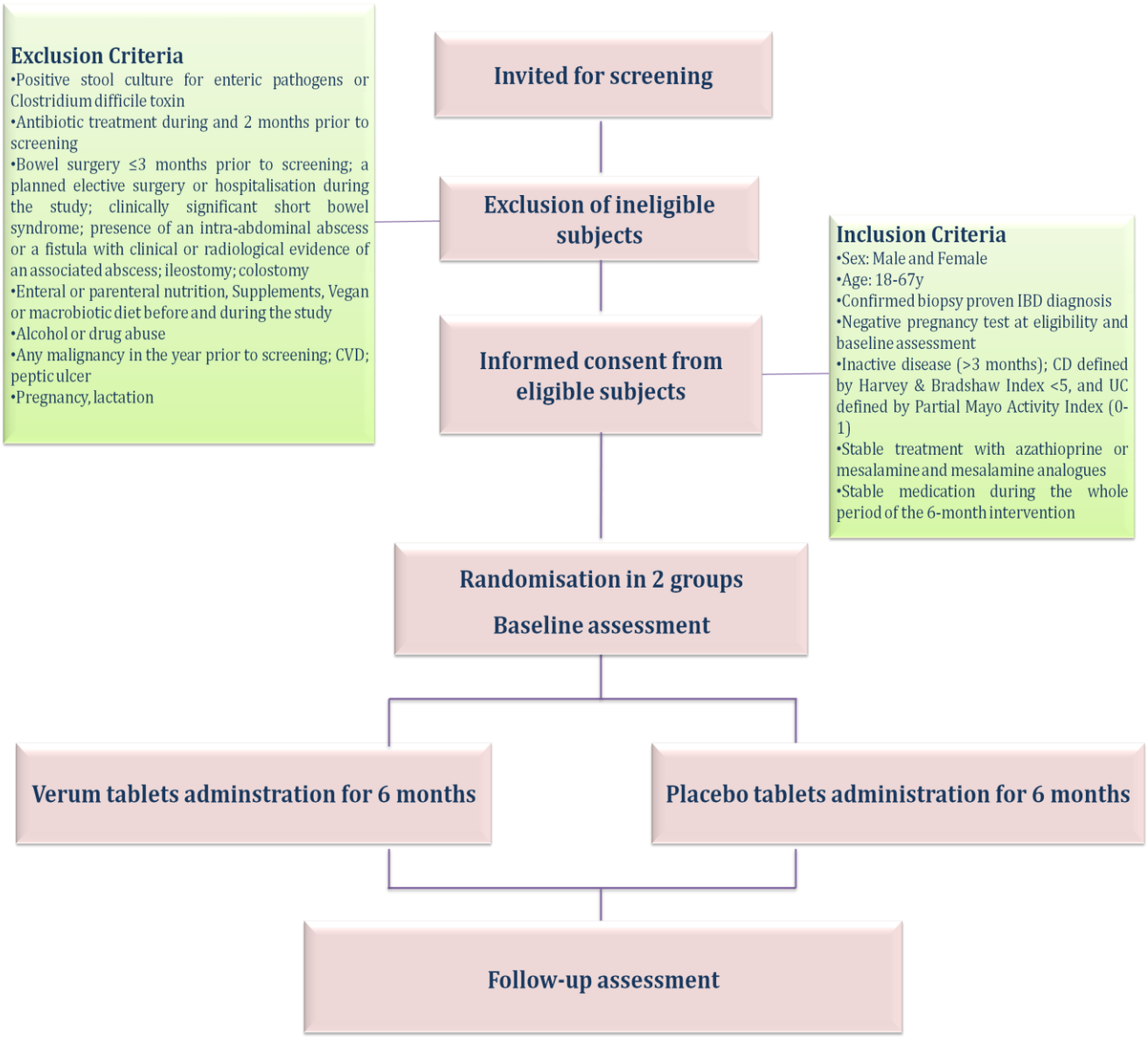


Figure 20. Study flowchart for patients in remission

2.5 Methods

2.5.1 Baseline assessment

Medical history

A medical history was recorded by the gastroenterologist, including general information (i.e. allergic reactions, smoking habits, etc.) as well as specific data regarding IBD (i.e. brief history of IBD, age of diagnosis, complications, treatment) (Appendix 2).

Disease activity

The HBI was calculated for CD patients and the PMS for UC patients by the gastroenterologist based on symptoms and clinical evaluation (Appendix 2).

Quality of life

Quality of life was assessed applying the IBDQ (Guyatt et al., 1989). IBDQ consists of 32 questions about bowel, social, systemic and emotional performance and is scored from 32 to 224 points. A higher score of IBDQ indicates a better quality of life. IBDQ is validated in Greek language (Pallis et al., 2001) (Appendix 2).

Anthropometry

Body weight was measured to the nearest 0.1 kg. Height was measured with a standard stadiometer to the nearest millimeter. Both measurements were performed twice. Body Mass Index was also calculated.

Dietary assessment

Adherence with Mediterranean diet was evaluated with MedDiet Score with higher scores indicating a greater adherence to the Mediterranean diet (Panagiotakos et al., 2006). A 24h recall was also collected by an experienced dietitian (Appendix 2).

Blood and stool sample collection

Standard Blood sampling (20mL) was performed. After collection, blood samples were centrifuged at 3000rpm for 10 minutes at 4◦C for plasma, serum, buffy coat and whole blood isolation. All samples were stored at -80◦C until further analysis. Additionally, patients provided a stool sample using a stool preparation system filled with extraction buffer IDK Extract® (Immundiagnostik, AG). Stool extracts were kept for a maximum of 9 days at -20◦C until further analysis.

2.5.2 Follow-up assessment

There was a biweekly telephone contact with the patients to check upon compliance and side effects. At the end of the intervention, all parameters of baseline assessment were evaluated again, including disease activity, quality of life, anthropometry, dietary assessment, stool and blood sample collection.

2.5.3 Laboratory analyses

Biochemical profile

Serum Amylase, Iron (Fe), Total & Direct Bilirubin, Glucose, Urea, Total Cholesterol, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Triglycerides (TG), Albumin, Serum Glutamic-Oxaloacetic Transaminase (SGOT), Serum Glutamic-Pyruvic Transaminase (SGPT), γ-Glutamyl Transferase (γ-GT), Alkaline Phospatase (ALP), Lactate Dehydrogenase (LDH) and Plasma Fibrinogen

were quantified with an automatic biochemical analyzer at baseline and at follow-up.

Inflammatory biomarkers in serum and stool

The levels of inflammatory biomarkers IL-6 and IL-10 in serum samples were quantified in an ELISA reader (Biotek PowerWave XS2) applying sandwich Elisa (R&D for IL-6, Origene for IL-10) at baseline and at follow-up. The method is based on the capacity of these molecules to bind to a specific capture antibody. Then a detection antibody is added, which is specific for the complex of each molecule with the capture antibody. Other proteins are washed out and, after adding enzyme-substrate, a reaction takes place, whose product absorbs at 450nm.

The same principal of ELISA sandwich was applied for the stool markers of inflammation calprotectin, lactoferrin and lysozyme. After collection with the specific stool preparation system, samples were centrifuged and assessed via the ELISA protocol (Immundiagnostik AG).

Oxidative stress assessment

Total serum oxidizability, oxLDL and uric acid were assessed in patients with active IBD as described in Section 1.5.4 at baseline and at follow-up. In addition, the ratios oxLDL/LDL and oxLDL/HDL were calculated at baseline and at follow-up.

Amino acids metabolic profiling

Amino acids levels in plasma were assessed as described in Section 1.5.3 at baseline and at follow-up.

2.6 Statistical analysis

Continuous variables are presented with mean and standard deviation (SD). Quantitative variables are presented with absolute and relative frequencies. For the comparison of proportions chi-square and Fisher's exact tests was applied. For the comparison of means between Mastiha and placebo groups Student's t-test was computed. Differences in changes of study variables from baseline to follow up period between the two groups were evaluated using repeated measurements analysis of variance (ANOVA). Variables with skewed distribution were log-transformed for the analysis of variance. All p values reported were two-tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 20.0).

PART C

RESULTS & DISCUSSION

CHAPTER 1

BIOAVAILABILITY OF THE MICROCONSTITUENTS OF NATURAL MASTIHA IN HEALTHY ADULTS

The results are presented in Paper 1.

1.1. Description of the study population

All the 17 participants completed the two phases of the study (wash-out and intervention). No adverse effects of Mastiha intake were reported. The baseline anthropometric and biochemical characteristics, which were among the reference ranges, are presented in Tables 8 and 9. As so, the volunteers recruited were in good health and no metabolic disorder was detected.

Table 8. Baseline anthropometric characteristics

Age (y)	27.1 ± 1.6
Height (cm)	179.6 ± 1.5
Weight (kg)	81.1 ±2.1
BMI (kg/m^2)	24.8 ± 0.7
Body fat (%)	17.7 ± 1.5
Total Body Water (%)	57.8 ± 1.2
Muscle mass (kg)	61.5 ± 1.0
Bone mass (kg)	3.2 ± 0.1

Data are presented as Mean ± Standard Error of Mean.

Table 9. Baseline biochemical characteristics

Red Blood Cells (/μL)	5026923.1 ± 103313.0
White Blood Cells (/μL)	7492.3 ± 696.5
Hemoglobin (g/dL)	15.7 ± 0.3
Hematocrit (g/dL)	45.5 ± 0.8
Mean Corpuscular Volume (fl)	90.6 ± 0.8
Mean Corpuscular Hemoglobin Concentration (g/dl)	34.6 ± 0.2
Mean Corpuscular Hemoglobin (pg/RBC)	31.3 ± 0.2
Glucose (mg/dL)	94.4 ± 2.9
Urea (mg/dL)	36.4 ± 1.8
Creatinine (mg/dL)	1.0 ± 0.0
Total Cholesterol (mg/dl)	184.8 ± 9.4
Triglycerides (mg/dL)	93.2 ± 14.6
High-Density Lipoprotein (mg/dL)	52.3 ± 3.2
Low-Density Lipoprotein (mg/dL)	113.9 ± 8.0
High Risk Rate	3.7 ± 0.3

Values are Mean ± Standard Error of Mean.

1.2. Profiling of total mastic extract without polymer

Total Mastiha Extract Without Polymer (TMEWP) is characterized by the presence of triterpenoids and in particular penta- and tetra-cyclic triterpenes with various substitution patterns such as 8-tirucallene and oleanene derivatives all eluted between 14.87 and 20.17 minutes.

The major peaks detected in TMEWP, are attributed to mastihadienonic (MNA), isomastihadienonic (IMNA), moronic acid (MA), oleanonic acid (OLEA) and olean-12,18-dien-3-olic acids. Concentrations of these triterpenic compounds are known, i.e. mastihadienonic acid 20.9mg/g mastiha and isomastihadienonic acid 19mg/g mastiha (Paraschos et al, 2007). The APCI(-) based peak chromatogram of TMEWP is presented in Figure 21.

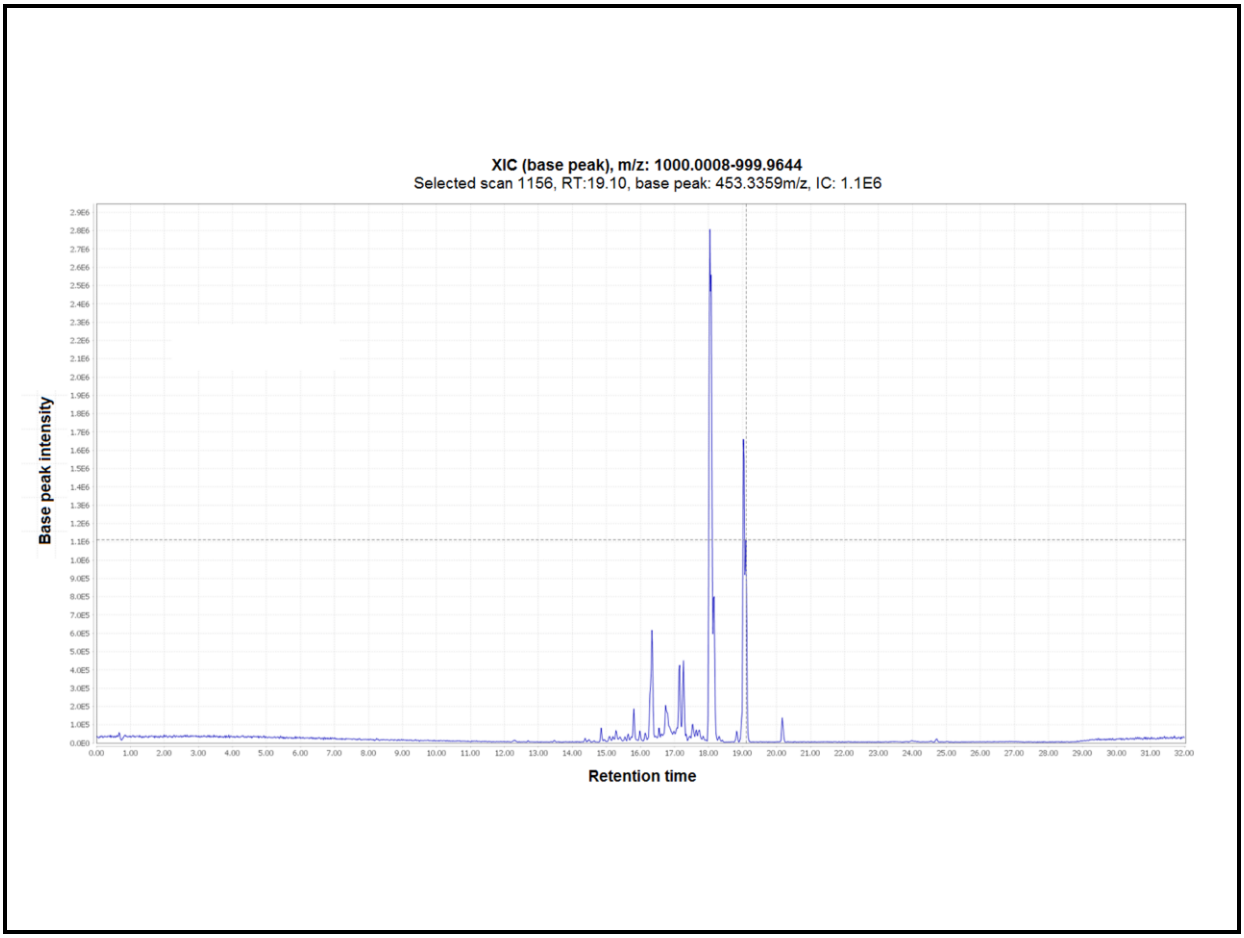


Figure 21. A typical APCI(-) based peak chromatogram in the m/z range of [100-1000] of the total mastic extract without polymer.

1.3. LC-MS analysis of plasma samples

Using extraction ion method (mass window: m/z 453.32-453.34) and based on the XICs (extraction ion chromatograms), major constituents of Mastiha's extracts MA and OLEA (Rt=12.96 and 13.18 min), MNA and IMNA (Rt=14.35 and 14.54 min) were detected in plasma samples. ESI(-) extracted ion chromatograms (m/z=453.32-453.34) of plasma samples of the same volunteer at different time points is depicted in Figure 22.

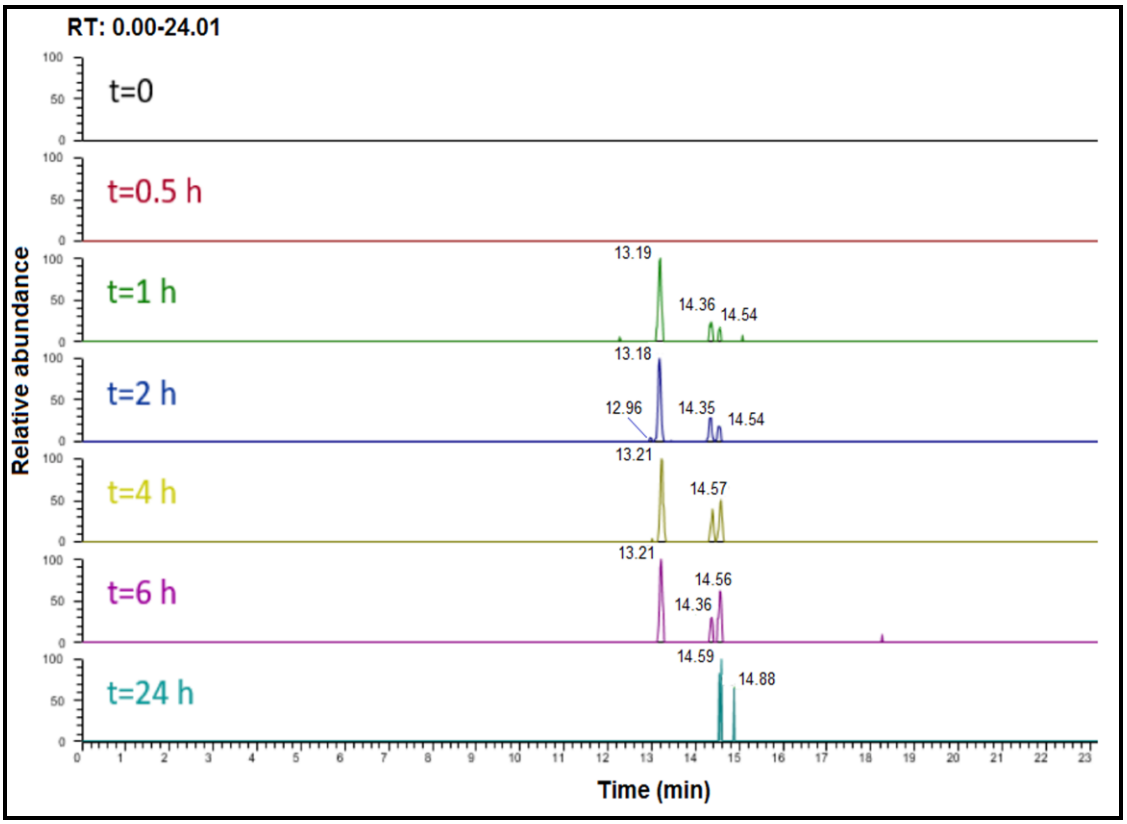


Figure 22. ESI(-) extracted ion chromatograms (m/z=453.32-453.34) of plasma samples of the same subject at different time points. Moronic and oleanonic acids eluted at Rt=13.18 min, mastihadienonic (MNA) and isomastihadienodic (IMNA) acids eluted at Rt=14.35 and 14.54 min.

All of these four terpenes were detectable 0.5h after Mastiha intake. MNA concentration at 0.5h (23.2 ±4.7ng/mL) increased significantly compared with baseline (p=0.005), reached its peak at 2h (106.1±41.8ng/mL) and followed a decreasing trend until 24h, when its concentration

(13.1±1.9ng/mL) was significantly increased compared with baseline (p=0.001). IMNA concentration reached a peak at 4h (212.4±74.2ng/mL) but no significant difference between different timepoints was observed. OLEA concentration followed a similar pathway with MNA, since its concentration at 0.5h (11.7±1.6ng/mL) and at 24h (10.3±1.0ng/mL) was significantly increased compared with baseline (p<0.001). Its peak concentration was detected at 4h. Last but not least, MA was detectable since timepoint 0.5h reaching a peak (9.1±5.0ng/mL) at 4h after Mastiha intake. The group mean plasma concentration-time curves are depicted in Figure 23.

