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Connecting the dots between diet, physical activity, gut microbiota and systemic lupus erythematosus

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2023

ACKNOWLEDGMENTS

This thesis is the final work of my Master's degree in Biochemistry and Biomedicine, which was essential to expand my scientific knowledge and lab skills, and to fuel my endless curiosity.

When choosing this project, I had in mind that studying the gut microbiota in a translational study would be exciting. However, I was far from knowing what an incredible experience it would be. I was lucky enough to be integrated into a reference research institute as Instituto de Medicina Molecular João Lobo Antunes is, and to learn from inspiring professionals, to whom I am deeply grateful.

Firstly, I would like to express my profound appreciation to Patrícia Costa Reis, my supervisor, who inspired me since the beginning of this project. Patrícia Costa Reis is a dedicated physician who, despite her hectic days as a clinician, is also an enthusiastic scientist. Her endless effort to make things happen and to push the projects forward is truly motivating, and her kindness and understanding were always reassuring in challenging times. For me, science is about fueling curiosity, constant learning, and hypothesis testing, but also about enthusiasm for not always viable ideas. Patrícia gave me room to explore all this without reservations, and because of it, I became a better researcher without losing creativity. Thank you for your care, generosity, trust, and encouragement in my participation in exciting new projects, and for supporting my future research and personal endeavors.

To Miguel Lyon de Castro, with whom I have shared the day-to-day work in the lab, for his dedication and support. It is always best to have someone to share ideas, the bench, the challenges, and the breakthroughs.

To João Eurico Fonseca for the opportunity to develop this work at the Rheumatology research unit, for the readiness and always prompt response to all my solicitations, and for the support in the steps towards my future research path.

To Ângelo Calado, Catarina Tomé, Rita Moura, Rui Teixeira, and Bruno Vidal, the Rheumatology research unit team, for their invaluable support and experience in showing my way around the lab, and for their readiness to help and teach whenever was necessary.

To Mário Ramirez and Inês Mendes, from the Microbiology unit at iMM, for their willingness to be part of this project and readiness to answer all our (many) questions. Thank you for your work in analyzing the complex microbiota data and for your professionalism and thoroughness when looking at the data.

To Catarina Sousa Guerreiro, for the enthusiasm to participate in this project, for facilitating access to the Nutrition lab equipment, without which the body composition and the dietary assessment would not have been possible, and for the valuable input regarding all the nutrition-related data.

To Nikita Khmelinskii, rheumatologist at Santa Maria hospital, for him receptivity to this project and for the continuous support and effort to accommodate the patients' recruitment in his busy professional days.

To Ângela Afonso, Andreia Lopes, and Ionela Toader, from Biobanco-iMM, for their friendliness and readiness to help whenever necessary, for their professionalism in taking care of our precious samples, and for their willingness to let me sneak peek at their days' work.

To Simão Teixeira da Rocha, and Adriana Vieira for their generosity, for welcoming me at X-Lupus project, and for all the support since then. Working and learning with such dedicated researchers has been a great privilege.

To Carla Motta, a skillful researcher at Instituto Nacional de Saúde Doutor Ricardo Jorge, for her cheerful and inspiring approach to science, and her readiness to teach and to be a part of (not always easy) new projects, despite the busy schedule.

To all the lupus patients that have given their time to make this project possible, despite the numerous tasks involved, for their enthusiasm and for allowing me to know first-hand their struggles and breakthroughs while living with lupus. To all the healthy controls that collaborated in this project and gave their samples and their time to help a scientific project.

To Fundação Amélia de Mello that awarded this project the D. Manuel de Mello grant, for its relevant financial support to Science.

To my family for the continuous and unconditional support, and for understanding my long absences. It can be challenging to make it all work smoothly.

To Inês Gomes, Diana Alexandre, Joana Ávila, and Filipa Pereira, my cheerleading team, for sharing my excitement for this project, and science, for reminding me that all the effort will be worth it because I am enjoying the path.

To João, for all the love and support, for understanding that I am at my best and happier when I am busy, learning, and pursuing something more challenging than the previous project.

TABLE OF CONTENTS

TABLES INDEX

FIGURES INDEX

ABBREVIATIONS

ACE - Angiotensin-converting enzyme
ACR - American College of Rheumatology
ALA- α-Linolenic acid
ANA - Antinuclear antibodies
ANOVA - Analysis of variance
anti-dsDNA- Antibodies to double-stranded DNA
anti-Sm – Anti-Smith antibodies
aPL - Antiphospholipid antibodies
ARA - Arachidonic acid
ARB - Angiotensin receptor blocker
BAFF - B-cell activating factor
BCR - B cell receptors
BMI - Body mass index
C - Complement
COX-2 - Cyclooxygenase-2
CRP - C-reactive protein
DCs - Dendritic cells
DHA- Docosahexaenoic acid
DLE - Discoid lupus erythematosus
DNA - Deoxyribonucleic acid
DRI - Dietary reference intake
DSS - Dextran sulfate sodium
EBV - Epstein-Barr virus
EFSA - European Food Safety Authority
ELISA - Enzyme-Linked Immunosorbent Assay
EPA- Eicosapentaenoic acid
EUA - United States of America
EULAR - European League Against Rheumatism
FADS - Fatty acid desaturase
F/B ratio – Firmicutes/Bacteroidetes ratio
FcγR - Fc gamma receptors
FCT - *Fundação para a Ciência e Tecnologia*
FDA - Food and drug administration
GALT - Gut-associated lymphoid tissue
GC - Glucocorticoids
GLP-1 - Glucagon-like peptide 1
GM-CSF - Granulocyte-macrophage colony-stimulating factor
GPCR - G protein coupled receptors
h - Helper
HC – Healthy controls
HCQ – Hydroxychloroquine
HDL - High-density lipoprotein
hsCRP - High-sensitivity C-reactive protein
HUFA - Highly unsaturated fatty acid
IAN-AF - *Inquérito Alimentar Nacional e de Atividade Física*

iBALT - Induced bronchial-associated lymphoid tissue
ICAM-1- Intercellular Adhesion Molecule-1
IECs - Intestinal epithelial cells
I-FABP - Intestinal fatty acid binding protein
IFN – Interferon
Ig - Immunoglobulin
IL – Interleukin
ILC3s - Type 3 innate lymphoid cells
IPAQ - International Physical Activity Questionnaire
jSLE - Juvenile-onset SLE
LDL - Low-density lipoprotein
LN - Lupus nephritis
LPS – Lipopolysaccharides
MALT - Mucosa-associated lymphoid tissue
MAPK - Mitogen-activated protein kinases
mDC – myeloid dendritic cells
METs - metabolic equivalents of task
miRNA – Micro-RNAs
MMF - Mycophenolate mofetil
MnSOD - manganese-superoxide dismutase
MRL - Murphy Roths large
MTHFR – Methylene tetrahydrofolate reductase
MUC - Mucin
MUFA - Monounsaturated fatty acids
n-3 - Omega-3 PUFA
n-6 - Omega-6 PUFA
NaCl - Sodium chloride
NAD - Nicotinamide adenine dinucleotide
NADPH - Nicotinamide adenine dinucleotide phosphate
NALT - Nasal-associated lymphoid tissue
NETs - Neutrophil extracellular traps
NF-κB – Nuclear factor κB
NK – Natural killer
NZB/NZW - New Zealand black x New Zealand white
PAMPs - Pathogen-associated molecular patterns
pDC - Plasmacytoid dendritic cells
PGA - Physician Global Assessment
PGE2 - Prostaglandin E2
PPAR-γ - proliferator-activated gamma receptor
PREDIMED - *PREvención con DIeta MEDiterránea*
PROMIS - Patient-Reported Outcomes Measurement Information System
PRRs - Pattern recognition receptors
PUFA - Polyunsaturated fatty acids
RA - Rheumathoid arthritis
RDI - Recommended daily intake
RegIIIγ - Regenerating islet-derived 3
RNA – Ribonucleic acid
ROR - acid-receptor-related orphan nuclear receptor

SCFA - Short-chain fatty acids
SFA - Saturated fatty acids
SGK1 - Serine/threonine protein kinase 1
SLE - Systemic lupus erythematosus
SLEDAI - Systemic lupus erythematosus disease activity index
SLICC - Systemic Lupus International Collaborating Clinics
SNPs - Single nucleotide polymorphisms
SOD - Superoxide dismutase
SSA - Anti-Ro antibodies
SSB - Anti-La antibodies
TCR - T cell receptors
T_{FH} - Follicular helper T cells
TGF - Transforming growth factor
Th – Thelper
TJ - Tight junctions
TLR – Toll-like receptor
TNF-α - Tumor necrosis factor-α
tRA - All-trans-retinoic acid
Treg – Regulatory T cells
UV light – ultraviolet light
VARA - All-trans-retinyl palmitate together with tRA
VCAM-1 - Vascular cell adhesion molecule-1
VDR – Vitamin D receptor

ABSTRACT

Introduction: Systemic lupus erythematosus (SLE) is an immune-mediated disease with a diverse phenotype that mainly affects young women and is associated with high morbidity and mortality. New treatment strategies are much needed.
Dysbiosis and a higher gut permeability may facilitate the translocation of microbes and their metabolites to the bloodstream, contributing to the chronic activation of the immune system seen in SLE.

Objectives: Study diet, physical activity, and gut microbiota in SLE.

Methods: Healthy controls (HC) and SLE patients who met the 2019 EULAR/ACR SLE criteria were included. Dietary intake was assessed by three 24-hour dietary recalls, adherence to the Mediterranean diet with PREDIMED and KIDMED questionnaires, physical activity with IPAQ questionnaire, body composition by air displacement plethysmography. The intestinal microbiota was sequenced by Next Generation Sequencing (16S rRNA), and glucagon-like peptide-1 concentrations were assessed by ELISA.

Results: This cross-sectional study included 33 SLE patients (87.9% women; 67% with lupus nephritis) and 13 age and gender-matched HC. SLE patients had lower adherence to the Mediterranean diet ($p=0.0222$), consumed less n-3 polyunsaturated fatty acids (PUFA) ($p=0.0248$), α-linolenic acid ($p<0.0001$), eicosapentaenoic acid ($p=0.0077$), and fiber ($p<0.0001$), and had a higher n-6/n-3 PUFA ratio than HC ($p=0.0042$). SLE patients had lower physical activity ($p=0.0302$), higher sitting time ($p=0.0479$), and fat mass ($p=0.0118$). SLE patients had a decreased gut microbiota α-diversity ($p=0.028$), which was associated with the Mediterranean diet ($p=0.014$), and with eicosapentaenoic and docosahexaenoic acid, two n-3 PUFA ($p=0.0127$). Finally, the *Rikenellaceae* bacterial family was increased in patients with lupus nephritis.

Conclusion: In this cohort of SLE patients, there was a low adherence to the Mediterranean diet, a low intake of several macro and micronutrients, low physical activity, high fat mass, and gut dysbiosis. Together, these findings support the hypothesis that rebalancing the gut microbiota could be a promising therapeutic target in SLE.

Key words: systemic lupus erythematosus, diet, body composition, gut microbiota, dysbiosis

RESUMO

Introdução

O lúpus eritematoso sistémico (LES) é uma doença crónica, imunomediada, com etiopatogenia complexa e fenotipicamente diversa. O LES afeta principalmente mulheres em idade fértil e é menos frequente, mas geralmente mais grave, em crianças do que em adultos.

O lúpus eritematoso sistémico tem um espectro diversificado de manifestações clínicas e apesar dos vários avanços no tratamento é ainda uma das principais causas de morte não-traumática em mulheres jovens. Apesar de todos os esforços para atenuar os efeitos desta doença, até à data, os doentes com LES ainda apresentam uma menor esperança de vida, maior taxa de desemprego e menores níveis de escolaridade. A falta de resposta aos tratamentos atuais e os efeitos adversos do uso crónico de imunossupressores não seletivos contribuem para a morbilidade e mortalidade dos doentes com LES.

A quebra da autotolerância e a inflamação crónica são fenómenos centrais no LES e refletem a interação entre fatores genéticos, epigenéticos e ambientais. Tendo em vista a minimização do impacto desta doença na qualidade de vida dos doentes, é essencial encontrar melhores estratégias terapêuticas.

Apesar dos conhecidos benefícios metabólicos de uma dieta equilibrada, os doentes com LES geralmente seguem uma dieta desequilibrada, ficando suscetíveis à insuficiência de macro e micronutrientes que pode também contribuir para uma pior composição corporal, uma maior atividade da doença e um maior uso de corticóides.

Além da dieta, o tempo sedentário também favorece uma maior atividade da doença, assim como uma maior frequência de recidivas e sintomas depressivos e ansiedade. Pelo contrário, a actividade física moderada a vigorosa diminui a pressão arterial, o risco de eventos cardiovasculares e fadiga, e melhora as funções físicas e executivas.

A dieta modula a microbiota intestinal, a integridade da barreira intestinal e a resposta imune intestinal aos patobiontes e comensais intestinais. De facto, a microbiota intestinal tem vindo a ser apontada como um potencial fator envolvido na patogénese do lúpus eritematoso sistémico. A disbiose da microbiota intestinal é frequentemente identificada em doentes com esta doença, o que, juntamente com uma alteração da barreira intestinal, pode favorecer a endotoxémia e a ativação crónica do sistema imunitário.

Objetivos

Avaliar a complexa interação entre fatores de estilo de vida e a microbiota intestinal no lúpus eritematoso sistémico. Para isso avaliámos a dieta, a composição corporal e a microbiota intestinal de doentes com lúpus eritematoso sistémico e de controlos saudáveis. Avaliámos ainda as concentrações de peptídeo semelhante a glucagon 1 (GLP-1), como possível novo marcador de permeabilidade intestinal.

Métodos

Incluíram-se controlos saudáveis e doentes com lúpus eritematoso sistémico (crianças e adultos), recrutados no Hospital de Santa Maria, entre Abril e Outubro de 2021, que cumpriam os critérios para LES da *European Alliance of Associations for Rheumatology*/*American College of Rheumatology* de 2019 (2019 *EULAR/ACR SLE*). A atividade da doença foi calculada de acordo com o índice *Systemic Lupus Erythematosus Disease Activity Index 2000* (SLEDAI-2K). Registaram-se os dados demográficos e clínicos. A ingestão alimentar foi avaliada por três inquéritos alimentares de 24 horas (dois dias úteis e um dia de fim-de-semana). A adesão à dieta mediterrânica foi avaliada com os questionários *PREvención con DIeta MEDiterránea* (PREDIMED) e *Mediterranean Diet Quality Index for children and adolescents* (KIDMED) e a atividade física com a versão curta do questionário *International Physical Activity Questionnaire* (IPAQ). A composição corporal foi avaliada por

pletismografia por deslocamento de ar (*BOD POD Gold Standard Body Composition Tracking System, COSMED*). A microbiota intestinal foi estudada por sequenciamento de nova geração (16S rRNA), e as concentrações de GLP-1 avaliadas por *Enzyme-Linked Immunosorbent Assay* (ELISA).

Resultados

Este estudo transversal avaliou 33 doentes com lúpus eritematoso sistémico (11 crianças e adolescentes e 22 adultos; 87.9% mulheres) e 13 controlos saudáveis. A maioria dos doentes tinha nefrite lúpica (67%). Os doentes com LES tinham uma idade mediana de vinte e nove anos (mínimo: 11 anos; máximo: 57 anos) e os controlos saudáveis de trinta e sete anos (mínimo:14 anos; máximo 50 anos). No momento da colheita das amostras a maioria dos doentes tinha baixa atividade da doença (SLEDAI-2K médio: 4). A maioria dos doentes (87,9%) encontrava-se a ser tratado com hidroxicloroquina e prednisolona e 59,4% com micofenolato de mofetil.

Na nossa coorte, 87,5% dos doentes com LES e 53,8% dos controlos saudáveis tinham uma baixa adesão à dieta mediterrânica, sendo a diferença entre os dois grupos estatisticamente significativa ($p=0,0222$). Ao analisar a ingestão alimentar dos participantes, verificou-se que os doentes com LES consumiam menos ácidos gordos polinsaturados ómega-3 ($p=0,0248$), em particular, ácido α-linolénico ($p<0,0001$) e ácido eicosapentaenoico ($p=0,0077$). Os doentes com LES apresentavam também uma proporção de ómega-6/ómega-3 superior aos controlos saudáveis ($p=0,0042$). Os doentes com LES tinham significativamente menor consumo de fibra ($p<0,0001$), potássio ($p=0,0062$), magnésio ($p=0,0004$), ferro ($p=0,0002$) e manganês ($p=0,0004$), assim como vitamina B6 ($p=0,0060$), vitamina K1 ($p=0.0235$) e folato ($p=0,0006$).

Ao avaliar a atividade física dos participantes, verificámos que os doentes com LES apresentaram uma menor atividade física ($p=0,0302$) e maior tempo sedentário ($p=0,0479$) que os controlos saudáveis. Os doentes com LES adultos apresentaram ainda uma maior percentagem de massa gorda que os controlos saudáveis adultos ($p=0,0118$).

Ao analisar a microbiota intestinal dos participantes, identificámos que a α-diversidade da microbiota intestinal dos doentes com LES se encontrava significativamente diminuída ($p=0,028$) e que este parâmetro foi influenciado pela dieta mediterrânica ($p=0,014$), mostrando que uma menor adesão a este padrão alimentar se associou a uma menor diversidade da microbiota intestinal. Para além da dieta mediterrânica, a diversidade da microbiota também foi influenciada pela ingestão de ácido eicosapentaenóico e ácido docosahexaenóico ($p=0,0127$), dois dos principais ácidos gordos polinsaturados ómega-3.

Os doentes com LES e os controlos saudáveis apresentaram uma maior proporção de bactérias do filo Firmicutes do que de Bacteroidetes. Contudo, os doentes com LES apresentaram uma proporção média Firmicutes/Bacteroidetes consideravelmente maior do que os controlos saudáveis ($\bar{x}$ Firmicutes/Bacteroidetes=126,12 *vs*. 17,47).

A avaliação taxonómica da microbiota intestinal, não mostrou diferenças significativas, no entanto, a família bacteriana *Rikenellaceae* estava aumentada em doentes com nefrite lúpica quando comparada com doentes sem esta manifestação e controlos saudáveis ($p=0,0385$).

Apesar de os doentes com LES apresentarem maiores concentrações GLP-1 do que os controlos saudáveis, estas diferenças não atingiram significância estatística. No entanto, encontrou-se uma forte correlação positiva entre as concentrações de GLP-1 e o consumo total de ómega-6 ($r=0,5538$, $p=0,0006$) e uma moderada correlação positiva com o consumo de ácido linoleico ($r=0,4741$, $p=0,0040$), um ácido gordo polinsaturado ómega-6. Encontrou-se ainda uma moderada correlação positiva entre as concentrações de GLP-1 e o tempo sentado ($r=0,4329$, $p=0,0094$), e uma moderada correlação inversa com a atividade física ($r=-0,3720$, $p=0,0278$).

Conclusão

Nesta coorte de doentes com lúpus eritematoso sistémico verificou-se uma menor adesão à dieta mediterrânea, uma menor ingestão de diversos macro e micronutrientes, incluindo menor consumo de ácido gordos polinsaturados ómega-3 e maior quociente ómega-6/ómega-3. Constatou-se que os doentes com LES tinham menor atividade física, maior massa gorda e disbiose intestinal. Verificou-se ainda que doentes com LES com nefrite lúpica apresentavam maiores concentrações bacterianas da família *Rikenellaceae* do que doentes sem esta manifestação. Demonstrou-se que uma maior adesão à dieta mediterrânica se associa a uma microbiota intestinal mais diversificada, salientando que um padrão alimentar saudável é benéfico para a modulação da microbiota intestinal.

Estes resultados mostram que a dieta influencia a diversidade da microbiota intestinal, podendo ter, juntamente com a atividade física, um papel no controlo da atividade desta doença. A disbiose da microbiota intestinal pode desempenhar um papel na patogénese do LES, ao prejudicar a integridade da barreira intestinal, favorecendo a endotoxemia e, através deste mecanismo, perpetuar a ativação crónica do sistema imunitário. Desta forma, o reequilíbrio da microbiota intestinal pode ser um alvo terapêutico promissor no lúpus eritematoso sistémico.

Palavras-chave: lúpus eritematoso sistémico, dieta, composição corporal, microbiota intestinal, disbiose

CHAPTER 1. Systemic Lupus Erythematosus: clinical characteristics

1.1. OVERVIEW

Systemic lupus erythematosus (SLE) is a prototypical systemic immune-mediated disease with a significant disease burden worldwide and an uneven prevalence among different genders, ethnicities, and age groups. It is more prevalent in women and has multiple clinical manifestations, affecting virtually every organ of the human body. An interaction between genetic and environmental factors seems to contribute to its pathogenesis (1), resulting in the dysregulation of innate and adaptative immune responses, influencing flares and disease activity (2).

SLE has highly heterogeneous manifestations between patients but also in the same patient during the course of the disease (3), which frequently results in delayed diagnosis (4). Nevertheless, constitutional, mucocutaneous, and musculoskeletal manifestations are the most common clinical manifestations. Organ damage occurs in up to 50% of the patients and results from the disease but also from its treatments. Despite the improvements in the available treatments, SLE remains a disease with high morbidity and mortality, greatly decreasing the patient's quality of life and productivity (4).

This chapter will review the main clinical characteristics of SLE, its diagnosis, treatment strategies, and outcomes.

1.2. EPIDEMIOLOGY

SLE global incidence ranges between 1.5 and 11 per 100,000 person-years, with disproportional differences between world regions. While in Europe, its incidence varies between 1.5 and 7.4 per 100,000 person-years, in the United States, it ranges between 3.7 and 49 per 100,000 person-years (5)(6)(7).

The World prevalence of SLE is rising, affecting between 13 and 7713.5 per 100,000 individuals. While in the United States, the global prevalence varies between 48 and 366.6 per 100,000 individuals, in Europe, it fluctuates between 29 and 210 per 100,000 individuals (5). Better access to medical care that facilitates SLE diagnosis and improvement in survival may explain the discrete increase in SLE prevalence (5)(7). The discrepancies between studies may reflect differences in sex distribution, genetic background, environmental factors, and access to health care. Furthermore, the different studies used different methodologies for case definitions, which has a significant impact on the results (4)(5)(8).

Although improving, the life expectancy of SLE patients is lower than the rest of the population (73 vs. 81 years), mainly due to infections and cardiovascular and renal diseases, and especially in the first year after diagnosis (5)(9). Considering that SLE is four to nine times more prevalent in women than in men, and it develops earlier in life, females with SLE have a 2.6-fold increase in mortality than the general population and a higher mortality risk than males, especially if Black, Hispanic or Asian (5)(6)(8)(10). In fact, in the United States, SLE ranks among the fifteen main causes of non-traumatic death of young women (15 to 44 years) (10).

The genetic background is a relevant determinant of SLE course and outcome. Although the insufficient data from African and Asian countries hinders a comprehensive picture of the SLE impact, it is well known that populations with an African and Asian background have a higher incidence of the disease and worse outcomes (5)(11-13).

The development of SLE during childhood or adolescence (juvenile SLE) is relatively rare. It occurs in 15% to 20% of patients (14). Juvenile SLE has a more severe phenotype, greater organ damage at the time of diagnosis, and greater renal, cardiovascular, and neurologic involvement, resulting in higher morbidity and mortality due to the duration of the disease and the burden of its treatments

(12)(13)(15). Similarly to what is observed in adults, juvenile SLE is more prevalent in females than males (14) and African-Americans, Hispanics, and Asians (13).

1.3. CLINICAL MANIFESTATIONS

SLE is a chronic disease with multiple clinical manifestations. It can affect any organ or tissue of the human body and is characterized by relapses or flares followed by periods of remission (16). Although it has heterogeneous manifestations, musculoskeletal and mucocutaneous manifestations are very frequent, while renal involvement is rare but greatly impacts prognosis (4).

1.3.1. Constitutional manifestations

Fever, fatigue, anorexia, and weight loss are common SLE symptoms (17). Fatigue is multifactorial and one of the most predominant SLE manifestations (4)(18). It is an important, disabling symptom present in most SLE patients and in a severe form in over 30% (18). Although its mechanisms are poorly understood, pain, organ damage, and high doses of glucocorticoids (GC) aggravate this symptom, as well as fibromyalgia, depression, anxiety, and reduced levels of physical activity (17-19).

Fever occurs in a significant number of SLE patients, usually in early stages (20)(21). It can be attributed to infection, disease activity, drug reactions, vasculitis, or concomitant illnesses (17)(20).

Weight changes are also frequent. Weight loss usually occurs before the diagnosis due to the disease activity (22), while weight gain is more commonly associated with corticosteroid use (17).

1.3.2. Musculoskeletal manifestations

Arthritis is one of the most common SLE manifestations. It is frequently present at diagnosis and affects the great majority of SLE patients (69% to 95%) (17)(23). Arthralgia, morning stiffness, tenderness without swelling, and, less frequently, swelling of the joints are the main symptoms (17)(23)(24). It can occur in any joint, but the small joints (metacarpal phalangeal, proximal and distal interphalangeal, and knees) are the most affected (23). Although lupus arthritis is usually non-deforming and non-erosive, Jaccoud arthropathy, which is characterized by joint deformities and ligament laxity, may occur in 3 to 13% of the patients (23)(25).

Avascular necrosis results from bone ischemia leading to bone necrosis (3)(23). It occurs in up to 40% of SLE patients (3)(23)(26). Patients receiving GC treatment, especially in high doses, have a higher risk of developing this complication, which can greatly impair patients' quality of life (3)(26).

While myalgia affects nearly 75% of the patients (27), myositis is less frequent (27)(28).

1.3.3. Mucocutaneous manifestations

Around 80% of SLE patients have skin involvement (17)(29). Malar rash is the most frequent presentation of acute cutaneous lupus erythematosus (17)(29)(30). It is localized, transient, nonscarring, and it is triggered by sun exposure (17)(29)(30). It presents as an erythema in both cheeks and nasal bridge but spares the nasolabial fold (17)(29)(30).

The subacute cutaneous lupus erythematosus lesions occur mainly in areas exposed to the sun (29)(30). Dyspigmentation may occur, but its active form presents as nonscarring papulosquamous lesions and annular plaques with a longer duration than acute cutaneous lupus erythematosus lesions (29)(30).

Discoid lupus erythematosus (DLE) affects 20% of SLE patients and is the most frequent presentation of chronic cutaneous lupus erythematosus (29)(30). It can have a localized presentation involving the scalp, nose, cheeks, and ears and, less frequently, a generalized presentation (29)(30). DLE lesions are characterized by discoid plaques, with peripheric hyperpigmentation and central depigmentation that, when involving the scalp, cause scarring alopecia (29)(30).

1.3.4. Hematologic manifestations

Leukopenia, lymphopenia, anemia, and thrombocytopenia are common in SLE (17). Leukopenia and lymphopenia are the most frequent and are associated with the disease activity (17)(31)(32).

Thrombocytopenia can precede SLE diagnosis by several years and is associated with greater morbidity and mortality despite severe forms being infrequent (31).

Anemia is present in 50% of SLE patients. It may be immune-mediated or non-immune-mediated, and it can have different causes, such as anemia of chronic disease, blood loss, nutritional deficiencies, myelotoxicity associated with drugs used for SLE treatment, and infection (33). Anemia of chronic disease is the most common form. Autoimmune hemolytic anemia is associated with disease activity (31)(33)(34).

1.3.5. Cardiovascular manifestations

Patients with SLE have a higher risk of cardiovascular diseases(1)(5)(35), including ischemic heart disease and stroke, which are responsible for up to 52% of deaths and are a relevant cause of morbidity (5). Cardiovascular manifestations may occur due to SLE-specific causes, treatment, and traditional risk factors such as hypertension, dyslipidemia, smoking, diabetes mellitus, and obesity (1)(35). Cardiovascular manifestations can result not only from the systemic inflammation seen in SLE but also as an adverse effect of corticosteroids (36).

Pericarditis affects nearly 25% of the patients and is frequently associated with pleuritis (36). SLE patients can also have myocarditis and endocarditis, although less frequently.

Vasculitis occurs in 50% of SLE patients involving small, medium, and less frequently large vessels (38).

1.3.6. Lupus nephritis

Lupus nephritis (LN) is a major determinant of prognosis (17)(37). Despite the various treatment strategies currently available, end-stage renal disease still occurs in 10% to 30% of LN patients (17)(35)(37)(38). The mortality of patients who develop LN is still higher than that of patients without renal involvement (5)(37)(39)(40).

LN is more prevalent among women, non-Caucasians, and children with juvenile SLE, but men have a higher risk for end-stage renal disease (5)(37-39)(41). Its' clinical manifestations range from asymptomatic hematuria or/and proteinuria to rapidly progressive glomerulonephritis (17)(37). LN usually occurs in the first year after diagnosis (38)(41).

1.3.7. Neurolupus

Neuropsychiatric manifestations impact patients' quality of life and are associated with poor prognosis, increasing morbidity and mortality (2)(42)(43). The central and/or peripheric nervous system can be involved (2)(43).

Cognitive dysfunction is among the most frequent neuropsychiatric manifestations, affecting up to 80% of patients (2). It can cause changes in memory, attention, executive function, and psychomotor speed (2)(34)(42). Mood and anxiety disorders, such as depression, are also prevalent and may be related to the impact of the diagnosis on patients' quality of life or the disease activity itself (2). Seizures occur in 20% to 25% of SLE patients (2). Cerebrovascular disease is frequent and an important cause of death (2).