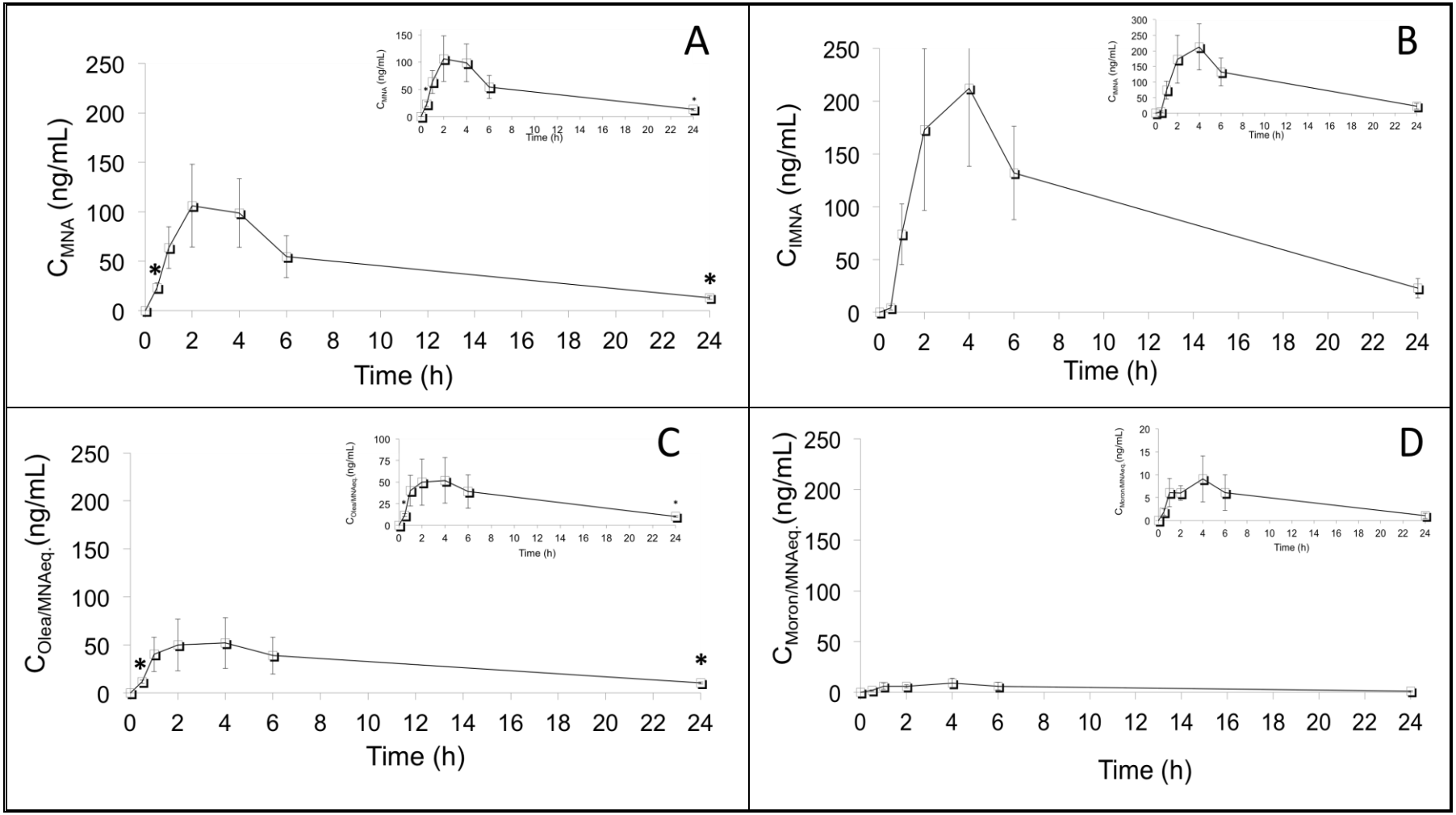


Figure 23. Plasma concentration-time curves for Mastihadienonic Acid (A), Isomastihadienonic Acid (B), Oleanonic Acid (C) and Moronic Acid (D). Values are presented as mean ± standard error of mean. * Mean values were significantly different from baseline ($p<0.05$).

Plasma kinetic parameters, namely Cmax, Tmax and AUC are presented in Table 10. The highest Cmax was observed for IMNA (293.1 ± 81.2ng/mL), whereas the lowest Cmax was observed for MA. Tmax was similar for IMNA, OLEA and MA. The highest AUC was observed for IMNA and the lowest for MA.

Table 10. Kinetic variables of plasma Mastihadienonic Acid, Isomastihadienonic Acid, Oleanonic Acid and Moronic Acid after Mastiha administration in healthy men.

Terpenes	Cmax (ng/mL)	Tmax (hrs)	Area Under Curve (ng x hrs/mL)
Mastihadienonic Acid	179.6±48.0	2.7±0.4	1077.5±274.3
Isomastihadienonic Acid	293.1±81.2	4.5±1.3	2269.1±696.3
Oleanonic Acid	173.8±69.8	4.1±1.3	695.8±210.3
Moronic Acid	19.3±6.2	4.1±1.3	102.9±39.4

Values are Mean ± Standard Error of Mean.

1.4. Oxidative stress biomarkers

Serum resistance to oxidation is expressed as lag time (tLAG) in seconds, and more specifically as the difference (ΔT) of tLAG of each time point from tLAG 0h. An increasing trend of serum resistance to oxidation was observed since time point 0.5h. This increase was statistically significant on time point 4h (402.3 ± 65.0sec), reached a peak on time point 6h (524.6 ± 62.9sec) and remained statistically significant until 24h post-ingestion (424.2 ± 48.0sec) ($p<0.05$). oxLDL levels (expressed as % change from 0h), were reduced significantly from time point 1h to time point 6h post-ingestion (Figure 24).

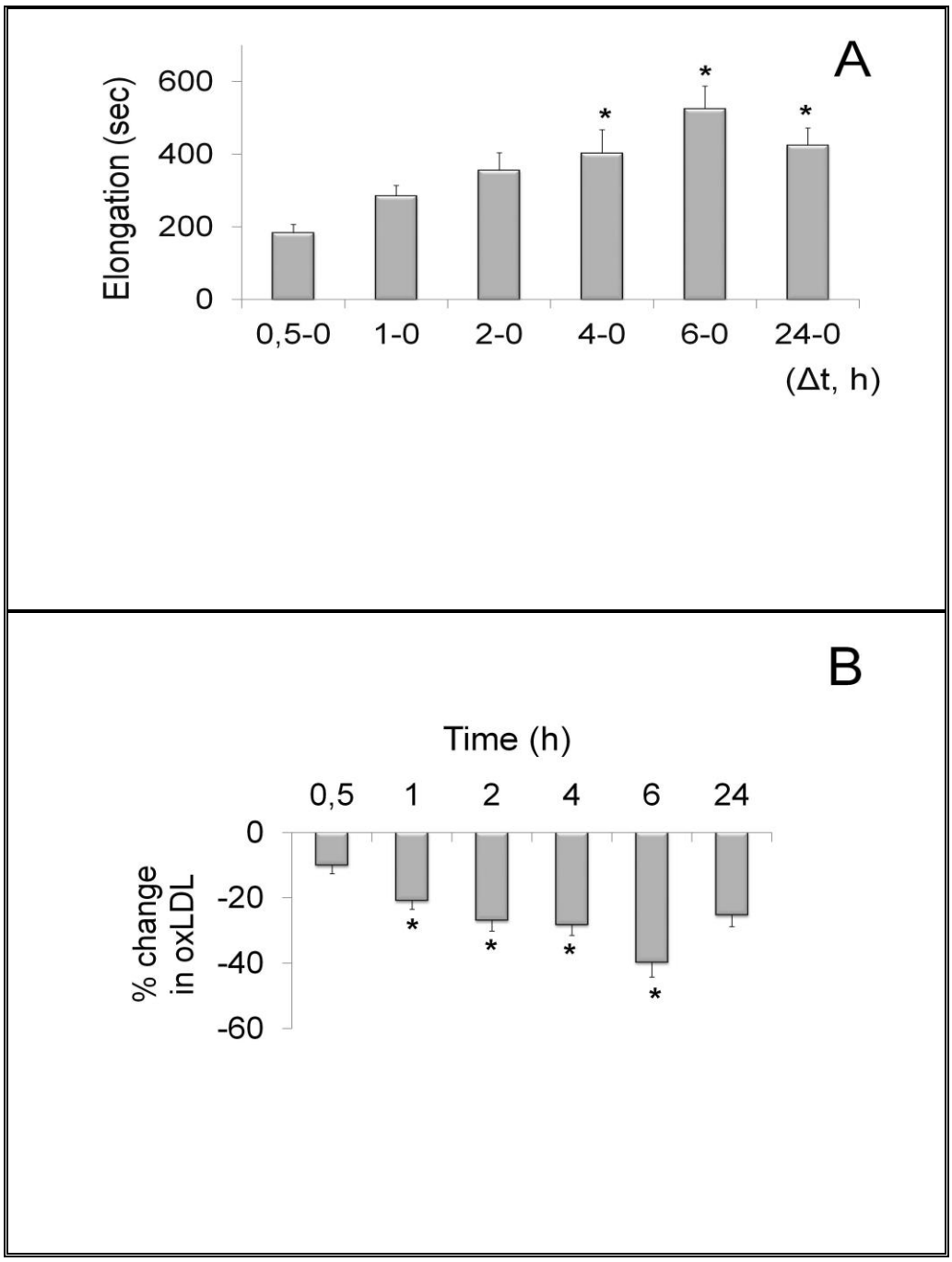


Figure 24. Evaluation of oxidative stress. A) Serum resistance to oxidation was estimated applying the copper sulphate oxidation assay. B) Levels of oxidized LDL were measured applying a sandwich Elisa Assay. Significance level was set at $p<0.05$.

Table 11 presents uric acid levels on different timepoints. UA concentration increased from T_0 to T_1 and peaked at T_2, whereas it decreased until T_{24}. No significant differences were observed between these timepoints.

Table 11. Uric acid concentrations on timepoints 0, ½ , 1, 2, 4, 6 and 24 hours after Mastiha intake.

Time points (hrs)	C (mg/dL)
T_0	3.6±0.9
$T_{1/2}$	3.4±0.8
T_1	3.7±1.1
T_2	4.3±2.1
T_4	3.6±0.7
T_6	3.5±0.8
T_{24}	3.5±0.7

Values are mean± standard deviation.

1.5. Discussion

The investigation of the absorption and bioavailability of several phytochemicals and their metabolites is of great importance the last decade due to their several health benefits (Neacsu et al., 2017). Terpenes have attracted a wide research interest worldwide. Their presence in diet and particularly in foods of plant origin and in herbs with beneficial properties has lead to further exploration of their bioavailability in the present survey. Mastiha selected herein is a natural product of the Mediterranean Diet rich in terpenes and found with several health-beneficial properties. Its composition is known; consisting of 30% polymer, as well as an acidic and a neutral fraction. The main compounds identified are MNA and IMNA, constituents of both the acidic and neutral fractions (Assimopoulou & Papageorgiou, 2005; Paraschos et al., 2007). The acidic fraction, rich in MNA and IMNA has been mainly attributed with anti-inflammatory and antibacterial properties.

This is the first study in healthy humans to evaluate the bioavailability of Mastiha's terpenes applying LC-MS/MS. MS-based approaches, especially when hyphenated to chromatographic techniques such as LC, offer sensitive and selective alternatives that play a critical role in the metabolomic investigation (Sumner et al., 2006). UHPLC has provided considerable improvements in terms of resolution, repeatability and time of analysis for high throughput investigation of complex mixtures (Guillarme et al., 2007). The application of this method provides the study with a significant methodological advantage.

These results demonstrate that the major terpenes of Mastiha, namely MNA and IMNA, but also MA and OLEA are bioavailable already 0.5h after intake reaching their peaks between 2h and 4h. Enzymatic transformation such as cyclizations, deglycosylations and oxidation/hydroxylations take place in terpene metabolism. As regards terpenes contained in Mastiha, existing data are derived from an animal study where Mastiha was administered in diet and terpenes peaked between 0.5 and 1h (Lemonakis et al., 2011). No data in humans were available to the best of our knowledge. The lack of meal or any food or formula matrix, the potential enterohepatic circulation and intermediate metabolism may explain the identified time points. It is rather possible that metabolites of the targeted compounds peak in different time points depending on the pathways

in which they are implicated.

The above-mentioned terpenes are considered quantitatively and qualitatively as marker compounds of TMEWP (Lemonakis et al., 2011; Assimopoulou et al., 2005). Also, other structurally close compounds, which are abundant in lower concentration levels according to the method applied, were detected. Five main peaks were detected corresponding to m/z 467.31 ($C_{30}H_{43}O_4$, RDBeq.: 9.5), suggesting that the extract is rich in 11-oxo-mastihadienonic acid and structural isomers according to Assimopoulou and Papageorgiou (2005).

When TMEWP was profiled, more terpenes were detected apart from those mentioned above as also detected by Paraschos and colleagues (2007). This could explain the relatively lower number of identified terpenes in plasma. Medicinal plant matrices play a crucial role in the bioavailability of terpenes, since they are subjected to digestion within the mouth and stomach before accessing the small intestine. Mechanical and enzymatic actions, different pH conditions, as well as transformations into usually more water-soluble and more readily excreted in the urine compounds affect this process. These transformations appear mainly in the liver, but also in the gastrointestinal tissue, lungs, kidneys, brain and blood (Furtado et al, 2017).

Regarding oxidative stress, most studies on bioavailability of phytochemicals have shown a peak in plasma antioxidant capacity 1-2h after intake, coming partly into agreement with our results. A postprandial study of Kanellos and colleagues (2013) investigated the absorption and bioavailability of phytochemicals in raisins and their impact on serum oxidation resistance in 15 healthy volunteers. It was shown that serum resistance to oxidation and plasma total phenolics reached their peak 1h after raisin ingestion. In the present study, serum resistance to oxidation increased significantly at 4h. However, oxLDL decreased significantly since 1h after Mastiha's administration, indicating that Mastiha may act as an antioxidant. Oxidised LDL in blood is considered the most specific biomarker for oxidative stress evaluation in humans; most recently its measurement applying immunological methods (i.e. antibodies) has been recognized by European Food Safety Authority (EFSA) as a reliable method to assess oxidative damage with appropriate specificity (2011). It is also important to mention that in the study of Kanellos and colleagues (2013) a total of 17 phytochemicals were identified and quantified in plasma including oleanolic

acid (a pentacyclic triterpenoid), but also phenolic compounds such as vanillic acid, gallic acid, tyrosol and quercetin, that have been previously identified in Mastiha as well. Each compound followed a different kinetic pathway, nevertheless 13 of 17 phytochemicals peaked in plasma 1h after administration. In the present protocol, even if the kinetic pathways were similar between the four terpenes detected, IMNA, OLEA and MA reached their peak 4h after administration, whereas MNA peaked at 2h coinciding possibly with the peak of uric acid levels in plasma at 2h. The significant increase in serum resistance to oxidation at 4h, 6h and 24h and the significant decrease in oxLDL at 1h, 2h, 4h and 6h could coincide with the detection and the peak concentrations of Mastiha's terpenes between 2h and 4h. However, whether the terpenes quantified in plasma or their metabolites could affect directly oxidative stress in humans is questionable since plasma endogenous antioxidants may have also been involved in increase of antioxidant capacity. Our hypothesis that differences in antioxidant capacity in plasma are owed to terpenes bioavailability stems from the fact that terpenes have proven antioxidant properties in vivo (González-Burgos et al., 2012), as well as the main fraction of Mastiha, which is rich in terpenes rather than phenolic compounds. Even if phenolic compounds have been detected in leaves and in fruits of Mastiha tree (Amel et al., 2016), only simple phenols in low concentrations or even in traces have been detected in the resin (Kaliora et al., 2004).

Another study evaluated the postprandial changes in plasma oxygen radical absorbance capacity (ORAC), as well as in concentrations of tocopherols, catechins, oxidized LDL, and malondialdehyde (MDA) in response to pecan meals. Sixteen healthy volunteers were allocated to 3 meal groups composed of whole pecans, blended pecans, or an isocaloric meal of equivalent macronutrient composition in a crossover design. Following the whole and blended pecan test meals, plasma concentrations of γ-tocopherols doubled at 8h and hydrophilic- and lipophilic-ORAC increased 12 and 10% at 2h, respectively. After whole pecan consumption, oxidized LDL significantly decreased 30, 33, and 26% at 2, 3, and 8h, respectively, following a similar pattern to our findings. Epigallocatechin-3-gallate concentrations at 1h and 2h were significantly increased at 1h compared with baseline and after the control test meal, showing that these phytochemicals are bioavailable after 1h of consumption (Hudthagosol et al., 2011). However, these data should be compared to our results with caution as the food matrix (i.e. carbohydrates, lipids) of both raisin and pecans

affects the bioavailability kinetics.

This fact was also pointed out by several researchers investigating the kinetics of boswellic acids (pentacyclic terpenes), which are constituents of Boswellia serrata gum resin extract. They demonstrated that boswellic acids are bioavailable but food intake as well as type of formula affects their bioavailability demonstrating that food matrix plays a critical role (Skarke et al., 2012; Sharma et al., 2004; Sterk et al., 2004). The analysis of different formulations of curcumin for improved relative oral bioavailability in humans by Purpura and coworkers (2017) showed that an innovative γ-cyclodextrin curcumin formulation significantly improved the absorption of curcuminoids in healthy humans subjects. Quercetin aglycone derived from onion skin extract powder was shown significantly more bioavailable than that from quercetin dihydrate powder filled hard capsules (Burak et al., 2017). Even if in the present protocol, the participants received the oral dose of Mastiha after overnight fasting, those studies could give a future direction for further investigation of terpenes bioavailability through different types of formulas and/or in the presence of different meals.

Apart from the plant matrix, food matrix, type of formula, gut microbiota, sex, age another factor that affects the bioavailability and biological responsiveness on phytochemicals is genetic/epigenetic variability (Manach et al., 2017). A recent postprandial study on 33 healthy males that consumed a test meal containing tomato puree with 9.7 mg of all-trans lycopene demonstrated that the ability of to respond to lycopene was explained partially by a combination of 28 SNPs in 16 genes (Borel et al., 2015a). Further research upon the interindividual variability in dietary β-carotene bioavailability in healthy men showed that 69% of the variance in the postprandial chylomicron β-carotene response was explained by 25 SNPs in 12 genes (Borel et al., 2015b). These data could also lead to further exploration of terpenes bioavailability in the context of genetic variability.

Since gender differences or the menopause in females have been shown to affect bioavailability of phytochemicals (Domínguez-Perles et al., 2017; García-Villalba et al., 2014), recruitment of males herein empowers the study design and outcomes. In addition, this protocol has several more strengths such as the rigorous selection of the participants according to the inclusion and exclusion

criteria that leaded to a homogenous sample, the application of cutting edge methodology (i.e. LC-MS/MS, oxLDL quantification) and the adequate power. Nevertheless, the fact that the successful washout was self-declared and was evaluated through a 24h recall is a limitation of our protocol.

In conclusion, this protocol demonstrates that triterpenic acids are bioavailable and may exhibit antioxidant effects on humans after oral administration of a natural terpene-rich product, such as Mastiha. Further developments are needed to characterize both the absorption/kinetic as well as its implication on the general metabolism including pathways analysis. Since terpenes exhibit a plethora of beneficial properties on human health, the deeper knowledge of the factors affecting their bioavailability and responsiveness will improve their use towards public health improvement.

CHAPTER 2

DETECTION AND QUANTIFICATION OF PLASMA FREE AMINO ACID RESPONSE TO ORAL SUPPLEMENTATION WITH MASTIHA

The results are presented in Paper 2.

2.1 Quantification of amino acids

A total of 26 plasma free AAs were detected and 25 of them were quantified. Table 12 presents plasma concentrations of free AAs on different time-points. Analysis with repeated measures ANOVA showed some significant differences in plasma concentrations of valine, proline and ornithine. More specifically, the BCAA valine was significantly lower on T4 compared with baseline. Proline concentration decreased significantly on T6 compared with baseline and T2, whereas it was significantly lower on T4 compared with T1. Ornithine levels were significantly decreased on T2 compared with baseline.

Table 12. Plasma concentrations of free amino acids at different time-points in healthy men.

Amino acid	C_0 (nmol/mL)	$C_{1/2}$ (nmol/mL)	C_1 (nmol/mL)	C_2 (nmol/mL)	C_4 (nmol/mL)	C_6 (nmol/mL)	P
Alanine	283.5±79.4	271.1±83.6	266.9±75.8	271.2±62.0	253.4±64.3	248.4±69.6	NS
Aloisoleucine	63.6±15.9	59.1±11.6	55.3±8.9	56.0±7.0	55.2±8.4	53.9±9.7	NS
α-Aminoapidic acid	ND	ND	ND	ND	ND	ND	NS
α-Aminobutyric acid	25.2±7.7	25.4±7.6	25.9±8.1	26.7±8.0	25.6±8.2	26.4±8.0	NS
β-Aminobutyric acid	99.46±3.2	98.9±3.1	98.4±2.7	97.7±2.2	98.8±2.5	99.0±3.6	NS
Asparagine	47.7±12.4	48.8±12.4	48.2±11.8	47.6±10.4	50.2±12.1	48.0±10.9	NS
Aspartic acid	14.3±7.4	13.7±6.4	13.4±7.5	14.3±8.5	14.4±9.2	14.3±6.5	NS
Cysteine	30.0±3.4	31.1±2.8	31.7±2.9	32.1±5.6	30.1±1.6	30.4±3.4	NS
Glutamic acid	26.9±21.6	23.3±15.6	23.1±21.9	12.2±9.1	13.3±10.9	9.1±6.8	NS
Glutamine	352.8±96.0	351.5±81.2	356.1±86.1	373.0±118.6	326.4±94.9	334.0±114.2	NS
Glycine	205.9±24.0	206.6±26.9	205.0±29.3	212.6±30.1	213.7±34.1	198.2±56.0	NS
Histidine	77.9±18.1	76.6±21.9	80.3±22.2	77.6±26.2	78.8±22.3	76.1±18.9	NS
Hydroxyproline	16.7±3.9	17.5±4.1	17.2±4.6	16.5±3.3	16.2±3.2	15.5±2.6	NS
Isoleucine	74.2±18.2	69.0±13.3	64.4±10.4	65.6±8.4	64.6±9.6	63.2±11.1	NS
Leucine	148.7±26.9	140.8±17.9	135.0±15.3	136.6±12.7	140.6±13.4	141.9±16.8	NS
Lysine	169.6±48.5	164.6±42.0	166.3±52.8	160.6±57.2	173.9±44.4	172.7±40.0	NS

Amino acid	C_0 (nmol/mL)	$C_{1/2}$ (nmol/mL)	C_1 (nmol/mL)	C_2 (nmol/mL)	C_4 (nmol/mL)	C_6 (nmol/mL)	P
Methionine	23.3±7.6	23.7±6.1	22.6±5.2	22.7±4.3	23.9±4.7	21.7±5.4	NS
Ornithine	54.2±12.0[1]	50.9±10.4	49.8±11.1	45.8±9.6[1]	47.9±10.9	47.1±8.8	[1]p=0.043
Phenylalanine	66.2±11.7	65.4±8.4	62.4±10.6	62.0±7.5	67.2±14.7	63.4±8.1	NS
Proline	238.9±115.6[2]	238.1±102.8	233.4±113.4[3]	229.3±99.1[4]	218.0±109.5[3]	194.9±102.7[2,4]	[2]p=0.028 [3]p=0.036 [4]p=0.040
Serine	111.8±20.8	113.7±22.0	115.8±26.2	110.1±17.4	112.6±18.4	106.9±33.5	NS
Thioproline	ND	ND	ND	ND	ND	ND	NS
Threonine	124.4±24.1	130.4±26.7	126.9±34.3	122.3±30.4	124.8±20.6	117.0±25.5	NS
Tryptophane	67.6±13.5	67.3±11.1	64.2±15.1	60.9±12.2	61.4±12.1	59.5±11.4	NS
Tyrosine	61.5±21.7	60.7±18.3	55.5±16.7	52.2±14.1	52.4±12.2	48.2±9.7	ns
Valine	409.3±65.7[5]	389.6±57.2	379.5±59.1	380.9±42.9	354.8±55.1[5]	344.3±55.8	[1]p=0.014

Values are presented as mean ± standard deviation. Mean values sharing the same number in superscript were significantly different ($p<0.05$). ND: not detected; NS: not significant

2.2. Correlation of amino acids with oxidative stress biomarkers

Levels of free amino acids in plasma were correlated with oxidant/antioxidant status in blood (Table 13). Alpha-aminobutyric levels were positively and moderately correlated with UA on T0 and T4. Leucine and serine concentrations were moderately associated with UA at baseline, and there was a strong positive correlation between cysteine and uric acid on T2. Additionally, total serum oxidizability was positively correlated on T2 with valine, and negatively with phenylalanine, methionine, tryptophane and cysteine levels. Furthermore, leucine levels were positively correlated with serum oxidizability on T4, whereas threonine, phenylalanine, ornithine, lysine, histidine, tyrosine and tryptophane were negatively associated with serum oxidizability on T6.

Levels of amino acids were also correlated with concentrations of MNA, IMNA, MA and OLEA on different timepoints as presented at Table 13. Alanine levels were positively correlated with MNA, IMNA and OLEA concentrations, β-aminobutyric acid was positively associated with OLEA and MA, whereas leucine was positively correlated with MNA and OLEA. Aloisoleucine and isoleucine concentrations were positively associated with MNA, IMNA, OLEA and MA, whereas proline levels were positively associated with OLEA and MA concentrations. Methionine levels were positively associated with IMNA and OLEA, glutamine was positively associated with OLEA and MA, and ornithine was positively correlated with IMNA, OLEA and MA. Lysine was positively correlated with MA levels and cysteine was negatively associated with MNA. Last, histidine levels were negatively related with MNA, IMNA, OLEA and MA.

Table 13. Pearson coefficients for amino acids correlations with uric acid, serum oxidizability and terpene levels on different time points

Amino acid (nmol/L)	Uric acid	Serum oxidizability	MNA	IMNA	OLEA	MA
Alanine			R=0.484(T_6)*	R=0.540 (T_6)*	R=0.491 (T_4)*	
Aloisoleucine			R=0.642 (T_1)*	R=0.522 (T_1)*	R=0.656 (T_1)**	R=0.491 (T_1)* R=0.514 (T_2)*
α-Aminobutyric acid	R=0.522 (T_0)* R=0.524 (T_4)*					
β-Aminobutyric acid					R=0.574 ($T_{1/2}$)*	R=0.575 ($T_{1/2}$)*
Aspartic acid		R=0.542 (T_0)* R=0.540 ($T_{1/2}$)*				
Cysteine	R=0.736 (T_2)**	R=-0.629 (T_2)*	R=-0.607 (T_2)*			
Glutamine					R=0.516 (T_4)*	R=0.565 (T_4)*
Histidine		R=-0.613 (T_6)*	R=-0.504 (T_1)* R=-0.574 (T_2)*	R=-0.576 (T_2)*	R=-0.519 (T_2)*	R=-0.548 (T_2)*
Isoleucine			R=0.646 (T_1)**	R=0.523 (T_1)*	R=0.654 (T_1)**	R=0.494 (T_1)* R=0.501 (T_2)*
Leucine	R=0.520 (T_0)*	R=0.555 (T_4)*	R=0.532 (T_1)*		R=0.490 (T_1)*	
Lysine		R=-0.718 (T_6)**				R=0.531 ($T_{1/2}$)*
Methionine		R=-0.563 (T_2)*		R=0.561 (T_6)*	R=0.537 ($T_{1/2}$)*	
Ornithine		R=-0.545 (T_6)*		R=0.699 ($T_{1/2}$)**	R=0.641 ($T_{1/2}$)*	R=0.726 ($T_{1/2}$)**
Phenylalanine		R=-0.553 (T_2)* R=-0.613 (T_6)**				
Proline					R=0.705 ($T_{1/2}$)**	R=0.647 ($T_{1/2}$)**
Serine	R=0.589 (T_0)*					
Threonine		R=-0.717 (T_6)**				
Tryptophane		R=-0.496 (T_2)* R=-0.633 (T_6)**				
Tyrosine		R=-0.703 (T_6)**				
Valine		R=0.604 (T_2)*				

(*p<0.05; **p<0.01).

2.3. Discussion

Herein, we have used targeted GC-MS plasma-free AA analysis to identify alterations in AAs in healthy non-obese subjects in response to terpenes.

Interestingly, we detected some significant changes in valine, proline and ornithine. Valine, a BCAA with direct effects on glutamine and arginine synthesis and on the balance of BCAAs (Wu, 2009), was significantly decreased at T4 after mastiha administration. BCAAs, including valine, have been associated with metabolic dysfunctions (Wang et al., 2011; Newgard et al., 2009). Therefore, the observed decrease in valine might be of importance. In addition, proline, an AA with direct and indirect effects on collagen synthesis, cellular redox state, stress response and immunity (Wu, 2009), was significantly decreased at the time point 6 h. Upregulation of proline biosynthesis is an oxidative stress response in mammalian cells (Krishnan et al., 2008), and so proline is increased in subjects with metabolic dysfunctions (Guevara-Cruz et al., 2018). In this acute trial design, the decrease in proline levels is indicative of the regulation in proline metabolism due to increase in serum antioxidant potential 6 h post-ingestion (Papada et al, 2018). Furthermore, ornithine was decreased at time point 2 h compared with baseline. Ornithine participates in ammonia detoxification as an intermediate of the urea cycle and contributes to mitochondrial integrity (Wu, 2009). Recently, an inverse association of plasma ornithine with low-grade chronic inflammation in healthy humans was reported (Pietzner et al., 2017). Herein, the change reported in plasma ornithine at T2 post-ingestion coincides with a peak in plasma concentration of MNA (Papada et al., 2018).

UA, which is the end product of purine metabolism, is a potent antioxidant acting as free radical scavenger and a chelator of transition metals. Nevertheless, it is also considered as a pro-oxidant since reactive oxygen species (ROS) are byproducts of its synthesis, and it seems to stimulate synthesis of proinflammatory molecules (Pasalic et al., 2012). Although, we did not detect any significant changes in UA levels, α-aminobutyric acid, leucine and serine were positively associated with UA at baseline. Additionally, α-aminobutyric acid correlated with UA at T4 and cysteine was strongly associated with UA at T2. This relationship is supported by a recent study in Japanese subjects with or without lifestyle-risk factors for future cardiovascular disease exploring the association between plasma-free AAs and high levels of UA. Apart from significant correlations of

plasma-free AAs with UA, significant inverse associations with high UA levels were observed for arginine, asparagine and threonine in healthy subjects as well, indicating the possibility of interplay between plasma-free AAs and UA (Mahbub et al., 2017). However, hereby, whether the changes in AAs have a direct relationship with antioxidant status or whether the relationship between plasma-free AAs and UA is causal remain unanswered.

An increase in lag time in serum lipoprotein oxidation assay following administration with mastiha terpenes was shown, as described in Results section 1.3. Herein, the AAs found significantly modulated after acute administration—valine at T4, proline at T6 and ornithine at T2—were not correlated with increase in lag time in serum lipoprotein assay or with UA or with terpenes quantified in plasma (Papada et al., 2018) at the respective time points. However, some significant correlations between the identified plasma-free AAs and lag time or UA or plasma terpenes were observed at different time points as presented in Table 14.

As regards oxidative stress, related biomarkers are presented in literature with several controversies. Uric acid itself is characterized as a "controversial" marker since its antioxidant effects are manifested only in the hydrophilic environment of biological fluids, such as serum or plasma, acting as a powerful scavenger of carbon-centered and peroxyl radicals, while it loses its ability to scavenge lipophilic radicals or break the radical chain propagation within lipid membranes. Serum lipid oxidizability assay is an ex vivo challenge model that is dependent on the circulating bioactive compounds and represents a biomarker of a highly relevant aspect of human health as oxidative stress. On the other hand, while glutathione is an important antioxidant, its measurement in our protocol of acute administration and measurement of antioxidant status post-ingestion would not be applicable, due to its high turnover time in plasma (Fläring et al., 2009).

Data on the effect of terpenes in the metabolic profile of humans are limited. To the best of our knowledge, limonene—a lipophilic monoterpene—is the only terpene examined as regards metabolic changes in humans. The study of Miller and co-workers has shown that limonene supplementation regulates a series of plasma metabolites (Miller et al., 2015). In our study terpene administration was found to modulate the AA profile in healthy humans. In the study of Schmedes et al. (2018) a significantly reduced level of valine and isoleucine was observed after a lean-seafood

intake. The authors hypothesized that this effect was rather due to direct endogenous effects on the host's own metabolic pathways rather than to the contained AAs in diets, as the differences in AAs between control and intervention diets were insignificant (Schmedes et al., 2018). Herein, administration of mastiha terpenes, namely MNA and IMNA, may have resulted in modulation of plasma-free AAs due to the formation of hydrogen bonds with AAs. As such, hydrogen bonds were detected between MNA and IMNA with AAs in the study of Vuorinen et al. (2015).

Even if our study has some limitations, such as the absence of the full metabolic profile identification, these limitations are counterbalanced by several strengths. The wash-out period of phytochemicals and the overnight fasting before the acute experiment contributed to a clearer pattern of the metabolic response of AAs to terpenes, since dietary AAs were absent. In addition, our homogenous sample of healthy and under-no-medication young men (20–40 years old) of the same ethnicity and within the normal body mass index range is an important strength, since previous (or literature) data show that plasma-free AAs profile of adults differ by gender, age, body mass index and ethnicity.

This study showed that plasma-free AAs are modulated in response to terpenes in healthy non-obese adult males. Further research is needed to clarify whether modulation of AAs by phytochemical compounds may be of physiological relevance.

CHAPTER 3

A STUDY ON THE EFFECTS OF A NUTRITIONAL SUPPLEMENT WITH NATURAL MASTIHA IN PATIENTS WITH ACTIVE INFLAMMATORY BOWEL DISEASE

The results are presented in Papers 3 and 5.

3.1. Description of the study population

Sixty IBD patients (N=60) met our criteria for recruitment (Figure 25). Out of the 60 patients, 27 (45.0%) were randomised to the placebo group and 33 (55.0%) to the verum group, while 40 (66.7%) of them were diagnosed with CD and 20 (33.3%) with UC.

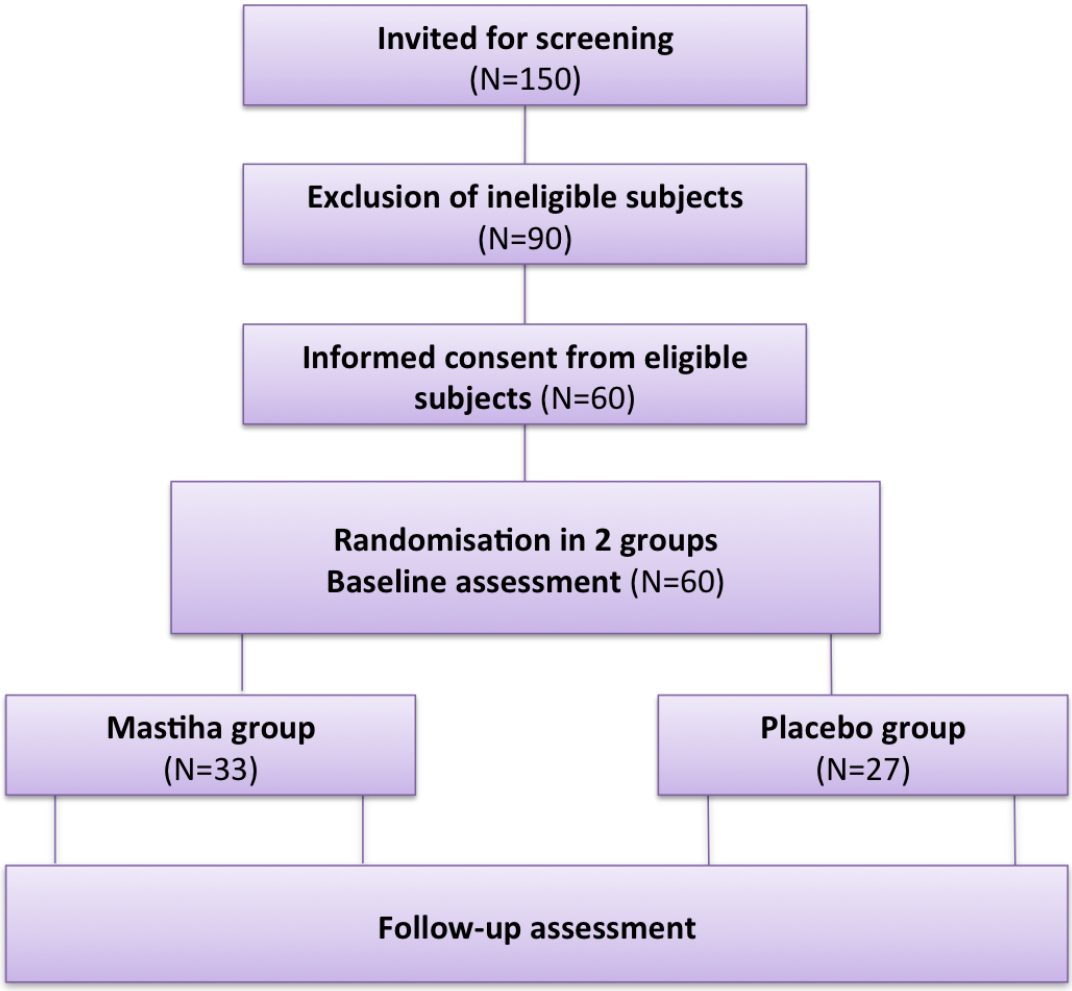


Figure 25. Study population in relapse

Clinical, anthropometric and demographic characteristics for all groups are presented in Tables 14 and 15. Patients of both placebo and verum groups were similar in total sample number and in each type of disease regarding their demographics.

Table 14. Demographics in all groups. Results are given as N (%) of the total number.