1.3.8. Other manifestations

Ocular manifestations are present in up to 30% of patients at diagnosis and are correlated with the disease activity (44). Keratoconjunctivitis sicca, associated with Sjögren's syndrome, and retinal vasculitis are the most frequent ocular manifestations, which can have variable clinical presentations and severity (44). Moreover, several drugs used in SLE treatment, such as GC and hydroxychloroquine (HCQ), show ocular toxicity, especially associated with long-term use (44)(45).

Gastrointestinal manifestations can be unrecognized and affect up to 60% of the patients. They are one of SLE's first subclinical manifestations and are usually mild, but if undiagnosed, they can be life-threatening, causing ischemia, perforation, and infarction (46)(47). Disease activity and high doses of GC are causes of gastrointestinal damage (47), with diverse, heterogeneous, and unspecific manifestations, such as nausea, vomiting, and anorexia (47). Acute pancreatitis is more prevalent in children (46). It has a mortality rate between 30% to 45% and has been associated with active disease (46)(47).

Pleuropulmonary manifestations are frequent and usually concurrent to other organ involvement, and when present can significantly impact survival (48)(49). Pleuritic pain, cough, and dyspnea are frequent symptoms of pulmonary involvement (48).

1.4. AUTOANTIBODIES

In SLE, a myriad of autoantibodies target self-antigens, resulting in the deposition of immune complexes in several tissues, causing inflammation (2)(17). Autoantibodies are present several years before the diagnosis (50)(51). Antinuclear antibodies (ANA), anti-Ro (SSA), anti-La (SSB), and antiphospholipid antibodies (aPL) are the first to be detected, even when no clinical manifestations are identified. In contrast, anti-Smith and anti–nuclear ribonucleoprotein antibodies occur closer to diagnosis (50).

ANAs are frequent in the population, even in healthy individuals, who will never develop an immune-mediated disease. Thus, ANAs lack specificity for SLE, and are unable to assess prognosis and SLE disease activity (51-53). Although between 20% and 30% of the healthy population is ANA positive, 95% to 99% of SLE patients are positive at any point in the disease (51). In fact, positivity to ANA is a classification criterion for SLE in the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) classification (51).

Antibodies to double-stranded DNA (anti-dsDNA) are more specific markers for SLE and are also associated with disease activity, nephropathy, hemolytic anemia, and fever (16)(51-53). Anti-dsDNA antibodies are rarely present in healthy individuals. However, they are less sensitive than ANA (50)(51), and their titers can fluctuate with flares and remission (51)(53).

SSA are present in 20% to 30% of SLE patients. Although having low specificity for SLE, they are highly sensitive and one of the earliest serological markers (16)(51)(53). SSB are frequently associated with SSA and seem more prevalent in the presence of malar rash, subacute cutaneous lesions, photosensitivity, arthritis, serositis, and thrombosis (16). SSA and SSB can be expressed in other rheumatic diseases (51).

Anti-Smith is specific for SLE and a classification marker associated with disease activity and photosensitivity (50)(53). These antibodies are rarely present in healthy individuals and occur in 10% of SLE patients, especially in the presence of oral ulcers and myositis (16)(51).

aPL antibodies (lupus anticoagulant, anticardiolipin antibodies, and/or anti-β_2-glycoprotein-I antibodies) are present in 30% to 40% of SLE patients and are associated with thrombosis, miscarriages, and thrombocytopenia (16)(17)(34). Moreover, SLE patients aPL-positive show a higher prevalence of valve disease, pulmonary hypertension, hemolytic anemia, renal lesions, cognitive impairment, worse quality of life, and higher risk of organ damage (34).

1.5. CLASSIFICATION CRITERIA

Diagnosing SLE is challenging, as this disease has diverse clinical manifestations. Delayed diagnosis is, therefore, common, particularly in children (16)(54).

Classification criteria for SLE diagnosis were developed to standardize clinical trials in SLE patients. In 1982, the ACR developed classification criteria revised in 1997 and more recently in 2019, together with EULAR, reflecting the advances in disease knowledge (21). In the 2019 EULAR/ ACR classification, one criterion should only be weighted if SLE is considered the most likely cause of that manifestation (21). It has ten weighted, hierarchical domains, with positive ANA ≥1:80 at any time as an entry criterion. A score ≥ 10 points corresponds to the disease being classified as SLE (21)(55). The definitions for the 2019 EULAR/ ACR SLE classification criteria are shown in **Appendix 1.**

The Systemic Lupus International Collaborating Clinics (SLICC) is an international research group in SLE that, in 2012, developed a classification that addressed some weaknesses regarding the 1982 ACR criteria (21)(56). The SLICC classification has a higher sensitivity than the 1997 ACR but lower specificity (21). SLE is assumed when four of the seventeen criteria are present, including one clinical and one immunological, or if LN, assessed by biopsy, with positive ANA or anti-dsDNA antibodies is present (56). The clinical and immunologic criteria used in the SLICC classification are shown in **Appendix 2.**

The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was developed in 1985 and modified and validated in 2000 (SLEDAI- 2K), reflecting clinicians' observations in the assessment of SLE disease activity (57). The SLEDAI-2K score is shown in **Appendix 3.**

1.6. JUVENILE-ONSET SLE

Childhood-onset SLE or juvenile-onset SLE (jSLE) occurs below 18 years old. Due to its low incidence, it is considered a rare disease that affects 15% to 20% of SLE patients (14)(39)(55)(58). jSLE is usually more aggressive than adult-onset SLE, presenting higher disease activity, more severe organ involvement, and consequently higher medication burden and morbidity and mortality (39)(55)(58). There is a higher prevalence of single gene mutations in children than in adults (7-8% vs. 1-3%) (55). Similarly to what is seen in adults, jSLE is more prevalent and severe in non-Caucasian populations (55)(59).

In jSLE, high organ damage is frequent at the time of the diagnosis. In fact, up to 55% of the patients develop LN in the first year after diagnosis, of which up to 50% develop end-stage kidney disease (14)(58). The peak onset of jSLE occurs at 12.6 years. The characteristics of the disease vary between age groups (14)(55). Children with very early onset are more likely to have an atypical presentation, more severe disease, and poorer prognosis (14)(55). In younger patients, disease severity increases over time, and the treatment burden tends to be higher (14)(39).

jSLE has a high burden associated with the disease and its treatments, as it frequently requires intense immunosuppression to achieve remission (39). The use of high doses of immunosuppressant drugs also increases the risk of infection, which is also associated with mortality in jSLE, and impairs growth in a significant number of patients (39). Moreover, children and adolescents have to grow physically, cognitively, socially, and emotionally while facing the challenges of a chronic disease, making them feel different from their peers.

1.7. TREATMENT STRATEGIES

Considering the diversity of organs affected, SLE treatment requires a multidisciplinary approach (45). The treatment strategies aim to achieve disease remission or low disease activity, prevent flares and organ damage, improve long-term survival and quality of life, and minimize treatments' side effects (45).

Flares, defined as an increase in disease activity, are frequent when SLE diagnosis occurs at early ages, when there is low compliance to treatment, persistent generalized disease activity, and serological activity (45).

Non-pharmacological measures to manage SLE include sun protection with high sun protection factor sunscreens since ultraviolet (UV) B light sensitivity affects the majority of patients and may induce flares (45)(60-62). The reduced exposure to sunlight may influence 25-hydroxycholecalciferol serologic levels once sunlight exposure is the main source of vitamin D (63)(64). Although low levels of vitamin D are frequent in the general population (64), sub-optimal levels are more prevalent in SLE patients (63)(65). Since adequate vitamin D levels seem to impact the severity of rheumatic diseases and prevent GC-induced osteoporosis, its levels should be periodically monitored, and supplementation considered when insufficiency is present (66).

Smoking cessation is also advised, as tobacco is a risk factor for SLE and negatively influences the course of the disease (67)(68). In addition, it was found that smoking can reduce the efficacy of treatments with belimumab (69) and HCQ in cutaneous lesions (70).

Physical exercise is essential for a healthy lifestyle, reducing cardiovascular risk and decreasing fatigue in SLE patients. Physical exercise should be recommended to all SLE patients. (71)

Regarding the pharmacologic interventions, if no contraindication exists, HCQ is recommended for all SLE patients (45). HCQ is an anti-malarial drug used in rheumatic diseases since 1955 (72). It modulates innate and adaptative immune responses, inhibiting several pathways, such as endolysosomal activities, cytokine signaling, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase signaling, and calcium mobilization from the endoplasmic reticulum, resulting in the reduction of inflammatory responses (73)(74). HCQ has several beneficial effects, reducing flares and disease activity, improving survival, endothelial dysfunction, and the metabolic profile (72)(73). Toxicities are rare (73). Nevertheless, retinal toxicity is one of the main concerns associated with prolonged use, affecting up to 2% of the patients after ten years and 20% after 20 years (74).

In SLE, the use of GC depends on the type and severity of the organ involvement (45). High doses should be used when severe organ involvement occurs (45). For chronic use, the dose should be the lowest possible, preferably ≤7.5 mg/day prednisone equivalent to prevent irreversible organ damage, and weaning and withdrawal should be considered individually as soon as the disease is controlled (45)(75). GC adverse effects depend highly on dose and treatment duration (76). Osteonecrosis is a well-known side effect of GC use in SLE patients (76)(77). Other adverse effects of GC are weight gain, diabetes, hypertension, dyslipidemia, increased risk for cardiovascular events and infection, all associated with higher doses and prolonged treatment (76).

Immunosuppressive drugs such as methotrexate, mycophenolate mofetil (MMF), and azathioprine are used when disease manifestations are not controlled with HCQ alone or in combination with GC or when dose reduction of GC cannot be achieved (45).

The immunosuppressive effect of MMF is achieved by inhibiting T and B lymphocyte proliferation (78)(79). MMF's most frequent side effects are gastrointestinal symptoms, such as diarrhea, usually dose-related, and bone marrow suppression, which requires close monitoring (78)(79). MMF is generally safer and better tolerated than cyclophosphamide (79). MMF is highly recommended for induction and maintenance treatment of proliferative lupus nephritis (45)(80).

Cyclophosphamide is also an immunosuppressant that can be considered when no favorable response is achieved with other immunosuppressive drugs (45). It should be carefully considered in patients of fertile age due to the risk of infertility, and low doses have comparable efficacy and lower gonad toxicity than higher doses (45). The main side effects of cyclophosphamide are dose-dependent and include infection, bladder toxicity, leukopenia, and a higher risk of malignancy (81).

LN treatment has two major phases: induction of remission that can last up to 6 months and includes a more intense immunosuppression regimen, and a more prolonged maintenance phase that

aims to prevent relapse and progression, requiring lower doses of immunosuppressants (45)(78)(82). MMF and azathioprine can be used in the maintenance phase, depending on the patient's characteristics and the drug used for induction (45). Maintenance with azathioprine may have a higher risk of relapse when compared to MMF (83), but this observation is not consensual (82).

Azathioprine is a prodrug that induces apoptosis of activated T cells, exerting an immunomodulatory effect (83). It is a drug used in the maintenance phase of hematologic and renal disease or in the absence of response to HCQ or inability to reduce GC dose (45). Although MMF seems superior in maintaining remission of renal disease, azathioprine is compatible with pregnancy (45)(84). However, hepatotoxicity and myelotoxicity may occur (83)(84).

Tacrolimus inhibits T and B cell activation, suppresses the transcription of interleukin (IL)-2, and reduces the synthesis of tumor necrosis factor-α (TNF-α), interferon-γ, IL-6, and IL-10 by activated T cells (81). This agent has been used in kidney transplantation and seems a good option for the induction phase of LN treatment (81)(85).

Belimumab was the first biologic agent approved for SLE treatment of adults and children and the only food and drug administration (FDA)-approved drug for SLE in 50 years (69)(86). It is an immunoglobulin (Ig)G1-λ monoclonal antibody that binds to the B-cell activating factor (BAFF), a cytokine belonging to the TNF-α family implicated in SLE pathogenesis (69). Belimumab has shown good control of the disease activity with long-term use, few safety concerns, and improved health-related quality of life (87).

Rituximab is a CD20 monoclonal antibody that depletes $CD20^+$ B cells (86). Although currently used off-label, it has shown promising results in controlling disease activity and LN (86). It is used when more than one immunosuppressant drug has been inefficient in controlling disease activity or refractory or relapsing LN (45). However, the EXPLORER trial showed no differences between rituximab and placebo for moderate-to-severe active SLE (88). In another randomized trial, 144 patients received rituximab or placebo. Although rituximab led to greater reductions in anti-dsDNA antibodies and increased complement (C)3 and C4 levels, it did not improve clinical outcomes after one year of treatment (89). Despite the lack of success of the clinical trials reported so far, rituximab continues to be a valuable off-label drug, particularly important in the treatment of refractory hematologic and renal disease.

Several new drugs are being developed, including biological drugs tailored to interfere with interferon α, a major cytokine in SLE pathogenesis. Hopefully, more robust treatment strategies with better safety profiles will be available in the near future, and a more personalized treatment adjusted to the patient will be used.

1.8. OUTCOMES

Since 1970, the improvement in available treatments increased SLE patients' survival. However, this improvement has decelerated in the last years (9). In addition, the mortality rates are still 2 to 3 fold higher than those of the general population (5)(9) and also of other systemic immune-mediated diseases (5). SLE-associated mortality is greatly influenced by sex, ethnicity, and socio-economic factors (5)(70). In this regard, black-American SLE patients die 13 years sooner than white-American patients (5). Several factors contribute to this disparity and less favorable outcomes (70). Frequently, there is lower compliance with the medication, differences in healthcare access (11), and also in the disease characteristics, as non-Caucasian patients tend to develop more severe disease phenotypes (70).

Women are four to nine times more affected by SLE than men and represent up to 93% of all SLE patients (5). Moreover, women have a 2.6 higher risk of dying from SLE than men, which makes SLE one of the leading causes of death among young women (5)(10).

jSLE is associated with increased disease-related morbidity and mortality and with higher medication burden (5)(13). It occurs with more severe manifestations at diagnosis and a higher incidence of renal, cardiovascular, and neuropsychiatric involvement (13), greatly influencing prognosis (70). In a 5-year evaluation of the outcomes in jSLE patients with end-stage kidney disease between 1995 and 2006, 22% of the patients listed for renal transplant died (58). On the other hand, late-onset (diagnosis above 50 years old) is also associated with worse outcomes and higher mortality, mostly associated with higher co-morbidities (70).

Clinical remission is the desired outcome in SLE. When achieved, even for short periods, it decreases the rate of organ damage (70)(90). Low disease activity is the most realistic outcome, and it also positively impacts organ damage, quality of life, and mortality (70)(90).

Patients with SLE have an increased risk of co-morbidities, greatly impacting prognosis and mortality (70). Renal and cardiovascular complications and infection, through the course of the disease, are associated with worse outcomes (5)(9)(70). In fact, cardiovascular disease and infection are the two main causes of mortality and morbidity in this population (5)(70). Neuropsychiatric lupus is also a relevant cause of morbidity and mortality (70).

Mortality and morbidity rates are far from ideal, enhancing the need for more research to better understand SLE's pathogenesis, find new therapeutic targets, and new treatment strategies, ultimately improving the outcomes (9)(70).

CHAPTER 2. Pathogenesis of SLE

2.1. OVERVIEW

SLE is an immune-mediated disease whose great variability in prevalence, incidence, and manifestations reflects the influence of both environmental and genetic factors. Several exogenous and endogenous stimuli can trigger SLE in genetically susceptible individuals (68), fueling a vicious circle of autoantigen exposure, autoantibody production, chronic inflammation, and tissue damage (91). Despite the complex pathogenesis of SLE, its onset and progression involve the presence of autoreactive B and T cells that recognize self-antigens originated in impaired clearance of apoptotic debris and neutrophil extracellular traps (NETs) (92)(93). The over-exposure to self-antigens leads to the break of self-tolerance by producing pathogenic autoantibodies that lead to tissue damage.

In this chapter, we will look at the several factors involved in the pathogenesis of SLE.

2.2. DIFFERENCES BETWEEN GENDERS

SLE is mainly a female disease, as it is up to nine times more frequent in women than men (94). The reason is still not fully understood, but sex hormones and genetic and epigenetic factors are certainly involved (94-96).

Some facts point to the hormone involvement in SLE pathogenesis. First, SLE is more prevalent in women of fertile age. Although serological hormone levels are usually within normal ranges at diagnosis, longer exposure to sex hormones (early menarche and nulliparity) increases the risk for SLE (97). Moreover, pregnancy may lead to disease exacerbation (96), and hormone replacement therapy with estrogen increases the risk of flares (68). In addition, men untreated for hypogonadism have an increased risk for SLE (97).

Immune cells express estrogen receptors, thus being candidates for estrogen-induced immune modulation (96)(98). Treatments with estrogen have been associated with hypomethylation and a decrease in apoptosis of T and B cells in SLE, the latter favoring prolonged persistence of autoreactive cells (98). In addition, estrogen can enhance the production of ds-DNA antibodies and increase monocytes' antigen presentation (96)(98). Estrogen can also favor pro-inflammatory cytokines production (96). On the other hand, progesterone and testosterone do not seem to aggravate the risk for SLE. They may even counteract the estradiol effects by inhibiting type I interferon (IFN) responses and immunoglobulin production (96).

SLE gender bias is also present before puberty and after menopause (99), suggesting that other factors besides hormone exposure may be implicated in SLE pathogenesis. Interestingly, men with Klinefelter syndrome (47,XXY) have a similar risk for SLE than women with unaltered karyotypes (96)(99). Women 47,XXX have nearly a 2.5-fold increase in the predicted prevalence for SLE than women 46,XX (99), while women with Turner syndrome (46,X) have a lower risk for developing the disease (92). These findings suggest that variations in the X chromosome may be implicated in SLE pathogenesis.

Epigenetic modifications may also contribute to gender skewing in SLE (96). The expression of some micro (mi) ribonucleic acids (RNAs) (non-coding RNAs that regulate gene expression post-transcription) can be changed by estradiol, and some miRNAs, such as miR148a, are located in the X chromosome and are upregulated in T lymphocytes of SLE patients, resulting in DNA hypomethylation and, consequently, overexpression of pro-inflammatory genes (96). Other genetics and epigenetic factors will be explored next in more detail.

2.2.1. GENETICS AND EPIGENETICS

Genetics has a significant role in SLE pathogenesis. SLE is more prevalent in first-degree relatives, and there is also a higher risk between monozygotic than dizygotic twins (95)(100).

SLE can be monogenic, resulting from a single gene variant or, more frequently, polygenic (95)(101). Monogenic SLE usually occurs at younger ages and presents with more severe manifestations (101). Genes associated with monogenic SLE codify proteins that participate in metabolic pathways such as nucleic acid sensing and degradation, type I IFN responses, complement cascade, phagocytosis, apoptosis, RAS-mitogen-activated protein kinases (MAPK) signaling, carbohydrate metabolism, aminopeptidase activity, self-tolerance, proteasome, and amino acid transport (95)(100)(101).

Susceptibility for polygenic SLE has been associated with more than 100 loci (68)(100)(101). Although most of these changes have a small effect on the risk for SLE, when associated with other risk factors, it increases its susceptibility (68)(100)(101). Interestingly, compared to women, men have a higher threshold for these accumulated risk factors before developing SLE (96). Several candidate loci have been associated with impaired immune responses. Some encode proteins related to B and T cell function, potentially influencing autoantibody production and immune complex deposition. Others influence phagocytosis, maintenance of self-tolerance, deoxyribonucleic acid (DNA) repair, and the regulation of type I IFN responses (100)(102-104)

The fact that monozygotic twins only have a concordance rate of 25% highlights that genetic factors alone are insufficient to explain the pathogenesis of this disease (68)(105). The environment and epigenetic factors also play a role in SLE pathogenesis. Epigenetic modifications occur in response to environmental stimuli (106) and contribute to the SLE phenotype through mechanisms such as DNA methylation, non-coding RNA, and histone modifications, which regulate gene expression (68)(95)(100)(105).

2.2.2. ENVIRONMENTAL FACTORS

Environmental factors, such as UV light exposure, infections, and tobacco, cause oxidative stress that triggers epigenetic changes, which, combined with genetic susceptibility, contributes to SLE occurrence (106)(107).

UV light is a known trigger for SLE, and up to 93% of the patients have photosensitivity throughout the course of the disease (62). UV light can induce DNA damage, decrease DNA repair, and drive apoptosis, favoring the exposure of auto-antigens leading to SLE in susceptible individuals (102). Moreover, UV light exposure induces the production of reactive oxygen species (107) and IFN I responses (60), which are also involved in SLE pathogenesis.

Viral infections, especially with Epstein-Barr virus (EBV), can trigger SLE in predisposed patients, stimulating the production of auto-antibodies with molecular mimicry between self-proteins and EBV proteins (68)(100)(108)(109). EBV is a highly prevalent virus in the general population, infecting mainly memory B cells (109). Patients with SLE have more EBV-infected B cells in the peripheral blood than healthy controls, higher EBV viral loads, and higher immune responses to this virus (109).

Smoking tobacco likely contributes to SLE occurrence and its course (108), especially in current smokers or smokers who quit recently (110). Several mechanisms may explain the influence of smoking on SLE. Smoke increases the expression of FAS (CD95), a membrane receptor involved in lymphocytes' apoptosis (110). Higher apoptosis can overburden the clearance of apoptotic material (110), a mechanism already impaired in SLE. Furthermore, the benzopyrenes in cigarette smoke can activate the hydrocarbon receptor, shaping Thelper (Th)17 and Th22 cell functions, both involved in SLE pathogenesis (111)(112). Smoke may also induce epigenetic modifications by forming reactive oxygen species and free radicals (110), which are already increased in SLE (107).

Diet and gut microbiota may influence SLE pathogenesis, but both factors will be discussed in detail in other chapters of this thesis.

2.3. DISEASE MECHANISMS

2.3.1. APOPTOSIS, NETOSIS AND OPSONIZATION

In SLE, impaired cell death mechanisms, such as apoptosis and NETosis, are sources of autoantigens and are believed to be central steps of auto-immunity (91)(93).

Programmed cell death or apoptosis is a silent process essential for homeostasis during development and aging (91)(93). Apoptosis is initiated after physiological or pathogenic stimulation, resulting in the phagocytosis and degradation of the apoptotic cells, followed by an efficient clearance of the resulting cellular debris, also known as efferocytosis (91)(113). Usually, autoreactive B cells can appear during somatic hypermutation but immediately suffer apoptosis once autoantigens are not present. In SLE, the differentiation of $CD34^+$ hematopoietic stem cells in phagocytes is diminished, and macrophages show impaired phagocytic activity (103), low adherence, and lower expression of CD44, a cell adhesion receptor involved in clearance processes (91). In this context, the nuclear material and modified autoantigens accumulate, and an altered cell membrane leads to the release of nuclear material (91)(93).

Besides UV light exposure, pathogens can also favor exposure to self-antigens. After an encounter with a pathogen, neutrophils prevent further invasion by rapidly accumulating at sites of tissue injury (91). They also participate in phagocytosis, cytokine secretion, production of antimicrobial agents, and NETs (91). NETs are webs of nucleic acids, histones, and granular antimicrobial proteins used by neutrophils to capture and eliminate pathogens (91)(105). Once NETs expose extracellular DNA at inflammatory sites, they become potential sources of autoantigens, thus favoring the occurrence of immune-mediated diseases (91)(105). NETosis is a specialized and regulated form of cell death by neutrophils that includes NETs formation induced by pathogens and reactive oxygen species (91). It occurs after pathogen stimulation or pro-inflammatory stimuli, such as the production of TNF-α, IL-8, IL-17, IFN-α, and IL-1β (91)(93). In non-pathological processes, NETs are processed extracellularly by DNase I and C1q and cleared by monocyte-derived macrophages. However, the impaired clearance of NETs, seen in SLE, leads to the accumulation of these traps, leaving them available to form complexes with other proteins (91)(93). In fact, uncleared NETs are a major source of dsDNA in SLE patients (93) and do not seem to be efficiently degraded in some of these patients, making them more susceptible to renal involvement (114)(115).

Opsonization is another clearance mechanism of cellular debris that depend on opsonins such as C-reactive protein (CRP), serum amyloid P component, C1q, IgM, and mannose-binding lectin (91). Alterations in any of these proteins may lead to inefficient clearance or accumulation of apoptotic debris, favoring autoimmunity (62)(91).

In conclusion, the impaired clearance of potentially immunogenic material, such as apoptotic debris or NETs, may be a source of autoantigens that favor SLE occurrence.

2.3.2. NUCLEIC ACID SENSORS

Impaired clearance of apoptotic and NETosis debris can activate nucleic acid-responsive endosomal TLRs and TLR-independent nucleic acid sensors in B cells (68)(92).

TLRs are pattern recognition receptors (PRR) involved in innate immune responses and autoimmune responses by recognizing pathogen-associated molecular patterns (PAMPs) or endogenous molecules released from damaged tissues, respectively (116-118).

TLRs are located on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or the membrane of endosomes (TLR3, TLR7, TLR8, and TLR9) (117). The TLRs located on the cell surface detect microbial products, such as lipopolysaccharides (LPS) of gram-negative bacteria (TLR4), bacterial flagellin (TLR5), and PAMPs (TLR2). In contrast, the ones located on the endosomal membranes

recognize synthetic or microbial nucleic acids, such as single-stranded RNA (TLR7 and TLR8) and unmethylated cytosine-phosphate-guanosine motifs within dsDNA (TLR9) (116)(117).

Endosomal TLRs participate in several immune-mediated diseases (104)(116). When self-antigens activate endosomal TLRs, they change the production of type I IFN (IFN-α, IFN-β) and type II IFN (IFN-γ), and IFN-inducible genes are upregulated (116). In line with this observation, the expression of TLR3, TLR8, and TLR9 and IFN-α, IFN-β, and IFN-γ are upregulated in SLE patients (116). Furthermore, TLR7, which is expressed in plasmacytoid dendritic cells (pDCs), monocytes, and B cells (92), can contribute to SLE pathogenesis by activating the production of type I IFN, promoting the activation of autoreactive B cells and activating the nuclear factor-κB (NF-κB)-inducible pathways (68)(118). In fact, in mice, higher concentrations of TLR7 induced lupus-like disease, increased disease activity, and decreased survival, while deletion of TLR7 reduced lupus development (92)(118).

TLR9 is expressed in B cells, myeloid cells, and dendritic cells (DCs) (119). It has a protective effect, so when decreased, it can favor SLE occurrence (92)(117). In a murine model of lupus, while deletion of TLR9 in B cells resulted in disease progression, overexpression improved disease outcomes (119). Moreover, overexpression of TLR9 was accompanied by a higher ratio of anti-nucleosome/RNA antibodies, showing an alteration in TLR7-TLR9 balance (119).

The mechanisms through which TLR8 can influence the pathogenesis of SLE need clarification. While in some mouse studies, multiorgan inflammatory response, autoimmunity, and arthritis were associated with TLR8 overexpression (116), in others, TLR8 had a protective role (120)(121). In fact, X chromosome deletion of TLR8 accelerated LN and splenomegaly and enhanced the production of circulating antibodies (121), simultaneously modulating the activation of B cells and splenic DCs (121).

2.3.3. SOLUBLE MEDIATORS

Impaired apoptosis may activate pDCs and the INF pathway, triggering the production of autoantigens and favoring the altered cytokine profile seen in SLE (93).

IFNs have a relevant role in SLE pathogenesis. These cytokines mediate Th1 responses, contributing to chronic inflammation and tissue damage (102). In SLE, type I IFN influences antigen presentation, DCs maturation, and lymphocyte differentiation and induces chemokine and co-stimulatory molecule expression (116). Although the expression of IFN-β can be increased in SLE patients (116), the hallmark of SLE is the higher production of IFN-α (95). The expression of genes induced by IFN-α is increased in the peripheral blood mononuclear cells of SLE patients (122), and nearly 50% have high levels of IFN-α (123).

Several mechanisms can favor the IFN-α signature in SLE. First, exposure to uncleared NETs induces the production of IFN-α by pDCs (91). Second, exposure to UV light may favor genomic DNA modifications, inducing monocytes to secret IFN-α (62). Interestingly, LPS can also induce IFN-α production (124). IFN-α expression and serum levels are associated with levels of TLR7, disease activity (116), and survival (102)(118). Moreover, there may be immunological differences between patients with high and low IFN-α (124).

IFN-γ is present in higher concentrations in SLE patients and is associated with higher disease activity (93). It increases antigen presentation, regulates macrophage activation, and inhibits regulatory T (Treg) cell differentiation (116). Moreover, B cells' sensitivity to TLR7 is increased by IFN-γ, contributing to SLE's pathogenesis (92)(118).

Other cytokines are altered in SLE (124). IL-6 is a pro-inflammatory cytokine involved in systemic autoimmunity and inflammatory processes (125). It can induce naive B cell maturation into plasma cells and stimulate cytotoxic T-cell differentiation and autoantibody production (125). IL-6 can also favor Th17/Treg imbalance, a known mechanism involved in SLE, by favoring the increase in Th17 cells and impairing Treg differentiation (125). Several studies identified higher levels of serum IL-6 in

SLE patients than in healthy controls, which seems to be correlated with disease activity (125)(126) and associated with joint involvement (127). In SLE, elevated IL-6 was also identified in the cerebrospinal fluid when neuropsychiatric involvement occurred (128), in the keratinocytes induced by UVB exposure (62), and in the urine of patients with LN (128). Nevertheless, at this time, blocking IL-6 does not seem to bring relevant clinical effects (128).