	IBD (N=60)			CD (N=40)			UC (N=20)		
	Placebo (27)	Mastiha (33)	P	Placebo (18)	Mastiha (22)	P	Placebo (9)	Mastiha (11)	P
Sex									
Females	9 (33.3)	18 (54.5)	0.100[+]	7 (38.9)	11 (50)	0.482[+]	2 (22.2)	7 (63.6)	0.092[++]
Males	18 (66.7)	15 (45.5)		11 (61.1)	11 (50)		7 (77.8)	4 (36.4)	
Age (yrs), mean (SD)	45 (17.4)	38.2 (11.9)	0.076[‡]	48.1 (17.6)	36 (10.7)	0.011[‡]	38.9 (16.1)	42.5 (13.6)	0.597[‡]
Marital status									
Unmarried/Divorced	14 (51.9)	14 (42.4)	0.466[+]	9 (50)	11 (50)	1.000[+]	5 (55.6)	3 (27.3)	0.362[++]
Married	13 (48.1)	19 (57.6)		9 (50)	11 (50)		4 (44.4)	8 (72.7)	
Education (yrs)									
1-9	3 (11.1)	7 (21.2)	0.510[+]	2 (11.1)	2 (9.1)	0.884[++]	1 (11.1)	5 (45.5)	0.326[++]
10-12	5 (18.5)	7 (21.2)		3 (16.7)	6 (27.3)		2 (22.2)	1 (9.1)	
>12	19 (70.4)	19 (57.6)		13 (72.2)	14 (63.6)		6 (66.7)	5 (45.5)	
Smoking[#]									
No	20 (74.1)	23 (69.7)	0.653[+]	11 (61.1)	15 (68.2)	0.641[+]	9 (100)	8 (72.7)	0.211[++]
Yes	7 (25.9)	10 (30.3)		7 (38.9)	7 (31.8)		0 (0)	3 (27.3)	

[#] Smoking habits are presented herein at baseline, [+]Pearson's chi-square test; [++]Fisher's exact test; [‡]Student's t-test

Table 15. Clinical and anthropometric characteristics for all groups.

	IBD (N=60)		P	CD (N=40)		P	UC (N=20)		P
	Placebo (27)	**Mastiha (33)**		**Placebo (18)**	**Mastiha (22)**		**Placebo (9)**	**Mastiha (11)**	
BMI (kg/m²) mean (SD)	24.5 (6.6)	23.4 (5.3)	0.481‡	24.4 (6.1)	22 (4.2)	0.149‡	24.7 (7.8)	26.2 (6.2)	0.628‡
Normal-weight subjects	17 (63.0)	23 (69.7)	0.863++	11 (61.1)	17 (77.3)	0.292++	6 (66.7)	6 (54.5)	0.830++
Overweight subjects	5 (18.5)	6 (18.2)		3 (16.7)	4 (18.2)		2 (22.2)	2 (18.2)	
Obese subjects	5 (18.5)	4 (12.1)		4 (22.2)	1 (4.5)		1 (11.1)	3 (27.3)	
Disease duration (yrs), mean (SD)	13.9 (10.7)	9.4 (7.0)	0.061‡	14.8 (12.0)	10.2 (6.9)	0.131‡	12.0 (7.7)	7.9 (7.4)	0.240‡
Age of first symptoms, mean (SD)	29.6 (16.1)	27.0 (11.9)	0.480‡	31.2 (16.6)	25.0 (10.3)	0.152‡	26.4 (15.7)	31.6 (14.2)	0.463‡
Age of diagnosis, mean (SD)	32.0 (17.4)	27.9 (11.9)	0.290‡	34.5 (18.0)	25.8 (10.6)	0.065‡	26.9 (15.9)	32.4 (13.9)	0.431‡
Disease extent									
Ileal				9 (50.0)	8 (36.4)				
Ileocolonic				7 (39.0)	9 (40.9)				
Colonic				1 (5.5)	3 (13.6)				
Colonic and duodenum				1 (5.5)	0 (0.0)				
Colonic and upper GI-				0 (0.0)	2 (9.1)				
Pancolitis							6 (66.7)	8 (72.7)	
Left-sided							3 (33.3)	2 (18.2)	
Rectitis							0 (0)	1 (9.1)	
Surgical therapy in the past	12 (44.4)	12 (36.4)	0.525+	10 (55.5)	10 (45.5)	0.525+	2 (22.2)	2 (18.2)	1.000++
Concomitant treatment									
Mesalazine	11 (40.7)	13 (39.4)	0.916+	5 (27.8)	4 (18.2)	0.705++	6 (66.7)	9 (81.8)	0.617++
Azathioprine	7 (25.9)	7 (21.2)	0.668+	5 (27.8)	4 (18.2)	0.705++	2 (22.2)	3 (27.3)	1.000++
Corticosteroids	9 (33.3)	14 (42.4)	0.807+	7 (38.9)	10 (45.5)	0.676+	2 (22.2)	4 (36.4)	1.000++

+Pearson's chi-square test; ++Fisher's exact test; ‡Student's t-test; *was not computed due to no distribution.

3.2. Effects of intervention on disease activity and quality of life

Table 16 presents quality of life, disease activity indices and MedDiet score for the two groups. IBDQ score improved significantly at follow-up only in the intervention arm, although the mean change was not statistically different between the two groups. Concerning HBI values, a significant decrease was found only in the Mastiha arm, no significant difference was reported in mean changes between groups. As regards adherence to Mediterranean Diet, the mean change in Med Diet score was not statistically different between the two groups. Nutritional intake is presented in Appendix 3 and did not differ between the groups at baseline and at follow-up.

Table 16. Quality of life, disease activity indices and Med Diet score at baseline and follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
IBDQ score					
Placebo	144.9 (29.0)	155.1 (33.3)	10.2 (43.6)	0.137	0.380
Mastiha	145.2 (27.3)	163.4 (30.6)	18.3 (26.4)	**0.004**	
P[3]	0.975	0.319			
HBI[+]					
Placebo	6.1 (1.8)	4.7 (2.6)	-1.4 (2.6)	0.055[‡]	0.635[‡]
Mastiha	7.8 (2.3)	4.7 (3.8)	-3.1 (4.1)	**<0.001[‡]**	
P[3]	0.134[‡]	0.691[‡]			
PMS[++]					
Placebo	3.2 (2.0)	2.2 (1.6)	-1.0 (2.1)	0.055	0.324
Mastiha	2.8 (1.8)	2.0 (1.3)	-0.9 (2.0)	0.481	
P[3]	0.114	0.355			
Med Diet score					
Placebo	26.81 (3.83)	28.95 (4.35)	2.13 (4.79)	**0.034**	0.059
Mastiha	27.59 (5.65)	27.18 (4.59)	-0.41 (5.32)	0.646	
P[3]	0.546	0.135			

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; [‡]based on logarithmic transformations; [+] only in patients with CD; [++]only in patients with UC

3.3. Effects of intervention on biochemical indices

Table 17 describes routine biochemical data at baseline and at follow-up for Mastiha and placebo. Also serum Fe, albumin and fibrinogen are presented. There were no significant differences at baseline between the two groups. At follow-up, only serum Fe was found to be significantly different between verum and placebo, with the verum having greater values. The mean change in plasma fibrinogen differed significantly between the two groups. More specifically, plasma fibrinogen decreased significantly only in the Mastiha group at follow-up. Additionally, serum Fe increased significantly only in the verum group at follow-up, while serum glucose decreased significantly.

Table 17. Biochemical data at baseline and at follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P^1	P^2
Plasma fibrinogen (mg/dL)					
Placebo	288.1 (66.9)	276.7 (83.2)	-11.4 (83.7)	0.441	**0.018**
Mastiha	281.5 (58.1)	243.3 (54.4)	-38.3 (69.9)	**0.006**	
P^3	0.687	0.067			
Serum Fe (μg/dL)					
Placebo	53.9 (27.0)	56.9 (24.0)	3.0 (24.7)	0.614	0.278
Mastiha	61.1 (30.5)	72.8 (29.2)	11.7 (34.9)	**0.032**	
P^3	0.343	**0.027**			
Serum albumin (g/dL)					
Placebo	4.3 (0.3)	4.2 (0.6)	-0.1 (0.5)	0.364	0.427
Mastiha	4.3 (0.4)	4.3 (0.5)	0.0 (0.6)	0.858	
P^3	0.474	0.764			
Serum amylase (IU/L)					
Placebo	68.2 (23.0)	68.8 (23.5)	0.6 (21.1)	0.871	0.577
Mastiha	67.6 (20.3)	65.3 (20.7)	-2.2 (17.8)	0.514	
P^3	0.909	0.546			
Serum urea (mg/dL)					
Placebo	29.9 (7.7)	28.2 (6.9)	-1.8 (6.3)	$0.238^{‡}$	$0.797^{‡}$
Mastiha	31.9 (10.1)	29.0 (7.2)	-2.9 (8.7)	$0.094^{‡}$	
P^3	$0.504^{‡}$	$0.621^{‡}$			

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Serum LDH (U/L)					
Placebo	154.8 (41.1)	188 (66)	33.2 (65.6)	**0.006‡**	0.975‡
Mastiha	143.9 (30.9)	183.5 (119.7)	39.5 (117.9)	**0.003‡**	
P[3]	0.284‡	0.421‡			
Serum total bilirubin (mg/dL)					
Placebo	0.32 (0.24)	0.48 (0.45)	0.15 (0.52)	0.275‡	0.361‡
Mastiha	0.43 (0.41)	0.55 (0.71)	0.12 (0.71)	0.877‡	
P[3]	0.221‡	0.899‡			
Serum direct bilirubin (mg/dL)					
Placebo	0.15 (0.12)	0.19 (0.13)	0.04 (0.15)	0.050‡	**0.001‡**
Mastiha	0.23 (0.17)	0.15 (0.15)	-0.08 (0.21)	**0.003‡**	
P[3]	0.281‡	0.251‡			
Serum SGOT (IU/L)					
Placebo	17.5 (9.3)	18.5 (8.6)	0.9 (12.8)	0.381‡	0.095‡
Mastiha	14.8 (4.8)	19.3 (7.0)	4.5 (6.7)	**0.001‡**	
P[3]	0.182‡	0.414‡			
Serum SGPT (IU/L)					
Placebo	18.3 (12.5)	15.3 (7.7)	-3.1 (9.4)	0.208‡	0.810‡
Mastiha	18.9 (10.3)	17.4 (8.6)	-1.6 (7.6)	0.299‡	
P[3]	0.519‡	0.317‡			
Serum γ-GT (IU/L)					
Placebo	19.2 (11.6)	18.2 (8.9)	-0.9 (9.9)	0.942‡	0.221‡
Mastiha	21.4 (13.3)	17.1 (6.2)	-4.3 (10.5)	0.059‡	
P[3]	0.330‡	0.921‡			
Serum ALP (IU/L)					
Placebo	64 (17.8)	65.6 (15.8)	1.6 (9.4)	0.550	0.107
Mastiha	68.3 (21.4)	64 (16.9)	-4.2 (16.4)	0.081	
P[3]	0.408	0.720			

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; ‡based on logarithmic transformations

3.4. Effects of intervention on inflammatory markers

Table 18 illustrates levels of inflammation markers in serum and in stools for the Mastiha and placebo groups. The change in faecal lysozyme was significantly different between the two groups. Even when excluding patients who deviated from the protocol, lysozyme levels were found to be significantly different in the Mastiha group after treatment (p=0.027), the change between the two groups being significant after treatment (p=0.036). Particularly in UC patients, a significant difference in faecal lysozyme was reported at follow up between these enrolled into the placebo and verum groups (p=0.048). More specifically, there was a significant decrease only in the Mastiha group at follow-up. IL-6 increased in both study groups. Faecal calprotectin and lactoferrin increased significantly in the placebo group at follow-up, while in the verum group they remained similar to baseline.

Table 18. Serum and faecal inflammatory markers at baseline and at follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Serum CRP (mg/L)					
Placebo	6.4 (7.6)	5.3 (4.8)	-1.1 (8.0)	0.767‡	0.791‡
Mastiha	6.9 (8.2)	5.7 (5.9)	-1.2 (8.3)	0.946‡	
P[3]	0.616‡	0.788‡			
Serum IL-6 (pg/mL)					
Placebo	14.4 (16.8)	24.3 (43.8)	9.9 (33.6)	**0.030**	0.955
Mastiha	11.5 (12.3)	15.7 (13.3)	4.2 (9.7)	**0.021**	
P[3]	0.552	0.502			
Serum IL-10 (pg/mL)					
Placebo	8.8 (18.9)	9.5 (20.1)	0.6 (3.4)	0.454	0.607
Mastiha	6.1 (2.7)	6.1 (2.7)	0.0 (3.6)	0.951	
P[3]	0.920	0.713			
Faecal lysozyme (μg/g)					
Placebo	11.7 (10.6)	15.6 (15.8)	3.9 (18.2)	0.326	**0.021**
Mastiha	18.8 (21.2)	10.3 (5.2)	-8.4 (21.8)	**0.018**	
P[3]	0.075	0.208			
Faecal calprotectin (μg/g)					
Placebo	2170.6 (4444.4)	3598.5 (3620.4)	1427.9 (5606.1)	**0.029**	0.348
Mastiha	1688.6 (1712.4)	2744 (4910.6)	1055.4 (5043.1)	0.289	
P[3]	0.825	0.357			
Faecal lactoferrin (μg/g)					
Placebo	102.4 (128.7)	306.3 (373.7)	203.9 (376.4)	**0.001**	0.130
Mastiha	130.4 (140.2)	165.6 (150.8)	35.2 (179.9)	0.109	
P[3]	0.479	0.240			

[1]p-value for time effect (based on logarithmic transformations); [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA) (based on logarithmic transformations); [3]p-value for group effect (based on logarithmic transformations)

3.5 Effects of intervention on oxidative stress

Table 19 presents oxidative stress markers in the two groups before and after the intervention. The levels oxLDL, oxLDL/HDL and oxLDL/LDL decreased signficantly in the intervention group. The mean change was significantly different between the control and intervention group for oxLDL/HDL and oxLDL/LDL. Serum resistance to oxidation and uric acid did not changed significantly in both groups.

Table 19. Oxidative stress markers at baseline and at follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
oxLDL (U/L)					
Placebo	135.3 (48.38)	135.45 (38.92)	0.15 (52.76)	0.989	0.136
Mastiha	160.42 (34.26)	140.12 (41.91)	-20.3 (51.7)	**0.031**	
P[3]	**0.022**	0.659			
oxLDL/HDL					
Placebo	2.44 (1.22)	2.63 (1.38)	0.19 (1.67)	0.568	**0.044**
Mastiha	3.06 (0.91)	2.37 (1.42)	-0.69 (1.63)	**0.020**	
P[3]	**0.028**	0.474			
oxLDL/LDL					
Placebo	1.62 (0.88)	1.7 (0.88)	0.07 (0.82)	0.654	**0.015**
Mastiha	1.84 (0.73)	1.3 (0.85)	-0.54 (1.03)	**0.005**	
P[3]	0.307	0.082			
Serum resistance to oxidation (lag time, s)					
Placebo	4279.83 (2573.79)	4889.15 (2094.22)	609.31 (2159.4)	0.155	0.893
Mastiha	4271.12 (2236.36)	4959.46 (1760.57)	688.34 (2327.7)	0.099	
P[3]	0.989	0.888			
Uric acid (mg/dL)					
Placebo	4.99 (1.92)	4.45 (1.22)	-0.54 (1.79)	0.130	0.551
Mastiha	5.76 (4.9)	4.64 (1.11)	-1.12 (4.82)	0.189	
P[3]	0.443	0.539			

[1]p-value for time effect; [2]Effects reported include differences between the groups in the degree of change (repeated measurements ANOVA); [3]p-value for group effect

3.6 Effects of intervention on amino acids levels

Levels of amino acids are presented in Table 20. Aspartic acid was significantly decreased only the mastiha group. Glutamic acid was significantly increased in both groups the mean change being insignificant. Lysine decreased only in the placebo group, while cysteine increased only in the verum group. No other signicant changes were reported.

Nevertheless, when the changes in amino acids levels were studied in the subgroups of CD and UC patients, there were some interesting results regarding UC patients. The mean changes of leucine ($p<0.05$), allo-isoleucine ($p<0.05$), isoleucine ($p<0.05$), serine ($p<0.05$), glutamine ($p<0.05$), ornithine ($p<0.05$), tryptophane ($p<0.05$), lysine ($p<0.01$) and tyrosine ($p<0.01$) between the two groups were significantly different. More specifically, the levels of the following amino acids were significantly decreased in the placebo group compared with baseline, whereas in the mastiha group remain unchanged: allo-isoleucine (77.9±51.7 nmoL/mL versus 50.1±15.4 nmoL/mL, p=0.024), isoleucine (91±60.9 nmoL/mL versus 57.4±17.4 nmoL/mL, p=0.019), lysine (209.9±31.1 nmoL/mL versus 155.6±26.3 nmoL/mL, p=0.002), tyrosine (71±18.4 nmoL/mL versus 58.1±17.2 nmoL/mL, p=0.045) and tryptophane (65.3±12.3 nmoL/mL versus 57±12.8 nmoL/mL, p=0.005). Tyrosine was significantly increased in the verum group compared with baseline (50.4±18.6 nmoL/mL versus 63.3±11.9 nmoL/mL, p=0.029).