In SLE, the production of IL-10 is usually enhanced and has been positively correlated with anti-dsDNA antibody titers, disease activity, disease severity, and prognosis (126)(129-131). IL-10 can target different cells, mostly monocytes and macrophages, due to their high levels of IL-10 receptors (132). In addition, immune complexes in circulation can also favor IL-10 synthesis (130). In this way, IL-10 can contribute to the perpetuation of the inflammatory cycle seen in SLE, increasing B cell survival, proliferation, differentiation, the production of antibodies, and suppressing apoptosis of auto-reactive B cells (126)(129-131). The pathogenic role of IL-10 (131) and its increase with disease progression has been demonstrated (132). The pro-inflammatory function of IL-10 may be induced by high concentrations of type I IFN (133), which may explain the improvement in SLE severity by administering an IL-10 antagonist (132). Nevertheless, the role of IL-10 in the pathogenesis of SLE is not consensual. The results from an *in vitro* and lupus mouse model study show that IL-10 may also have an anti-inflammatory action, meaning that this cytokine may have different contributions to SLE, depending on the micro-environment and the stage of the disease (132) or even on the cells that produce it (131).

IL-2 is a cytokine produced by activated $CD4^+$ and $CD8^+$ T cells (134). This cytokine activates T cells, their proliferation, cytotoxicity, and the control of lymphoproliferation after clonal expansion (135). IL-2 is also relevant in maintaining Treg cells and their proliferation (68)(134). Not surprisingly, SLE patients can have decreased concentrations of Treg cells (135), and reduced production of IL-2 (68)(135), exacerbated in active disease (135) and when renal involvement is present (134). Treatment with low-dose IL-2 has been explored as a therapeutic option (134)(136)(137). It has been well tolerated, with promising results in SLE with (137) and without renal involvement (134)(136). Thus, it can be a valuable tool in treating SLE patients with low Treg cells (138). Paradoxically, in a population of jSLE, the levels of IL-2 were increased, associated with disease activity, and significantly higher in active than in inactive disease (139), reinforcing the heterogeneity of SLE.

IL-21 belongs to the IL-2 superfamily (135), and it is involved in the proliferation and maturation of B cells, plasma cell differentiation, and antibody production (98)(140). IL-21 also participates in T cell proliferation and differentiation (98)(135)(140). IL-21 polymorphisms have been associated with SLE susceptibility (140).

IL-17A is a pro-inflammatory cytokine secreted by activated Th17 cells, double-negative T cells, macrophages (141), and neutrophils after IL-6 stimulation (140). It interacts with other pro-inflammatory cytokines, inducing IL-6 production and cooperating with IL-23 in the tissue damage seen in SLE (141). It also activates B cells and promotes germinal center formation (142). Increased IL-17A levels have been reported in lupus mouse models (142) and SLE patients (127). Furthermore, patients with LN have increased IL-17 levels in the kidney (127). To reinforce this cytokine's role in SLE, it was shown in a lupus mouse model that IL-17 deficiency attenuated nephritis and improved inflammatory response (142).

IL-23 is involved in the pathogenesis of SLE, as it can stimulate pro-inflammatory cytokines, activate T memory cells, and trigger Th1 responses (143). Macrophages and DCs produce IL-23, which is implicated in the expansion and survival of Th17 cells (143)(144). The production of IL-17 by Th17 cells is enabled and enhanced by IL-23 (144), a mechanism through which autoimmune responses can be exacerbated (143). SLE patients have increased IL-23 levels in circulation (145)(146), which are higher in active disease, particularly in patients with active nephritis, dermatitis, and arthritis (145). However, these findings are not consensual (143), which may be related to the variability of SLE (145).

In lupus mouse models, IL-23 receptor deletion improved LN (147). Nonetheless, the phase 3 trial for the use of ustekinumab in active SLE, a monoclonal antibody that inhibits IL-12 and IL-23, has been suspended due to lack of efficacy, highlighting the need for further investigation elucidating the mechanisms through which IL-23 can influence this disease (148).

BAFF belongs to the TNF ligand superfamily and is key for B lymphocytes' survival, differentiation, and proliferation (93)(149). BAFF is produced by macrophages, neutrophils and DCs, TLR9-activated pDCs, and IL2-activated natural killer (NK) cells (93)(149). Autoreactive B cells greatly depend on BAFF for survival (150), and in fact, BAFF is increased in the serum of SLE patients, especially in patients with higher disease activity (151). Finally, as mentioned previously, belimumab, a monoclonal antibody that binds to BAFF, was the only FDA-approved drug for SLE in the last 50 years (86). It reduces circulatory B cells and plasma cells without reducing memory B cells and T cells (150). The effect of belimumab occurs by targeting soluble forms of BAFF and promoting its inhibition by directly binding to BAFF receptors. It also disrupts the 60-mer BAFF form, one of the major oligomeric states of soluble BAFF (150).

2.3.4. MAJOR CELL TYPES

2.3.4.1. Neutrophils

Neutrophils participate in innate immune responses to microorganisms through phagocytosis, production of reactive oxygen species, secretion of cytokines and chemokines, and release of NETs, eliminating microorganisms and removing apoptotic neutrophils and damaged tissue (152). In SLE, the increase in apoptosis results in apoptotic burden that, together with poor apoptotic clearance by macrophages, contributes to the accumulation of apoptotic debris (152).

In SLE patients, neutrophils show altered gene expression and enhanced response to type I IFN compared with healthy controls (153). A subset of neutrophils shows a pro-inflammatory phenotype associated with organ damage (153).

As mentioned previously, NETs are defense structures used by neutrophils to capture and eliminate pathogens (91)(105). They are implicated in the pathogenesis of SLE (152), contributing to tissue damage (153). Anti-dsDNA positive SLE patients showed increased NETosis compared to negative anti-dsDNA patients (154).

Neutrophils can be influenced by several molecules (cholesterol, glucose, lipid, and fatty acids) due to their multiple receptors. Glucose is essential for neutrophils' phagocytic function and NETosis, while fatty acid metabolism is involved in neutrophils' apoptosis (155). However, under pathophysiological conditions or when glucose supply is limited, the regulation of phagocytosis occurs via glutaminolysis and fatty acid metabolic pathways (155), meaning that in SLE, phagocytosis by neutrophils may happen through these pathways.

The gut microbiota may also have a role in modulating neutrophils' function. Short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, produced by the gut microbiota, can induce neutrophil migration toward an intruder/injury site (chemotaxis) (155). Furthermore, it has been recently shown that lactate, which can also be a product of bacterial metabolism, might influence NETosis (155) and that, in fact, SLE neutrophils have increased intracellular basal lactate levels (156). Moreover, other bacterial molecules may influence neutrophil activity. *In vitro* studies highlight that NETosis can be induced by LPS from *Escherichia coli* and other gram-negative bacteria (157). However, LPS concentrations may dictate if NETosis is induced (high concentrations) or if the neutrophils' lifespan is increased (low concentrations), and even different bacteria may have a distinct capacity to promote NETosis (157). Even *Caenorhabditis elegans*, a non-pathogenic bacteria, can induce NET formation, and the same occurs with the parasites' antigens in the absence of the live and intact microorganism (158).

2.3.4.2. Monocytes

Monocytes secret several chemokines and cytokines leading to further recruitment of immune cells to damaged tissues (133). In addition, monocytes can recognize PAMPs (159), which is essential for phagocytosis activation and pathogen clearance (160).

In SLE, monocytes may be decreased in the blood (133), possibly due to an altered development of these cells, higher recruitment to inflammation sites (159), and high apoptosis (154). In fact, healthy monocytes treated with IgG-anti-dsDNA antibodies showed increased apoptosis (154).

The involvement of dysfunctional monocytes in the pathogenesis of atherosclerosis is well known. As previously discussed, this is a relevant mortality and morbidity factor in SLE (106). Moreover, monocytes participate in the pathogenesis of lupus nephritis. The accumulation of CD16+ monocytes in the glomeruli of patients with active lupus nephritis correlates with impaired renal function and anti-dsDNA autoantibodies levels (161).

Polymorphisms in Fc gamma receptors (FcγR) are susceptibility factors for SLE (162). Alterations in these cell surface molecules may alter monocytes' response to stimuli (159). In SLE, there is a higher expression of the FcγRI/CD64 in monocytes, which is higher in advanced stages of renal disease and positively correlated with creatinine, proteinuria, and anti-dsDNA antibodies (163). In these monocytes, higher doses of prednisone decreased the expression of the receptor (163), highlighting its contribution to SLE.

2.3.4.3. Macrophages

Macrophages are plastic, phagocytic antigen-presenting cells that, depending on the conditions of the local environment, are polarized by LPS and/or Th1 cytokines into M1 macrophages (more pro-inflammatory) or by Th2 cytokines into M2 macrophages (more anti-inflammatory) (100)(133)(160)(164). A predominance of different polarization profiles, hence a disruption in the M1/M2 balance, has been identified in several immune-mediated diseases (133), including SLE (100). M1 macrophages are sources of inflammatory cytokines (160), and high levels of IFN-γ and TNF-α are frequently seen in SLE (133). In fact, SLE patients have distinct macrophage activation signatures from healthy controls (165), showing an upregulation of M1 macrophages (166). In opposition, patients with inactive disease show an upregulation of M2 macrophages than patients with active SLE (166). M1 and M2 macrophages have different metabolic signatures involving glucose, amino acid, iron, and folate metabolism (160). While in M1 macrophages, IFN-γ and microbial LPS induce glucose and suppress fatty acid metabolism, M2 macrophages show high mitochondrial activity and oxidative phosphorylation (160).

Comparably to monocytes, macrophages can also recognize PAMPs and damage-associated molecular patterns, triggering their migration to inflammation sites and recruiting other immune cells to tissues by secreting chemokines and cytokines (133). Altered migration, activation, and phagocytosis of macrophages may contribute to SLE pathogenesis (133).

2.3.4.4. Dendritic Cells

Dendritic cells are antigen-presenting cells that activate T and B cells (167). DCs in circulation can be pDCs or myeloid DCs (mDCs) (167). pDCs are major and fast producers of IFN-I following a viral or bacterial infection through TLR7 or TLR9 stimulation and have been implicated in SLE pathogenesis (91)(119)(168). SLE patients show reduced pDCs in the peripheral blood (100), especially when high IFN levels are present, which may be explained by the migration of these cells to the tissues (124). Supporting this observation, increased pDCs were found in the glomerulus and cutaneous lesions in SLE (100)(167). Despite the reduced number of pDCs in circulation, their high IFN-I production after

activation by autoantibodies or DNA-containing NETs explains the contribution to SLE pathogenesis (169).

2.3.4.5. B cells

In SLE, several changes in B-cell homeostasis can lead to the deposition of immune complexes (170) and altered B-cell responses (152). Lymphopenia (< 1000 lymphocytes /mm^3) is a frequent finding and diagnostic criteria for SLE (56)(95). B-cell lymphopenia occurs in SLE patients (171)(172), especially in the presence of active disease (171). Moreover, there are alterations in B-cell subsets in SLE, such as decreased naive B cells (95)(171), increased plasma cells (171)(172), increased double negative memory B cells, increased $CD11c^{hi}$ B cells (173)(172). SLE patients with active disease may show further changes in B-cell populations (173).

Altered B-cell activation also favors SLE (170) and is influenced by several cytokines, such as BAFF (also known as BLyS), IL-6, and IL-21 (149). The influence of these cytokines in SLE pathogenesis has been discussed previously.

Loss of B-cell tolerance and the consequent production of autoreactive B cells is a central phenomenon in immune-mediated diseases, including SLE (149). Alterations in the receptors that allow antigen recognition, B cell receptors (BCR), and T cell receptors (TCR) can determine immune-mediated responses (174). In fact, the changes seen in SLE B cells may reflect an anergic B-cell phenotype induced by a continuous BCR stimulation without T cell-derived co-stimulation, favoring B cell hyperactivity and, consequently, autoimmunity (175). However, in SLE, BCR signaling reduction is more pronounced than the alterations in other immune-mediated diseases, but the anergic state can be overcome by CD40 activation of B cells (175).

2.3.4.6. T cells

T cells contribute to the pathogenesis of SLE, excessively activating B cells, secreting pro-inflammatory cytokines, and promoting and amplifying inflammation (68)(147)(176)(177). Furthermore, in SLE, T cells display signaling alterations (175). Antigen recognition in T-cells depends on the TCR and CD3 protein complex (177), and changes in the CD3ζ subunit contribute to the altered signaling of the TCR (177). SLE patients have low levels of the CD3ζ chain, favoring T-cell signaling changes (178)(179). Interestingly, the reconstitution of the CD3ζ chain restored T-cell signaling and increased IL-2 production (179).

Follicular helper T cells (T_{FH}) cells ($CD4^+$ T cells) are a subset of effector T cells that have a key role in the interaction between T and B cells, stimulating B-cell differentiation into plasma cells, favoring the production of antibodies (95)(142)(180). T_{FH} cells are initially increased in SLE (68)(142)(143)(181)(182) but decrease throughout the course of the disease, independently of the immunosuppressive treatment, which suggests that these cells may be critical in SLE initial phases of the disease (181). Paradoxically, patients with class IV and V LN have increased levels of T_{FH} cells, positively correlated with disease activity (183).

In SLE patients, the particular subset of IL-17-producing T_{FH} cells is also increased (143)(182)(184). The Th17-cell increase in SLE has been positively correlated with IgG anti-dsDNA production, urine albumin/creatinine ratios, and disease activity (182). Th17 cells produce IL-21, IL-6, IL-17A cytokines, and soluble CD40 ligand (140). These cells influence vascular inflammation, leukocyte recruitment, and B-cell activation, contributing to glomerular damage and chronic inflammation in SLE (183). Interestingly, IL-17 deficiency can reduce SLE severity by increasing Treg cells and IL-10 production (142). Moreover, reducing Th17 cells can improve nephritis (182), highlighting their contribution to SLE pathogenesis.

Tregs maintain tolerance, inhibiting autoreactive B-cell accumulation and controlling T-cell responses (180)(185), but their survival, growth, and homeostasis are IL-2-dependent (135-137). The results regarding the variation of Tregs in SLE are conflicting (185). While some authors found lower Tregs in SLE patients than in healthy controls (184)(185), especially when LN was present (134), others did not find changes (186), and others saw an increase in these cells (127). Treatment with belimumab altered the Treg proportion and brought the Th17/Treg ratio closer to the one of healthy controls (184).

Some dietary long-chain fatty acids promote the generation of Th1 and Th17 cells. In contrast, SCFA promotes the differentiation and maintenance of Treg cells in the gut, showing that lifestyle habits may also influence SLE pathogenesis (176).

$CD8^+$ T cells, or cytotoxic T cells, have cytolytic activity by producing granzymes and perforins towards infected or damaged cells or cytotoxic T cells (180). In SLE, there is a decreased cytotoxic capacity, leading to impaired defense mechanisms against infectious agents (95) and an altered distribution in $CD8^+$ T cell subtypes positively correlated with disease activity (187). SLE patients show decreased production of cytotoxins, leading to decreased cytolytic function (180), impairing the removal of autoreactive B-cells, and increasing the risk of infection (187). Furthermore, prolonged exposure to IFN-α and TCR stimulation in SLE alters mitochondrial function and morphology, decreasing the nicotinamide adenine dinucleotide $(NAD)^+$/NADH ratio and the metabolic fitness of $CD8^+$ T cells that will show higher spontaneous death (188). Interestingly, restoring NAD^+ availability improved $CD8^+$ T cell viability (188).

CHAPTER 3. Gut

3.1 OVERVIEW

The small and large intestines are complex organs with a highly relevant role in immunity. A complex orchestra of structures and molecules in the gut preserves gut homeostasis and the gut barrier, eliminating potential pathogens while keeping a beneficial partnership with the commensal microbiota (189). A disruption of the tight junction proteins, promoted by altered gut microbiota, pathogens, diet, and medication, can lead to an impaired gut barrier that favors the passage of microbial metabolites to circulation, inducing systemic immune responses and, ultimately, favoring the occurrence of immune-mediated diseases.

This chapter will explore the main components that maintain gut health and the mechanisms that may explain their role in immune-mediated diseases, particularly in SLE.

3.2 GUT COMPONENTS OF IMMUNITY

3.2.1. Intestinal Mucus Layer, Goblet cells, and Mucins

The intestinal mucus layer is produced by goblet cells. These specialized epithelial cells secrete, maintain and stabilize the mucus layer (190), which is one of the critical components of the gut barrier (191). It favors gut homeostasis, protecting epithelial cells from physical and chemical damage and pathogen invasion, simultaneously allowing ions, water, and nutrients to reach enterocytes (190)(191). The turnover of the mucus layer is tightly regulated to ensure the maintenance of its protective function (191). Besides host-related factors, external factors, such as pathogens, diet, and medication, influence mucus barrier characteristics (191).

The mucus layer is mainly composed of water (90%-95%), lipids (1%-2%), and mucins (191), which are glycoproteins produced by goblet cells responsible for the gel-like properties of mucus (191). Mucin (MUC)2 is the main and the best characterized gel-forming mucin in the gut (191)(192), which expression is regulated by hormones, neurotransmitters, bile acids, prostaglandins, and butyrate (191).

The gut microorganisms can maintain the gut barrier homeostasis (193) or compromise it. The impairment of the gut barrier by microorganisms occurs through the degradation of mucins, reduction of their expression, or inhibition of mucus production. This will increase gut permeability (191) and allow commensals and pathogens to reach the gut epithelium, causing inflammation (191)(193). The translocation of luminal substances to the lamina propria through goblet cell-associated antigen passages, can also induce an immune response. These substances can reach antigen-presenting cells and generate antigen-specific T-cell responses (190). There is, therefore, a complex interaction between the microbiota and the intestinal mucus layer.

3.2.2. Gut Epithelium: M cells and Paneth cells

The gut epithelium comprises a large number of specialized intestine epithelial cells (IECs) (190) that constantly crosstalk with immune cells to maintain gut homeostasis (194). M cells and Paneth cells are two of the main IEC. M cells are located at the mucosal epithelium and Peyer's Patches (195). They capture immunogenic particles, such as antigens from viruses and bacteria, at the apical surface of the lumen and transfer them to DCs for antigen presentation, inducing mucosal immune responses or tolerance (196). M cells have B and T lymphocytes and myeloid cells at the basolateral pockets (196)(195). B lymphocytes, in particular, are required for M cell maturation and function in the Peyer's patchs and also seem relevant for transcytosis (the transport of luminal particles across the mucosal barrier) and delivery to the submucosal tissues (196).

Paneth cells produce and secrete antimicrobial peptides and enzymes (α-defensins, Reg3 lectins, phospholipase A2, and lysozyme), protecting the gut barrier from invasion by commensals and pathogens (197-199). Alterations in Paneth cells' number or functionality have been associated with

dysbiosis (197)(200), suggesting they may influence the microbiota composition in the gut lumen (198)(199). In a mouse model deficient in Paneth cells, gavage with lysozyme, an antimicrobial peptide secreted by Paneth cells that can be produced independently of the microbiota, prevented dysbiosis associated with the depletion of these cells (197). Similarly, in an acute pancreatitis mouse model, the long-term reduction of Paneth cells led to a decrease in the mRNA expression of lysozyme, increased intestinal permeability and endotoxemia, and the inhibition of IECs proliferation (200). However, lysozyme administration improved all these phenomena by regulating the gut microbiota (200).

Finally, there is a link between changes in the production of antimicrobial peptides and immune-mediated diseases (201-203). Since gut dysbiosis can change the expression of antimicrobial peptides, strategies targeting gut microbiota modulation, such as probiotics, may modulate their secretion (204). Considering that antimicrobial peptides can modulate complement pathways involved in immune-mediated diseases, such as SLE, treatment strategies involving these molecules may be promising (202).

3.2.3. Gut Epithelium: Enterocytes

Enterocytes in the small intestine and colonocytes in the colon are the predominant cells of the gut epithelium and are part of the gut barrier (198)(205). The nutrient and water absorption functions of enterocytes are optimized by the microvilli that increase their absorptive cell surface and have high concentrations of digestive enzymes such as glycosidases, peptidases, and lipases (205)(206).

Several mechanisms protect enterocytes from microorganisms. Glycocalyx, a glycan-rich diffusion barrier, protects the cell membrane (192). Enterocytes and colonocytes produce antimicrobial molecules that are barriers to the microbiota (207). The regenerating islet-derived 3 (RegIIIγ) restricts the gram-positive bacterial colonization of the small intestinal mucosal surface, limiting the activation of adaptative immune responses and maintaining homeostatic interactions between the microbiota and its host (207). In the colonocytes, the domain containing 8 (Lypd8) binds to the flagella of commensal bacteria, suppressing their motility and impairing their invasion ability (208). Furthermore, bacterial growth can be restricted by the secretion of IgA into the lumen (207)(209).

Enterocytes also express receptors that recognize components of commensal microorganisms, such as TLR1-5 (209)(210). The TLR expression is not uniform along the gastrointestinal tract (211). While the small intestine and colon have similar levels of TLR3 (211), TLR1, TLR2, TLR4, and TLR5 are highly expressed in the colon, and TLR1, TLR2, and TLR4 are lowly expressed in the small intestine (211)(212). There seems to be a microbiota-mediated contribution of TLR5 to SLE in mice and humans. In mice, *Tlr5* deficiency led to increased flagellated bacteria and dysbiosis (210). Interestingly, in SLE patients, a polymorphism in *TLR5* (rs5744168 TT and TC genotype) and higher renal TLR5 expression led to higher renal involvement, probably due to an increased inflammatory reaction to the microbiota leading to a TLR5-dependent LN progression (213). Besides TLR5, an altered expression of TLR4 can also favor SLE occurrence. In the gut, TLR4 signaling influences the presence of Paneth cells and α-defensins in the small intestine and regulates α-defensins' expression, participating in the protection against epithelial injury, inflammation, and bacterial invasion (212). However, mice overexpressing TLR4 showed dysbiosis and a systemic translocation of the gut bacteria, culminating in increased colonic inflammation (212).

In summary, TLRs recognize PAMPs, initiating a signaling cascade that activates transcription factors such as NF-kB and interferon regulatory factors. This leads to the expression and secretion of various cytokines and chemokines, such as TNF-α, IL-6, IL-8, IL-18, and chemokine (C-C motif) ligand (CCL)20, stimulating local immune responses by recruiting neutrophils, macrophages, DCs, and T cells, that will promote mucosal repair (198)(212). Thus, enterocytes modulate inflammatory and antimicrobial responses, impacting bacterial colonization (205).

Epithelial tight junctions (TJ), adherens junctions, and desmosomes are part of the intercellular complex that maintains the gut barrier integrity (194). These transmembrane and cytoplasmic proteins

seal the intercellular space of the intestinal epithelium and regulate the diffusion of solutes and bacteria through the paracellular pathway (194)(205)(214)(215), while dendrites of DCs pass through the TJ to sample soluble antigens (205)(216).

TJ are controlled by signaling molecules and can be influenced by the gut microbiota. For example, an increase in proteolytic bacteria may lead to proteolytic digestion of the TJ, impairing the barrier integrity (217). Furthermore, luminal components, some of which from the diet, can also regulate TJ (205)(218). While, in mice, vitamin D deficiency altered TJ structure (219), fiber intake improved the gut barrier (220).

Zonulin is the only described endogenous regulator of intestinal permeability (218). Interestingly, dysbiosis and gluten trigger its release, highlighting the connection between diet, gut microbiota, and gut permeability. After zonulins' release, a signaling cascade promotes the disassembling of the TJs and the opening of the paracellular pathway. The consequent passage of gut microorganisms and their antigens to the subepithelial tissues may facilitate the activation of the host immune system, ultimately favoring immune-mediated diseases in genetically predisposed individuals (214).

3.2.4. Gut Epithelium: Enteroendocrine cells and GLP-1

Enteroendocrine cells are dispersed through the gastrointestinal tract (198), have a high turnover rate, and their sub-types are defined by the hormones they secrete (221). These hormones influence food digestion and absorption, insulin secretion, and appetite (198)(221)(222). These cells are also essential to modulate innate immune responses against pathogens and commensal bacteria and to maintain mucosal homeostasis (223). Some enteroendocrine cells have the apical surface facing the lumen, detecting particles through sensory proteins, such as G protein coupled receptors (GPCR), which are responsive to nutrients and microbial metabolites. The function of enteroendocrine cells reflects, therefore, the dietary intake and the gut microbial composition (221)(222).

Glucagon-like peptide 1 (GLP-1) is a gut hormone whose concentration increases after food intake (222). The enteroendocrine L-cells secrete GLP-1 in the small intestine upon stimulation by sugars, amino acids, and long-chain fatty acids (224). Besides its anorexigenic activity, GLP-1 regulates gastric emptying and glucose-dependent insulin secretion (222)(225). The release of GLP-1 in the distal small intestine and colon is regulated by the gut microbiota, its metabolites, and neurohormonal pathways (222)(225). The influence of the gut microbiota in GLP-1 production becomes clear when the absence of microbiota leads to a quick increase in plasmatic GLP-1 along with an increase in colonic L cells (222)(224) and an alteration in their transcriptomic profiles (226).

Several hypotheses may explain the association between GLP-1 secretion and the gut microbiota. First, increasing GLP-1 delays gastrointestinal transit, increasing nutrient absorption (224). The rise of GLP-1 may be a compensatory mechanism to deal with a decrease in butyrate, an important short-chain fatty acid (SCFA), and a fundamental energy source for colonocytes (221). Second, inflammation may favor GLP-1 increase. Intestinal barrier impairment in mice and humans promoted LPS access to L cells, leading to a transitory rise in IL-6, IL-10, and TNF-α and an increase of the expression and secretion of GLP-1, the latter through a TLR4-dependent mechanism (223). Furthermore, in mice, IL-6 (227-229), LPS, and IL-1 (227)(229) also promoted GLP-1 increase (227). In this case, IL-6 acted directly on gut endocrine cells to promote GLP-1 secretion (227). The LPS-induced increase in GLP-1 might be either a mechanism to cope with the glucose rise associated with inflammatory conditions, such as sepsis, or a mechanism to maintain gut homeostasis following intestinal injury (221). Interestingly, blocking the GLP-1 receptor signaling led to an increase in the LPS pyrogenic effect (227). Moreover, the absence of GLP-1 receptors, expressed in Paneth cells (230) and intestinal intraepithelial lymphocytes (227)(230), increased the severity of intestinal damage induced by

dextran sulfate sodium (DSS) (223)(227). These results highlight the anti-inflammatory potential of endogenous GLP-1 (227).

In summary, GLP-1 can attenuate pro-inflammatory cytokine expression in intraepithelial lymphocytes, regulate genes associated with gut homeostasis, modulate the gut microbiota, and adequate host immune responses to intestinal injury (227). GLP-1 can be, therefore, a promising biomarker of dysbiosis, intestinal barrier integrity, and inflammation (223)(227).

3.2.5. Gut Epithelium: Intraepithelial lymphocytes and Plasma cells

Intraepithelial lymphocytes are a phenotypically and immunologically heterogeneous population of cells located at the basolateral side of enterocytes (194)(231). These cells have a relevant immune function against luminal microorganisms, limiting bacterial translocation (194)(231).

Intraepithelial lymphocytes differ from systemic T cells in phenotype and immune function (232) and are considered partially activated T cells, promoting a quick response against invasive microorganisms while limiting autoimmunity (194)(231). Because of their cytotoxic (232) and immune regulatory functions, intraepithelial lymphocytes help maintain gut barrier homeostasis (227). Although gut bacteria are not essential for intraepithelial lymphocyte development, they seem relevant for their motility patterns in the gut mucosa (231). On the other hand, commensal viruses are critical for intraepithelial lymphocyte homeostasis and can prevent tissue damage through their regulation (233). Interestingly, in the gut, intraepithelial lymphocytes express GLP-1 and GLP-2 receptors, as well as integrins, TJ proteins, and adhesion molecules, showing that these cells are well integrated into the intestinal epithelium (232) and can use TJ proteins to navigate within the intraepithelial compartment (234). In addition, the gut barrier can be modulated by cytokines produced by the intraepithelial lymphocytes (205), such as IFN-γ, TNF-α, and IL-2, that have a pro-inflammatory action in the gut, and IL-4, IL-10, and transforming growth factor (TGF)-β, that can prevent exacerbated inflammatory responses (194)(233). Thus, a dysregulation between Th1 and Th2 cytokines can culminate in gut barrier impairment (205). All these data show that intraepithelial lymphocytes play a relevant role in gut homeostasis, tightly intertwined with the gut microbiota (205).

Plasma cells from the gastrointestinal tract are the main IgA-producing cells (235). Secretory IgA is transported to the lumen to integrate the first line of defense against bacteria, viruses, and toxins (235-237). The gut microbiota is also coated with IgA antibodies, which may function as a host-copping mechanism to maintain beneficial bacteria with pro-inflammatory potential (238). IgA production can shape the gut microbiota composition (238)(239) and helps maintain the interplay between commensals, epithelium, and the immune system, which is fundamental to maintain systemic immune homeostasis (237). The importance of IgA in maintaining gut homeostasis becomes clear when looking at patients with selective IgA deficiency who show significant changes in the gut microbiota composition (239). These patients have increased inter-individual variability of the gut microbiota, which may be related to frequent deviations from a healthy microbial composition, leaving them more prone to develop immune-mediated or infectious diseases (239). In SLE, some authors found fewer IgA-producing plasma cells (240) and decreased fecal and serum IgA levels before the occurrence of LN (241). However, others found an increase in IgA in fecal samples of SLE patients correlated with zonulin (242). More studies are necessary to better understand the role of secretory IgA in immune-mediated diseases and in lupus in particular.