Table 20. Amino acids levels (nmoL/mL) at baseline and at follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P^1	P^2
Alanine					
Placebo	320.3 (86.2)	334.7 (80.8)	14.3 (86)	0.409	0.714
Mastiha	317.5 (81.1)	322.9 (92.3)	5.4 (97.7)	0.712	
P^3	0.866	0.530			
Allo-isoleucine ‡					
Placebo	60.5 (36.6)	50.9 (14.6)	-9.6 (35.3)	0.206	0.271
Mastiha	51.3 (19.3)	49.9 (11.2)	-1.4 (17.7)	0.810	
P^3	0.271	0.907			
α-Aminobutyric acid					
Placebo	14.6 (6.3)	16.1 (5.4)	1.5 (5.7)	0.261	0.177
Mastiha	16.2 (6.1)	15.3 (4.6)	-0.9 (7.4)	0.437	
P^3	0.325	0.551			

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
β-Aminobutyric acid ‡					
Placebo	102.4 (23.4)	98.5 (3)	-3.9 (23.4)	0.245	0.303
Mastiha	97.9 (1.6)	98.4 (2.4)	0.5 (2.3)	0.804	
P[3]	0.283	0.886			
Asparagine ‡					
Placebo	45.1 (12.6)	43.5 (7.7)	-1.5 (14.7)	0.791	0.928
Mastiha	43.4 (10.8)	42.5 (7.5)	-0.9 (11.6)	0.874	
P[3]	0.623	0.602			
Aspartic acid					
Placebo	7 (4.1)	6.7 (4.2)	-0.3 (6.2)	0.814	0.104
Mastiha	8.2 (4.5)	5.3 (3.3)	-2.9 (6)	**0.009**	
P[3]	0.284	0.149			
Glutamic acid ‡					
Placebo	21.2 (24.9)	22.3 (14.9)	1.1 (24)	**0.039**	0.994
Mastiha	22.6 (16.3)	27 (10.1)	4.4 (14.7)	**0.022**	
P[3]	0.142	**0.011**			
Glutamine ‡					
Placebo	426.8 (75.8)	396.4 (85.5)	-30.5 (100.9)	0.156	0.270
Mastiha	376.8 (110)	368.7 (70.3)	-8.1 (102.3)	0.944	
P[3]	**0.036**	0.247			
Glycine					
Placebo	239.5 (70.2)	238.7 (49.2)	-0.8 (64)	0.948	0.399
Mastiha	224.5 (60.1)	237.3 (50.6)	12.9 (60.1)	0.238	
P[3]	0.377	0.918			
Cysteine ‡					
Placebo	28.6 (3.3)	28.2 (2.5)	-0.4 (3.2)	0.596	0.059
Mastiha	27 (2.2)	28.2 (2.8)	1.2 (2.9)	**0.027**	
P[3]	**0.030**	0.977			
Histidine ‡					
Placebo	77.1 (16.3)	75.8 (15.5)	-1.3 (20.8)	0.748	0.499
Mastiha	72.2 (19.9)	73.2 (13.7)	1 (18.4)	0.514	
P[3]	0.238	0.617			
Hydroxyproline ‡					
Placebo	17.6 (3.7)	16.5 (2.4)	-1.1 (3.8)	0.218	0.233
Mastiha	17.4 (4.5)	17.4 (3)	0 (4.8)	0.678	
P[3]	0.681	0.203			
Isoleucine ‡					
Placebo	70.2 (42.9)	59.5 (16.7)	-10.7 (41.5)	0.241	0.244
Mastiha	59.8 (22)	59.2 (13.1)	-0.6 (20.8)	0.660	

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
P[3]	0.306	0.898			
Leucine ‡					
Placebo	125.5 (38.8)	125.6 (30.8)	0 (36)	0.830	0.681
Mastiha	119.9 (34)	121.9 (20.7)	2.1 (32.2)	0.397	
P[3]	0.545	0.779			
Lysine ‡					
Placebo	187.4 (48.1)	160.5 (29.8)	-26.9 (43.2)	**0.025**	0.587
Mastiha	187 (54.3)	163.8 (29)	-23.3 (53.5)	0.088	
P[3]	0.812	0.621			
Methionine ‡					
Placebo	22.3 (6.7)	19.6 (4.3)	-2.6 (8.2)	0.149	0.629
Mastiha	19.8 (5.3)	18.5 (4.5)	-1.3 (7.4)	0.376	
P[3]	0.133	0.329			
Ornithine ‡					
Placebo	93.2 (31)	85.8 (21.2)	-7.4 (35.2)	0.382	0.362
Mastiha	81.5 (23.3)	81.7 (18.9)	0.2 (24.6)	0.694	
P[3]	0.126	0.438			
Phenylalanine ‡					
Placebo	72.8 (20.7)	69.3 (15.8)	-3.5 (22.1)	0.481	0.658
Mastiha	68.1 (18.4)	66 (11.2)	-2.1 (16.4)	0.903	
P[3]	0.326	0.445			
Proline					
Placebo	257.1 (67.9)	248.3 (71.8)	-8.8 (87.5)	0.602	0.285
Mastiha	237.2 (73.3)	252.8 (64.1)	15.6 (87.1)	0.308	
P[3]	0.284	0.798			
Serine					
Placebo	109.7 (33.9)	110.4 (20.9)	0.7 (29.6)	0.887	0.626
Mastiha	97.2 (28.5)	101.4 (26.1)	4.2 (25.2)	0.378	
P[3]	0.126	0.150			
Thioproline					
Placebo	16.7 (4.1)	14.1 (2.3)	-2.7 (5.2)	**0.006**	0.170
Mastiha	18.1 (4.2)	13.7 (2.8)	-4.4 (4.6)	**<0.001**	
P[3]	0.216	0.539			
Threonine ‡					
Placebo	128.1 (39.5)	125.6 (27.5)	-2.5 (42.6)	0.949	0.902
Mastiha	122.8 (44)	120.8 (29.3)	-2 (48.5)	0.799	
P[3]	0.531	0.468			
Valine					
Placebo	328.5 (99)	325.2 (68.9)	-3.2 (71.9)	0.893	0.999

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Mastiha	330.5 (89.5)	324.8 (58)	-5.7 (80.3)	0.880	
P[3]	0.980	0.970			
Tyrosine					
Placebo	62.9 (22)	62.1 (16.5)	-0.8 (20.2)	0.824	0.523
Mastiha	55.5 (19.4)	57.9 (15.3)	2.4 (18.7)	0.480	
P[3]	0.171	0.313			
Tryptophane					
Placebo	60.7 (13)	59.1 (10.5)	-1.6 (14.3)	0.513	0.319
Mastiha	56.8 (12.3)	58.5 (9.4)	1.7 (11)	0.444	
P[3]	0.238	0.808			

[1]p-value for time effect; [2]Effects reported include differences between the groups in the degree of change (repeated measurements ANOVA); [3]p-value for group effect; [‡]based on logarithmic transformations

3.7 Discussion

Herein, we investigated the effect of a natural supplement prepared with Mastiha to influence the clinical course of IBD. There are pre-clinical and clinical data supporting its administration in IBD (Kaliora et al., 2007a, 2007b; Gioxari et al., 2011; Papalois et al., 2012) but this is the first study, to our knowledge, evaluating the effectiveness of Mastiha on the basis of a Phase-II randomised placebo-controlled clinical trial of both CD and UC patients in relapse.

In order to assess our primary outcome, namely improvement in quality of life, we used the IBDQ, an established and validated tool (Alrubaiy et al., 2015), which is fully validated in the Greek language as well in the original English (Pallis et al., 2001). Despite a lack of significant difference in mean changes between the study groups, the IBDQ improved significantly in the Mastiha group but not in the placebo group. Although the mean score at follow-up in the verum group did not reach the usual range of patients in remission (170-190 points), this increase shows that Mastiha supplementation may improve quality of life in IBD patients in relapse when given as an adjunct to conventional medical therapy.

When evaluating disease severity, we observed a significant decrease in HBI at follow-up compared with baseline only in the Mastiha group. However, the detected decrease should be interpreted with caution due to the subjective variables comprising the score (e.g. intensity of abdominal pain and general well-being). A longer period of treatment could possibly induce a further reduction in HBI and a significant mean change between groups, as well as a reduction in PMS for UC patients. However, the nature of active IBD with frequent changes in medical treatments with fluctuations in dosages would jeopardise the compliance with our inclusion and exclusion criteria.

To avoid patient discomfort as well as increased drop-out rate and public health cost due to repeated colonoscopies, we evaluated serum and faecal biomarkers of inflammation. No significant changes in CRP or IL-6 have been identified. However we detected noteworthy regulation in lysozyme, lactoferrin and calprotectin levels. Previous research suggested that faecal lysozyme is increased in IBD patients and correlates with disease activity, especially in colonic IBD (van der Sluys Veer et al., 1998). Our study showed a significant decrease in the lysozyme levels of the Mastiha group, most importantly it showed a significant difference in mean change between

the two groups. When analyzing lysozyme in UC or CD individually, significantly lower levels at follow-up for the Mastiha group in UC patients were reported. Lysozyme, an antimicrobial protein that regulates innate immune response, is expressed in both entities, CD and UC, mainly in small intestine, but markedly also in the colon, as shown in both experimental animals (Coulombe et al., 2016) and humans (Rubio, 2011; Fahlgren et al., 2003). Since increased lysozyme expression is correlated with dysbiosis and with inflammation (Coulombe et al., 2016), herein we could hypothesise that the effect of Mastiha is rather a prebiotic one related to the contained phenolic compounds and arabinogalactanes, both reported to have prebiotic activities (Williamson et al., 2017; Dion et al., 2016). In addition, faecal calprotectin, a protein present in granulocytes and neutrophils, is released into the intestinal lumen after leucocyte epithelial migration into the inflamed gut mucosa and has good diagnostic accuracy in assessing mucosal healing in IBD patients (Kochhar & Lashner, 2017). Furthermore, faecal lactoferrin is usually elevated in patients with active IBD detecting inflammation and correlates well with mucosal healing and histological improvement (Abraham & Kane, 2012). In our study, levels of calprotectin and lactoferrin significantly increased in the placebo but not in the Mastiha group. Significantly lower levels of lactoferrin at follow-up were found in UC patients under Mastiha treatment.

Regarding biochemical markers, fibrinogen is an acute phase reactant participating in thrombi formation and its levels increase in inflammatory conditions. Increased fibrinogen levels are related with higher plasma viscosity and platelet activation, additionally to the microcirculation of the inflamed intestine and among other serological markers, fibrinogen levels are predictive of CD's endoscopic activity (Hudson et al., 1996; Miranda-Garcia et al., 2016). Higher levels of fibrinogen have been detected previously in IBD patients and increasing levels were associated with disease activity (Dolapcioglu et al., 2014). In our study, plasma fibrinogen levels decreased significantly in the Mastiha group compared with baseline, notably the change in mean values between the two groups remained significant as well. A significant decrease of fibrinogen in the Mastiha group at follow up (250.2 ± 57.4 mg/dl) compared with baseline (288.3 ± 65.5 mg/dl, p= 0.022) was reported in CD but not in UC patients. These findings come into agreement with a study investigating the antidiabetic effects of two pentacyclic triterpenic acids, madecassid acid and rotundic acid, on a mouse model. Apart from beneficial effects on oxidative and inflammatory

stress, madecassic acid induced a significant reduction in fibrinogen levels and plasminogen activator inhibitor (Hsu et al., 2015). Furthermore, another protocol on a mouse model of diabetes showed that the triterpenoids asiatic and maslinic acids, act as anti-coagulants, by lowering fibrinogen levels among others (Hung et al., 2015). Even though these results refer to mouse models and should not be directly extrapolated to humans, we could hypothesise that terpenes in Mastiha possibly exhibit favorable effects on homeostatic imbalance of IBD patients.

Serum Fe levels significantly increased in the Mastiha group compared with baseline and compared with the follow-up levels in the placebo group. Additionally, serum Fe levels increased significantly in the Mastiha group at follow up (75.3±32.2 μg/dl) compared with baseline (61.3±30.7 μg/dl, p=0.034) in CD but not in UC patients. Even though this increase could be partially attributed to increased food intake due to symptoms improvement, better nutrient absorption or less intestinal losses, this finding should be interpreted with caution, since several nutrients may fluctuate in serum as positive or negative acute phase reactants as a part of inflammatory response (Forbes et al., 2017).

As regards to oxidative stress markers, oxLDL is considered the most specific biomarker for oxidative stress evaluation in humans; most recently its measurement by immunological methods (i.e. antibodies) has been recognised by the European Food Safety Authority (EFSA) as a reliable method to assess oxidative damage with appropriate specificity (EFSA, 2011). In our study it fell significantly only in patients receiving Mastiha, even if the mean change between the two groups was not significant. However, data on oxLDL levels in IBD patients are limited. A previous study in eight patients with active CD and eight controls showed that CD patients had elevated levels compared with the controls reflecting the increased oxidative stress and the higher risk for atherosclerosis (Grip et al., 2004). However, when oxLDL was measured in paediatric IBD patients no values significantly different from healthy controls were reported (Pac-Kożuchowska et al., 2016). The decreased levels of oxLDL after Mastiha intervention could point towards an antioxidant effect of this natural supplement in active IBD patients, and future research on its mechanism of action is justified, since its main bioactive compounds, mostly triterpenes (i.e. mastihadienonic, isomastihadeinonic acids), are bioavailable and exhibit antioxidant effects in vivo

(Papada et al., 2018).

Another interesting result of the present study was the significant reduction in the ratios of oxLDL/LDL and oxLDL/HDL in the Mastiha arm compared with the placebo arm. The level of oxLDL and the ratios oxLDL/LDL and oxLDL/HDL seem to be better biomarkers than total cholesterol, triglycerides, HDL and LDL for discrimination between patients with increased cardiovascular risk and healthy subjects (Huang et al., 2008). In addition they seem to be the most useful clinical parameters of lipoprotein oxidation in other pathologies as well, such as type-II diabetes, reflecting the association between lipids in the state of oxidative stress (Motamed et al., 2016). A study in elderly patients with type II diabetes showed that oxLDL/HDL was directly associated with advanced glycation end products and advanced oxidation protein products, which enhance oxidative stress (Gradinaru et al., 2013). A more recent study in a population with a high prevalence of cardiovascular disease and type-II diabetes showed that these ratios were significantly associated with HbA1c, glucose and CRP (Harmon et al., 2016). Data for these ratios in IBD patients are scarce and given the fact that IBD patients are at increased risk of cardiovascular disease [Kirchgesner et al., 2018; Yarur et al., 2011), the present findings might be of importance. To our knowledge, this is the first study evaluating these ratios in patients with active IBD in the context of a randomized clinical trial evaluating the effectiveness of a natural supplement. Our results could point towards a favourable effect of an antioxidant supplement on the mechanisms of systemic inflammation that increase the risk of cardiovascular disease.

Amino acids are both substrates and regulators in several metabolic pathways. Circulating AA levels may reflect not only nutritional status, but also inflammatory state and disease activity. In our study of patients with active IBD there were no significantly different changes between the groups at baseline and at follow-up. However, we have shown some stimulating results regarding AAs changes in the subgroup of patients with active UC. Levels of allo-isoleucine, isoleucine, cysteine and tryptophane significantly decreased in the placebo group, whereas in the Mastiha group they remain unchanged, with the mean changes between the groups being significant. Trends were also observed for ornithine and tyrosine. The mean changes between the two UC groups differed significantly for leucine, serine and glutamine. Plasma free AA normalisation was

not however correlated with improvement in oxidative stress biomarkers ($p>0.05$).

Data on circulating AAs in active IBD patients are limited and sometimes conflicting, however it seems that the AA profile differentiates IBD patients from healthy controls pointing towards a link between the AAs and the disease process (Hisamatsu et al., 2012; Ooi et al., 2011; Zhang et al., 2013). The general tendency for plasma AAs to decrease in our UC patients on placebo may indicate increased de novo AA synthesis in the presence of inflammation. The fact that AA alterations were present predominantly in the group of UC patients may indicate a more favourable effect of Mastiha in patients with active UC.

Regarding the role of AAs in oxidative stress, it seems that several AAs are implicated in these mechanisms. Cysteine, (significantly lower in our placebo group), is a precursor of glutathione and correlated negatively with levels of oxLDL ($r=-0.26$, $p=0.044$). This is in accordance with previous findings that oxidative stress in vivo could be translated as a deficiency of glutathione and/or cysteine (Atkuri et al., 2007). Tryptophane, which was significantly decreased in our placebo group, is metabolized along the oxidative kynurenine pathway leading to the generation of quinolinic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, all of which are known to generate free radicals (Forrest et al., 2004). In addition, proline is upregulated as an oxidative stress response in mammalian cells (Krishnan et al., 2008). Previous studies in intestinal cell culture models have shown that the presence of sulfhydryl-containing AAs or BCAAs or heterocyclic group-containing AAs or other AAs, is associated with attenuation of oxidative stress-induced inflammation (Katayama & Mine, 2007; Son et al., 2005).

While our study has interesting results and the quality of being double-blinded, it has limitations, including the absence of endoscopy at follow-up, precluding our ability to comment on histological alterations, and the absence of the full metabolic profile identification. It is also compromised by the incomplete separation of patients with UC and CD and the consequent need for post facto subgroup analysis. However, these limitations are compensated by the very tight control of the verum and placebo groups to ensure compliance of patients with the protocol, as well as the adequate power of the study overall ensuing from the total number of participants recruited.

The above findings could also point toward anti-inflammatory properties of the Mastiha terpenes, which have been shown to be absorbed and bioavailable in the systemic circulation in healthy humans (Papada et al., 2018).

While this study reports interesting findings, it has some limitations, such as the absence of colonoscopy at follow-up, precluding our ability to comment on any histological alterations. On the other hand, this limitation was compensated by the very tight control of the verum and placebo groups to ensure compliance of patients with the protocol, as well as the adequate number of participants that allowed for a power of 0.90 for the between-subjects main effect at an effect size of 0.37; a power of 0.95 for the within-subjects main effect at an effect size of 0.25; and a power of 0.95 for the interaction effect at an effect size of 0.25.

Our results showed for the first time that a natural supplement prepared with Mastiha could serve as an innovative treatment approach as an adjunct to conventional medical therapy. Mechanistically, regulation of faecal lysozyme secondary to a prebiotic effect is proposed.

CHAPTER 4

A STUDY ON THE EFFECTS OF A NUTRITIONAL SUPPLEMENT WITH NATURAL MASTIHA IN PATIENTS WITH QUIESCENT INFLAMMATORY BOWEL DISEASE

The results are presented in Paper 4.

4.1 Description of the study population

Sixty-eight IBD patients (N=68) met our criteria for recruitment (Figure 26). A total of 34 patients were randomized to placebo group and 34 patients to the Mastiha group, while 46 of them were diagnosed with CD and 22 with UC.

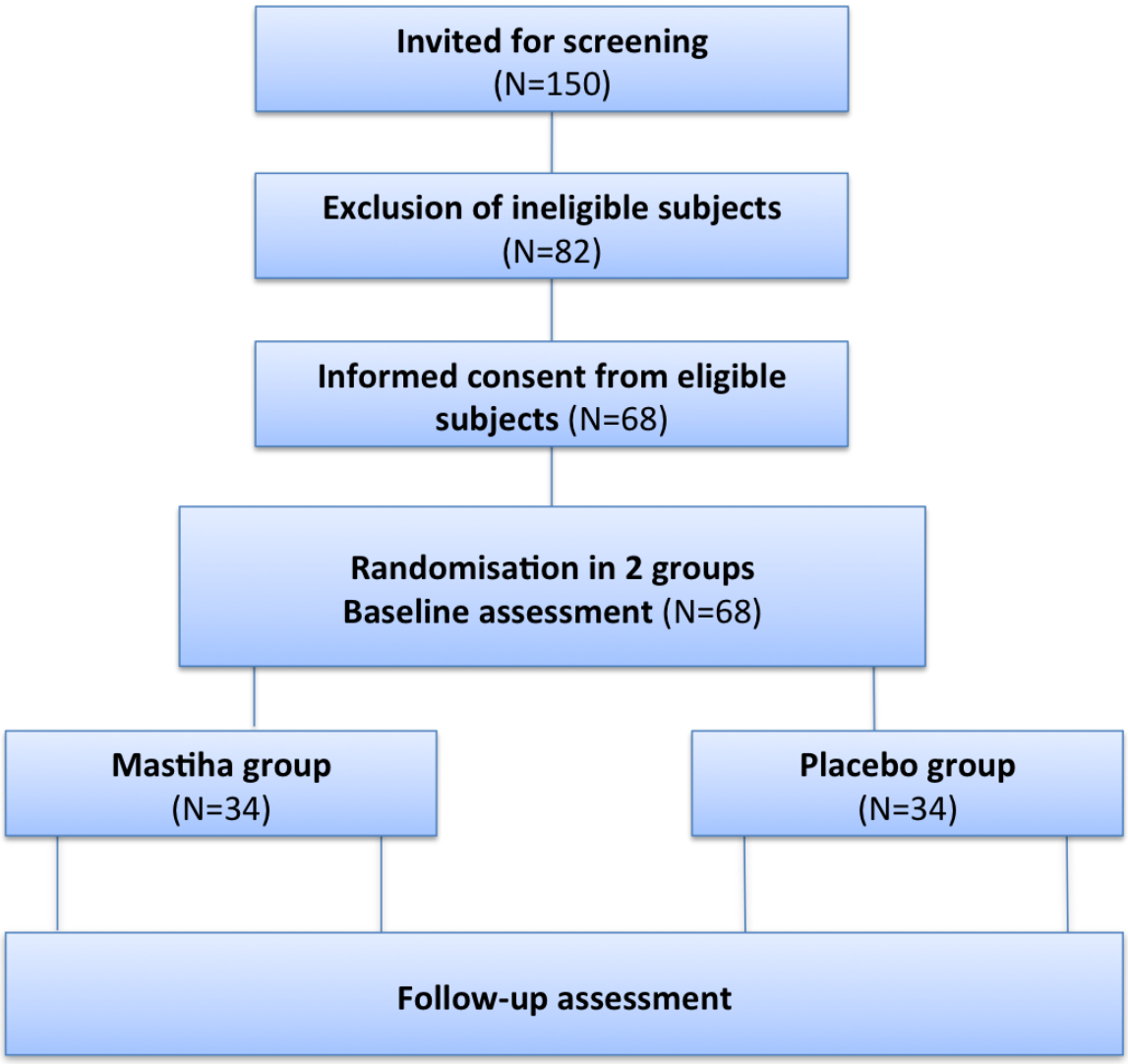


Figure 26. Study population in remission

Clinical, anthropometric and demographic characteristics for all groups are presented in Tables 21 and 22. Patients of both placebo and verum groups were similar in total sample number and in each type of disease regarding their demographics.