3.2.6. Gut Associated Lymphoid Tissue (GALT)

The mucosa-associated lymphoid tissue (MALT) can be subdivided into gut-associated lymphoid tissue (GALT), nasal-associated lymphoid tissue (NALT), or induced bronchial-associated lymphoid tissue (iBALT) (235).

GALT is a complex structure that includes isolated lymphoid follicles, Peyer's patches, cryptopatches, and the appendix, as well as M cells, lymphocytes, plasma cells, DCs, and macrophages (235)(240)(243). GALT is essential for maintaining immune tolerance to the gut microbiota despite being simultaneously dependent on these microorganisms for its development (243). Microbiota metabolites, such as SCFA, can regulate GALT's immune responses by epigenetic mechanisms (243), making food a potential regulator of immunity through the modulation of antigen-presenting cells such as DCs and macrophages (244). After antigen presentation, these cells lead to Th and Treg differentiation, inhibiting food-initiated inflammatory responses and responses to commensals and environmental antigens (244).

Cryptopatches are located at the base of the intestinal villi and are mainly composed of type 3 innate lymphoid cells (ILC3s) and $CX3CR1^{+}$ myeloid cells (245)(246). ILC3s are the main source of IL-22 and also express IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF), the first two after microbial stimulation (247)(248). Metabolites such as vitamin-A-derived retinoic acid and soluble aromatic hydrocarbons also activate ILC3s (249). The first promotes the expression of the transcription factor retinoic acid-receptor-related orphan nuclear receptor γ (RORγ) (249). The second senses microbiota-derived (tryptophan metabolism) or diet-derived soluble aromatic hydrocarbons and are relevant for ILC development and IL-22 production (249).

Peyer's patches are located in the small intestine, and there are similar structures in the colon (colonic patches) and cecum (cecal patches) (249). In Peyer's patches, there are enterocytes and M cells (248), as well as DCs ($CD11b^{+}$) and lysozyme-secreting cells (250). In addition, it is in these structures that IgA is produced in a T cell-dependent process (248)(249). The Peyer's patches' involvement in immune responses mediated by bacteria was well demonstrated in an arthritis mouse model, in which segmented filamentous bacteria increased T follicular helper cells in these structures. These cells migrated and stimulated auto-antibody production, modulating autoimmunity and ultimately showing that the gut microbiota, particularly segmented filamentous bacteria, can trigger immune-mediated arthritis (251).

In conclusion, in the gut, there is a tight equilibrium between the cells in the gut barrier, the microbiota, and the immune system.

CHAPTER 4. Gut microbiota

4.1 OVERVIEW

The human gastrointestinal tract hosts a diverse and large community of microorganisms, the microbiota (252). The gut microbiota comprises bacteria, archaea, viruses, and fungi, living in symbiosis with their host (221)(253). The gut microbiota has an important role in nutrition, prevention of pathogenic colonization, regulation of gut homeostasis, and modulation of the innate and adaptive immune system (252). Several factors can modulate the gut microbiota composition, such as genetics, type of birth, gender, lifestyle, diet, and medication. Furthermore, the gut microbiota can influence several metabolic and immune processes. When it is in equilibrium, the gut microbiota is associated with health, but when altered, it has been linked to several diseases, including immune-mediated disorders (254). In SLE, dysbiotic gut microbiota has been identified by several authors, in many cases related to disease progression and worst outcomes, suggesting that it has a critical role in the pathogenesis of this disease (131).

In this chapter, we will look at factors that influence gut microbiota composition and mechanisms through which it can favor the occurrence of immune-mediated diseases, and SLE, in particular.

4.2 DEFINING THE GUT MICROBIOTA

The human gut microbiota is a heterogeneous and complex set of symbiotic microorganisms that colonize the small intestine and colon (221)(253). The microbiome comprises the microbiota genome, estimated to contain nearly 130 times more genes than the human genome (252)(254).

The gut microbiota has a commensal relationship with its host, contributing significantly to nutrient and drug metabolism, detoxification, vitamin synthesis maturation, and modulation of the immune system (254). The heterogeneity of the gut microbiota is a marker of the bacterial communities' stability and ecological fitness (237). Gut microbial homeostasis is associated with health (237) since the competitiveness between symbionts and pathogens protects the host against the latter (252).

Alterations in the composition and function of the gut microbiota (dysbiosis), driven by external and host-related factors, have been associated with several diseases (255), including inflammatory bowel disease (254), malignancies (255), and immune-mediated diseases (255). Dysbiosis can have several causes, and it is characterized by one or more of the following parameters: overgrowth of pathobionts, loss or decrease in the number of commensals by the death of the microorganisms or reduction in their proliferation, and decrease in the gut microbiota diversity (α-diversity) (255). Besides bacterial diversity, the Firmicutes and Bacteroidetes (F/B) ratio is also considered a predictive marker of health or disease (254).

Next-generation sequencing technology allowed a deeper knowledge of the gut microbiota (254). Although there is inter-individual variability regarding the genera that make up the gut microbiota, there are similarities between individuals (221). The dominant phyla are Bacteroidetes and Firmicutes, accounting for nearly 90% of the gut microbiota, and Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (252)(254)(256). The most common genera in adults are *Bifidobacterium* (phylum Actinobacteria), *Bacteroides* and *Prevotella* (phylum Bacteroidetes), *Clostridium, Lactobacillus, Streptococcus* and *Ruminococcus* (phylum Firmicutes), *Escherichia* (phylum Proteobacteria) (252)(254).

The microbiota varies throughout the gastrointestinal tract, according to the microenvironment of each organ (252). While the stomach has few bacteria (257), in the small intestine, there are differences between its fractions. In the duodenum, Firmicutes and Actinobacteria are predominant, while in the jejunum, gram-positive aerobic bacteria, such as *Lactobacilli, Enterococci*, and *Streptococci*, are the most abundant (254). In the proximal zone of the ileum, there are mostly aerobic

species, whereas in the distal zone, gram-negative anaerobic organisms, similar to those found in the colon, predominate (254). Most bacteria are located in the colon (257), where anaerobic bacteria, Firmicutes and Bacteroidetes, have a higher presence.

4.3 FACTORS INFLUENCING THE GUT MICROBIOTA COMPOSITION

4.3.1 BIRTH, AGE AND GENDER

The gut microbiota changes during its host life cycle (258). It begins to be defined in the uterus, shaped by the pregnant woman's metabolic profile and gut microbiota composition (259). Some authors argue that the environment during pregnancy influences GALT development and, ultimately, affects health outcomes (259). Besides the mode of delivery (252)(260), birth gestational age, breastfeeding (252)(261)(262), early use of antibiotics, and place of birth also impact the gut microbiota colonization, which at this stage is greatly susceptible to early life events (259).

Initially, after birth, the gut microbiota is less diverse (261), increasing its diversity when solid foods are introduced (260). Until 12 months, it is not fully matured, and during this time, it is affected by factors like daycare attendance, siblings, pacifier use, and pets in the household (261)(260). The gut microbiota becomes more diverse, stable, and resistant to change in children 2 - 5 years old (263). However, some authors argue that it may only be truly stabilized after puberty (258). During adulthood, it remains relatively stable despite being susceptible to environmental and lifestyle factors (264), such as host phenotype, body mass index (BMI), geographic location, diet, stress, diseases, use of drugs, and, in particular, exposure to antibiotics (221). Despite the apparent stability in adulthood, some authors identified age-related changes in the gut microbiota associated with several diseases (265). Changes consistent with loss of stability in the microorganisms are identified in older adults, impacting digestive function (264). In this period, the interindividual variability is greater than in younger adults. Factors such as reduced ability to chew, reduced esophageal and oropharyngeal motility, increased prevalence of gastroesophageal reflux, increased duration of intestinal transit, altered nutrient absorption, and increased prevalence of malnutrition, negatively impact the diversity of the gut microbiota at this time (264). Interestingly, in a very healthy population of Chinese elderly (> 94 years old), the microbiota composition remained similar to younger adults (266), meaning that these microorganisms may have a role in health and longevity.

The existence of gender-specific gut microbiota is not consensual (267). However, some authors identified gender variability (262)(268-270), which may be associated with the gender bias seen in some diseases (268)(269)(271), such as SLE. When differences exist, generally, women have higher diversity than men, more pronounced in younger ages (267)(269)(272). These alterations may be favored by hormonal factors (269)(271), particularly estrogens (273)(274).

4.3.2 GENETICS

The influence of human genetics on the composition and modulation of the gut microbiota is a new focus of research (275). Several polymorphisms have been associated with α and β-diversity and specific bacteria (275-278). One of the strongest associations between genetics and the gut microbiota is a greater relative abundance of *Bifidobacterium* in adults with lactase gene (*LCT*) single nucleotide polymorphisms (SNPs) associated with lactase-non persistence (GG genotypes) versus lactase persistence (AA/AG genotypes) (275-278). Moreover, a recent study identified 31 genetic variants associated with 39 bacterial taxa (276). The gut microbiota can also be indirectly influenced by genetics through the modulation of dietary preferences, gut architecture, innate and adaptive immunity, and BMI (275-278). For example, *NOD2* variants can alter sensing and response to the gut microbiota, while *FUT2* variants affect the composition and functional properties of glycans in mucosal tissues, greatly affecting the gut microbiota composition (275).

4.3.3 ETHNICITY AND PLACE OF LIVING

Ethnicity and place of living impact gut microbiota composition, but it is challenging to isolate these factors from the socioeconomic environment, diet, lifestyle, and cultural traditions (265)(280). Nevertheless, in a large sample of 528 healthy Amerindians from the Amazonas of Venezuela, residents of rural Malawian communities, and inhabitants of metropolitan areas of the United States of America (EUA), there were significant differences in the gut microbiota composition of the three populations, regardless of age (280). In another study with 1020 healthy individuals from 23 populations, there was a positive correlation between Firmicutes abundance and latitude and a negative between Bacteroidetes and latitude (281). Finally, two different communities of the Bantu village in Southwest Cameroon, with the same ancestry but different dietary habits, had different gut microbiota profiles (282), highlighting that in many cases, lifestyle habits may overrule the ethnic background (283).

4.3.4. MEDICATION

The gut microbiota metabolizes drugs, potentially influencing treatment efficacy (284). It is broadly recognized that antibiotics can alter the gut microbiota (272), but not all antibiotics are the same (285). While first-generation quinolones only affect a few bacterial species, fourth-generation ones inhibit nearly all tested bacteria (285). In addition, some antibiotics, such as macrolides and tetracyclines, have a profound effect on the gut microbiota composition even at lower concentrations (285), and with others, the impact can last up to 2 years, affecting persisting communities such as *Bacteroides* even with just seven-day use (286).

Several other drugs can also alter the gut microbiota (287). Proton pump inhibitors, for instance, have anti-commensal activity, suggesting that altering the stomach pH promotes bacterial migration from the oral cavity (288), changing the bacteria in the gut (287). Furthermore, proton pump inhibitors increase the risk of *Clostridium difficile*-associated diarrhea (288).

Metformin can also affect the bacterial composition of the gut microbiota without necessarily affecting diversity (288). Several studies observed that metformin increased *Akkermancia muciniphila*'s abundance, generally considered a benefic mucin-degrading bacteria, and several SCFA-producing bacteria, meaning that some of the therapeutic effects of this drug may occur due to an improvement in the gut-barrier integrity (288).

Several medications used to treat immune-mediated diseases, including SLE, can change the gut microbiota. In mice, high doses of HCQ (100mg/kg) altered α-diversity and the gut microbiota composition (289). Methotrexate has a dose-dependent influence on the gut microorganisms (284). MMF causes changes in the gut microbiota diversity and composition that are sustained after treatment suspension (290). This drug decreases SCFAs, essential metabolites for colonic homeostasis (291). Several MMF gastrointestinal adverse effects may be mediated by gut microbiota due to modifications in the colonic epithelium that promote inflammation, weight reduction, and body fat and muscle mass alterations (290)(291). Finally, GCs can also alter the gut microbiota, reducing the F/B ratio and increasing *Lactobacillus* and *Bifidobacterium* (292). These are relevant effects since *Lactobacillus* can be reduced in SLE patients, and *Bifidobacterium* was inversely correlated with disease activity (293). Moreover, both *Lactobacillus* and *Bifidobacterium* produce SCFA, which have an anti-inflammatory action on epithelial and immune cells (292), improving the gut barrier function. In fact, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* strains can improve the gut barrier function (294) and induce Treg cells (295).

4.4 GUT MICROBIOTA AND IMMUNE-MEDIATED DISEASES

Although the exact mechanisms through which the gut microbiota contribute to immune-mediated diseases are not fully known, some have been proposed. The impairment of the gut barrier and

increased bacterial translocation is seen in several immune-mediated diseases (296-298) and may be caused by alterations in the gut microbiota composition (296). Patients with rheumatoid arthritis (RA) have increased gut barrier dysfunction markers such as serum LPS, LPS binding protein, and intestinal fatty acid binding protein (I-FABP), and in mice, arthritis development and activity has been linked to gut permeability and inflammation (296). Furthermore, in a RA mouse model, treatment with larazotide, a zonulin antagonist, prevented the gut barrier disruption, decreased intestinal and systemic inflammation, and reduced joint swelling, which once more highlights the role of gut permeability in RA. Besides higher gut permeability, patients with ankylosing spondylitis showed an upregulation of zonulin, modulated by bacteria in the ileum, and higher expression of gut barrier dysfunction markers, as was observed in RA patients (298).

Bacterial metabolites can also activate the immune system, influencing immune-mediated diseases (299). An altered metabolic profile was identified in RA patients (300) and in axial spondyloarthritis (301). In the latter, there was an increase in the byproducts of tryptophan metabolism, two of which potentially microbially generated and able to modulate the host barrier and immune functions (301).

Finally, SCFA are involved in immune tolerance by activating Treg cells (302). These metabolites are produced by the fermentation of indigestible complex carbohydrates by the gut bacteria (303). Individuals at risk for developing RA but who did not progress to RA had higher mean baseline serum levels of total SCFA, butyrate, and acetate than individuals who developed RA, showing that SCFA can reduce RA risk (304).

Molecular mimicry is another mechanism through which the gut microbiota can affect immune responses. In this case, there is a similarity between self-antigens and bacterial antigens (pathogens or commensals) that drive the cross-activation of effector immune cells to react to self-antigens (299)(305). In RA, a wide spectrum of microorganisms can elicit RA-specific autoreactive T cells, between which constituents of the gut and oral microbiota, such as *Bacteroides fragilis*, *Prevotella* strains, and *Capnocytophaga gingivalis* (305). For example, in type 1 diabetes, exposure to a *Parabacteroides distasonis* peptide, similar to insulin, may contribute to the onset and progression of the disease (306).

Although gut microbiota dysbiosis has been identified in several immune-mediated diseases, it is yet to be established if it is a cause or a consequence of these diseases (255), substantiating further research in this regard.

4.5 THE GUT MICROBIOTA AND SLE

Several authors have looked at the role of the gut microbiota in SLE. However, the results are frequently contradictory, possibly due to the heterogeneity of the lupus-mouse models and studied populations, the latter regarding ethnicity, gender ratio, disease activity, chronicity, and medication. Nevertheless, most reported gut microbiota dysbiosis, although it remains to clarify if it is a cause or a consequence of the disease or its treatments.

4.5.1. Gut microbiota in SLE mouse-models

As already mentioned, α-diversity is an indicator of microbial species diversity, and it considers the variety of species (richness) and the evenness of species abundance (307). There are heterogeneous results regarding the α-diversity in lupus mouse-models. Six-week-old murphy roths large (MRL)/*lpr* mice have a significantly lower α-diversity, while MRL+/+ mice, the decrease was not significant (308). On the other hand, in New Zealand black (NZB)/New Zealand white (NZW)F1 mice and NZB/NZW F1 mice induced by HCMVpp65$_{422-439}$, a viral peptide of human cytomegalovirus, the diversity of the microbiota increased after SLE onset (309)(310).

Differences in the microbial community composition (β-diversity) also exist in SLE mouse-models (295)(308)(310). Changes in β-diversity were identified in 16-week-old SNF1 mice housed littermates but not in 4-week-old mice (311), showing that disease progression affects the gut microbiota.

The F/B ratio has been considered a marker of gut homeostasis, and a higher or lower ratio is indicative of dysbiosis (312). Nevertheless, it should be considered together with other markers of gut microbiota diversity and composition (313). Within the phyla Firmicutes and Bacteroidetes, several bacteria may contribute to an altered F/B ratio.

Both increased (309) and decreased (308)(314) F/B ratios were found in SLE mouse-models. In 6-week-old MRL/*lpr* mice, for instance, the lower F/B ratio may have contributed to early disease onset and was attributed to the decreased abundance of *Peptostreptococcaceae* and *Lactobacillaceae* (phylum Firmicutes) and increased abundance in *Rikenellaceae* (phylum Bacteroidetes) (308).

In SNF1 mice, depleting the gut microbiota with an antibiotic led to a lower expression of pro-inflammatory cytokines without affecting immune regulatory cytokines, such as IL-10 (311). Moreover, after treatment with antibiotics, only 40% of the female mice developed severe nephritis; without antibiotics, 80% of the mice developed this manifestation (311). Interestingly, the microbiota depletion in male mice did not alter the disease incidence, immune phenotype, disease stage, or renal involvement (311). In addition, castrated male SNF1 mice had distinct microbial communities at the genus level than non-castrated mice and showed higher lupus incidence and earlier onset (although not statistically significant) (311). This work highlights that, in SLE, there may be an interplay between the gut microbiota and hormonal factors (311) and that the gut microbiota may have a role in the higher SLE incidence in females.

In female and castrated male *lpr* mice, colonization with *Lactobacillus* spp., before disease onset, improved renal function and survival by increasing the expression of TJ proteins and decreasing endotoxemia (315). The improvement of the gut barrier and consequent decrease in bacterial translocation and activation of immune responses was corroborated by the reduction in T cell migration to the gut lamina propria (315). Moreover, *Lactobacillus* spp. also increased the systemic production of IL-10. Hence, these bacteria may have a systemic anti-inflammatory effect (315). Furthermore, treatment with *Lactobacillus* spp. increased the influx of $CD8^+$ T cells and $Foxp3^+$ T reg cells to the kidney while decreasing Th17 cells, attenuating LN, and improving the Treg-Th17 balance in castrated mice but not in non-castrated ones. These results suggest that androgens attenuate the alterations induced by *Lactobacillus* spp., and in this mouse model, the control of LN produced by the gut microbiota was sex hormone-dependent (315).

It is important to note that distinct bacterial communities are present in the gut microbiota of different strains of lupus mouse models and at diferente disease time points (308). Depending on the disease status, bacteria may have multiple roles (295)(308).

Despite variations in the gut microbiota composition between mouse studies, microbiota changes seem to be associated with lupus-like symptoms (309)(314) and variations in cytokines' concentrations (292)(309), meaning that interfering with inflammatory cytokines production may be one of the mechanisms by which the gut microbiota influences SLE pathogenesis (316).

When studying the gut microbiota in SLE mouse-models the results are often contradictory, possibly due to the heterogeneity of the mouse models used. Characteristics, such as the production of autoantibodies, the timing of disease onset, or the organs involved, differ between models. Although mouse models have been essential in improving the knowledge about SLE, human SLE characteristics are difficult to mimic in mice. In humans, SLE is a highly heterogeneous disease, whose pathogenesis is influenced by several factors beyond genetics. It is, therefore, challenging to develop a mouse model that fully represents it.

4.5.2. Gut microbiota in SLE patients

The differences in gut microbiota between lupus-mouse models and SLE patients are striking, as well as between different SLE populations, making it difficult to establish consensual conclusions about SLEs' gut microbiota profile. Considering that in any population, several internal and external factors impact the gut microbiota (317) and that in SLE patients, both the disease and treatments seem to influence its composition and diversity (318), different results should be expected.

Similarly to the results in mouse models, the α-diversity alterations in SLE patients are also heterogeneous, varying between lower α-diversity (292)(293)(310)(316)(319-323), increased α-diversity (324) or no changes compared with healthy controls (295)(325-328). In some contexts, disease duration, disease activity, and LN did not alter α-diversity (321).

In some studies, SLE patients had an altered β-diversity when compared with controls (293)(295)(316)(321)(323)(324)(326), but not in all (327). Furthermore, β-diversity was also altered by disease activity (316)(329), although not consensually (321).

Data assessing the F/B ratio in SLE patients are, once more, heterogeneous. The F/B ratio can be identical (310)(320), lower (292)(327)(329), or increased (326) than healthy controls, and in some cases, it is associated with active disease (292)(309).

When looking at the taxonomic alterations in the gut microbiota of SLE patients, several authors identified changes (293)(308)(309)(322)(329). Still, heterogeneous disease duration, severity (295)(310) and activity (316)(330)(320) may explain the differences in the bacteria involved. For example, while some bacteria may influence disease activity (293)(316)(320), others may increase SLE risk (*Bacilli, Eggerthella*, and *Lactobacillales*), and others can be protective (*Coprobacter, Bacillales*, *Lachnospira*) (331).

Ruminococcus gnavus is a gram-positive bacterium that can be increased in SLE patients and was connected to lupus nephritis and disease activity (316)(323). However, one study reported a lower abundance of *Ruminococcus gnavus* in SLE patients with active disease (293). Genetics, diet, and other lifestyle factors should be considered when looking at these differences.

Besides *Ruminococcus gnavus*, other bacteria can favor SLE occurrence. Certain strains of *Bacteroides fragilis,* which are increased in some SLE patients (323), produce a toxin that binds to a colonic epithelial cell receptor and alters gut permeability (332). Furthermore, *Akkermancia muciniphila* was increased in a group of SLE patients in which a bacterial peptide mimicking an extracellular part of the human FAS, which can bind IgG, showed a positive correlation with serologic markers of inflammation (323).

4.5.3. How can the gut microbiota influence SLE pathogenesis?

Despite the heterogeneity of the results assessing gut microbiota variations in lupus, it seems undeniable that the gut microbiota is involved in SLE pathogenesis (319). Although the precise mechanisms still need to be fully known, some aspects connecting SLE and the gut microbiota are already minimally elucidated.

The gut mucosa immunity is key to host homeostasis (333). Its important role is corroborated by studies in germ-free mice in which, without the gut microorganisms, there is a lower production of cells involved in defense and inflammation, such as intraepithelial lymphocytes, as well as a decrease in Tregs (333). This finding shows that the production of bacterial components is essential for maintaining gut mucosal immunity. On the other hand, in female lupus-prone mice, depleting the gut microbiota suppressed pro-inflammatory gut immune phenotype and decreased disease incidence and progression, demonstrating the involvement of the gut microbiota in SLE, possibly through interactions with the gut mucosa (311). Furthermore, other authors reported that, although the gut microbiota did not seem to contribute to the development of lupus phenotype in BXD2 and SKG mice, especially in older

mice, it favors the maintenance of the immune dysregulation, influencing the disease progression (334)(335).

The gut microbiota participates in host defense, producing antimicrobial components that will prevent colonization by pathobionts (336), which means that alterations in the gut microbiota composition may lead to an increase in pathogenic bacteria that can induce immune responses. However, the gut microbiota can also stimulate the production of autoantibodies and activate immune cells (325). Fecal transfer from TC lupus-prone mice (B6.*Sle1*.*Sle2*.*Sle3*) to B6 mice induced autoimmune phenotypes but only when the TC donor mice were older and exhibited autoimmunity (325). In this model, diet had a clear role in autoimmunity. Low dietary tryptophan prevented autoimmune pathology in TC mice, whereas high dietary tryptophan exacerbated the disease (325). Reducing dietary tryptophan changed gut microbiota patterns in both lupus mouse models (325). Furthermore, fecal transfer from TC mice fed a high tryptophan diet but not a low tryptophan diet induced autoimmune phenotypes in germ-free B6 mice (325).

In another mouse model, fecal transfer from lupus mice also induced anti-dsDNA antibodies and led to increased stimulation of B cells and decreased Treg cells, highlighting that feces and, consequently, the gut microbiota can induce gut mucosal immune responses (319). Furthermore, the influence of the gut microbiota is not limited to the gut mucosa, as peripheral immune responses were also identified, as well as higher gene expression of type I interferon-related genes and susceptibility genes for lupus (319).

Finally, higher gut permeability may favor gut microorganisms' passage and their antigens through the epithelium, activating the host immune system and favoring immune-mediated diseases in genetically predisposed individuals (214). Increased levels of LPS were identified in SLE patients (337) associated with SLE progression (294), showing that gram-negative bacteria may favor SLE pathogenesis (294). In fact, LPS-producing bacteria can enter circulation and have systemic effects by triggering immune-mediated responses (323).

To summarize, the gut microbiota can shape intestinal mucosal immunity and gut permeability by adjusting TJ protein levels. These mechanisms will affect endotoxemia and the chronic activation of the immune system. Some mechanisms through which the gut microbiota participate in SLE pathogenesis are highlighted in **Figure 4.1**.

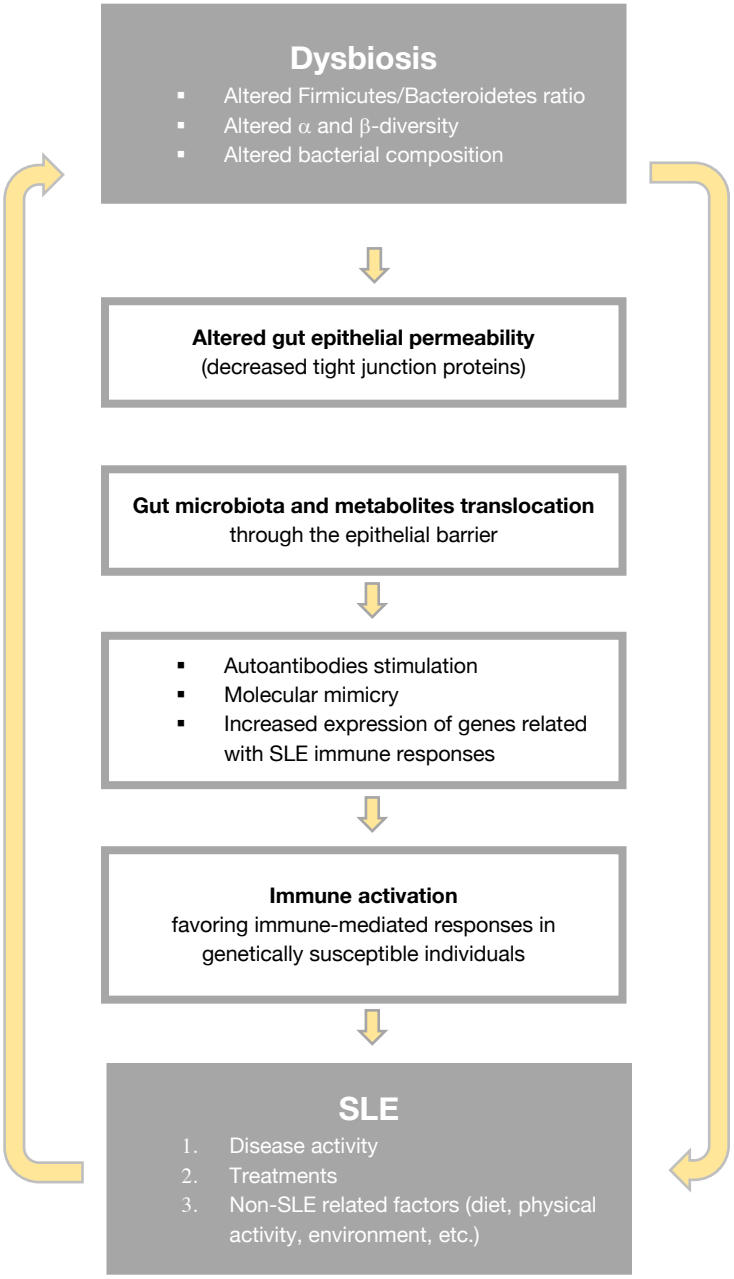


Figure 4.1. Diagram showing some of the mechanisms through which the gut microbiota can influence systemic lupus erythematosus (SLE).

CHAPTER 5. Diet, body composition and physical activity in SLE

5.1 OVERVIEW

Westernized urban living brought significant changes in lifestyle and dietary habits, which have been implicated in the increased incidence of non-communicable diseases (338). Diet, in particular, modulates inflammation and immunity. In fact, the shift from a diet high in plant-based foods and fiber to a high-protein, high-fat diet led to an increase in chronic inflammatory diseases (338), which may be explained by the impact of diet on gut microbiota composition and diversity. Typically, individuals who follow a Mediterranean dietary pattern have a diverse gut microbiota, with adequate proportions of beneficial bacteria, that produce metabolites, such as SCFA, with a favorable systemic influence on health (339). Moreover, altered body composition and decreased physical activity have also been associated with worse health outcomes in several immune-mediated diseases (340-343). In this chapter, the contribution of diet, body composition, and physical activity to immune-mediated diseases, particularly SLE, will be detailed.