Table 21. Demographics in all groups. Results are given as N (%) of the total number.

	IBD (N=68)			CD (N=46)			UC (N=22)		
	Placebo (34)	Mastiha (34)	P	Placebo (21)	Mastiha (25)	P	Placebo (13)	Mastiha (9)	P
Sex									
Females	19 (55.9)	19 (55.9)	1000[+]	13 (61.9)	14 (56.0)	0.685[+]	6 (46.2)	5 (55.6)	1.000[++]
Males	15 (44.1)	15 (44.1)		8 (38.1)	11 (44.0)		7 (53.8)	4 (44.4)	
Age (yrs), mean (SD)	38.1 (11.8)	36.4 (9.9)	0.513[‡]	38.5 (12.6)	35.9 (10.7)	0.455[‡]	37.6 (10.8)	37.9 (7.3)	0.948[‡]
Marital status									
Unmarried/Divorced	20 (58.8)	17 (50.0)	0.465[+]	12 (57.1)	14 (56.0)	0.938[+]	8 (61.5)	3 (33.3)	0.387[++]
Married	14 (41.2)	17 (50.0)		9 (42.9)	11 (44.0)		5 (38.5)	6 (66.7)	
Education (yrs)									
1-9	5 (14.7)	4 (11.8)	0.755[++]	4 (19.0)	4 (16.0)	0.305[++]	1 (7.7)	0 (0.0)	0.765[++]
10-12	5 (14.7)	8 (23.5)		2 (9.5)	7 (28.0)		3 (23.1)	1 (11.1)	
>12	24 (70.6)	22 (64.7)		15 (71.4)	14 (56.0)		9 (69.2)	8 (88.9)	
Smoking[#]									
No	18 (54.5)	23 (67.6)	0.271[+]	12 (57.1)	17 (68.0)	0.447[+]	6 (50.0)	6 (66.7)	0.660[++]
Yes	15 (45.5)	11 (32.4)		9 (42.9)	8 (32.0)		6 (50.0)	3 (33.3)	

[#] Smoking habits are presented herein at baseline, [+]Pearson's chi-square test; [++]Fisher's exact test; [‡]Student's t-test

Table 22. Clinical and anthropometric characteristics for all groups.

	IBD (N=68)		P	CD (N=46)		P	UC (N=22)		P
	Placebo (34)	**Mastiha (34)**		**Placebo (21)**	**Mastiha (25)**		**Placebo (13)**	**Mastiha (9)**	
BMI (kg/m^2) mean (SD)	24.8 (4.1)	23.9 (3.7)	0.339‡	25.7 (4.9)	23.9 (3.6)	0.169‡	23.5 (2.1)	23.8 (4.2)	0.821‡
Normal weight subjects	19 (59.4)	19 (55.9)	0.486++	10 (47.6)	14 (56.0)	0.154++	10 (76.9)	5 (55.6)	0.454++
Overweight subjects	10 (31.3)	14 (41.2)		8 (38.1)	11 (44.0)		3 (23.1)	3 (33.3)	
Obese subjects	3 (9.4)	1 (2.9)		3 (14.3)	0 (0.0)		0 (0.0)	1 (11.1)	
Disease duration (yrs), mean (SD)	9.9 (7.2)	8.8 (6.1)	0.478‡	11.4 (8.3)	8.6 (5.2)	0.167‡	7.6 (4.1)	9.4 (8.4)	0.502‡
Age of first symptoms, mean (SD)	26.1 (9.7)	26.2 (10.6)	0.973‡	24.8 (10.0)	25.5 (10.8)	0.819‡	28.2 (9.2)	28.1 (10.4)	0.992‡
Age of diagnosis, mean (SD)	28.2 (10.6)	27.6 (10.3)	0.818‡	27.1 (11.5)	27.3 (10.3)	0.945‡	30 (9.2)	28.4 (10.8)	0.720‡
Disease extent									
Ileal				4 (19.0)	7 (28.0)				
Ileocolonic				10 (47.6)	12 (48.0)				
Colonic				6 (28.6)	5 (20.0)				
Colonic and upper GI-				1 (4.8)	1 (4.0)				
Pancolitis							8 (61.5)	6 (66.7)	
Left-sided							3 (23.1)	2 (22.2)	
Sigmoeid							0 (0)	1 (11.1)	
Rectitis							2 (15.4)	0	
Surgical therapy in the past	8 (24.2)	8 (23.5)	0.945+	7 (35.0)	8 (32.0)	0.823+	1 (7.7)	0 (0.0)	1.000++
Concomitant treatment									
Mesalazine	17 (51.5)	17 (50.0)	0.901+	5 (25.0)	10 (40.0)	0.289+	12 (92.3)	7 (77.8)	0.544++
Azathioprine	11 (33.3)	10 (29.4)	0.729+	8 (40.0)	7 (28.0)	0.396+	3 (23.1)	3 (33.3)	0.655++

+Pearson's chi-square test; ++Fisher's exact test; ‡Student's t-test

4.2 Effects on relapse rate, disease activity indices and quality of life

Relapse was defined by a score HBI higher than 4 and a score of PMS higher than 1. No significant differences in relapse rates were detected between the two groups at follow-up (Table 23).

Table 23. Remission rate in placebo and verum group. Results are given as N (%) of the total number.

	Placebo (34)	**Mastiha (34)**	**P**
Baseline	0 (0.0)	0 (0.0)	-
Follow-up	8 (23.5)	6 (17.6)	0.549

Additionally, disease activity indices and IBDQ score did not alter significantly at follow-up in both groups. As regards adherence to Mediterranean Diet, Med Diet score decreased significantly only in placebo group at follow-up, although the mean change was not statistically different between the two groups (Table 24). Nutritional intake is presented in Appendix 3 and did not differ between the groups at baseline and at follow-up.

Table 24. Quality of life, disease activity indices and MedDiet scores at baseline and follow up.

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
IBDQ score					
Placebo	173.2 (24.8)	178.4 (23.5)	5.2 (22.0)	0.249	0.572
Mastiha	180.1 (28.6)	181.7 (16.0)	1.6 (26.5)	0.720	
P[3]	0.290	0.496			
HBI[+ ‡]					
Placebo	2.24 (1.48)	2.70 (1.82)	0.46 (1.81)	0.355	0.436
Mastiha	2.36 (1.25)	2.67 (2.14)	0.31 (1.88)	0.887	
P[3]	0.567	0.805			
PMS[++ ‡]					
Placebo	0.77 (0.44)	1.44 (1.37)	0.67 (1.16)	0.234	0.366
Mastiha	0.67 (0.50)	0.76 (1.04)	0.10 (1.19)	0.857	
P[3]	0.616	0.269			
MedDiet Score					
Placebo	31.4 (6.2)	29.6 (6.2)	-1.7 (4.6)	**0.036**	0.426
Mastiha	30.3 (5.5)	29.6 (4.9)	-0.7 (5.6)	0.438	
P[3]					

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; [‡]based on logarithmic transformations; [+] only in patients with CD; [++]only in patients with UC.

4.3 Effects on biochemical indices

All biochemical markers assessed were within the normal range at baseline and at follow up. Serum albumin increased significantly in Mastiha arm. The mean change in serum albumin differed significantly between the two groups. Likewise, total cholesterol and LDL cholesterol increased significantly in placebo but not in Mastiha arm, resulting in a significant difference of mean changes between the two study arms. In addition, serum glucose decreased significantly, while LDH increased significantly in placebo group (Table 25).

Table 25. Biochemical data at baseline and at follow-up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P^1	P^2
Plasma fibrinogen (mg/dL) ‡					
Placebo	247.5 (62.8)	225.3 (51.0)	-22.2 (70.1)	0.109	0.879
Mastiha	264 (70.6)	242.8 (57.3)	-21.2 (72.1)	0.080	
P^3	0.295	0.191			
Serum Fe (μg/dL) ‡					
Placebo	72.4 (34.6)	73.7 (25.3)	1.3 (36.9)	0.534	0.912
Mastiha	73 (63.8)	71.2 (30.5)	-1.8 (67.7)	0.437	
P^3	0.618	0.621			
Serum albumin (g/dL) ‡					
Placebo	4.38 (0.35)	4.36 (0.54)	-0.02 (0.48)	0.639	**0.016**
Mastiha	4.32 (0.3)	4.61 (0.49)	0.28 (0.56)	**0.004**	
P^3	0.482	**0.049**			
Serum glucose (mg/dL)					
Placebo	87.1 (18.1)	77.9 (15.2)	-9.2 (21.4)	**0.004**	0.204
Mastiha	85.7 (13.5)	82.1 (10.4)	-3.6 (13.5)	0.246	
P^3	0.728	0.186			
Total cholesterol (mg/dL) ‡					
Placebo	171.7 (43.7)	188.8 (46.9)	17 (39.9)	**0.004**	**0.032**
Mastiha	178.5 (42.6)	175.9 (32.9)	-2.6 (29.6)	0.916	
P^3	0.453	0.262			

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
LDL cholesterol (mg/dL) ‡					
Placebo	96.4 (38.4)	111.4 (39.5)	15.1 (33.3)	**0.002**	**0.045**
Mastiha	106.4 (36.6)	105.7 (27.3)	-0.7 (25.9)	0.693	
P[3]	0.173	0.744			
Serum amylase (IU/L) ‡					
Placebo	68 (24.2)	69.3 (16.1)	1.3 (24)	0.351	0.124
Mastiha	71.1 (24.7)	64.4 (11.4)	-6.7 (21.3)	0.211	
P[3]	0.562	0.221			
Serum urea (mg/dL)					
Placebo	30.7 (7.7)	31.2 (6.3)	0.5 (8.3)	0.703	0.242
Mastiha	32.5 (7.2)	30.8 (4.7)	-1.7 (6.7)	0.202	
P[3]	0.326	0.777			
Serum LDH (U/L) ‡					
Placebo	149.7 (33.7)	165.1 (36.2)	15.4 (35.4)	**0.015**	0.463
Mastiha	146.8 (27.8)	154.7 (26)	7.9 (31.1)	0.150	
P[3]	0.765	0.292			
Serum total bilirubin (mg/dL) ‡					
Placebo	0.40 (0.31)	0.42 (0.59)	0.20 (0.63)	0.100	0.112
Mastiha	0.39 (0.22)	1.24 (3.25)	0.85 (3.25)	0.217	
P[3]	0.663	0.077			
Serum direct bilirubin (mg/dL) ‡					
Placebo	0.18 (0.1)	0.18 (0.17)	0 (0.16)	0.106	0.965
Mastiha	0.18 (0.1)	0.2 (0.21)	0.02 (0.2)	0.119	
P[3]	0.707	0.870			
Serum SGOT (IU/L) ‡					
Placebo	16.8 (4.5)	18.1 (4.5)	1.3 (6.1)	0.166	0.744
Mastiha	18.2 (9.6)	19.2 (5.7)	1 (8.4)	0.067	
P[3]	0.813	0.410			
Serum SGPT (IU/L) ‡					

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Placebo	19.6 (10.5)	16.9 (6.9)	-2.7 (9.7)	0.190	0.114
Mastiha	17.1 (9.1)	17.1 (5.7)	0.1 (7)	0.350	
P[3]	0.249	0.675			
Serum γ-GT (IU/L) ‡					
Placebo	18.3 (10.3)	19.2 (8.4)	0.9 (11.4)	0.356	0.955
Mastiha	29.3 (72.4)	26.7 (49.6)	-2.6 (25.4)	0.317	
P[3]	0.799	0.723			
Serum ALP (IU/L) ‡					
Placebo	65.4 (19)	69.2 (16.5)	3.8 (20.2)	0.217	0.642
Mastiha	67.8 (41)	73 (39.3)	5.2 (19.1)	0.061	
P[3]	0.780	0.870			

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; ‡based on logarithmic transformations

4.4. Effects on inflammatory markers

Levels of serum and faecal inflammatory markers in two study groups are given in Table 26. Serum IL-6 increased significantly in placebo arm. Also, faecal calprotectin and lactoferrin increased significantly in the placebo group at follow-up, while in Mastiha group they remained unaffected. No significant difference in mean changes between the two groups was reported in any serum or faecal biomarker.

Table 26. Serum and faecal inflammatory markers at baseline and at follow up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P^1	P^2
Serum CRP (mg/L) ‡					
Placebo	4.4 (8.3)	5.2 (9.8)	0.8 (5.6)	0.161	0.859
Mastiha	5.4 (9.1)	4.9 (5.5)	-0.5 (8.6)	0.099	
P^3	0.510	0.357			
Serum IL-6 (pg/mL) ‡					
Placebo	6.8 (16.6)	11.3 (12.7)	4.5 (12.4)	**0.005**	0.360
Mastiha	6.5 (7.7)	10.3 (12.2)	3.8 (14)	0.093	
P^3	0.279	0.916			
Serum IL-10 (pg/mL) ‡					
Placebo	7 (3.6)	6.2 (3)	-0.8 (2.5)	0.222	0.770
Mastiha	7.2 (10.1)	5.8 (3)	-1.4 (9.3)	0.416	
P^3	0.334	0.465			
Faecal lysozyme (μg/g) ‡					
Placebo	12.2 (9.8)	9.1 (6.1)	-3.1 (12)	0.155	0.614
Mastiha	15.7 (23.6)	9.4 (3.3)	-6.3 (25.6)	0.472	
P^3	0.973	0.359			
Faecal calprotectin (μg/g) ‡					
Placebo	940.9 (988.8)	5221.3 (18634.4)	4280.3 (18705.7)	**0.002**	0.117
Mastiha	1396.6 (4547.6)	977.63 (960.3)	-419.0(4756.2)	0.145	

P[3]	0.817	**0.026**			
Faecal lactoferrin (μg/g) ‡					
Placebo	112.4 (210.2)	141.4 (155.5)	29 (267.5)	**0.035**	0.419
Mastiha	65.7 (98.3)	86.12 (143.7)	-20.4(155.5)	0.060	
P[3]	0.715	0.156			

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; ‡based on logarithmic transformations.

4.5. Effects on amino acids levels

Table 27 shows the levels of plasma free amino acids in IBD patients pre- and post-intervention. Alanine, glycine, alpha-aminobutyric acic, valine, leucine, allo-isoleucine, isoleucine, threonine, serine, proline, asparagine, phenylalanine, ornithine, tyrosine, tryptophane and cystine increased significantly in the placebo group, while in the Mastiha group changes were insignificant. The mean changes in alanine, valine, proline and tyrosine differed significantly between the two groups at follow up. Furthermore, glutamine increased significantly in patients treated with placebo and decreased significantly in patients receiving Mastiha, with the mean change between the two arms being significant at follow up. Glutamic acid increased significantly in Mastiha group.

Table 27. Amino acids levels (nmol/mL) at baseline and at follow-up.

	Baseline	**Follow-up**	**Change**		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Alanine ‡					
Placebo	278.9 (77.9)	329.1 (86.4)	50.2 (101)	**0.002**	**0.006**
Mastiha	343.9 (80.4)	324.5 (40.2)	-19.4 (91.5)	0.418	
P[3]	**<0.001**	0.767			
Allo-isoleucine ‡					
Placebo	50 (24.8)	58.6 (19.3)	8.6 (31.9)	**0.004**	0.122
Mastiha	54.8 (18.9)	55.4 (6.9)	0.6 (21)	0.438	
P[3]	0.146	0.698			
α-Aminobutyric acid ‡					
Placebo	13.9 (5.4)	16.4 (5.8)	2.5 (7.3)	**0.018**	0.401
Mastiha	16 (6.5)	17.2 (6.6)	1.2 (7.1)	0.220	
P[3]	0.157	0.561			
β-Aminobutyric acid ‡					
Placebo	97.8 (2.3)	98.6 (2.6)	0.8 (3.5)	0.722	0.249
Mastiha	98.1 (2.3)	104.6 (29)	6.5 (32.1)	0.157	
P[3]	0.607	0.192			
Asparagine ‡					
Placebo	42.2 (12.4)	47.7 (11.8)	5.5 (15.3)	**0.005**	0.062
Mastiha	47.7 (12.8)	47.1 (6.3)	-0.7 (11.2)	0.844	
P[3]	**0.050**	0.918			
Aspartic acid ‡					
Placebo	7.7 (4.2)	7.4 (4.4)	-0.3 (5.8)	0.547	0.964
Mastiha	9.2 (5.9)	8.1 (4.4)	-1.2 (8.6)	0.591	
P[3]	0.723	0.730			
Cysteine ‡					
Placebo	27 (2.6)	28.5 (3.1)	1.5 (3.9)	**0.023**	0.338