5.2 DIET

Diet is one of the lifestyle factors that can impact immune-mediated diseases (344). Nutrients can have a direct immune modulatory effect or an indirect one through the gut microbiota (345)(346). Diet's potential influence on immune modulation or inflammation becomes clear when studying rural and urban cohorts (338). In a cohort of healthy Tanzanian adults, the urban population had a higher pro-inflammatory cytokine production than the rural population, which was linked to diet composition (338). The gut microbiota can extract energy from the diet. Thus, nutrients can indirectly promote or limit the growth of certain microorganisms, modulating the microbiota (347). On the other hand, the direct influence that diet can have on the gut microbiota composition is undeniable when looking at the significantly different taxonomic distribution it has in urban and rural populations (347)(348). The main differences are related to the fiber intake (higher in rural areas), which translated into higher SCFA production (348). Furthermore, when comparing the gut microbiota between different ethnic groups, the intake of vegetables, red meat, and overall diet quality induced significant differences (349).

5.2.1 Mediterranean Diet

The Mediterranean diet is a dietary pattern characterized by high consumption of olive oil, pulses, whole grain cereals, fruits and vegetables, moderate consumption of wine, fish, dairy products, poultry, meat, and its products, and low consumption of highly processed foods, refined grains, and sugars. It has several health benefits, including the prevention of chronic diseases, such as cardiovascular diseases, obesity, metabolic syndrome, type 2 diabetes, and immune-mediated diseases, as well as an increase in life expectancy (344-346).

Given the potential to prevent cardiovascular events attributed to the Mediterranean diet, this dietary pattern is even more relevant in rheumatic diseases. These patients have increased cardiovascular risk due to conventional risk factors, chronic inflammation, and underlying endothelial damage (345)(350).

In SLE, adherence to a Mediterranean diet was inversely associated with the risk of active disease and damage (345). In this cohort, patients with high adherence to the Mediterranean diet had lower BMI and fat mass than patients with medium and low adherence. In opposition, low adherence was linked to obesity and increased corticoid use (345). However, these results are far from being consensual, and other authors could not find definitive evidence that diet alters SLE risk (350)(351).

The benefits of the Mediterranean diet in other immune-mediated diseases have also been assessed. RA patients with high adherence to this dietary pattern had lower disease activity and better

gut microbiota composition (346). Moreover, in a population of patients with Sjögren's syndrome, adherence to the Mediterranean diet was inversely correlated with disease activity (344).

Although the exact mechanisms behind the immune effects of the Mediterranean diet are still not known, there are undeniable metabolic improvements (350), which may be due to some particular foods such as olive oil (350), nuts, and legumes (351). Interestingly, when assessing the association between four dietary quality scores and the risk of SLE, women with a higher intake of nuts/legumes had a 41% decrease in the risk of developing this disease (351). Furthermore, consuming monounsaturated fatty acids (mainly present in olive oil) as part of a Mediterranean dietary pattern was an independent variable for RA remission (352). All this data points out that the Mediterranean diet has numerous benefits for patients with immune-mediated diseases.

5.2.2 Diet: Fatty acids, Sugars, and Fiber

Rather than individual dietary components, the dietary pattern comprehending the interactions between its nutrients brings the most benefits. However, some central macro and micronutrients have immune and gut microbiota modulatory characteristics.

5.2.2.1 Fatty acids

When considering the potential contribution of dietary fat to disease, it is important to note that not all fats are equal. While saturated fatty acids (SFAs) can have inflammatory potential, polyunsaturated fatty acids (PUFA), such as n-3, activate anti-inflammatory pathways (353). For instance, SFAs C12:0 and C16:0 can induce the expression of cyclooxygenase-2 (COX-2) and IFN-α in macrophages and activate TLR-mediated pro-inflammatory pathways, while the PUFA docosahexaenoic acid (DHA) countered this effect (353). Interestingly, SFAs can activate PRRs such as TLR4 and TLR2, increasing the expression of IL-8 (353), one of the main mediators of an inflammatory response that can be elevated in SLE patients (354). Cells are particularly susceptible to the activation of TLR4 and TLR2 by SFAs when the production of reactive oxygen species increases (354). Considering that in SLE, there is a high production of these compounds, patients are probably more susceptible to the activation of PRRs by SFAs.

In animal studies, monounsaturated fatty acids (MUFA), and in particular the oleic acid of olive oil, can suppress immune cell functions such as lymphocyte proliferation and NK cell activity (355). Although similar results were not observed in humans, which may be explained by differences in the doses considered, olive oil consumption seems useful for managing the chronic immune activation seen in immune-mediated diseases (356). In an RA mouse model, a diet enriched in extra virgin olive oil reduced disease prevalence, joint edema, and cartilage destruction (357). Interestingly, extra virgin olive, due to its phenolic fraction and fatty acid profile, reduced IL-17 production and improved the expression of the mitogen-activated protein kinases (MAPK) (JNK and p38), known for initiating inflammatory responses (357). In another immune-mediated disease, multiple sclerosis patients showed reduced serum concentrations of oleic acid. In addition, stimulation of Tregs isolated from these patients with oleic acid restored defects in Tregs' suppressive function, reduced the expression of IL-12 and inhibited the development of Th1-like Tregs (358). Despite being partial effects, they show that oleic acid is relevant in regulating inflammatory signals. Finally, in na SLE mouse model, a diet supplemented with extra virgin oil induced a lower increase in paw swelling, spleen and thymus enlargement, proteinuria, and histological renal damage, as well as a lower increase in cytokines usually elevated in SLE, such as TNF-α, IL-6, IL-10 and IL-17 (359).

Humans cannot synthesize PUFA, which are considered essential fatty acids, meaning that their daily needs must be met through diet (360)(361). Omega-6 (n-6) PUFA and omega-3 (n-3) PUFA are the main PUFA families, but while increased consumption of n-3 PUFA is known to improve

inflammatory responses, preventing various pathologies (361), high n-6 PUFA concentrations have the potential to increase inflammation (362).

The n-3 PUFA exist mainly in the esterified form and are associated with phospholipids in the cell membrane or with triacylglycerols in lipid storage. After ingestion, α-linolenic acid (ALA, C18:3) is converted into the bioactive forms (363): eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic DHA (C22:6), in a relatively inefficient process in mammals (361)(364). This conversion occurs mainly in the liver through desaturation and elongation reactions (363). The activity of desaturase and elongase enzymes is regulated by nutritional and hormonal status and influenced by genetic polymorphisms [fatty acid desaturase 2 and 1 (FADS 2 and 1)] (363). Importantly, as the conversion of the linoleic acid (C18:2, n-6) into arachidonic (ARA, C20:4) and osbond acid (C22:5) uses the same Δ-6 and Δ-5-desaturase enzymes, there is a competition between ALA and ARA conversions (363). This is particularly relevant as Δ6-desaturase is a rate-limiting enzyme, meaning that its activity determines the rate of the metabolic pathway. Although Δ6-desaturase has a higher affinity for ALA, because linoleic acid is present in higher concentrations in most Western diets (363)(365), n-6 PUFA will potentially limit n-3 PUFA concentrations in plasma and cells (361). Thus, despite being essential to maintain a balanced intake of both n-6 and n-3 PUFA, in Western diets, the n-6/n-3 PUFA ratio hovers around 15/1 and 16.7/1, far from the ideal 1/1 to 4/1 ratio (365).

The anti-inflammatory properties of n-3 PUFA (366) occur through mechanisms such as reduction of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) and adhesion molecules (363); increase in anti-inflammatory cytokines (IL-10), and activation of the proliferator-activated gamma receptor (PPAR-γ), a transcription factor with anti-inflammatory functions (363)(366); reduction of prostaglandin E2 (PGE2) synthesis, by competition with ARA in cell membranes, thus reducing the substrate available for the production of PGE2 by COX-2 (363); interfering with the activation of NF-κB, one of the main pro-inflammatory transcription factors, which triggers a signaling cascade of various pro-inflammatory stimuli (363)(366); modulation of the gut microbiota (366)(367), and modulation of endotoxemia (368). Interestingly, n-3 PUFA increase butyrate-producing bacteria, such as *Lactobacillus, Bifidobacteria, Lachnospira,* and *Roseburia*, and decrease LPS-producing bacteria with pro-inflammatory action while also improving the gut barrier integrity and decreasing local inflammation (366)(367).

In lupus-prone mice, diets rich in n-6 PUFA and n-9 MUFA, increased plasma autoantibodies, proteinuria, and glomerulonephritis and all these three endpoints were markedly attenuated in mice that consumed the n-3 PUFA diet (369). Of note, the doses of EPA and DHA used in this trial, if extrapolated to human intake, would represent 18g/day in a 2,000 kcal/day diet (369), a much higher intake than usually recommended (370). Other mouse studies, however, have shown that smaller doses can induce similar effects. In lupus-prone NZBWF1 female mice, repeated intranasal exposure to silica triggers premature initiation of autoimmune responses in the lungs and kidneys. Interestingly, the consumption of n-3 PUFA docosahexaenoic acid prevents this phenotype. Mice were fed isocaloric AIN-93G diets containing 0.0, 0.4, 1.2, or 2.4% docosahexaenoic acid, which was incorporated into erythrocytes, lungs, and kidneys in a concentration-dependent fashion. The docosahexaenoic acid intake inhibited pro-inflammatory cytokines, B-cell proliferation factors, IgG, and anti-dsDNA antibodies in the bronchoalveolar lavage fluid and plasma. It also reduced proteinuria and glomerulonephritis. A human eating 2,000 kcal/day would require 2, 6, and 12 g/day to correlate with the amounts consumed in this study, which clearly shows that smaller doses are also beneficial (371).

The incorporation of n-3 PUFA in cells peaks six weeks after diet initiation and remains stable for several weeks (372). Interestingly, in SLE, DHA-rich diets can suppress the increase in macrophages, neutrophils, and lymphocytes in the bronchioalveolar lavage fluid, suppress plasma auto-antibodies, and prevent renal involvement (372)(373). Furthermore, diets supplemented with DHA also suppressed interferon-related gene responses in the lungs, alleviated silica-induced cytokine increase and cell

infiltration in the bronchoalveolar lavage fluid (371)(373), and systemic autoantibody and cytokine production (371).

The n-3 highly unsaturated fatty acid (HUFA) score and the n-3 PUFA index are valuable tools for assessing the necessary dietary intake (374). n-3 HUFA score considers the percentage of n-3 PUFA from the total PUFA (n-3, n-6, and n-9) in erythrocytes, while the omega-3 index considers the percentage of EPA+DHA from the total fatty acids in erythrocytes (374). An n-3 HUFA score above 40% was associated with reduced lung interferon-regulated gene expression, decreased TNF-α, diminished macrophage and lymphocyte numbers in the bronchioalveolar lavage fluid, and delayed disease progression (374). The omega-3 index showed similar results. Both biomarkers can, therefore, predict DHA's preventive effects (374).

The importance of n-3 PUFA in SLE has been studied not only in mouse models, but also in humans. In a group of juvenile-onset SLE, in which 35% had dyslipidemia at baseline, a 9-month dietary intervention program reduced energy, carbohydrates, total fat, saturated fat, and trans-fat intakes, improved high-density lipoprotein (HDL) function and prevented the body mass index increase (375). The results are particularly relevant, showing that a well-planned dietary intervention may modulate cardiovascular risk factors. Although in this group of juvenile-onset SLE patients, the dietary intervention did not alter the disease activity (375), another group identified an inverse association between EPA and DHA concentrations in the adipose tissue and disease activity (376). Moreover, carotid plaque prevalence showed a small negative correlation with n-3 PUFA and a small positive correlation with n-6 adipose tissue concentrations (376). Interestingly, carotid plaques can incorporate PUFA, and decreased concentrations of DHA and EPA have been correlated with symptomatic carotid atherosclerotic plaques and increased inflammation (377). Moreover, a higher intake of total n-3 PUFA, particularly EPA and DHA, can decrease the risk of carotid plaque progression (378).

Recently published trials have assessed the benefits of n-3 PUFA intake in SLE patients. In this population, 3g of n-3 PUFA (1.8g EPA, 1.2g DHA) for 12 weeks significantly declined the inflammatory marker vascular cell adhesion molecule-1 (VCAM-1). However, there were no changes in disease activity, endothelial function, fasting lipid profile, and other inflammatory markers (379). Patients' dietary intake was not registered in this study, which impairs the assessment of n-3 PUFA and n-6 PUFA total consumption and n-6/n-3 PUFA ratio. This is particularly relevant once, as previously mentioned, high n-6 PUFA intake influences n-3 PUFA metabolization (380), thus potentially impacting health outcomes.

Another group reported that in SLE patients with low disease activity, ~1.3g of n-3 PUFA (1.08g EPA, 0.2g DHA) led to a decrease in C-reactive protein (CRP) serum levels, with no changes in interleukin (IL)-6, IL-10, leptin, and adiponectin levels (381).

In a 6-month randomized, single-blind, placebo-controlled trial, SLE patients taking 2.25g of EPA and 2.25g of DHA improved global disease activity assessed by Physician Global Assessment (PGA), and erythrocyte sedimentation rate, increased IL-13, and decreased IL-12 concentrations (382). IL-13 is an anti-inflammatory cytokine with a complex role in SLE, as polymorphisms in the IL-13 gene have been associated with increased IL-13 plasma levels and risk for SLE (383). However, these authors mention that there may be a beneficial effect of IL-13-induced Th2 skewing in SLE (382). On the other hand, IL-12 is a pro-inflammatory cytokine frequently elevated in SLE (382)(384).

In another trial, 3g of n-3 PUFA (1.8 g EPA, 1.2 g DHA) significantly improved disease activity (assessed with SLEDAI), increased adiponectin levels and decreased leptin levels (385).

When comparing the results of these studies, it is important to consider that the trials' duration and n-3 PUFA doses may affect the outcomes. Several authors did not assess the fatty acids concentration (385), impairing a real picture of the actual PUFA presence in the cell membranes, which individual differences may influence absorption and metabolism (380). n-3 PUFA concentration on cell membranes is a relatively stable biomarker (380) that correlates with the n-3 PUFA content in other

tissues (373). In a group of 68 SLE patients, the percentage of n-6 PUFA and n-3 PUFA, assessed in erythrocytes' membrane, was associated, respectively, with higher and lower CRP values (380), which once more highlights the inflammatory potential of high n-6 PUFA intake and the anti-inflammatory potential of n-3 PUFA. In a large cohort of SLE patients (n=456), self-reported lupus disease activity (Systemic Lupus Activity Questionnaire - SLAQ) was associated with the dietary n-6/n-3 PUFA ratio (386). Moreover, lupus self-reported disease activity and Patient-Reported Outcomes Measurement Information System (PROMIS) sleep disturbance scores were significantly lower with each 1-gram/1,000 kcal increase of n-3 fatty acids (386).

Finally, SLE patients taking n-3 PUFA had higher low-density lipoprotein (LDL) cholesterol concentrations than the placebo group, with no alterations in the LDL/HDL ratio (379). Although higher doses of EPA and DHA can increase LDL concentrations, the European Food Safety Authority does not consider that it can significantly increase cardiovascular risk (387).

In general, all studies reported improvement outcomes associated with n-3 PUFA intake in SLE. Nevertheless, in both human and mouse studies, the high variability of factors such as n-3 PUFA dose, composition in DHA and EPA, n-3 PUFA assessment method, trial duration and n-6/n-3 PUFA ratio can all influence trials' outcomes, making it difficult to reach a consensus regarding n-3 PUFA benefits in SLE (373). Thus, an n-3 PUFA formulation high in DHA will have different outcomes than a formulation low in DHA, even if the total n-3 PUFA is similar. Studies in lupus-mouse models showed that n-3 PUFA doses above the usually used in humans might be necessary to improve outcomes in SLE. It is important to note that although doses close to 5g/day are generally above the recommended daily intake for the general population (370), they are considered safe by the European Food Safety Authority (387). Nevertheless, more studies are needed to better understand the effect of n-3 PUFA intake in SLE and the necessary doses that should be used.

5.2.2.2 Sugars

Sugars, starches, and fibers are carbohydrates that compose a healthy diet and are available in fruits, vegetables, grains, and milk products (388). Lactose, sucrose, and maltose are disaccharides that result from the first phases of the carbohydrates' digestion, while glucose, fructose, and galactose are monosaccharides and the end-products of the digestible carbohydrates' metabolism in the small intestine (388). In humans, glucose can be used as an energy source, stored as glycogen in the liver and muscle, or converted into body fat (388). Fructose, on the other hand, has a higher sweetener power than glucose (389). High fructose corn syrup is one of the main sweeteners used by the food industry and, for this reason, is highly consumed in Western diets (389).

The World Health Organization recommends that the intake of free sugars does not exceed 10% of the total energy daily intake, mentioning that an intake below 5% can bring additional health benefits (390). However, in Europe, the intake of free sugars ranges from 15% to 21% in adults and 16% to 26% in children (391). In Portugal, a national diet questionnaire (*Inquérito Alimentar Nacional e de Atividade Física* IAN-AF) 2015-2016 showed that the mean consumption of free sugars represents 7.5% of the total energy daily intake, and the mean consumption of mono and disaccharides represents 18.5% of the total energy daily intake (392).

The high sugar intake, especially glucose and fructose, is particularly concerning once it has been considered a potential risk factor for many non-communicable diseases (389). One possible mechanism associating high sugar intake with the risk for chronic non-communicable diseases is low-grade chronic inflammation mediated by TLR4, high-sensitivity C-reactive protein (hsCRP), IL-6, IL-18, and TNF-α, besides others (393). In fact, both high consumption of sucrose and fructose can increase these inflammation markers (393), probably due to their metabolic similarity (394).

The modulation of the gut microbiota can also explain the association between sugar and non-communicable diseases. In mice, a high-fructose or high-glucose diet decreased the gut microbiota

diversity, particularly decreasing the abundance of Bacteroidetes and increasing Proteobacteria without weight variation, despite promoting fat deposition in the liver (395). In addition, these mice also showed increased gut permeability, with lower expression of TJ proteins and higher expression of inflammatory cytokines in the colon (395). In humans, a 7-day simple sugar diet decreased duodenal microbiota diversity and increased gut permeability (396). High sugar intake can promote inflammation by altering the diversity and composition of gut microbiota, affecting SCFA and inflammatory cytokines production (389). In a cohort of 186,900 women, consuming more than one sugar-sweetened beverage/day increased the risk for seropositive RA. In contrast, no increase in RA risk was found with the intake of low-calorie beverages (397). These results were independent of weight change (397). Finally, SLE patients with active disease had a higher sugar intake than patients with inactive disease, and free sugars intake was associated with the number of complications, disease activity, and dyslipidemia (398).

5.2.2.3 Fibers

Fibers are carbohydrates resistant to digestion and absorption in the small intestine (360). Fibers are essential for maintaining the gut's health and microbiota equilibrium. With the wide adoption of Western diets, there has been a decrease in fiber consumption associated with several non-communicable diseases (365)(399). One of the mechanisms behind this shift may be the negative impact of low fiber-diets on gut microbiota diversity (400). Glycans, such as resistant starch, inulin, lignin, pectin, cellulose, and fructooligosaccharides, are indigestible carbohydrates mainly derived from vegetables and are central nutrients to gut microbiota growth (347). Although the human genome only encodes a limited number of glycan-degrading enzymes, bacteria encode a high number of these enzymes, in particular the *Bacteroides, Bifidobacterium,* and *Ruminococcus* genera which are primary glycan degraders (347). The degradation and anaerobic fermentation of fiber by the gut microorganisms results in the formation of the SCFA, acetate, propionate, butyrate, lactate, and succinate, which are sources of energy for the gut microbiota (347)(360) and colonocytes (399), and enhance Treg cell function and epithelial barrier integrity (400).

In lupus, a low-fiber diet can facilitate immune dysregulation and disease progression, favoring an inflamed phenotype of adaptative and innate immune cells (401). On the other hand, a high-fiber diet can improve gut barrier integrity and increase Mucin-2 production, improving the mucus layer (401). Further, in a lupus-mouse model (TLR7-Tg), mice in a resistant starch diet were protected from the disease and showed reduced IFN-I signaling and pDCs in the secondary lymphoid organs (402). Resistant starch increased *Clostridiales*, reduced gut permeability, and the fecal concentration of *L. reuteri*, preventing its translocation (402). In addition, *in vitro* incubation of *L. reuteri* with SCFAs, particularly butyrate, prevented its growth (402). These data suggest that *L. reuteri* may participate in SLE pathogenesis and that fiber, particularly resistant starch, can modulate the gut microbiota, influencing SLE occurrence and progression.

5.2.3 Diet: minerals

Besides macronutrients, dietary micronutrients (minerals and vitamins) can increase and modulate immune functions (360)(403) and maintain an antioxidant balance that protects cells against oxidation (403). Several factors can influence vitamins and minerals' bioavailability, and bioaccessibility such as food processing (404) and interindividual variability (405) that includes digestive function (406), gut microbiota composition (407), circadian rhythm (405), BMI (405), and genetic polymorphisms (405). Since vitamins and minerals have a tight connection with innate and adaptative immune responses, a closer look at the interplay between them is warranted.

Zinc is an essential nutrient for the optimal function of granulocytes (408), NK cells (403)(408), and lymphocytes, especially T lymphocytes (408). Tregs, in particular, have higher levels of intracellular free zinc than other T cell subsets, hence needing higher zinc concentrations for adequate function (409).

Since both zinc excess and insufficiency seem prejudicial, adequate concentrations must be maintained. In the presence of zinc deficiency, granulocytes show altered phagocytosis, cytokine secretion, oxidative burst, granule release, chemotaxis (410), and NETosis (411). Zinc supplementation may be a strategy to consider in T cell immune-mediated diseases (409)(412) since it can suppress Th1 (IFN-γ), Th2 (IL-5), and Th17 (IL-17) cytokine production (412).

Iron is essential for macrophages, neutrophils (413), NK cells, and lymphocyte function (414). Iron deficiency, as frequently seen in the presence of inflammation, delays the proliferation and activation of T cells in immune responses (414), suppresses neutrophiles' reactive oxygen species production and phagocytic capacity (413), and increases NETosis (413)(415), an important process in SLE pathogenesis. In an *in vitro* study that used human blood-derived neutrophils, iron supplementation reduced NET formation. However, it is yet to be established if chronic iron deficiency or iron overload induces NETosis abnormalities in patients (415).

Salt or sodium chloride (NaCl) influences several immune cells (416). Extracellular salt concentrations favor the differentiation of M1 macrophages (417). Further, high salt concentrations can accelerate autoimmunity onset, promoting Th17 cell differentiation in the gut lamina propria (417). In fact, in a population of healthy adults, an increased salt intake (15g of salt or 256 mmol of Na/day) increased Th17 cells and decreased Treg cells, also increasing inflammatory cytokines production (418). These alterations were reversed with a low salt diet (5g of salt or 85.5 mmol of Na/day) (418).

Magnesium is essential to manage inflammation. Thus, an intake below 50% of the recommended daily intake (RDI) seems to increase markers of inflammation, such as TNF-α, and increase oxidative stress in rats (419). Moreover, macrophage-like cells cultured in a low-magnesium medium showed increased LPS-induced IL-1β release (420).

5.2.3.1 Minerals, gut barrier, and microbiota

Minerals are also essential to maintain the gut barrier integrity. *In vitro* and *in vivo* studies demonstrated that depletion of intracellular zinc decreased occludin and claudin-3 expression, leading to an impaired gut barrier (421), the same being observed in mice in a low potassium diet, leding to increased endotoxemia (422). In line with these findings, zinc supplementation *in vitro* improved epithelial barrier function, cell growth, and differentiation (423). Magnesium can also impact the gut barrier integrity since, when insufficient, it can decrease cecal *Bifidobacteria* (424), an alteration that, in mice, led to lower TJ expression, higher gut permeability, and higher portal LPS concentrations (424).

Manganese is a cofactor of manganese-superoxide dismutase (MnSOD), a mitochondrial enzyme that mediates the production of reactive oxygen species (425). MnSOD is activated by LPS, cytokines, UV radiation, and reactive oxygen species and is involved in several inflammatory diseases (425). A single nucleotide polymorphism in *SLC39A8* encoding the variant A391T (426), which is necessary to maintain manganese levels, has been associated with increased BMI, higher risk of coronary artery disease, cardiovascular death, inflammatory bowel disease, SLE with Sjögren syndrome, and inflammatory progression of osteoarthritis, beyond others (427). Mice with this single nucleotide polymorphism were manganese-deficient in the colon, which was associated with transmembrane mucin glycoproteins disruption and impaired intestinal barrier (426). In fact, a manganese-insufficient diet increases the susceptibility to intestinal barrier disruption (426) and alters TJ protein expression, even if followed for a short time (428). These data show that manganese is essential for MnSOD activity, management of oxidative stress, and intestinal barrier homeostasis (428).

Minerals also influence gut microbiota diversity and composition. In a large Chinese cohort, energy-adjusted sodium, potassium, and sodium/potassium ratio were associated with β-diversity and bacterial taxa linked to cardiovascular risk (429). While in mice, a low-potassium diet induced dysbiosis (422). Zinc deficiency and zinc excess can both favor dysbiosis (430)(431). Moreover, both iron

deficiency (432) and excess (433) can be detrimental. A randomized trial with a population of Kenyan infants demonstrated that non-absorbed iron favored the growth of potentially pathogenic bacteria, increased intestinal inflammation, and decreased the beneficial *Bifidobacteria* (433).

Sodium can also shape the gut microbiota composition and indirectly influence immune cell function and inflammatory conditions (416). In mice, a high salt diet decreased *Lactobacillus*, particularly *Lactobacillus murinus* (434). These results were corroborated in a cohort of healthy volunteers, in which a high salt intake (13.8 ± 2.6 g /day) led to decreased *Lactobacillus* concentrations, an increase in blood pressure, and Th17 cell frequency in the peripheral blood (434).

Finally, mice in a magnesium-deficient diet showed a lower gut microbiota diversity and an altered bacterial composition correlated with anxiety-like behavior (435). Furthermore, in a mouse model of colitis, mice in a magnesium-deficient diet had more severe disease than mice in a diet with high magnesium content (436). In this work, magnesium supplementation induced bacterial changes in the gut microbiota independently of colitis (436).

5.2.3.2 Minerals and immune-mediated diseases

Changes in mineral serum concentrations, such as zinc (437)(438), iron (439), and copper (438), are common in patients with immune-mediated diseases (437). Interestingly, in an experimental model of RA, supplementation with zinc reduced inflammatory cytokines' concentration and lymphocytes (440). SLE patients show decreased serum zinc levels (441-444), inversely correlated with disease activity (441) and proteinuria (442). Lower zinc intake may explain the low zinc levels in SLE patients (445). Still, the oxidative stress seen in SLE may increase zinc needs since it is a co-factor of superoxide dismutase-1 (SOD1). Patients with SLE should have a normal serum zinc level, and zinc supplementation might be needed to reach this target.

Regarding iron, patients show altered iron metabolism in RA (438)(439), Sjögren's syndrome (446), and juvenile idiopathic arthritis (447). In European SLE patients, SLE risk was inversely associated with serum iron, ferritin concentrations, and transferrin saturation levels and positively associated with transferrin concentrations (448). Nevertheless, SLE patients can also have high ferritin levels associated with inflammation (449). When looking at serum iron concentrations, some authors found them increased (441), others reported similar concentrations between SLE patients and controls (443), and others observed lower concentrations in SLE patients than in healthy controls (450)(451). More studies are, therefore, needed to determine the exact role of iron in SLE.

As previously mentioned, high sodium intake may alter the Th17/Treg balance, thus influencing immune-mediated diseases. In both humans and in a mouse model of collagen-induced arthritis, higher salt intake aggravated inflammatory arthritis, inducing Th17 polarization (452). Despite the differences in methodologies, not all authors found similar associations in RA and SLE patients (453). However, in a lupus mouse model (MRL/lpr), high salt intake promoted increased glomerular injury and proteinuria and decreased survival, accompanied by changes in Th1 and Th17 cells and increased Th17/Treg ratio (454). The increase in Th1 cells leads to an imbalance in the Th1/Th2 ratio, which has been suggested to be involved in lupus pathogenesis (454). Moreover, in this lupus mouse model, a high salt diet increased anti-dsDNA antibodies, when compared with a regular diet (454). In addition, $CD4^+$ T cells from healthy controls, as well as SLE patients, when treated with high salt concentrations, increased Th17 in a process mediated by glucocorticoid-inducible serine/threonine protein kinase 1 (SGK1), a regulator of cellular NaCl homeostasis (454). These data collectively support that a low salt diet should be recommended for SLE patients, a well-known reno-protective measure.

RA patients have a lower magnesium intake than healthy controls, which is not beneficial considering that dietary magnesium is inversely correlated with RA (455). However, in other immune-mediated diseases, such as SLE and Sjögren's syndrome, the patient's magnesium levels do not seem to be decreased (443). Interestingly, magnesium intake seems only beneficial when moderate, as higher

intakes may increase RA risk (455). However, in some cases, a short-term (14-day) low-magnesium diet may improve RA severity by influencing T cell subset differentiation (456). Paradoxically, when looking at osteoarthritis (457), magnesium has a protective role in a dose-dependent manner (458), demonstrating that the particular characteristics of each immune-mediated disease may influence the magnesium effects.

SLE patients also showed decreased zinc serum levels (441-443), inversely correlated with disease activity (441), and lower in patients with proteinuria (442). In this population, other minerals were also decreased, such as calcium (443), potassium (443), selenium (442), and iron. As previously mentioned, anemia is one of the most frequent SLE manifestations (33). Thus, in SLE, iron may have a protective role (448).