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Mastiha	27.1 (2.9)	27.6 (2.4)	0.6 (3.8)	0.340	
P[3]	0.972	0.203			
Glutamic acid ‡					
Placebo	18.2 (19.6)	22.2 (14.1)	4 (22.7)	0.143	0.688
Mastiha	18.5 (11.5)	24.7 (12.3)	6.2 (15.8)	**0.044**	
P[3]	0.319	0.120			
Glutamine ‡					
Placebo	355.9 (76.3)	405.5 (68)	49.6 (91.3)	**0.003**	**<0.001**
Mastiha	422 (90.1)	365.9 (59.7)	-56 (102.3)	**0.005**	
P[3]	**0.003**	**0.014**			
Glycine ‡					
Placebo	212 (65.4)	237.4 (54.5)	25.4 (75.1)	**0.008**	0.115
Mastiha	238.9 (55)	239.9 (25.5)	1 (52.7)	0.229	
P[3]	**0.045**	0.499			
Histidine ‡					
Placebo	78.2 (23.4)	83.2 (17.7)	5 (27.5)	0.140	0.336
Mastiha	78.2 (21.7)	76.8 (12.6)	-1.4 (23.6)	0.903	
P[3]	0.894	0.135			
Hydroxyproline ‡					
Placebo	17 (5.4)	18.1 (4.8)	1.1 (7.8)	0.207	0.074
Mastiha	19.7 (5.7)	18.4 (6.8)	-1.3 (6.6)	0.201	
P[3]	**0.028**	0.295			
Isoleucine ‡					
Placebo	58.8 (28.5)	67.3 (22.1)	8.5 (36.8)	**0.011**	0.159
Mastiha	64.4 (21.6)	64.5 (8.2)	0.1 (24)	0.547	
P[3]	0.139	0.831			
Leucine ‡					
Placebo	118.5 (44.2)	143.1 (41.7)	24.6 (60.7)	**<0.001**	0.118
Mastiha	127.5 (32)	135.9 (15.8)	8.4 (35.2)	0.137	
P[3]	0.146	0.632			
Lysine ‡					
Placebo	177 (80.5)	187.2 (60.4)	10.2 (91.6)	0.100	0.098
Mastiha	193.7 (68.4)	177.6 (21.8)	-16.1 (73.2)	0.483	
P[3]	0.150	0.672			
Methionine					
Placebo	20.7 (8.7)	21.8 (7.8)	1.1 (11.2)	0.437	0.879
Mastiha	20.9 (6)	21.7 (4.1)	0.8 (5)	0.576	
P[3]	0.928	0.923			
Ornithine ‡					

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Placebo	78.3 (27.1)	89.2 (27.3)	10.9 (38.7)	**0.032**	0.085
Mastiha	89.8 (44)	82.3 (10.6)	-7.4 (47.9)	0.778	
P[3]	0.163	0.279			
Phenylalanine ‡					
Placebo	62.4 (18.6)	74.1 (15.3)	11.7 (25.6)	**0.001**	0.188
Mastiha	65.8 (13.2)	71.4 (8.1)	5.7 (16.6)	0.082	
P[3]	0.206	0.544			
Proline ‡					
Placebo	215.2 (65.5)	277.2 (73.4)	62 (85.6)	**<0.001**	**0.022**
Mastiha	269 (104.1)	277.8 (71.8)	8.8 (88.6)	0.241	
P[3]	**0.018**	0.922			
Serine ‡					
Placebo	101.5 (29.6)	117.1 (32.6)	15.5 (34.4)	**0.003**	0.105
Mastiha	110 (31)	111.1 (16.9)	1.2 (25.8)	0.458	
P[3]	0.238	0.550			
Thioproline ‡					
Placebo	16.3 (4)	13.8 (2.9)	-2.5 (5)	0.146	0.831
Mastiha	17.8 (6.4)	14.6 (3.8)	-3.2 (7.3)	0.081	
P[3]	0.856	0.406			
Threonine ‡					
Placebo	117.4 (41)	139 (46.1)	21.6 (56.6)	**0.004**	0.210
Mastiha	124.9 (34.8)	131.2 (25.3)	6.3 (32.1)	0.238	
P[3]	0.321	0.591			
Tryptophane ‡					
Placebo	57.3 (16.1)	66.3 (16.5)	9 (26.2)	**0.006**	0.278
Mastiha	59.1 (15.2)	62.1 (9.2)	3 (15.7)	0.207	
P[3]	0.575	0.275			
Tyrosine ‡					
Placebo	50.6 (17.8)	65.9 (18.5)	15.4 (26.8)	**<0.001**	**0.043**
Mastiha	54.9 (15.3)	59.2 (10.5)	4.3 (20.2)	0.190	
P[3]	0.180	0.100			
Valine ‡					
Placebo	311.7 (93.8)	363.4 (88)	51.7 (120.7)	**0.001**	**0.047**
Mastiha	346.9 (75.7)	354.1 (39.6)	7.2 (80.7)	0.454	
P[3]	**0.045**	0.848			

[1]p-value for time effect; [2]Effects reported include differences between the groups in mean changes (repeated measurements ANOVA); [3]p-value for group effect; ‡based on logarithmic transformations

4.6. Discussion

The chronic nature of IBD compromises the quality of life of patients. Even though there are many pharmacological agents targeting the maintenance of remission, a significant proportion of patients are primary non-responders or lose response over time. Additionally, adverse effects of current medications can make compliance with therapy a challenging issue for both patients and clinicians. Thus, supplementation of diet with natural treatments for periods of some months is under investigation in maintaining remission. Particularly curcumin seems to be a promising and safe medication for maintaining remission (Hanai et al., 2006), but studies with probiotics have shown conflicting results (Kruis et al., 1997; Schultz et al., 2004).

Mastiha, the resinous secretion of the shrub Pistacia lentiscus, is well known for its antioxidant and anti-inflammatory properties, with preclinical (in TNBS-colitis) and clinical data (in active CD patients) supporting its beneficial effects (Kaliora et al., 2007a; 2007b; Gioxari et al., 2011; Papalois et al., 2012). However, the present study is the first, to our knowledge, to evaluate the effects of Mastiha in preventing relapse in CD and UC patients with quiescent disease on the basis of a Phase-II randomized placebo-controlled clinical trial.

The relapse rates at 6 months (23.5% versus 17.6%) with no significant differences between the groups, did not show that Mastiha was superior to placebo, but interesting results in the free plasma AA responses after oral Mastiha were observed. AAs and their metabolites act as both substrates and regulators in various metabolic paths. Different circulating AA profiles have been reported in a plethora of diseases including IBD, with the differences in the plasma AA of IBD patients reflecting not simply nutritional state, but also inflammatory status and disease activity (Hisamatsu et al., 2012). For example, the essential AA valine has been found at significantly increased levels in DSS-treated mice compared to controls (Shiomi et al., 2011), and the non-essential AA proline has been found to be upregulated in both CD and UC patients compared to healthy controls (Hisamatsu et al., 2012).

In our study, we detected significant changes in the levels of valine and proline, and also in those of alanine, glutamine and tyrosine between the two groups at follow up, significantly increasing in the placebo arm, while remaining unchanged under treatment with Mastiha. Glutamine increased

significantly in patients treated with placebo and decreased significantly in patients receiving Mastiha, with the mean change between the two arms being significant. Data on the circulating levels of AAs in inactive IBD are very limited, and most data are derived from studies in active disease or from experimental animals (Hisamatsu et al., 2012; Shiomi et al., 2011; Gupta et al., 2012; Zhang et al.; 2013). However Williams and colleagues found significantly decreased levels of serum alanine in inactive IBD patients compared with controls, and although not investigated in their cohort, the authors speculated that this was due to increased protein catabolism and intestinal permeability (Williams et al., 2012). A different AA profile was detected in IBD patients by Ooi et al. (2011) but their results are confusing as the data presented refer to both active and inactive patients. The increase in circulating AAs in the plasma of our placebo patients is thus of considerable potential importance, as it may demonstrate the need for de novo AA synthesis in patients with increasing in inflammation as depicted by increased levels of IL-6, faecal calprotectin and lactoferrin. Alanine and glutamine account for a high proportion of the total AAs in muscles and their efflux during catabolism might well be reflected in levels of plasma free AAs, proposed as a sensitive marker for early prognosis of catabolism and inflammation (Storr et al., 2013). Although no significant differences were detected in precursors of alanine such as aspartic acid, it could also be hypothesized that increased catabolism provides the nitrogen source and contributes to the carbon pool from which AAs are synthesized. Interestingly, Mastiha treatment resulted in attenuation of the above-mentioned increase in plasma AAs, indicative of a protective role in relapse onset and in maintenance of remission. Additionally, unpublished data on plasma free AAs in healthy humans have shown that a decrease of plasma AAs occurs after Mastiha administration, and that this correlates strongly with enhanced human antioxidant capacity (data not shown).

Regarding other biochemical data, a significant increase was reported in albumin in the verum group. Although albumin is a very poor marker of nutritional status, its undoubted prognostic importance again indicates advantage for the Mastiha-treated patients. Additionally, total and LDL cholesterol significantly increased in the placebo group compared to the verum patients. Previous research has shown that Mastiha will lower total cholesterol (Kartalis et al, 2016), which could be of particular benefit to IBD patients who seem to be characterized by dyslipidemia and altered

lipoprotein profile (Sappati Biyyani et al., 2010).

While this design has some interesting results and the unusual quality for a nutrition study of being fully double-blinded, it has limitations, including the absence of colonoscopy at follow-up, precluding our ability to comment on any histological alterations. The study was however very tightly controlled to ensure compliance with the protocol, and had a final power of 0.90 for the between-subjects main effect at an effect size of 0.37.

Although superiority of Mastiha versus placebo in maintenance of remission in IBD could not be confirmed, the amelioration in increased plasma amino acids indicates an evident role in limiting disease activity. We are confident that the results demonstrated are of potential clinical significance and fully justify further exploration of the use of Mastiha in IBD.

PART D

CONCLUSIVE REMARKS

The incidence of chonic inflammatory diseases is increasing the last years not only in westernized populations but in other regions of the world as well. As such, IBD is a chronic inflammatory disease of the gastrointestinal tract, affecting about 100/100,000 persons in the general population and the incidence seems to increase worldwide. Since IBD has a significant impact on the quality of life of patients with serious complications, hospitalization, surgery and serious side effects caused by current medical treatment, there is an urgent need for management of IBD with less side effects, effectiveness and reduced cost for the public health.

All the above, led to further research towards natural supplements with beneficial properties for the human body. Several natural nutritional supplements are known since antiquity for their effectiveness in a plethora of pathologies. However, the evolution of laboratory techniques for the identification and quantification of their active compounds and phytochemicals has allowed the further investigation of their effects on the pathological conditions in preclinical and clinical context.

Mastiha is the resinous secretion of the shrub Pistacia lentiscus, growing exclusively on Chios Island, Greece. This natural product is rich mainly in terpenes and it is known since antiquity for its beneficial effects on the gastrointestinal system, skin inflammation and oral hugiene. Until now, Mastiha has been studied in several cell, animal and human studies and EMA has recognized Mastiha as a herbal medicinal product with the following indications, a) mild dyspeptic disorders, and b) symptomatic treatment of minor inflammations of the skin and as an aid in healing of minor wounds.

A Phase-I study of Kaliora and colleagues (2004a; 2004b) in active CD patients has shown safety and anti-inflammatory effects of Mastiha on inflammatory markers and disease activity. Based on this hypothesis and the gap of the current literature on the effectiveness of Mastiha on both CD and UC patients in relapse or in remission, the present Thesis aimed at investigating the effects of Mastiha administration in the context of a Phase-II clinical trial. But since the bioavailability of its phytochemicals in humans was not known, the present Thesis aimed also at discovering whether these phytochemicals are bioavailable in healthy humans.

In order to evaluate the human bioavailability of Mastiha's main phytochemicals, namely terpenes, seventeen healthy male volunteers followed a low-phytochemical diet for 5 days. Next, after overnight fasting, volunteers consumed Mastiha powder and blood samples were collected on time-points 0h (before ingestion) and 0.5h, 1h, 2h, 4h, 6h, 24h (post- ingestion). UHPLC-HRMS was applied for high throughput analysis of plasma. Serum resistance to oxidation, oxLDL levels and uric acids levels were measured.

UHPLC-HRMS/MS analysis showed that major terpenes were bioavailable since 0.5h after administration, reaching a peak between 2h and 4h. Serum resistance to oxidation, expressed as difference of tLAG (time point-0h), started to increase from 0.5h. This increase reached statistical significance at 4h, peaked at 6h and remained statistically significant until 24h. oxLDL levels, expressed as %change from 0h, were reduced significantly from time point-1h until time point-6h. These results demonstrate that terpenes of Mastiha are bioavailable in humans and potential mediators of antioxidant defence in vivo. Additionally it was shown that plasma free AAs detected and quantified with Gas Chromatography-Mass Spectrometry are modulated in response to terpenes intake in healthy subjects.

Regarding the study of the effects of Mastiha on active IBD, a randomised, double-blind, placebo-controlled clinical trial was designed. A total of 60 Inflammatory Bowel Disease patients were enrolled and randomly allocated to Mastiha (2.8g/day) or placebo groups for 3 months adjunct to stable medical treatment. Medical and dietary history, Inflammatory Bowel Disease Questionnaire, Harvey-Bradshaw Index, Partial Mayo Score, biochemical indices, oxidative stress, plasma free AAs, faecal and blood inflammatory markers were assessed. A clinically important difference between groups in IBDQ was defined as primary outcome. The results showed a significant improvement in Inflammatory Bowel Disease Questionnaire score in verum compared with baseline. There was a significant decrease in faecal lysozyme in Mastiha patients with the mean change being significant and significant increases of faecal lactoferrin and calprotectin in the placebo group. Fibrinogen reduced significantly with a significant mean change, whereas iron increased in Mastiha arm. Additionally, oxLDL was significantly decreased in Mastiha arm and the ratios of oxLDL/HDL and oxLDL/LDL were significantly decreased in the same group with the mean changes being

significantly different. The mean changes of AAs levels between the two groups did not differ significantly. However, it is of great importance that in UC patients an attenuation of the increase in the levels of several free AAs was observed in the Mastiha arm, showing a possible effect on AAs metabolic pathways related with inflammation, disease activity or muscle catabolism.

In addition, the effects of mastiha supplement in patients with inactive IBD were evaluated. A total of 68 patients were randomly allocated to Mastiha (2.8g/day) or placebo adjunct to stable medication. Free AAs were identified with GC-MS in plasma. Medical-dietary history, Inflammatory Bowel Disease Questionnaire, Harvey-Bradshaw Index, Partial Mayo Score, biochemical, faecal and blood inflammatory markers were assessed. Primary endpoint was the clinical relapse rate at 6 months. Although Mastiha was not proven superior to placebo in remission rate, attenuation in increase of free AAs levels in verum group was reported. Inflammatory markers had not a significantly different change between the two groups, even serum IL-6, faecal calprotectin and faecal lactoferrin increased only in the placebo group.

To sum up, the present Thesis resulted in the following conclusions:

- Mastiha's major terpenes are bioavailable in healthy humans
- Mastiha's terpenes are potential mediators of antioxidant defence in vivo
- Plasma free AAs are modulated in response to terpenes intake
- Regulation of faecal lysozyme after a 3-month administration of Mastiha in active IBD patients is shown and an effect secondary to a prebiotic potency is proposed.
- Mastiha inhibited an increase in plasma free AAs seen in patients with quiescent IBD and since change of AAs is considered an early prognostic marker of disease activity, this indicates a potential role of Mastiha in remission maintenance.
- Mastiha seems to exhibit a notable and favourable effect in UC patients in relapse that needs to be further explored.

The above-mentioned conclusions are of potential clinical significance and support the further

research of Mastiha use in IBD. Future research upon the mechanisms underlying the effects of Mastiha is necessary. For example, identification of changes in the metabolome of IBD patients supplemented with Mastiha may indicate a plethora of metabolites (i.e. lipids, TCA intermediate metabolites etc) regulated by this natural product. Additionally, the metagenome analysis of faecal samples of IBD patients will possibly reveal modifications in gut microbiota induced by Mastiha and will further investigate its potential prebiotic effect.

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APPENDICES

APPENDIX 1

BIOAVAILABILITY STUDY DOCUMENTS

GENERAL QUESTIONNAIRE

DATE:……

CODE:

- AGE:
- WEIGHT (KG):
- HEIGHT (CM):
- BMI :
- BOFY FAT PERCENTAGE:

- MEDICAL THERAPY/NUTRITIONAL SUPPLEMENTS

FORMULATION	DOSAGE	DURATION

- MEDICAL PROBLEMS LAST YEAR

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

- FOLLOWING A SPECIFIC DIETARY PATTERN;

How many portions/week do you eat?	
Whole grains (bread, pasta, rice etc)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Processed grains (bread, pasta, rice etc)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Potatoes	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Fruits	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Vegetables (raw or cooked))	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Legumes	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Fish	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Fatty fish (e.g. sardines, anchovies, salmon, mackerel, herring)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Olive oil use for cooking	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Red meats and products (veal, pork, sausage, lamb etc)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Poultry (chicken, turkey)	
Dairy whole fat (cheese, yoghurt, milk)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Cheese	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
Alcoholic drinks (number of glasses)	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily
	☐0 ☐0-1 ☐1-2 ☐2-3 ☐3-5 ☐ daily

Do you drink soft drinks …	Cola type ☐YES ☐NO Carbonated lemonade ☐YES ☐NO Carbonated juices ☐YES ☐NO Sugar free ☐YES ☐NO
How many glasses (250 ml) of soft drinks do you drink per day?	
How many times do you eat ready-to eat food as breakfast, lunch or dinner per week?	

Do you consume daily…	*Olive oil*	☐YES ☐NO
	Seed oil	☐YES ☐NO
	Butter	☐YES ☐NO
	Margarine	☐YES ☐NO

Do you prefer the milk or yoghurt to be:	☐*full-fat* ☐ *low-fat* ☐ *fat-free*
Do you prefer the cheese to be:	White : ☐ YES ☐ NO Yellow : ☐ YES ☐ NO Low fat: ☐ YES ☐ NO

Do you eat bread;	☐ YES ☐ NO	
If you answered YES…:		White ☐ YES ☐ NO Whole wheat ☐ YES ☐ NO

Do you drink alcohol regularly?	☐ YES ☐ NO
Do you drink coffee daily?	☐YES ☐NO
Coffee type:	☐greek ☐instant/ cappuccino ☐filtered

If you answered YES, how many cups do you drink daily?	☐0-1(60 g caffeine) ☐1-2 ☐3-5 ☐5+
Do you drink tea daily?	☐YES ☐NO
If you answered YES, how many cups do you drink daily?	☐0-1(60 gr teine) ☐1-2 ☐3-5 ☐5+

24h RECALL

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

PATIENT INFORMATION LEAFLET

Title of the study

«BIOAVAILABILITY OF THE MICROCONSTITUENTS OF NATURAL CHIOS MASTIHA IN HEALTHY ADULTS»

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 bioavailability of a natural unprocessed nutritional product, namely Mastiha.

Mastiha 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.. 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.

However, it is still unknown to which extent the microconstituents of Mastiha are bioavailable in the human body.

Screening assessments

After signing the Consent Form, we will check if you are eligible for this study (Screening assessment will include medical history, demographic data, complete blood count). If you are

eligible, you will be invited to follow a low in phytochemicals diet for five consecutive days as is it described on the attached brochure.

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 one time for the whole study period.

Assessment on the day of the intervention: Medical history, Dietary history, Body Weight (kg), height (cm), Body Mass Index (kg/m^2), Body fat percentage will be assessed. On the day of the experiment and after overnight fasting, you will consume 10g of natural Mastiha and blood samples will be obtained on timepoints 0h, 30min, 1h, 2h, 4h, 6h and 24h after Mastiha intake. Until timepoint 6h, you will be allowed to consume only water. Urine samples will be also collected on timepoints 0h, 4h, 8h and 24h.

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.

We hope that the results of this study will let us know which microconstituents of Mastiha are bioavailable. This will lead us to understand the mechanisms through which Mastiha exhibits its beneficial properties, improving its use to promote Public Health.

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.

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.

INSTRUCTIONS FOR A LOW-PHYTOCHEMICALS DIET

You are invited to follow these instructions for 5 consecutive days before conducting the experimental protocol for the assessment of bioavailability of Mastiha's constituents.