5.2.4 Diet: vitamins

5.2.4.1. Vitamin A

Retinol, or vitamin A, is a fat-soluble vitamin with immune-regulating functions (360). Retinoic acid, the main biologically active retinol metabolite (459), can increase the production of Th2 cytokines, balancing Th1/Th2 ratio towards Th2 responses (460). Retinoic acid also participates in DCs function, differentiation, and T-cell migration into the intestinal lamina propria and GALT (459). Moreover, vitamin A is important in the gut to produce the mucous layer, maintain the integrity of the gut barrier (461), and regulate commensal responses against pathogens (462). The segmented filamentous bacteria *Bifidobacterium bifidum* and *Bacillus cereus* produce retinoic acid involved in the host defense (462). In addition, gut commensals regulate retinoic acids' production and storage by up or down-regulating IL-22, thus achieving a balance that prevents colonization by pathogens and promotes symbiosis with commensals (463). However, dysbiosis reduces the expression of genes implicated in retinoic acid signaling in the ileum, colon, liver, and adipose tissue and of gut-homing genes, impairing lymphocyte homing to the gut, hence host defense (464).

Interestingly, in patients with immune-mediated diseases, such as multiple sclerosis and atherosclerosis, vitamin A supplementation decreased the gene expression of pro-inflammatory cytokines (IL-17, IFN-γ, and T-bet) and increased anti-inflammatory cytokines (TGF-β and FOXP3) (465). SLE patients have an insufficient intake of vitamin A (445) and show a higher incidence of hypovitaminosis A than healthy controls (466)(467), especially in the presence of renal involvement (466). Furthermore, there is a negative correlation between vitamin A levels and Th17 cell percentage in this population (467). Interestingly, when $CD4^+$ T cells from SLE patients with hypovitaminosis A were treated with retinoic acid, there was a decrease in Th17 cell percentage and Th17/Treg ratio and an increase in Treg differentiation (467).

All-trans-retinoic acid (tRA), a retinoic acid metabolite known to modulate immunity (468), has shown disease-specific effects in the treatment of several immune-mediated diseases (469) but has paradoxical effects in SLE (469). In a lupus mouse model, tRA treatment before lupus induction led to more severe renal lesions and higher renal lymph node enlargement, which may be due to the activated bone marrow dendritic cells and splenic T cells migration to the kidneys, favoring renal inflammation (469). On the contrary, tRA treatment after disease development did not induce these changes and even downregulated pro-inflammatory cytokines, and reduced the renal expression of TNFα (469). In addition, in a lupus mouse model, both tRA and all-trans-retinyl palmitate (a main ingredient in vitamin A supplements) together with tRA (VARA) worsened brain lupus-like disease, increased circulating autoantibodies and C3 accumulation, and increased IgG expression in the brain, which did not occur in control mice (470). Thus, this means that tRA and VARA-induced inflammation depend on a predisposed immunogenic environment (471). The same group has previously reported worsened skin and lung lupus-like disease with tRA supplementation, as well as an increase in anti-dsDNA and total IgG autoantibodies both with tRA and VARA supplementation (471).

In SLE patients, supplementation with tRA and rapamycin did not improve the outcomes obtained with rapamycin alone regarding Th17 and Treg cell counts, SLEDAI score, and prednisolone dose (468).

5.2.4.2. Vitamin B complex

The vitamin B family is composed of eight vitamins (B1, B2, B3, B5, B6, B7, B9, B12) that are enzymatic co-factors (403)(472). B vitamins are obtained from diet, but since they are water-soluble and thermolabile, cooking can destroy them (473). These vitamins can also be synthesized by gut bacteria (473)(474) and influence their diversity and composition (474). The main functions of B vitamins and their main bacterial producers in the gut are synthesized in **Table S12.1.**

SLE patients have decreased intake of folate and other B vitamins, such as biotin and pantothenic acid (445). In addition, SLE patients often show increased homocysteine levels (342)(475-477), in some cases associated with disease activity (477). Several mechanisms may explain this finding. First, chronic inflammation and altered immune responses can increase the production of reactive oxygen species, leading to higher oxidization of B vitamins and an increase in their nutritional requirements (476). Second, the exacerbated inflammation seen in SLE can increase immune cells' proliferation, increasing B vitamin turnover (476). Finally, renal impairment and genetic polymorphisms affecting the methylenetetrahydrofolate reductase (MTHFR) enzyme, which uses homocysteine, folate, and B vitamins to regenerate methionine, may also explain this phenomenon (475)(477). Even though some authors found unaltered folic acid, vitamin B6, and vitamin B12 serum levels in SLE (476)(477), others reported decreased levels of α-tocopherol and vitamins B5, B6 (478) and folic acid even when the intake was equivalent to healthy control's intake (342).

In SLE, there seems to be a negative correlation between homocysteine levels and folic acid (476) (477) and vitamin B6 levels (476), but not all authors reported these same findings (342). However, higher homocysteine in SLE increases the production of inflammatory mediators, enhancing cardiovascular risk (476). In addition, in comparison with healthy controls and rheumatoid arthritis patients, SLE patients have profound alterations in glutathione levels, a critical intracellular antioxidant (478), which can also favor a higher cardiovascular risk (479).

5.2.4.3. Vitamin D

There are two forms of vitamin D (calciferol): ergocalciferol (D2) and cholecalciferol (D3) (480). Exposure to UVB radiation stimulates the production of cholecalciferol in the skin (480). Cholecalciferol is firstly converted in the liver to the inactive intermediate 25-hydroxyvitamin D (25(OH)D), which is further converted in the kidney to the active form, calcitriol (1,25 $(OH)_2D$) (480). Vitamin D is mainly synthesized by the skin, and diet is a small contributor (480). Several immune cells, such as macrophages (480), DCs (481), B and T cells (482)(483), and several tissues, such as kidneys, colon, and the small intestine, express the vitamin D receptor (VDR) (484), meaning that vitamin D can regulate these cells responses (480). In fact, when T cell progenitors express VDR, the resulting T cells proliferate less than those derived from progenitors without this receptor when treated with calcitriol (484). In humans, vitamin D also increased Treg number and function when concentrations improved from 24.3 ng/dl to 57 ng/dl after 3 to 12 months of supplementation (485).

Vitamin D intervenes in gut mucosal immune defense, maintaining the gut barrier integrity (219)(486) and restoring its function when necessary (487), as well as maintaining the gut microbiota equilibrium (488) and limiting LPS-induced damage to the gut epithelium (486). All these effects inhibit gut bacterial translocation and favor gut homeostasis, which, when disrupted, contributes to several immune-mediated diseases. In a large randomized, double-blind, placebo-controlled trial, vitamin D supplementation, with 2,000 UI/day for five years, significantly decreased the risk for immune-mediated diseases (489). Some mechanisms may explain the positive effects of vitamin D in these diseases, such

as the regulation of genes associated with inflammation (489), down-regulation of inflammatory cytokines (490), or regulation of lymphocyte proliferation (482). Patients with Sjögren's syndrome (491), RA (492), SLE (65), and other immune-mediated diseases (66) frequently show sub-optimal levels of vitamin D. Genetic polymorphisms in the VDR gene have been associated with an increased risk for SLE (66). Moreover, increased levels of vitamin D, through supplementation, were associated with improved disease activity, decreased anti ds-DNA levels (64), and disease remission (65). Thus, maintaining adequate vitamin D plasma levels, which in SLE may require higher doses than for the general population (66), is relevant for bone health homeostasis but also for its immune-modulatory functions.

5.3 BODY COMPOSITION

The body composition assessment is a relevant tool in immune-mediated disease management, considering that obesity significantly contributes to these diseases (493). Interestingly, sarcopenia, characterized by low muscle quantity or quality, accompanied by low muscle strength and low physical performance (494), can coexist with overweight and obesity (495), and it is also frequently present in immune-mediated diseases (493). For some time, sarcopenia has been associated with aging. Still, it has more recently been identified in chronic non-communicable diseases and a contributing factor to complications and adverse outcomes in this context (496).

The white adipose tissue is an active endocrine organ that secretes numerous peptide hormones (adipokines), cytokines, and bioactive lipids (lipokines) that can act locally or systemically (497). The white adipose tissue comprises several immune cells, such as macrophages, eosinophils, neutrophils, and T and B cells (498). The crosstalk between adipocytes and immune cells warrants tissue homeostasis in lean individuals (498). On the other hand, overnutrition increases adipocyte expansion, leptin secretion, and infiltration of inflammatory cells (498).

The gut microbiota influences and is influenced by body composition (499), suggesting a potentially vicious circle. People with overweight or obesity show an altered gut microbiota (499). Despite BMI not being the best assessment tool for body composition, it has been demonstrated that children with a BMI corresponding to obesity have dysbiotic gut microbiota, with an altered F/B ratio and altered bacterial taxonomic distribution (500).

In immune-mediated diseases, obesity and gut microbiota seem to be interlinked. In a mouse model of multiple sclerosis, obesity increased disease severity through a gut microbiota-mediated mechanism, in which dysbiosis led to increased gut permeability and pro-inflammatory mediators (501). Moreover, in immune-mediated diseases, obesity affects the response to treatment (340), the likelihood of remission (341), and cardiovascular risk (342). In SLE, obesity has been associated with increased disease activity (502), increased incidence of LN during follow-up, and cumulative organ damage (503).

5.4 PHYSICAL ACTIVITY

According to the World Health Organization, physical activity is defined as "*any bodily movement produced by skeletal muscles that requires energy expenditure above resting levels"* (504). The World Health Organization's physical activity recommendations vary with age and health status and are shown, together with the sedentary time recommendations in **Table 5.1.**

Table 5.1. World Health Organization recommendations for physical activity and sedentary time (504).

Age	Physical activity	Sedentary time
5-17 years	≥60min/day of moderate to vigorous-intensity physical activity. AND ≥3 day/week: vigorous-intensity aerobic physical activity, as well as muscle and bone-strengthen physical activities.	Limit sedentary time, particularly recreational screen time
18-64 years (with or without chronic conditions)	≥150 to 300min/week of moderate-intensity aerobic physical activity. OR ≥75 to 150min/week of vigorous-intensity aerobic physical activity. AND ≥2 day/week of moderate to vigorous-intensity muscle-strengthening physical activities.	Limit sedentary time. Replace with more physical activity of any intensity.
≥65 years (with or without chronic conditions)	≥150 to 300min/week of moderate-intensity aerobic physical activity. OR ≥75 to 150min/week of vigorous-intensity aerobic physical activity. AND ≥2 day/week of moderate to vigorous-intensity muscle-strengthening physical activities. AND ≥3 day/week physical activity that emphasizes functional balance.	Limit sedentary time. Replace with more physical activity of any intensity.

Exercise has a known effect on the immune system, dependent on its intensity and duration (323). Acute moderate-intensity exercise enhances immunosurveillance and, when regular, confers multiple health benefits (323). Regular exercise decreases inflammatory markers such as C-reactive protein, IL-6, IL-18, and TNF-α, independently of BMI, meaning that it has an overall anti-inflammatory impact by improving inflammatory signaling pathways (323), decreasing adipose tissue (323) and visceral adiposity (324), and decreasing TLRs' expression (324). Another interesting finding is that the expression of TLRs can be modulated by exercise. In a cohort of 60 subjects, exercise significantly decreased the expression of TLR4 in $CD14^+$ cells and decreased LPS-stimulated IL-6 production, which may explain the anti-inflammatory effects of exercise (325).

The decrease in inflammatory markers achieved with exercise may also occur through the modulation of the gut microbiota, impacting the pathophysiology of immune-mediated diseases. In an osteoarthritis mouse model, exercise increased the gut microbiota diversity and altered its composition in mice fed a high-fat diet (326). Moreover, exercise reduced LPS concentrations in the synovial fluid and blood in these mice and improved parameters associated with cartilage degeneration, such as TLR4 (326). In humans, exercise improved disease activity, cardiovascular fitness, neutrophil migration, phagocytic capacity, and reactive oxygen species production in a small group of older RA patients (327). Moreover, in axial spondyloarthritis, sedentary time was associated with lower quality of life, while higher physical activity was associated with better spinal mobility and functional ability (328).

The beneficial effects of exercise and physical activity are also identified in SLE, independently of the type of exercise (329). However, patients tend to be sedentary (157)(330). Not surprisingly, higher sedentary time is inversely correlated with increased disease activity, flare frequency, and depressive and anxiety symptoms (157). On the contrary, moderate to vigorous physical activity is inversely associated with blood pressure and with a lower 10-year risk of cardiovascular events (331), as well as with fatigue (332), and improvement of physical and executive functions in SLE (333).

CHAPTER 6. Objectives

The main goal of this project was to study diet, physical activity, body composition, and gut microbiota in SLE. For this purpose, we studied, in SLE patients and age and gender-matched healthy controls:

- Adherence to the Mediterranean diet and dietary macro and micronutrients to evaluate the association between the different dietary components and SLE;

- Physical activity and sitting time to better understand their effects on this disease;

- Body composition to analyze differences between patients and controls and between patients with different immunologic profiles, clinical manifestations, and disease activity;

- α and β-diversity of the gut microbiota, F/B ratio, and bacterial populations present in the feces of SLE patients and healthy controls to assess if there was an association between dysbiosis or particular bacterial genera, families, or species and SLE diagnosis, lupus disease activity, clinical manifestations or immunologic profile.

The ultimate goal of this project is to establish foundations for the development of new treatment strategies for SLE that include the repair of the gut mucosa integrity, gut permeability decrease, and gut microbiota modulation through lifestyle changes or the use of drugs to restore gut integrity. These strategies might reduce the need for higher doses of immunosuppressants for disease control and avoid harmful side effects, improving the quality of life of lupus patients.

CHAPTER 7. Methods

7.1. Population

Adult SLE patients were recruited at the Rheumatology Division of Hospital de Santa Maria. Pediatric SLE patients were recruited at the Pediatric Rheumatology Unit of Hospital de Santa Maria. All the participants were recruited between April and October of 2021, and fulfilled the 2019 EULAR/ACR SLE classification criteria (21). Patients with antiphospholipid syndrome, Sjögren syndrome, inflammatory bowel disease, celiac disease, irritable bowel syndrome, metabolic disorders, cancer, or other immune-mediated diseases were not included. Patients who were treated with antibiotics in the last 4 weeks were also excluded. Disease activity was assessed at the time of diagnosis and at sample collection with SLEDAI-2K score (57) and the following categories were used: SLEDAI-2K >6, SLEDAI-2K: ≥3 - ≤6, SLEDAI-2K <3, and SLEDAI-2K <3 HCQ only (516,517). The patients' clinical and demographic data were obtained from the records of Hospital de Santa Maria. Healthy age, and gender-matched controls were recruited after an interview with a physician to exclude possible undiagnosed symptoms that could preclude the participation.

This project was approved by the Ethics Committee of *Centro Académico de Medicina de Lisboa* (**Appendix 4)**. Detailed oral and written explanation about the study was given, after which written informed consents were obtained. Children and teenagers gave written assent and their legal representatives their written consent. Documents explaining the details of the study were adjusted for different age groups.

7.2. Dietary assessment

Dietary intake was assessed with three 24-hour diet recalls performed by a trained nutritionist, including two weekdays and one weekend day. Patients reported the amount of each food item consumed, menu preparation and intake of water, alcoholic and nonalcoholic drinks, dietary supplements and probiotics. Standard household measures and pictorial food models were used during the interview. Parents or caregivers of children under 18 years old participated in the interviews.

Food records were converted to nutrient intake using Microdiet® software. The results of the dietary assessment were analyzed, considering the following variables: energy, macronutrients (protein, lipids, carbohydrates), total sugars, glucose, fructose, sucrose, maltose, lactose, trans-fatty acids, total SFAs, total MUFA, and total PUFA, including total n-3 and n-6 PUFA, fiber, minerals (sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, selenium, manganese, iodin), vitamins (B1, B2, B6, B12, folate, C, D, E, K1, retinol, niacin, biotin, pantothenic acid), carotenes (α-carotene, β-carotene, cryptoxanthin), amino acids (isoleucine, lysine, methionine, cystine, phenylalanine, tyrosine, threonine, tryptophan, valine, arginine, histidine, alanine, aspartic acid, glutamic acid, glycine, proline, serine), SFAs (4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, 24:0), MUFA (10:1, 12:1, 14:1, 15:1, 16:1, 17:1, 18:1, 20:1, 22:1), PUFAs (18:2, 18:3 (ALA), 18:4, 20:4, 20:5 (EPA), 22:5, 22:6 (DHA), total phytochemicals, brassicasterol, β-sitosterol, campesterol; water, alcohol intake. In addition, the total intake of EPA and DHA together, and the n-6/n-3 PUFA ratio were calculated.

The compliance with the dietary reference intake (DRI) of some nutrients (carbohydrates, fiber, potassium, magnesium, phosphorus, iron, zinc, manganese, vitamins B1, B2, B6, B12, C, E, K1, A, niacin, biotin, pantothenic acid, folate, and EPA+DHA) were assessed using the European Food Safety Authority (EFSA) dietary reference values (518). For this purpose, the population reference intake was used when available, otherwise, the average requirement or the adequate intake was considered.

The adherence to the Mediterranean diet was assessed in adults with the PREDIMED questionnaire (*PREvención con DIeta MEDiterránea*)(519) and in children/adolescents with the KIDMED questionnaire (520). PREDIMED is a 14-item questionnaire, which evaluates the intake of typical Mediterranean foods (olive oil, fruits, vegetables, fish, nuts, legumes, among others) and the

consumption of non-Mediterranean foods, such as soft drinks. A score ≥ 10 points corresponded to a good compliance with the Mediterranean dietary pattern (**Appendix 5)**. KIDMED is a 16-item questionnaire, similar to the adult version (521). In this questionnaire, a diet with a score higher than 8 represents an optimal compliance with the Mediterranean dietary pattern (**Appendix 6)**. The following categories were considered when assessing the adherence to the Mediterranean diet: insufficient adherence (KIDMED: <8; PREDIMED: <10), good adherence (KIDMED ≥8; PREDIMED ≥10) (522).

7.3. Anthropometry and Body Composition

Weight was measured to the nearest 0.1 kg on a medical scale or in the scale included in the whole-body air-displacement plethysmography equipment. Height was measured to the nearest 0.1 cm with a wall-mounted stadiometer with the head in the Frankfort horizontal plane and during inspiration. BMI was calculated as weight in kilograms divided by the square of height in meters. BMI was categorized in adults according to the World Health Organization guidelines (523)(524): underweight (<18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), obese class-I (30.0–34.9 kg/m^2), and obese class-II (35.0-39.9 kg/m^2). In children under 20 years old, the BMI categories considered were (525): nonobese (BMI < 85th percentile), overweight (BMI between the 85th-95th percentile), and obese (BMI ≥ 95th percentile).

Waist circumference was measured midway between the lowest ribs and the iliac crest. Hip circumference was obtained by measuring the maximum circumference of the buttocks. Both circumferences were measured with a nonelastic flexible tape, with the subjects standing, while arms were relaxed at the sides, at the end of a normal expiration. Waist circumference was categorized in adults into three classes according to the World Health Organization recommended cut-offs (524)(526): no risk of metabolic complications, high risk of metabolic complications (women > 80 cm and men > 94 cm), and very high risk of metabolic complications (women > 88 cm and men > 102 cm). Waist circumference in children was analyzed according to the age and sex-specific waist circumference reference data for Portuguese children and adolescents aged 10 to 18 years (525). The results were further analyzed considering the categories: no cardiovascular risk (women ≤ 80cm, men ≤ 94cm, children <P75) and with cardiovascular risk (women > 80 cm, men > 94 cm, children ≥P75). Waist/hip (≥ 18 years old) (526) and waist/height ratios were also calculated. For the waist/hip ratio the results were categorized as follows: no metabolic risk (women <0.85 and men < 0.90), and high metabolic risk (women ≥ 0.85 and men ≥ 0.90) (526). For the waist/height the results were divided into the following categories: no risk of obesity if < 0.5 or risk of obesity if ≥ 0.5 (527).

The fat mass, and fat-free mass of the participants were assessed by whole-body air-displacement plethysmography (BOD POD Gold Standard Body Composition Tracking System, COSMED) (528). The participants were asked not to drink water, eat food, or make exercise in the two hours prior to testing, as well as to remove all accessories before testing and to wear swim cap and lycra-type clothes or swimsuit during the assessment, according to the manufacturers' instructions. The following categories were used when analyzing the data in women: ≤ 18%; 18-22%; >22-30%; 30%-40%; >40%; and in men: ≤8%; 8-12%; >12-20%; >20-30%;>30%.

7.4. Physical Activity and Time Sitting

Physical activity was determined in children ≥ 14 years old, and in adults with the International Physical Activity Questionnaire (IPAQ) short form (529). This questionnaire assesses the physical activity of the seven days prior to the interview, and it consists of seven questions regarding time sitting, walking and time engaged in moderate and vigorous physical activity (**Appendix 7)**. The results were expressed as metabolic equivalents of task (METs)-minute per week according to the IPAQ scoring protocol, that considers the following values: light physical activity = 3.3 METs, moderate intensity

physical activity = 4.0 METs, and vigorous intensity physical activity = 8.0 METs (530). The participants' physical activity was classified in high, moderate, and low, according to the IPAQ scoring protocol. The time sitting was also assessed with IPAQ.

7.5. Other variables

Bristol stool scale, hours of sleep, and time fasting were also assessed. The Bristol stool scale is a simple tool to assess the rate of intestinal contents passage (531). It consists of an ordinal scale comprehending seven-points that characterize stools according to its consistency, and that has been translated into a pictorial model (532). Types 1 and 2, in conjunction with other symptoms, are considered indicative of constipation, types 6 and 7, in conjunction with other symptoms, are considered indicative of diarrhea, and types 3 and 4 are generally considered adequate consistency (532). The hours of sleep and time fasting were assessed on the three days of the dietary questionnaire, and the mean of the three days was considered.

7.6. Gut microbiota

Feces samples were self-collected by the participants with the OMNIgene® GUT kit (DNA Genotek). This kit allows for a quick homogenization and stabilization of the sample, and transport and storage at room temperature for 60 days, with no cold chain needed, minimizing bias related to microbial overgrowth and aliquoting, and improving reproducibility (533).

The genomic DNA was extracted with QIAamp® PowerFecal Pro kit (QIAGEN) according to the manufacturers' instructions. The extracted DNA was quantified by spectrophotometer NanoDrop ND-2000 (Thermo Scientific). The V4 region of the 16S rRNA gene was amplified with primers 515F-806R prior to sequencing on an Illumina MiSeq instrument for 500 cycles, at *Instituto Gulbenkian da Ciência*, Lisbon, Portugal. The taxonomic classification and analysis were performed with QIIME2® (Quantitative Insights Into Microbial Ecology) analysis package (version 2022.2.0, available at https://qiime2.org). To evaluate sequence quality, FastQC (version 0.11.9, available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used, and the results were summarized with MultiQC (534) (version 1.10.1). α and β-diversity were obtained with Qiime2 diversity core-metrics-phylogenetic plugin, using the "rooted-tree.qza" rooted tree file, the "table.qza" file obtained by DADA2 with the filtered data, and the metadata file as input (534).

7.7. Glucagon-Like Peptide-1

Blood samples were collected in the same day of the body composition analysis and feces sample delivery. Serum and plasma were isolated at Biobank-*Instituto de Medicina Molecular João Lobo Antunes*, Lisbon, Portugal, and stored at -80ºC. Serum samples were used to assess the total GPL-1 (7-36 and 9-36) by Enzyme-Linked Immunosorbent Assay (ELISA) with the EZGLP1T-36K kit (EMD Millipore), following the manufacturers' protocol, and using two biological replicates per sample. Briefly, antibodies specific for human GLP-1 were pre-coated onto the provided 96-well strip microplate. Wash buffer concentrate was diluted in 900 ml of de-ionized water. The microplate was washed three times with 300 µl of wash buffer, the solution matrix, and the assay buffer sequentially. The standards/quality controls and samples (50 µl) were added to the micro plate and incubated for 1.5 hours at room temperature, after which the wells were washed with wash buffer. 100 µl of detection antibody was added to the wells, and incubated for 1 hour at room temperature, followed by washing with the wash buffer. 100 µl of enzyme solution was added to the wells and incubated for 30 minutes at room temperature, after which the wells were once again washed with wash buffer. 100 µl of substrate

was added to the wells and incubated for 15 to 20 minutes at room temperature and then the stop solution was added to the wells. Absorbance values were recorded at 450 nm and 570 nm.

7.8. Statistical Analysis

Graph Pad Prism software (version 9.4.0) was used for the statistical analysis. The results were considered significant at the 5% significance level. Frequency analysis (n, %) was used to characterize the qualitative data. Quantitative data with non-normal distribution were presented as median (minimum, maximum), while mean and standard deviation was calculated for normally distributed variables. The Shapiro-Wilk test was used to test the normality of the data. The Chi-square test was used to study the relationship between qualitative variables when the applicability assumptions were verified, and the Fisher exact test or Chi square test for trend when they did not. The Spearman correlation coefficient was used to study the relationship between two quantitative variables, when conforming to normal distribution or Pearson correlation coefficient when non-conforming. The Mann-Whitney test was used to compare a quantitative variable between two independent groups, when the normality assumption was not verified ($p<0.05$), and the results are expressed as the median (minimum, maximum), and Student's t-test was used when the normality assumption was verified. Analysis of variance (ANOVA) was used to compare a quantitative variable between three or more independent groups, when the normality assumption was verified ($p<0.05$), and Kruskal-Wallis test when it was not. Dun's test was used for multiple comparisons correction.

7.9 Funding

This Project was funded by *Fundação para a Ciência e Tecnologia* (FCT), L'OREAL Women in Science, UNESCO, *Sociedade Portuguesa de Pediatria* e *Fundação Amélia de Mello*.

CHAPTER 8. Results

8.1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

This study included 33 SLE patients (11 children and 22 adults) and 13 healthy controls (HC). The gender distribution was similar in the two groups. Twenty-nine SLE patients were female (87.9%) and four were male (12.1%), while 11 HC were female (84.6%), and two were male (15.4%).

SLE patients had a median age of 29 years old (range:11-57), and HC had a median age of 37 years old (range:14-50).

The majority of SLE patients were Caucasian (n=24; 72.7%), four were black (12.1%), one patient was Asian (3%), and another was of mixed ethnicity (3%). All HC were Caucasian.

SLE patients had a median age at diagnosis of 20.5 years old (8.4-40), 15 being diagnosed during childhood (45.5%), and 18 in adulthood (54.5%). Disease duration was longer than two years in 81.9% of the patients. At diagnosis, patients showed active disease, with a median SLEDAI-2K of 21 (7-24), while at sample collection, the median SLEDAI-2K was 4 (0-20).

Table 8.1 shows the demographic characteristics of SLE patients and HC.

Table 8.1. Demographic characteristics of systemic lupus erythematosus (SLE) patients and healthy controls (HC). HCQ-hydroxychloroquine, n – total number of individuals, SLEDAI - Systemic lupus erythematosus disease activity index

SLE (n=33)		
		n (%)
Gender (n=33)	Male	4 (12.1)
	Female	29 (87.9%)
Ethnicity (n=33)	Caucasian	24 (72.7%)
	Asian	1 (3.0%)
	Black	4 (12.1%)
	Mixed	1 (3.0%)
	Not given	3 (9.1%)
Age (years)	Median (min-max): 29 (11-57)	
Age at diagnosis (years)	Median (min-max): 20.5 (8.4-40)	
Time since diagnosis (years)	Median (min-max): 7.5 (0.3-28.7)	
	n (%)	
<1	4 (12.1%)	18.1%
≥1-2	2 (6.0%)	
≥2-5	9 (27.3%)	81.9%
≥5-10	3 (9.1%)	
≥10	15 (45.5%)	

HC (n=13)		
		n (%)
Gender	Male	2 (15.4%)
	Female	11 (84.6%)
Ethnicity	Caucasian	13 (100%)
Age (years)	Median (min-max): 37 (14-50)	
SLEDAI-2K at Collection (n=32)	**Median** (min-max): 4 (0-20)	
	n (%)	
<3, HQC only	1 (3.1%)	
<3	13 (40.6%)	
≥3-6	10 (31.3%)	
>6	8 (25.0%)	
SLEDAI-2K at Diagnosis (n=11)	Median (min-max): 21 (7-24)	

The main clinical manifestations during the course of the disease were mucocutaneous (84.8%), arthritis (84.8%), hematological (75.8%), and constitutional, such as fever and tiredness (75%). Lupus nephritis occurred in 66.7% of SLE patients. Children had a higher incidence of constitutional manifestations (100%), hematologic manifestations (100%), such as hemolytic anemia (63.6%) and leukopenia (90.9%), arthritis (90.9%), malar rash (81.8%), and photosensitivity (81.8%) than adults. However, only hemolytic anemia, leukopenia, hematological disease, and constitutional manifestations reached statistical significance when comparing children and adults (**Figure 8.1, Table S12.2**). **Table 8.2** shows the clinical manifestations of this cohort.

Figure 8.1. SLE clinical manifestations that reached statistical significance between adults and children. A. Hemolytic anemia; B. Leukopenia; C. Hematological disease; D. Constitutional manifestations. n – Total number of individuals. Significant p-value < 0.05.

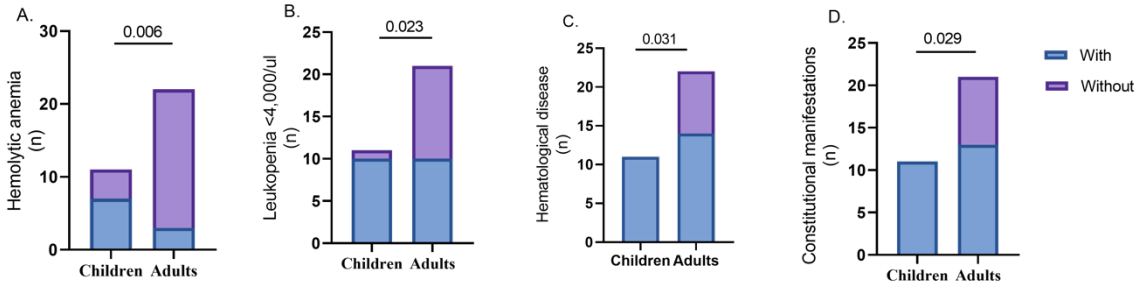


Table 8.2. Clinical manifestations of SLE patients. ACR - American College of Rheumatology; ANA - Antinuclear antibodies; anti-dsDNA - Antibodies to double-stranded DNA; C – complement; n – total number of individuals; EULAR - European League Against Rheumatism; SLE - Systemic lupus erythematosus; SLICC - Systemic Lupus International Collaborating Clinics.

ACR 1997	
	n (%)
Immunological disease	33 (100%)
ANA positivity	33 (100%)
Arthritis	28 (84.8%)
Hematological disease	25 (75.8%)
Malar rash	24 (72.7%)
Photosensitivity	23 (69.7%)
Lupus Nephritis	22 (66.7%)
Oral/nasal ulcers*	11 (34.4%)
Serositis*	6 (18.8%)
Discoid rash	4 (12.1%)
Neurological Disease*	1 (3.1%)

SLICC 2012 (items not included in ACR1997)	
	n (%)
Low complement (C3 and/or C4)	27 (81.8%)
Acute or subacute cutaneous lupus	24 (72.7%)
Positive Direct Coombs test**	9 (69.2%)
Leukopenia (<4,000/mm³)*	20 (62.5%)
Lupus nephritis + ANA / anti-dsDNA positivity*	18 (54.5%)
Alopecia	15 (45.5%)
Thrombocytopenia (<100,000/mm³)*	11 (34.4%)
Hemolytic anemia	10 (30.3%)
Chronic cutaneous lupus	3 (9.1%)

EULAR/ACR 2019 SLE (items not included in ACR1997 nor SLICC2012)	
	n (%)
Mucocutaneous	28 (84.8%)
Low complement (C3 or C4)*	25 (78.1%)
Constitutional manifestations (fever >38.3°C, tiredness)*	24 (75%)
Low complement (C3 and C4)*	21 (65.6%)
Neuropsychiatric*	1 (3.1%)

* n=32; ** n=13

Low complement (C3 and/or C4) occurred in 81.8% (n=27) of SLE patients. All patients were ANA positive at diagnosis, as required by the ACR/EULAR 2019 SLE classification criteria. The more prevalent antibodies were anti-ds DNA (84.4%, n=27), lupus anticoagulant (56.3%, n=18), anti-RNP (38.7%, n=12), anticardiolipin IgA, IgG or IgM (37.5%, n=12), anti-Sm (37.5%, n=12), and anti-β2-glycoprotein I (37.5%, n=12). All children had anti-ds DNA antibodies and all had low complement (C3 and/or C4). The immunologic profile of this cohort is described in **Table 8.3.**

Table 8.3. Immunological status of SLE patients. Anti-dsDNA - Antibodies to double-stranded DNA; Anti-Sm - Anti-Smith antibodies; Ig – Immunoglobulin; n – total number of individuals; RNP – ribonucleoprotein.

Immunologic Status	
	n (%)
Anti-dsDNA*	27 (84.4%)
Lupus anticoagulant*	18 (56.3%)
AntiRNP**	12 (38.7%)
Anticardiolipin IgA or IgG or IgM*	12 (37.5%)
Anti-Sm*	12 (37.5%)
Anti–B2-glycoprotein I (IgA or IgG, or IgM)*	12 (37.5%)
AntiRo (anti SSA)**	10 (32.3%)
AntiLa (anti SSB)**	1 (3.2%)

* n=32; ** n=31

Regarding treatment, at sample collection, 87.9% (n=29) of the patients were being treated with HCQ and prednisolone, 59.4% (n=19) with MMF, 15.6% (n=5) with azathioprine, and 12.1% (n=4) with methotrexate. Regarding biologics, 18.2% (n=6) of SLE patients were being treated with belimumab and 3.1% (n=1) with rituximab. Tacrolimus was part of the current treatment in 6.3% (n=2) of the patients. Cyclophosphamide, intravenous immunoglobulin, and cyclosporine were not part of any patients' treatment at the time of sample collection. However, 18.8% (n=6) of the SLE patients in this cohort had been treated in the past with cyclophosphamide, 9.4% (n=3) with intravenous immunoglobulin, and 3.1% (n=1) with cyclosporine. Angiotensin-converting enzyme (ACE) inhibitor and angiotensin receptor blocker (ARB) were being used by 37.5% (n=12) and 6.3% (n=2) of SLE patients, respectively. **Figure 8.2** and **Table S12.3** show treatment strategies currently used and used in the past in this cohort of SLE patients.

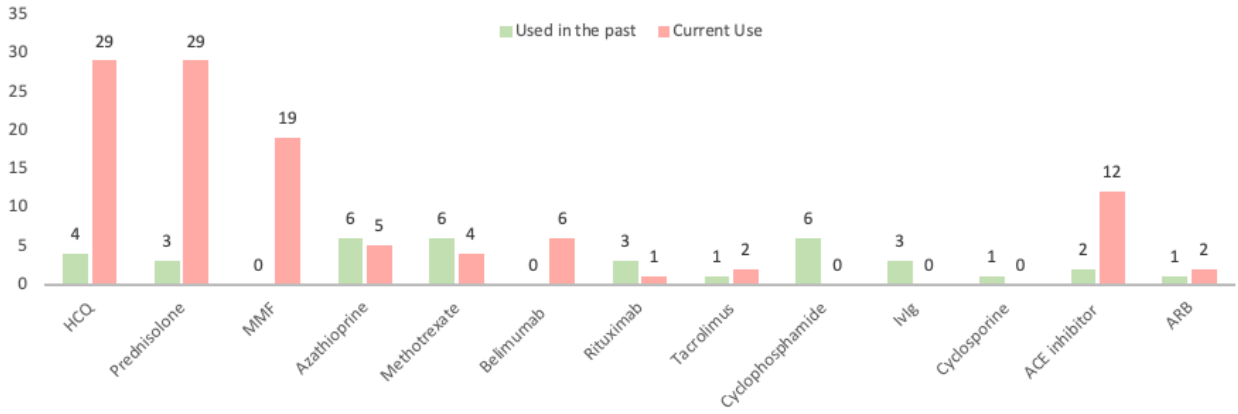


Figure 8.2. Treatment strategies of SLE patients. ACE - Angiotensin-converting enzyme; ARB - Angiotensin receptor blocker, HCQ – Hydroxychloroquine; MMF - Mycophenolate mofetil; IvIg - Intravenous immunoglobulin.

8.2. MEDITERRANEAN DIET

In immune-mediated diseases, such as SLE, adherence to the Mediterranean diet has been inversely correlated with disease activity (206). However, the results are not consensual (207), warranting further investigation.

In our cohort, SLE patients had significantly lower adherence to the Mediterranean diet than HC (p=0.0222, Fisher's exact test, **Figure 8.3A, Table S12.4)**, particularly adults (p=0.0007, Fisher's exact test, **Table S12.4)**. Insufficient adherence to the Mediterranean diet occurred in 87.5% of the patients and 53.8% of HC (**Table S12.5**). Patients who were adults when diagnosed (p=0.0278, Fisher's exact test, **Figure 8.3B, Table S12.4)** and patients who had disease duration longer than two years (p=0.0076, Fisher's exact test, **Figure 8.3C, Table S12.4)** had lower adherence to the Mediterranean diet. Patients with and without LN at diagnosis had similar adherence to the Mediterranean diet (**Table S12.4)**. It was not possible to assess the association between adherence to the Mediterranean diet and disease activity due to the small sample size.

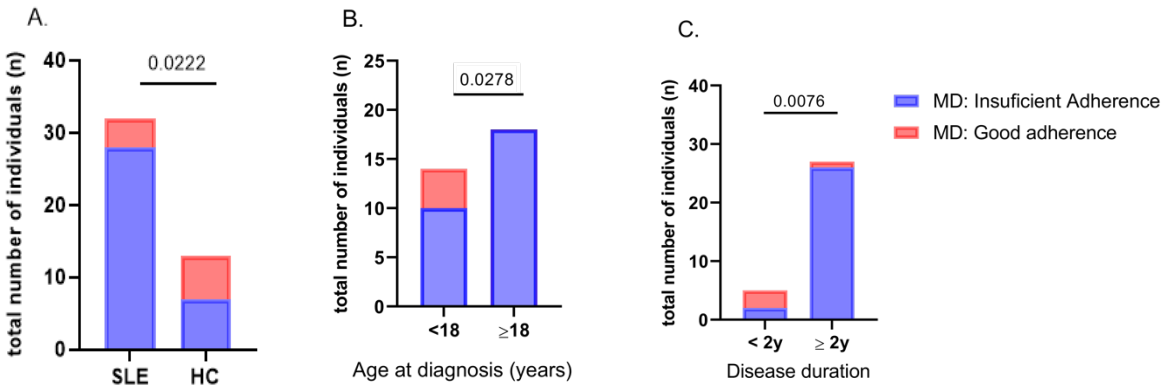


Figure 8.3. Adherence to the Mediterranean diet (A) in SLE patients (n=32) and HC, (B) According to age of diagnosis, (C) According to disease duration. HC – healthy controls (n=13), MD – Mediterranean diet, SLE – systemic lupus erythematosus; n – total number of individuals, y- years. p-value significant < 0.05.

Ethnicity, immunological status and treatment (**Figure S13.1**) did not influence the adherence to the Mediterranean diet.

Interestingly, when looking at the PREDIMED/KIDMED answers regarding the main food groups that characterize a Mediterranean diet, SLE patients ate significantly less vegetables, fruits, legumes, and nuts than HC (**Table 8.4**).

Table 8.4. PREDIMED and KIDMED answers regarding the main food groups that characterize a Mediterranean diet (results significantly different between groups). HC- healthy controls, n-total number of individuals, SLE- systemic lupus erythematosus.

Answers PREDIMED and KIDMED						
	SLE insufficient intake (n)	HC insufficient intake (n)	SLE sufficient intake (n)	HC sufficient intake (n)	Test	p-value
Intake of Vegetables	28	3	4	10	Fisher's exact test	<0.0001
Intake of Fruits	25	5	7	8	Fisher's exact test	0.0163
Intake of Legumes	26	6	6	7	Fisher's exact test	0.0300
Intake of Nuts	24	5	8	8	Fisher's exact test	0.0374

8.3. NUTRIENTS INTAKE

Considering that several individual dietary components can have immune-modulatory characteristics, the dietary macro and micronutrients were calculated after converting the food intake of three 24h-recalls (two weekdays and one weekend day).

8.3.1 Macronutrients

There were no differences between the caloric intake of SLE patients and HC (p=0.0557, Mann-Whitney test, **Figure 8.4A**), nor in the intake of protein (p=0.3275, t-test, **Figure 8.4B**), carbohydrates (p=0.0818, Mann-Whitney test, **Figure 8.4C**), or total lipids (p=0.2976, Mann-Whitney test, **Figure 8.4D**). SLE patients consumed less fiber (p<0.0001, t-test, **Figure 8.4E**), glucose (p=0.0027, t-test, **Figure 8.4F)** and fructose (p=0.0006, t-test, **Figure 8.4G)** than HC (**Table S12.6**).

The intake of other macronutrients was similar between SLE patients and HC (**Table S12.6**).

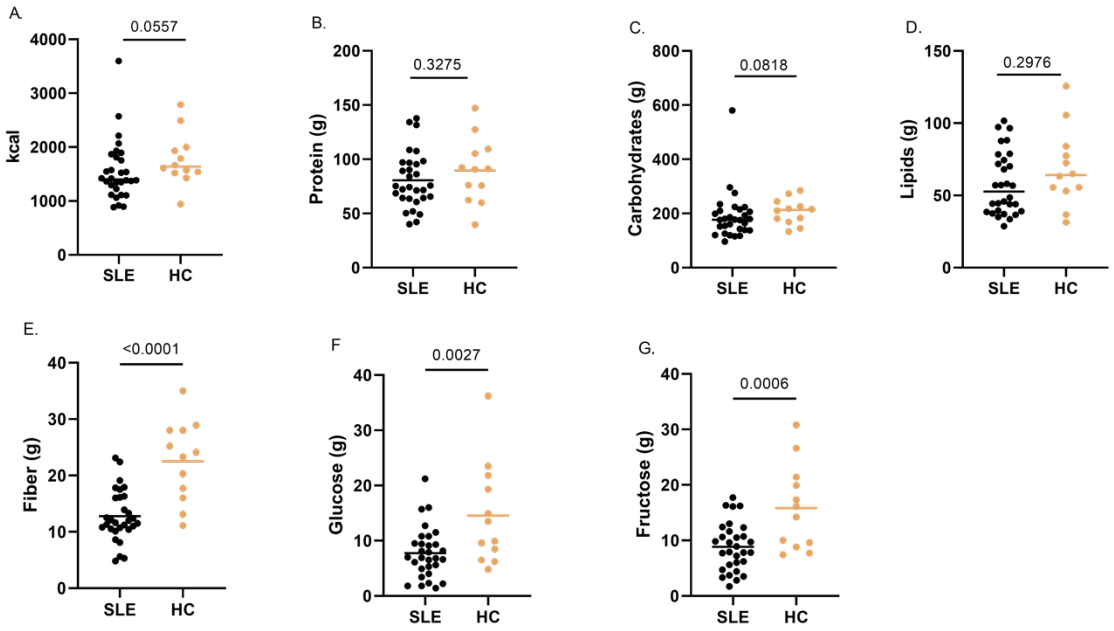


Figure 8.4. Intake of macronutrients significantly altered between SLE patients and HC. (A) Energy (calories – kcal), (B) Protein, (C) Carbohydrates, (D) Lipids, (E) Fiber, (F) Glucose, (G) Fructose. HC – healthy controls (n=12), SLE – systemic lupus erythematosus (n=30); g- grams, n – total number of individuals. p-value significant < 0.05.

Despite the intake of total SFA being similar between SLE patients and HC, some specific SFA were altered between these two groups. HC consumed more myristic acid (SFA14:0) than SLE patients (p=0.0455, t-test, **Figure 8.5A**), more arachidic acid (SFA 20:0) (p=0.0461, Mann-Whitney test, **Figure 8.5B**), and behenic acid (SFA 22:0) (p=0.0160, Mann-Whitney test, **Figure 8.5C).** All data regarding SFA consumption are detailed in **Table S12.7**.

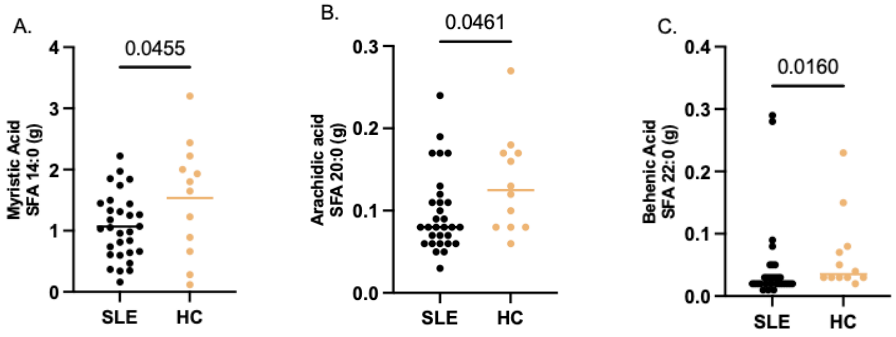


Figure 8.5. Dietary SFAs significantly altered between SLE patients and HC. (A) Myristic acid (SFA 14:0). (B) Arachidic acid (SFA 20:0). (C) Behenic acid (SFA 22:0). HC – healthy controls (n=12), SLE – systemic lupus erythematosus (n=30); g- grams, n – total number of individuals. p-value significant < 0.05.

When looking at MUFA intake, SLE patients also had lower intakes of palmitoleic acid (MUFA 16:1) (p=0.0105, Mann-Whitney test, **Figure 8.6A**), oleic acid (MUFA 18:1) (p=0.0461, Mann-Whitney test, **Figure 8.6B**), eicosenoic acid (MUFA 20:1) (p=0.0045, Mann-Whitney test, **Figure 8.6C**) and erucic acid (MUFA 22:1) (p=0.0028, Mann-Whitney test, **Figure 8.6D**). All data regarding MUFA consumption are detailed in **Table S12.8**.

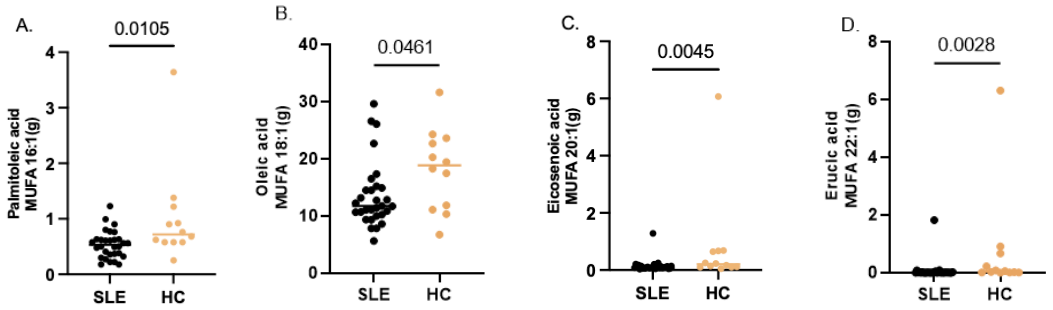


Figure 8.6. Dietary monounsaturated fatty acids (MUFA) significantly altered between SLE patients and HC. (A) Palmitoleic acid (MUFA 16:1), (B) Oleic acid (MUFA 18:1), (C) Eicosenoic acid (MUFA 20:1), (D) Erucic acid (MUFA 22:1). HC – healthy controls (n=12), SLE – systemic lupus erythematosus (n=30); g- grams, n – total number of individuals. p-value significant < 0.05.

Total PUFA consumption was similar between SLE and HCs (p=0.0805, Mann-Whitney test, **Table S12.6**). Interestingly, there was a lower intake of total n-3 PUFA (p=0.0248, Mann-Whitney test, **Figure 8.7A**), ALA (p<0.0001, t-test, **Figure 8.7C**), and EPA (p=0.0077, Mann-Whitney test, **Figure 8.7D**) by SLE patients than HC. Stearidonic acid (PUFA 18:4) was also significantly less consumed by SLE patients than HCs (p=0.0171, Mann-Whitney test, **Figure 8.7F**). The statistical significance was maintained after the outliers' removal (p=0.0287, Mann-Whitney test). All data regarding PUFA intake are detailed in **Table 8.4**.

Table 8.4. Dietary intake of polyunsaturated fatty acids (PUFA). ALA- α-linolenic acid, DHA- docosahexaenoic acid, EPA- eicosapentaenoic acid, g-grams, HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.

		n	Median/Mean	Min - Max	Test	p-value
Linoleic acid (LA) PUFA 18:2 (g)	SLE	30	3.245	0.94-13.47	Mann-Whitney test	0.2755
	HC	12	3.600	1.78-9.30		
ALA PUFA 18:3 (g)	SLE	30	0.275	0.08-0.68	t-test	**<0.0001**
	HC	12	0.5533	0.11-0.92		
Stearidonic acid PUFA 18:4 (g)	SLE	30	0.000	0-0.37	Mann-Whitney test	**0.0171**
	HC	12	0.010	0-0.31		
Arachidonic acid PUFA 20:4 (g)	SLE	30	0.04	0-0.31	Mann-Whitney test	0.6829
	HC	12	0.05	0-0.20		
EPA PUFA 20:5 (g)	SLE	30	0.025	0-0.75	Mann-Whitney test	**0.0077**
	HC	12	0.15	0-0.78		
Docosapentaenoic acid PUFA 22:5 (g)	SLE	30	0.020	0-0.27	Mann-Whitney test	0.6303
	HC	12	0.025	0-0.22		
DHA PUFA 22:6 (g)	SLE	30	0.06	0-0.66	Mann-Whitney test	0.0986
	HC	12	0.125	0.01-1.1		

The n-6/n-3 PUFA ratio translates the equilibrium between the intakes of PUFA with inflammatory potential and PUFA with anti-inflammatory potential, warranting a detailed analysis. In fact, SLE patients showed a higher n-6/n-3 PUFA ratio than HC (p=0.0042, Mann-Whitney test, **Figure 8.7G, Table S12.6**).

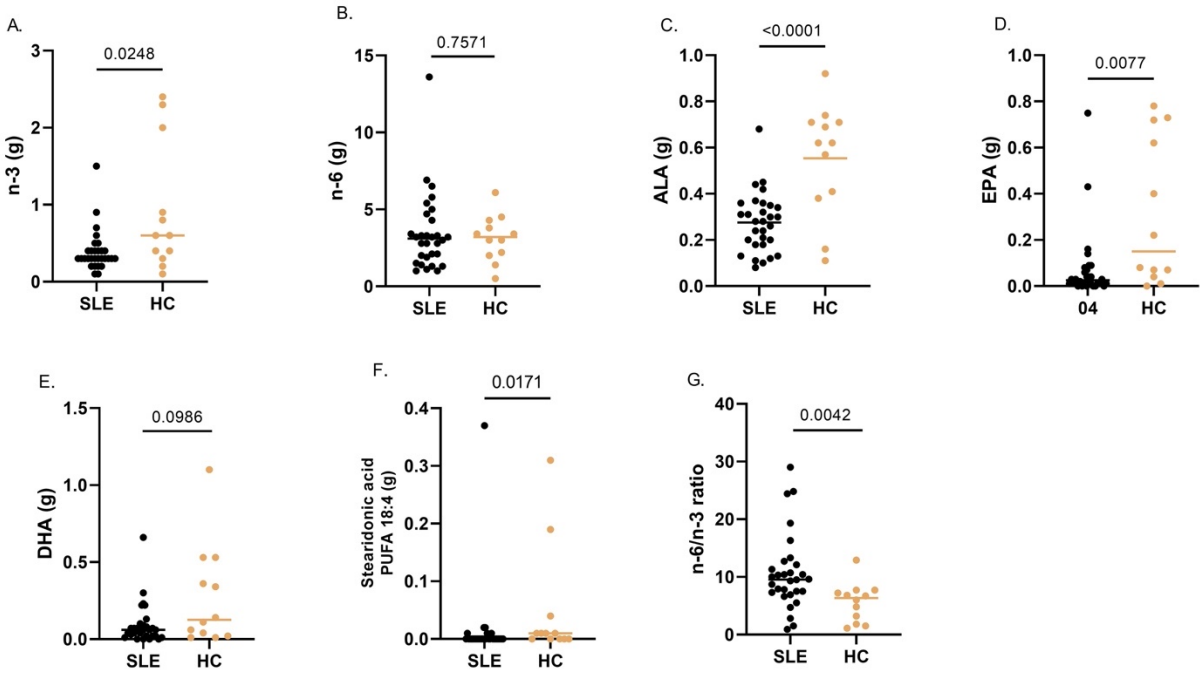


Figure 8.7. Dietary polyunsaturated fatty acids (PUFA). (A) Total omega-3 (n-3) PUFA, (B) Total omega-6 (n-6) PUFA, (C) α-linolenic acid (ALA, PUFA 18:3), (D) Eicosapentaenoic acid (EPA, PUFA 20:5), (E) Docosahexaenoic acid (DHA), (F) Stearidonic acid (PUFA 18:4), (G) Ratio n-6/n-3 PUFA. HC – healthy controls (n=12), SLE–systemic lupus erythematosus (n=30); g-grams, n–total number of individuals. p-value significant < 0.05.

The intake of amino acids, such as leucine, lysine, methionine, phenylalanine, tyrosine, tryptophan, arginine, glycine, beyond other, was similar between SLE patients and HC (**Table S12.9**).

8.3.2 Minerals and Vitamins

The dietary minerals and vitamins were also studied. SLE patients consumed less minerals than HC. Potassium (p=0.0062, t-test, **Figure 8.8A**), magnesium (p=0.0004, Mann-Whitney test, **Figure 8.8B**), iron (p=0.0002, Mann-Whitney test, **Figure 8.8C**), and manganese (p=0.0004, Mann-Whitney test, **Figure 8.8D**) were particularly less ingested by SLE patients than HC (**Table S12.10**).

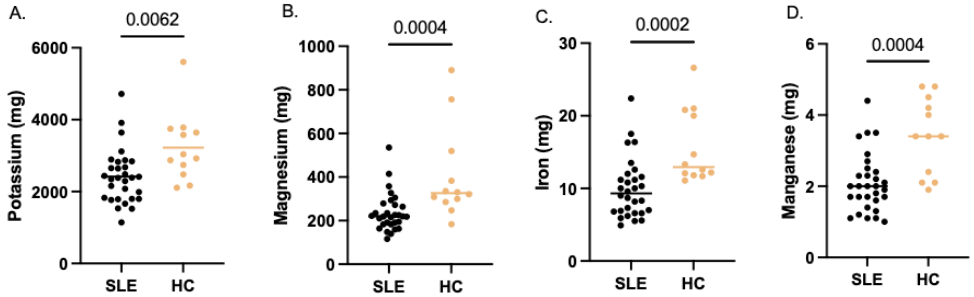


Figure 8.8. Dietary minerals significantly altered between SLE patients and HC. (A) Potassium, (B) Magnesium, (C) Iron, (D) Manganese. HC–healthy controls (n=12), SLE–systemic lupus erythematosus (n=30); g-grams, n–total number of individuals. p-value significant < 0.05.

When looking at vitamin intake, similarly to minerals, there was also a lower intake of vitamins by SLE patients than HC. In this regard, pyridoxine (vitamin B6) (p=0.0060, Mann-Whitney test, **Figure 8.9A**), folate (vitamin B9) (p=0.0006, Mann-Whitney test, **Figure 8.9B**), and phytonadione (vitamin

K1) (p=0.0235, Mann-Whitney test, **Figure 8.9C**) were lower in SLE patients' diet than in HC (**Table S12.11**).

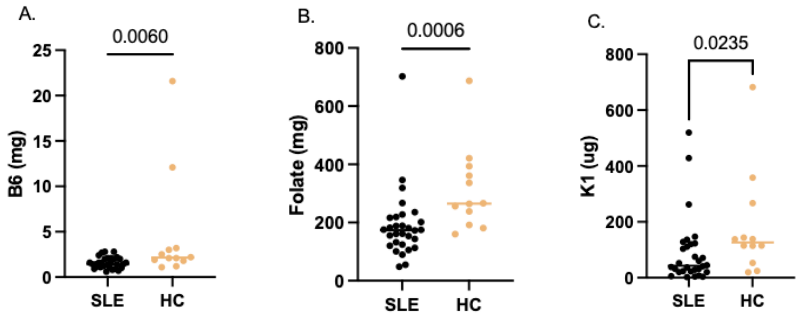


Figure 8.9. Dietary vitamins significantly altered between SLE patients and HC. (A) Vitamin B6, (B) Folate, (C) Vitamin K1. HC–healthy controls (n=12), SLE – systemic lupus erythematosus (n=30); mg-miligrams, ug-micrograms, n-total number of individuals. p-value significant < 0.05.

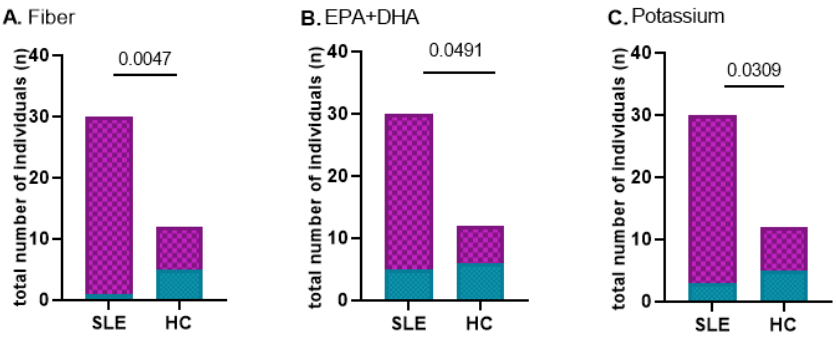
8.3.3 Dietary Reference Intake

The dietary compliance with the EFSA dietary reference intake of several macro and micronutrients was assessed, and all results are detailed in **Table S12.12**. The majority (96.7%) of SLE patients did not reach the fiber DRI, while 58.3% of HC did not reach the same DRI (p=0.0047, Fisher's exact test, **Figure 8.10A**). The total carbohydrate intake did not influence these results once there were no differences between SLE patients and HC in this regard (p=0.0818, Mann-Whitney test, **Table S12.6**).

Considering EPA together with DHA, 16.7% of SLE patients and 50% of HC reached the EFSA recommendations (p=0.0491, Fisher's exact test, **Figure 8.10B**).