Food groups	**Foods that are ALLOWED**	**Foods that are NOT ALLOWED**
Milk and dairy	Milk, yoghurt, cheese	Yoghurt with fruits (fresh or dried) or honey
Fruits	-	Fruits (fresh or dried) and their juices (fresh or packed)
Vegetables	-	Vegetables (raw or cooked), tomato sauce and other vegetable juices
Grains - Bread - Pasta	Breakfast cereals, cereal bars, rice, potatoes, bread, crisps, crackers, pasta	Legumes, breakfast cereals or cereal bar with chocolate or fruits, soy, tortellini with vegetables
Meat and products	Meat (pork, veal etc), poultry, fish, eggs, cold cuts	-
Fats and oils	Margarine, butter, mayonnaise, nuts, ollive oil and seed oils **in small quantity**	-
Sweets	Desserts/biscuits/cakes/croissants **without** chocolate and fruits (fresh or dried)	Chocolate, Desserts/biscuits/cakes/croissants **without** chocolate and fruits (fresh or dried)
Beverages - Drinks	Water, Cola type soft drinks (**in small quantity**), Coffee (≤ 1 cup/day)	Coffee, wine, beer, cocoa, tea and other herbal infusions, alcoholic drinks, fruit juices
Herbs and Spices	Salt, pepper, spices and vinegar **in small quantity**	Herbs

For any question you are pleased to contact Mrs Efstathia Papada, Dietitian-Nutritionist (mob. 6978195984). **We would like to thank you for your participation!**

INFORMED CONSENT

Within this document I consent to participate voluntarily in the trial «BIOAVAILABILITY OF THE MICROCONSTITUENTS OF NATURAL CHIOS MASTIHA IN HEALTHY ADULTS».

The study will be conducted at the facilities of Harokopio University of Athens. The aim of the trial is to investigate the actual absorption and bioavailability of the microconstituents of natural Mastiha in healthy adults.

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. Complete a questionnaire including:
   - demographics
   - health status (personal and family medical history)
   - smoking habits
   - dietary history
   - physical activity.
2. Undergo anthropometry measurements, namely height, weight and body fat percentage.
3. Undergo a blood examination at a private diagnostic center without any financial burden before the start of the intervention.
4. Follow a low-phytochemical diet for 5 consecutive days before the day of the experiment.
5. Consume 10g of natural Mastiha that will be administered by the study personell on the day of the experiment.
6. Undergo a collection of blood (20cc/collection).
7. Collect and deliver urine sample (20cc/collection).

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 1-7.
- **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

_____ _____

APPENDIX 2

RCT DOCUMENTS

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 disorder* *Embolic episode* *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;

MED DIET SCORE

Foods	Frequency	Portion size
Whole grain products *bread* *pasta* *rice* *breakfast cereals* *toasts*		
Potatoes *boiled* *baked* *fried*		
Fruits and juices *Fresh fruits* *Dried fruits* *Juices*		
Vegetables and salads *raw* *cooked*		
Legumes		
Fish and soups *baked* *boiled* *fried*		
Red meat and products *meat* *cold cuts*		
Poultry *meat* *cold cuts*		
Dairy (full fat) *milk* *yoghurt* *white cheese* *yellow cheese*		
Olive oil in daily cooking		
Alcohol drinks *wine* *beer* *other*		
Soft drinks	Cola type drinks ☐YES ☐NO Carbonated Lemonade ☐YES ☐NO Other Carbonated juices ☐YES ☐NO Sugar free ☐YES ☐NO	

Coffee daily consumption *Coffee type* *cups/day*	☐YES ☐NO ☐greek ☐Instant / cappuccino ☐filtered ☐0-1(60 gr caffeine) ☐1-2 ☐3-5 ☐5+
Tea daily consumption *cups/day*	☐YES ☐NO ☐0-1(60 gr theine) ☐1-2 ☐3-5 ☐5+

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

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 the study site 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 microbiota 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.

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 2.8g of Mastiha daily in the form of tablets or placebo 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

_____ _____

APPENDIX 3

Table 1. Nutritional intake in IBD patients in relapse before and after intervention.

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Energy consumption (kcal/day)					
Placebo	1727.31 (808.15)	1630.82 (592.11)	-96.48 (811.1)	0.760	0.917
Mastiha	1898.03 (595.84)	1828.86 (542.67)	-69.17 (698.07)	0.622	
P[3]	0.158	0.171			
Protein (g/day)					
Placebo	77.3 (48.76)	63.26 (38.48)	-14.04 (54.76)	**0.045**	0.247
Mastiha	93.37 (42.44)	95.76 (55.01)	2.39 (57.64)	0.605	
P[3]	0.062	0.015			
Protein (% of energy consumption/day)					
Placebo	17.86 (5.44)	15.27 (7.22)	-2.6 (9.02)	**0.037**	0.152
Mastiha	19.82 (7.37)	20.67 (8.05)	0.85 (10.54)	0.847	
P[3]	0.320	**0.041**			
Carbohydrates (g/day)					
Placebo	186.09 (86.43)	157.27 (98.63)	-28.82 (116.95)	0.063	0.843
Mastiha	215.37 (79.53)	171.28 (77.5)	-44.09 (111.36)	**0.020**	
P[3]	0.078	0.419			
Carbohydrates (% of energy consumption/day)					
Placebo	43.59 (8.35)	40.03 (20.69)	-3.56 (21.5)	0.058	0.884
Mastiha	46.21 (11.05)	37.93 (14.79)	-8.28 (20.44)	**0.022**	
P[3]	0.428	0.900			
Dietary fiber (g/day)					
Placebo	10.81 (6.95)	12.83 (7.61)	2.02 (8.94)	0.243	0.277
Mastiha	13.54 (7.9)	11.94 (4.63)	-1.6 (8.17)	0.741	
P[3]	0.170	0.980			
Sugars (g/day)					
- **Glucose (g/day)**					
Placebo	7.55 (6.8)	9.7 (6.59)	2.15 (6.96)	0.052	0.677
Mastiha	8.03 (6.08)	9.35 (5.1)	1.32 (7.6)	0.123	
P[3]	0.706	0.900			
- **Lactose (g/day)**					
Placebo	2.29 (3.7)	0.82 (1.42)	-1.47 (4.14)	0.146	0.257
Mastiha	5.02 (9.34)	3.85 (8.91)	-1.17 (6.72)	0.938	
P[3]	0.539	**0.016**			
- **Fructose (g/day)**					
Placebo	10.57 (10.18)	12.4 (8.31)	1.82 (10.79)	0.056	0.912
Mastiha	8.77 (7.32)	11.31 (6.74)	2.55 (9.39)	0.051	
P[3]	0.763	0.557			

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	p[1]	p[2]
Total fat (g/day)					
Placebo	75.69 (42.15)	52.43 (25.51)	-23.25 (40.63)	**0.012**	0.197
Mastiha	75.33 (41.6)	65.98 (28.79)	-9.35 (44.65)	0.363	
p[3]	0.810	0.091			
Total fat (% of energy consumption/day)					
Placebo	38.3 (12.04)	27.64 (9.96)	-10.65 (13.38)	**0.001**	0.094
Mastiha	34.52 (11.4)	30.79 (10.39)	-3.72 (16.25)	0.189	
p[3]	0.274	0.238			
Saturated Fatty Acids (g/day)					
Placebo	24.64 (15.05)	20.23 (10.79)	-4.41 (12.12)	0.193	0.210
Mastiha	23.95 (13.43)	25.43 (12.38)	1.48 (15.08)	0.665	
p[3]	0.873	0.134			
Monosaturated Fatty Acids (g/day)					
Placebo	33.28 (20.63)	25.22 (16.44)	-8.05 (23.45)	0.092	0.680
Mastiha	32.4 (21.74)	26.23 (15.7)	-6.17 (26.07)	0.208	
p[3]	0.917	0.696			
Polyunsaturated Fatty Acids (g/day)					
Placebo	11.76 (7.68)	8.84 (5.71)	-2.93 (8.76)	0.051	0.330
Mastiha	11.79 (7.41)	10.38 (4.93)	-1.41 (8.42)	0.432	
p[3]	0.767	0.163			
Trans Fatty Acids (g/day)					
Placebo	0.65 (1.64)	0.38 (0.57)	-0.27 (1.84)	0.638	0.612
Mastiha	0.75 (1.58)	0.32 (0.39)	-0.43 (1.59)	0.205	
p[3]	0.641	0.836			
α-carotene (μg/day)					
Placebo	228.29 (604.77)	746.39 (1815.55)	518.1 (1899.26)	0.085	0.567
Mastiha	324.71 (735.57)	717.5 (1431.87)	392.79 (974.14)	0.284	
p[3]	0.152	0.552			
β-carotene (μg/day)					
Placebo	895.72 (1357.95)	1976.11 (3621.34)	1080.4 (3615.63)	0.314	0.919
Mastiha	1521.31 (3087.64)	2176.2 (3363.52)	654.89 (2283.42)	0.335	
p[3]	0.559	0.658			
Vitamin D (μg/day)					
Placebo	3.6 (8.46)	1.31 (1.25)	-2.3 (8.69)	0.218	0.311
Mastiha	10.54 (46.65)	3.08 (3.59)	-7.46 (46.91)	0.884	
p[3]	0.549	**0.003**			
Vitamin E (mg/day)					
Placebo	1.7 (2.26)	1.41 (1.64)	-0.28 (2.89)	0.633	0.130
Mastiha	0.89 (0.84)	2.18 (3.39)	1.29 (3.42)	0.084	
p[3]	0.147	0.422			

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	p[1]	p[2]
Vitamin K (μg/day)					
Placebo	44.49 (40.74)	50.31 (48.87)	5.81 (50.3)	0.668	0.933
Mastiha	56.35 (78.19)	79.2 (132.02)	22.85 (129.08)	0.727	
p[3]	0.361	0.509			
Vitamin C (mg/day)					
Placebo	39.99 (44.44)	68.39 (57.11)	28.4 (63.38)	0.077	0.613
Mastiha	53.02 (57.61)	66.5 (51.05)	13.48 (51.78)	0.222	
p[3]	0.895	0.610			
Ca (mg/day)					
Placebo	839.27 (648.91)	713.56 (365.1)	-125.72 (534.96)	0.617	0.274
Mastiha	721 (419.59)	842.8 (468.91)	121.8 (532.53)	0.280	
p[3]	0.526	0.475			
Fe (mg/day)					
Placebo	10.64 (5.22)	10.83 (4)	0.19 (5.31)	0.603	0.532
Mastiha	11.9 (4.09)	11.82 (4.78)	-0.08 (5.9)	0.721	
p[3]	0.169	0.561			
Se (μg/day)					
Placebo	97.33 (63.48)	95.18 (48.92)	-2.16 (77.59)	0.804	0.533
Mastiha	114.99 (50.31)	141.62 (80.46)	26.63 (87.27)	0.231	
p[3]	0.069	**0.011**			
Lycopene (μg /day)					
Placebo	2982 (5090.63)	2123.65 (4159.34)	-858.36 (7054.55)	0.764	0.906
Mastiha	5142.7 (15708.33)	1581.52 (2144.9)	-3561.19 (15593.84)	0.875	
p[3]	0.491	0.559			
Caffeine (mg/day)					
Placebo	262.07 (402.6)	190.7 (241.77)	-71.37 (272.77)	0.059	0.392
Mastiha	112.17 (347.72)	98.04 (119.14)	-14.14 (327.34)	**0.001**	
p[3]	0.157	0.304			

Logarithmic transformations were used in all of the analyses except those for med diet score, where raw data were used.
[1]p-value for time effect; [2]Effects reported include differences between the groups in the degree of change (repeated measurements ANOVA); [3]p-value for group effect

Table 2. Nutritional intake in IBD patients in remission before and after intervention.

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	P[1]	P[2]
Energy consumption (kcal/day)					
Placebo	1959.99 (822.52)	1754.63 (616.3)	-205.36 (746.05)	0.257	0.489
Mastiha	2088.31 (742.42)	2009.31 (565.49)	-79 (803.33)	0.872	
P[3]	0.373	**0.038**			
Protein (g/day)					
Placebo	91 (37.19)	97.19 (44.3)	6.19 (49.53)	0.474	0.897
Mastiha	98.03 (41.77)	105.96 (46.86)	7.94 (57.8)	0.370	
P[3]	0.511	0.354			
Protein (% of energy consumption/day)					
Placebo	19.99 (8.25)	22.83 (7.91)	2.84 (11.2)	0.069	0.665
Mastiha	18.95 (5.77)	21.15 (6.71)	2.2 (9.53)	0.221	
P[3]	0.909	0.417			
Carbohydrates (g/day)					
Placebo	225.05 (151.76)	183.64 (84.65)	-41.41 (153.54)	0.224	0.810
Mastiha	229.84 (106.47)	184.4 (52.85)	-45.44 (109.01)	0.122	
P[3]	0.533	0.590			
Carbohydrates (% of energy consumption/day)					
Placebo	45.82 (24.42)	42.23 (12.99)	-3.59 (27.44)	0.464	0.379
Mastiha	43.42 (10.81)	37.52 (6.86)	-5.9 (12.19)	0.052	
P[3]	0.928	0.160			
Dietary fiber (g/day)					
Placebo	16.86 (13.41)	13.11 (6.88)	-3.75 (13.12)	0.261	0.764
Mastiha	18.13 (14)	15.51 (12.78)	-2.62 (13.42)	0.483	
P[3]	0.884	0.574			
Sugars (g/day)					
- **Glucose (g/day)**					
Placebo	10.66 (14.01)	8.6 (8.15)	-2.05 (14.48)	0.836	0.900
Mastiha	12.08 (11.47)	10.39 (6.52)	-1.69 (12.68)	0.976	
P[3]	0.182	0.121			
- **Lactose (g/day)**					
Placebo	2.69 (4.64)	2.43 (4.26)	-0.26 (5.11)	0.962	0.698
Mastiha	3.68 (6.18)	2.52 (2.78)	-1.17 (7.17)	0.552	
P[3]	0.823	0.378			
- **Fructose (g/day)**					
Placebo	12.55 (16.55)	9.51 (9.45)	-3.04 (16.76)	0.765	0.786
Mastiha	15.64 (16.8)	12.12 (9.13)	-3.51 (16.53)	0.932	
P[3]	0.122	0.072			
Total fat (g/day)					
Placebo	86.59 (47.64)	65.59 (30.32)	-21 (47.35)	**0.048**	0.099
Mastiha	83.61 (36.68)	83.82 (37.34)	0.21 (46.78)	0.724	

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	p^1	p^2
p^3	0.834	**0.012**			
Total fat (% of energy consumption/day)					
Placebo	38.32 (11.77)	33.17 (9.13)	-5.15 (13.36)	**0.045**	0.037
Mastiha	35.92 (10.41)	37.31 (7.52)	1.39 (11.75)	0.335	
p^3	0.488	**0.033**			
Saturated Fatty Acids (g/day)					
Placebo	25.69 (14.73)	24.4 (11.74)	-1.29 (13.07)	0.844	0.362
Mastiha	26.72 (14.07)	30.82 (14.75)	4.09 (19.03)	0.139	
p^3	0.538	**0.036**			
Monosaturated Fatty Acids (g/day)					
Placebo	38.88 (25.72)	26.35 (12.18)	-12.53 (26.2)	0.061	0.160
Mastiha	35.31 (18.2)	34.19 (18.04)	-1.12 (23.71)	0.917	
p^3	0.950	**0.026**			
Polyunsaturated Fatty Acids (g/day)					
Placebo	12.88 (10.77)	10.06 (7.74)	-2.81 (11.84)	0.110	0.164
Mastiha	14.43 (8.15)	14.13 (7.08)	-0.3 (10.41)	0.712	
p^3	0.304	**0.003**			
Trans Fatty Acids (g/day)					
Placebo	0.65 (1.03)	0.49 (0.71)	-0.16 (1.26)	0.646	0.831
Mastiha	0.38 (0.56)	0.32 (0.39)	-0.05 (0.73)	0.874	
p^3	0.279	0.286			
α-carotene (μg/day)					
Placebo	538.25 (1435.24)	287.2 (406.83)	-251.06 (1466.64)	0.419	0.568
Mastiha	539.87 (1285.49)	474.32 (890.34)	-65.55 (864.3)	0.109	
p^3	0.256	**0.047**			
β-carotene (μg/day)					
Placebo	1646.77 (3530.37)	2046.72 (3587.59)	399.94 (4926.6)	0.214	0.536
Mastiha	1802.99 (3098.75)	1985.32 (1963.53)	182.33 (2189.84)	**0.036**	
p^3	0.277	0.051			
Vitamin D (μg/day)					
Placebo	2.87 (3.68)	1.75 (2.18)	-1.13 (4.04)	0.083	0.044
Mastiha	1.98 (1.72)	2.35 (1.48)	0.38 (2.05)	0.258	
p^3	0.631	**0.033**			
Vitamin E (mg/day)					
Placebo	0.93 (1.4)	2.05 (2.24)	1.12 (2.71)	0.009	0.175
Mastiha	1.18 (1.03)	1.74 (2)	0.56 (2.07)	0.445	
p^3	0.173	0.523			
Vitamin K (μg/day)					
Placebo	53.92 (69.77)	128.13 (282.98)	74.21 (298.1)	0.152	0.974
Mastiha	60.83 (42.85)	72.96 (36.03)	12.13 (49.86)	0.140	

	Baseline	Follow-up	Change		
	Mean (SD)	Mean (SD)	Mean (SD)	p^1	p^2
p^3	0.187	0.204			
Vitamin C (mg/day)					
Placebo	59.56 (66.47)	187.55 (552.66)	127.98 (543.28)	**0.016**	0.125
Mastiha	181.63 (561.58)	94.82 (50.64)	-86.81 (566.23)	0.791	
p^3	**0.019**	0.653			
Ca (mg/day)					
Placebo	874.93 (639.26)	779.92 (395.31)	-95.02 (580.68)	0.862	0.841
Mastiha	863.9 (484.92)	876.12 (424.77)	12.21 (642.65)	0.648	
p^3	0.651	0.366			
Fe (mg/day)					
Placebo	14.41 (9.16)	11.01 (4.54)	-3.4 (9.38)	0.107	0.431
Mastiha	13.37 (5.8)	12.4 (5.15)	-0.97 (6.49)	0.607	
p^3	0.952	0.179			
Se (μg/day)					
Placebo	103.17 (65.35)	126.28 (69.41)	23.1 (69.11)	**0.012**	0.334
Mastiha	124.07 (62.6)	143.93 (72.58)	19.86 (85.7)	0.237	
p^3	0.071	0.160			
Lycopene (μg /day)					
Placebo	2020.76 (3805.52)	1487.61 (2509.1)	-533.15 (3917.65)	0.259	0.245
Mastiha	5572.83 (7706.23)	1732.59 (1471.26)	-3840.23 (7202.46)	0.604	
p^3	**0.001**	**0.034**			
Caffeine (mg/day)					
Placebo	328.81 (567.28)	71.07 (57.5)	-257.74 (553.42)	0.696	0.888
Mastiha	234.68 (344.26)	92.43 (89.6)	-142.25 (348.88)	0.555	
p^3	0.898	0.989			

Logarithmic transformations were used in all of the analyses except those for med diet score, where raw data were used.
[1]p-value for time effect; [2]Effects reported include differences between the groups in the degree of change (repeated measurements ANOVA); [3]p-value for group effect