Potassium, magnesium, phosphorus, iron, zinc, and manganese DRI were also assessed. 90% and 73.3% of SLE patients did not meet the DRI for potassium, and magnesium, respectively, while 80% did not meet the DRI for iron, zinc, and manganese. Significant differences were found between potassium (p=0.0309, Fisher's exact test, **Figure 8.10C**), magnesium (p=0.0061, Fisher's exact test, **Figure 8.10D**), and manganese (p=0.0014, Fisher's exact test, **Figure 8.10E**) intake between SLE patients and HC. On the other hand, the majority of SLE patients (97%) reached phosphorus DRI.

All SLE patients reached the DRI for B1 vitamin, and the majority reached the DRI for retinol (73.3%) and vitamins B3 (86.7%), B6 (53.3%), and B12 (53.3%). However, just a minority of SLE patients reached the DRI for vitamins B2 (23.3%), B5 (43.3%), B7 (20.0%), C (26.7%), E (26.7%), K1 (40%), and folate (6.7%). When comparing the number of SLE patients and HC that did not meet the vitamins DRI, the differences were only significant for folate (p=0.0138, Fisher's exact test, **Figure 8.10F**).



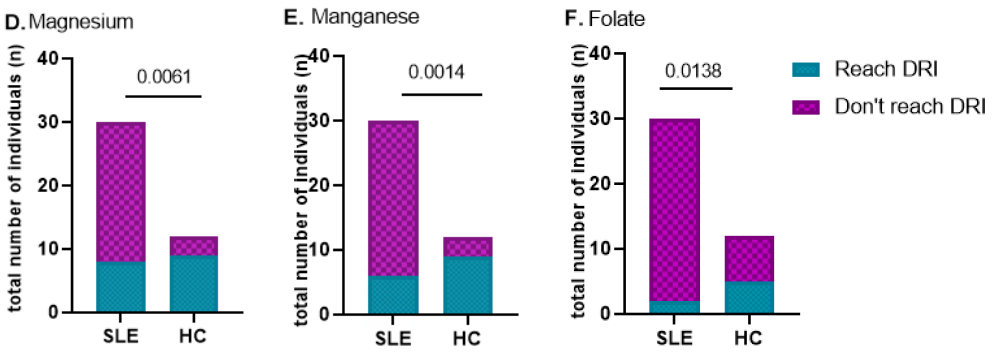


Figure 8.10. Recommended daily intake (DRI) significantly altered between SLE patients and HC. (A) Fiber, (B) EPA+DHA, (C) Potassium, (D) Magnesium, (E) Manganese, (F) Folate. HC–healthy controls (n=12), SLE–systemic lupus erythematosus (n=30), n–total number of individuals. p-value significant < 0.05.

Finally, SLE patients and HC intake of several macro and micronutrients were compared with the intake of the Portuguese population (**Table S12.5**). The data from the Portuguese population include children older than 3 months old and adults. SLE patients consumed less monounsaturated and polyunsaturated fatty acids, fiber, potassium, phosphorus, magnesium, iron, zinc, sodium, vitamins B2, B3, B6, folate, C, and E, and more SFA, vitamins A and B12, and calcium than the Portuguese population. While HC consumed less saturated fat, sodium, and vitamins B3, C, and E than the Portuguese population.

8.4. PHYSICAL ACTIVITY

A decreased physical activity has been associated with worse health outcomes (208). In addition, acute moderate-intensity exercise enhances immunosurveillance and, when regular, improves inflammatory signaling pathways (209). The physical activity and sedentary time were assessed with IPAQ questionnaire, validated for ages ≥ 14. Thus, the three patients younger than 14 years old were not included in this analysis. When assessing physical activity, all the daily activities were considered, as well as exercise, which is the planned, structured, and repetitive physical activity aiming to improve or maintain physical fitness (210).

SLE patients had lower physical activity than HC (p=0.0302, Mann-Whitney test, **Figure 8.11A, Table S12.13)**, and the difference was higher between adults (p=0.0366, Mann-Whitney test, **Table S12.13)**. Although female SLE patients had lower physical activity than female HC (p=0.0371, Mann-Whitney test, (**Table S12.13**), there were no differences between male participants (p>0.9999, Mann-Whitney test, (**Table S12.13),** which may be related to the very small number of male participants in this study. Other variables did not influence physical activity except anti-RNP antibodies (**Figure 8.12**, **Table S12.13, Table S12.14, Figure S13.2**).

SLE patients spent more time sitting than HC (p=0.0479, t-test, **Figure 8.11B, Table S12.15)**.

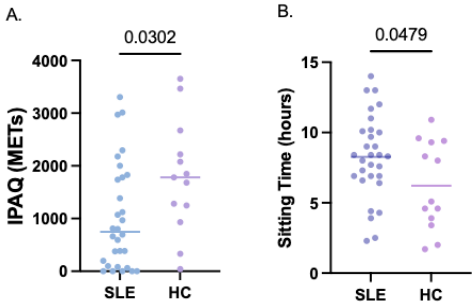


Figure 8.11. International physical activity questionnaire (IPAQ), validated for individuals with ages ≥ 14 years old. Results for physical activity (A) and sitting time (B). HC – healthy controls (n=13), METs - metabolic equivalents of task, SLE – systemic lupus erythematosus (n=30); n – total number of individuals. p-value significant < 0.05.

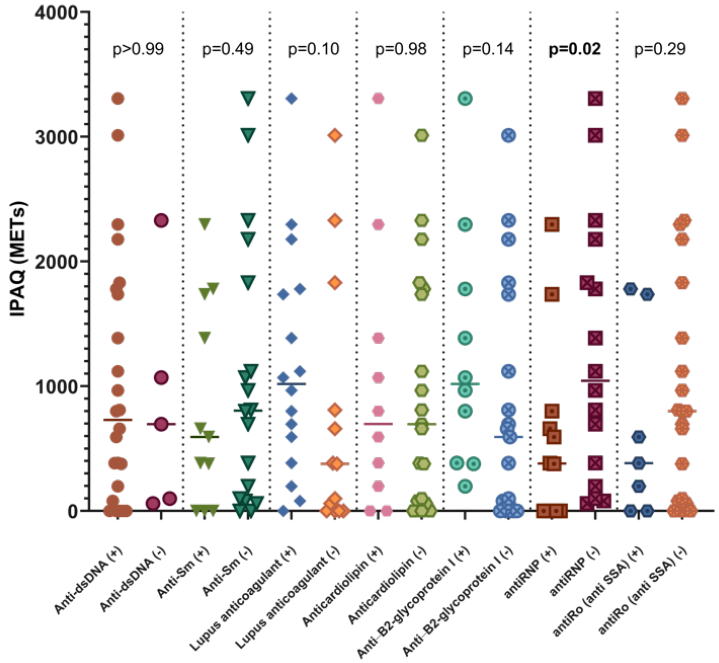


Figure 8.12. Physical activity and immunological status of SLE patients. Physical activity assessed with the International Physical Activity Questionnaire (IPAQ), validated for individuals with ages ≥ 14 years old, according to the patients' immune status. Only one patient was positive for anti-La antibodies. (+) positive, (-) negative, Anti-RNP- antinuclear ribonucleoprotein, Anti-Sm- anti-Smith, METs- metabolic equivalents of task. p-value significant < 0.05.

8.5. BODY COMPOSITION

Since obesity is associated with several chronic inflammatory and immune-mediated diseases (212), and increases the risk for SLE comorbidities, such as cardiovascular events (1)(35), we have assessed body composition in SLE patients and HC.

8.5.1 Body mass index

BMI is an easy and widely used tool to assess the prevalence of obesity in a given population, although it does not account for the fat mass and fat-free mass distribution (213). It is an index of weight for the square of the height (weight/ height^2), and the results are translated into a classification ranging from underweight to obesity (193). The mean BMI of adult SLE patients was 24.77 kg/m^2 (18.70-31.00 kg/m^2), while of HC was 22.33 kg/m^2 (18.20-27.70 kg/m^2), with no significant differences between the two groups (p=0.0899, t-test). Moreover, no significant differences were found between HC and SLE patients regarding overweight/obesity when the results of all participants (children and adults) were combined and compared (p=0.2833, Fisher's exact test). In adults, BMI influenced disease activity (p=0.0048, Kruskal-Wallis test, **Table S12.16**). Finally, no significant differences existed between genders, ethnic groups, age, disease duration, or presence of LN (**Table S12.16**).

8.5.2 Fat Mass

Since the BMI does not account for the fat mass distribution and quantification, the fat mass percentage was estimated by whole-body plethysmography.

Adult SLE patients had a mean fat mass of 36.16% (18.10-47.80%), and adult HC of 27.22% (13.40-38.80%), with the difference being statistically significant (p=0.0118, t-test, **Figure 8.13)**

As expected, there was a tendency towards a lower fat mass in males than in females (p=0.1313, t-test).

Other variables did not influence fat mass distribution (**Table S12.17**).

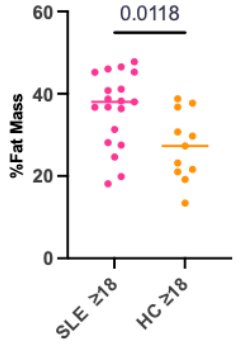


Figure 8.13. Percentage of fat mass between adult SLE patients (n=19) and HC (n=11). HC – healthy controls, SLE – systemic lupus erythematosus; n – total number of individuals. p-value significant < 0.05.

8.5.3 Waist Circumference

Intra-abdominal fat mass deposition increases the risk of unfavorable health outcomes (193). Waist circumference provides a simple method to assess abdominal fat and identify individuals at increased risk of obesity-associated illness due to abdominal fat deposition (193). The cardiovascular risk was assessed through the waist circumference, according to the World Health Organization categories, and the reference data for Portuguese children and adolescents aged 10 to 18 years (**Figure 8.14A)** (193)(195). The results were analyzed considering the categories: no cardiovascular risk (women ≤ 80cm, men ≤ 94cm, children <P75), and with cardiovascular risk (women > 80 cm, men > 94 cm, children ≥P75). An increased risk for cardiovascular events occurred in 51.5% of SLE patients, significantly higher than in HC (p=0.0143, Fisher's exact test, **Figure 8.14B, Table S12.18)**. Other variables did not influence waist circumference. No significant differences were found between genders, ethnicity, age, disease duration, disease activity, presence of LN, treatments, and immunological status (**Table S12.18**, **Table S12.19, Table S12.20**).

A.

		No CDV risk	With CDV risk
≤ 18 y.o		<P75	≥P75
> 18 y.o	Women	≤ 80 cm	> 80 cm
	Men	≤ 94 cm	> 94 cm

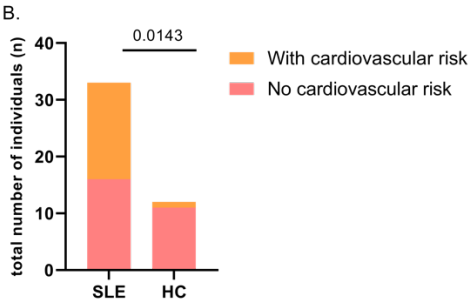


Figure 8.14. (A) Waist Circumference categories. (B) Risk of cardiovascular events assessed by the waist circumference. CVD-cardiovascular, HC–healthy controls (n=12), P-percentile, SLE–systemic lupus erythematosus (n=33). p-value significant < 0.05.

8.5.4 Waist/Hip and Waist/Height ratios

The waist-to-hip ratio is an important parameter to assess the risk of obesity (193) that, in some populations, may be a better predictor of abdominal adiposity than waist circumference (214). It is an easy-to-use measure and an indicator of visceral fat and risk for metabolic diseases (214). The waist/hip ratio was assessed only in adults, according to the World Health Organization recommendations (193). There were no significant differences between SLE patients and healthy controls regarding waist-to-hip ratio (p=0.0890, Fisher's exact test, **Table S12.21**). No differences were found between genders, ethnicity, disease activity, and presence of LN (**Table S12.21**).

The waist-to-height ratio is also a measure of central fat and metabolic risk (215) and was assessed in all participants. SLE patients had a higher ratio than HC (p=0.0303, t-test, **Figure 8.15A, Table S12.22**), with 48.5% showing metabolic risk. When the results were categorized between "no metabolic risk" (when the waist/height ratio was < 0.5) and "with metabolic risk" (when the waist/height ratio was ≥ 0.5), according to the World Health Organization recommendations (193), more SLE patients showed metabolic risk than HC (p=0.0168, Fisher's exact test, **Figure 8.15B, Table S12.22**).

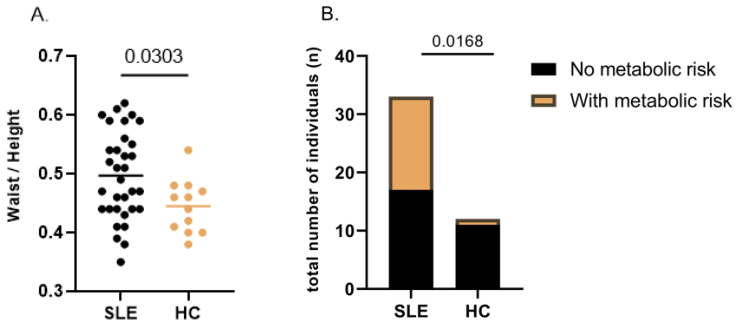


Figure 8.15. (A) Waist/Height ratio between SLE patients and HC. (B) Waist/Height ratio categorization according to the metabolic risk. HC–healthy controls (n=12), SLE–systemic lupus erythematosus (n=33). p-value significant < 0.05.

Fasting and sleep duration were also assessed. However, there were no differences between SLE patients and HC (**Table S12.15**).

8.6. GUT MICROBIOTA

The gut microbiota of 13 HC and 31 SLE patients was analyzed. The raw sequencing data were studied with Qiime2 summarize tool and visualized with the Qiime2 view tool (https://view.qiime2.org). On average, each sample contained 57,186 paired-end reads with an ununiform distribution. Two samples had half the median value of reads of other samples (**Figure S13.3**). The lower number of reads did not seem to affect the number of OTUs recovered (p-value=0.461, Kruskal-Wallis test), which means there was sufficient information. Thus, the samples were included in the analysis. From the mean quality score plot, there is a dip in quality below a Phred-score of 30 for the forward reads near position 230 and for reverse reads near position 200 (**Figure S13.4**).

8.6.1. Operational Taxonomic Units

Operational Taxonomic Units (OTUs) are analytic units in which similar 16S/18S rRNA sequences are clustered assuming that phylogenetically similar organisms share sequence similarity (216). A total of 1,461 unique OTUs were recovered, with a frequency ranging from 2 to 90,798 (median frequency of 76). The negative control contains 9,187 features (**Figure S13.5**), which was set as minimum sampling depth in the α and β diversity analysis. The representative sequences were aligned with the MAFFT plugin tool (217), and the highly variable positions were removed with Qiime2 alignment mask plugin to reduce noise.

8.2.2. Alpha and Beta-diversity

α-diversity is an indicator of microbial species diversity, and it considers the variety of species (richness) and the evenness of species abundance in a single sample (218). Variations in this parameter have been observed in several diseases (219). β-diversity considers the differences in the composition of microbial communities between samples (220).

For α-diversity the observed OTUs (community richness) were assessed. α-diversity was significantly decreased in SLE patients than in HC (p=0.028, Kruskal-Wallis test; **Figure 8.16**), meaning that the gut microbiota of SLE patients was less diverse than the gut microbiota of HC.

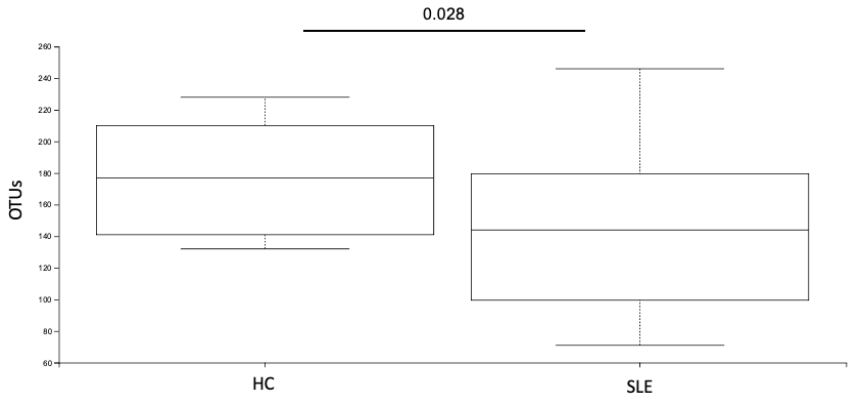


Figure 8.16. Observed OTUs (α-diversity) in SLE patients and HC. HC – healthy controls (n=13), SLE – systemic lupus erythematosus (n=31); n– total number of individuals, observed features– operational taxonomic units (OTUs). p-value significant < 0.05.

A significant difference in the number of observed OTUs was found between females and males (p=0.043, pairwise permanova test, **Table S12.23**). There were no significant differences in α-diversity between children and adults (p=0.617, pairwise permanova test) or between ethnic groups (p=0.116, permanova test). The number of observed OTUs was not different according to the Bristol scale results (p=0.168, pairwise permanova test).

Interestingly, the Mediterranean diet influenced the gut microbiota diversity. Categorizing the Mediterranean diet score, obtained with PREDIMED and KIDMED questionnaires, in two categories (1-insufficient adherence to the Mediterranean diet and 2-good adherence to the Mediterranean diet), a significant difference was found between the two groups (p=0.014, Kruskal-Wallis test; **Figure 8.17**), which means that higher compliance with the Mediterranean diet was associated with higher gut microbiota diversity.

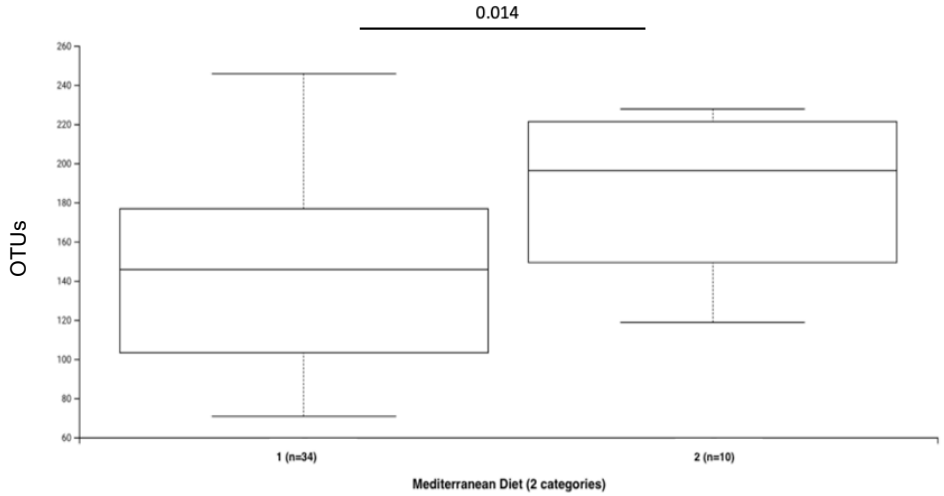


Figure 8.17. Observed OTUs (α-diversity) according to the adherence to the Mediterranean diet. 1- insufficient adherence to the Mediterranean diet (n=34), 2-good adherence to the Mediterranean diet (n=10). n – total number of individuals, observed features– operational taxonomic units (OTUs). p-value significant < 0.05.

Since the Mediterranean dietary pattern influenced the gut microbiota diversity, a closer look at some dietary macro or micronutrients was warranted to check if they were the main drivers of the gut microbiota alterations. Fructose (p=0.3709, Kruskal-Wallis test), total n-3 PUFA (p=0.8911, Kruskal-Wallis test), manganese (p=0.7408, Kruskal-Wallis test) and total fiber (p=0.4606, Kruskal-Wallis test) did not influence α-diversity. EPA, together with DHA consumption (n-3 PUFA), were categorized into four categories (1:0.00-0.05g; 2:0.05-0.07g; 3:0.07-0.12g; 4:>0.12g). EPA and DHA significantly influenced α-diversity (p=0.0127, Kruskal-Wallis test; **Figure 8.18**).

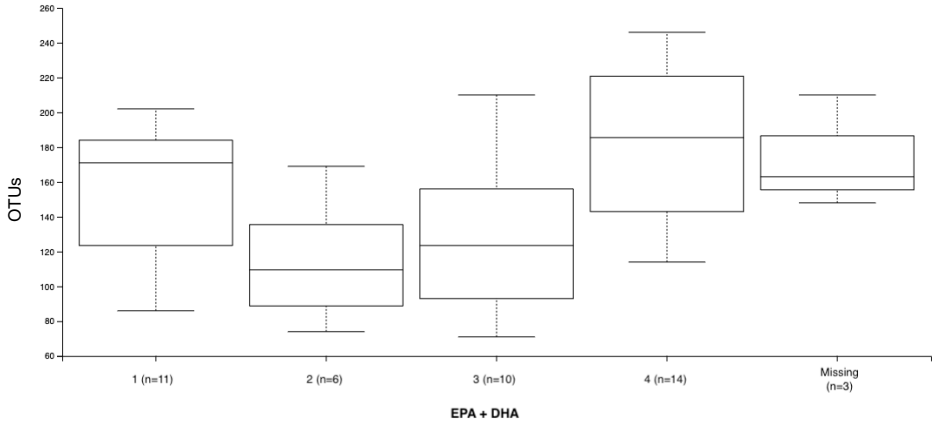


Figure 8.18. Observed OTUs (α-diversity) according to EPA+DHA categories (1- 0.00-0.05g; 2- 0.05-0.07g; 3- 0.07-0.12g; 4- > 0.12g) (p=0.0127). DHA - docosahexaenoic acid, EPA- eicosapentaenoic acid, n – total number of individuals, observed features– operational taxonomic units (OTUs). p-value significant < 0.05.

Age at diagnosis, time since diagnosis, presence of LN, disease activity score assessed with SLEDAI-2K, sedentary time and physical activity assessed with IPAQ questionnaire, fasting time, hours of sleep, body fat mass, waist circumference, BMI, waist/height ratio and GLP-1 levels did not influence the gut microbiota diversity.

For β-diversity, weighted UniFrac distance (a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features) was used. Regarding β-diversity, the principal component analysis plot for the unweighted UniFrac distance (**Figure 8.19**) did not show a clear separation between SLE patients and HC. However, the PERMANOVA test for the β-diversity within the two groups obtained a p-value of 0.024.

LN (p=0.055) and SLEDAI-2K (p=0.205) did not influence β-diversity. Interestingly, a cluster that included eleven SLE patients is clearly separated from the other patients and controls. When analyzing the main differences between the patients included in this cluster, there was a higher prevalence of LN, although without reaching statistical significance (p= 0.055; Fisher's exact test). No associations were found with SLEDAI-2K (p=0.41; Mann-Whitney test), physical activity (p=0.20; Mann-Whitney test), Mediterranean diet categories (p>0.99; Fisher's exact test), GLP-1 (p=0.51; t-test) or treatment with MMF (p=0.12; Fisher's exact test).

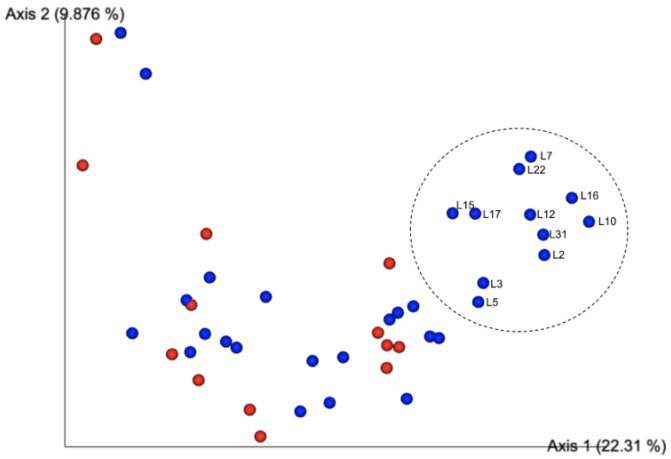


Figure 8.19. Principal component analysis plot for the unweighted UniFrac distance (β-diversity) between healthy controls (red) and SLE patients (blue).

8.6.3. Firmicutes/Bacteroidetes ratio

Both groups had a higher proportion of Firmicutes than Bacteroidetes. However, SLE patients showed a considerably higher average ratio ($\bar{x}$ Firmicutes/Bacteroidetes=126.12) than HC ($\bar{x}$ Firmicutes/Bacteroidetes=17.47). Despite this, due to the large variances, the differences were not enough to reject the null hypothesis that the average ratio is equal for both groups (p=0.4041, one-way ANOVA test, **Table S12.24**).

Considering SLE disease activity (SLEDAI-2K score), the same trend was observed. All groups show a higher proportion of Firmicutes than Bacteroidetes, with considerable differences between categories (**Table S12.25**). However, the differences are insufficient to reject the null hypothesis that the average ratio is equal for all groups (p=0.7335, one-way ANOVA test, **Table S12.25**).

8.6.4. Taxonomic classification

At the class level, the most abundant taxa in all samples were Clostridia, followed by Bacteroidia, Actinobacteria and Bacilli. However, there was a significant variation between samples. There was not a clear distinguishable taxonomic pattern distribution between SLE patients and HC (**Figure 8.20**).

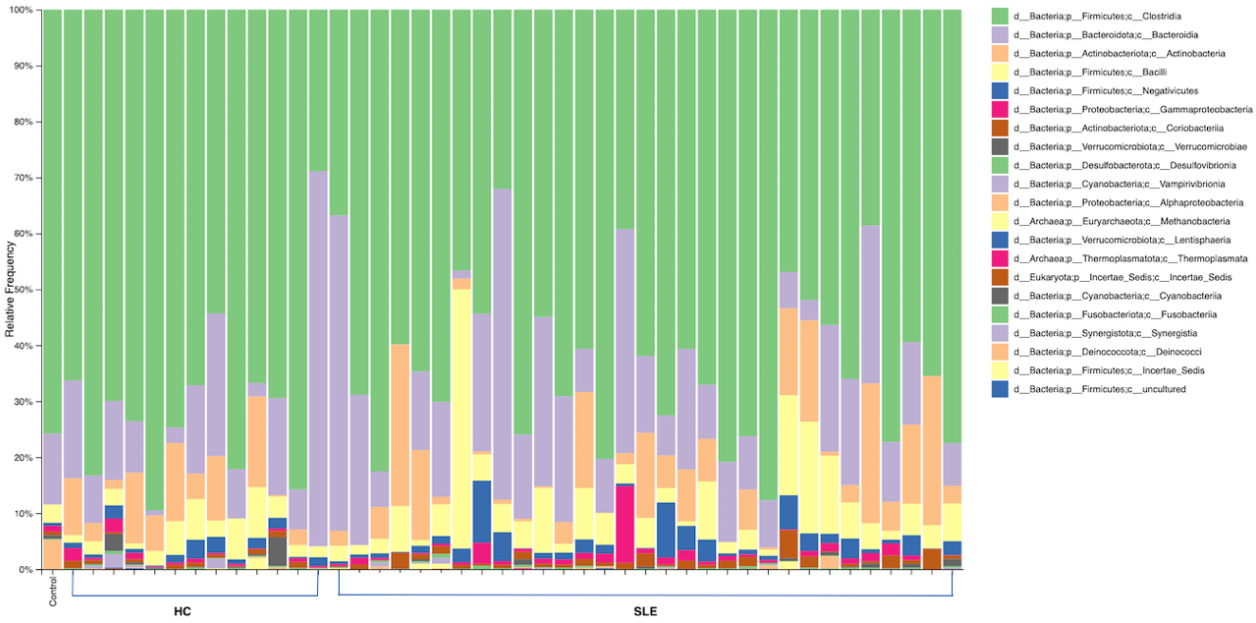


Figure 8.20. Relative frequency of the taxonomic classification between SLE patients and HC at the class level. HC – healthy controls, SLE – systemic lupus erythematosus.

When assessing the relative bacterial abundance at the class level, there were no significant differences between SLE patients and HC.

There were no significant differences between SLE patients and HC at the genus level. Nevertheless, the *Streptococcus* genus was the most altered between the two populations (**Figure S13.6**). When assessing differences in bacterial composition at this taxonomic level, between male and female SLE patients, the absolute abundance of *Butyricicoccaceae;g UCG-008* genus, belonging to the Clostridia class, was significantly different between the two groups (**Figure S13.7**).

Taxa previously reported to be altered in lupus, were also assessed. Proteobacteria phylum (p=0.3287, ANOVA), Lactobacillalles order (p=0.13969, ANOVA), *Ruminococcaceae* family (p=0.0951, ANOVA), *Ruminococcus* genus (p=0.18998, ANOVA), and species *Streptococcus anginosus* (p=0.2384, ANOVA), *Lactobacillus* spp. (p=0.7418, ANOVA), and *Ruminococcus torques* (p=0.7070, ANOVA) were not significantly altered between SLE patients and HC.

When assessing if there were taxonomic variations according to SLE disease activity (SLEDAI-2K), no significant variations were found in Lactobacillalles order (p=0.1206, ANOVA), and species *Streptococcus anginosus* (p=0.4356, ANOVA), *Lactobacillus* spp. (p=0.8555, ANOVA), and *Ruminococcus torques* (p=0.9651, ANOVA).

Assessing the differences between SLE patients with LN, patients without LN, and HC, the *Prevotellacear* family (p=0.6947, ANOVA), *Bacteroides uniformis* (p=0.5468, ANOVA), and *Ruminococcus gnavus* species (p=0.4364, ANOVA) were not significantly altered. However, the *Rikenellaceae* family was altered between these three groups (p=0.0385, ANOVA; **Figure 8.21, Table S12.26**). SLE patients with LN showed higher concentrations of *Rikenellaceae* bacteria than patients without LN and HC.

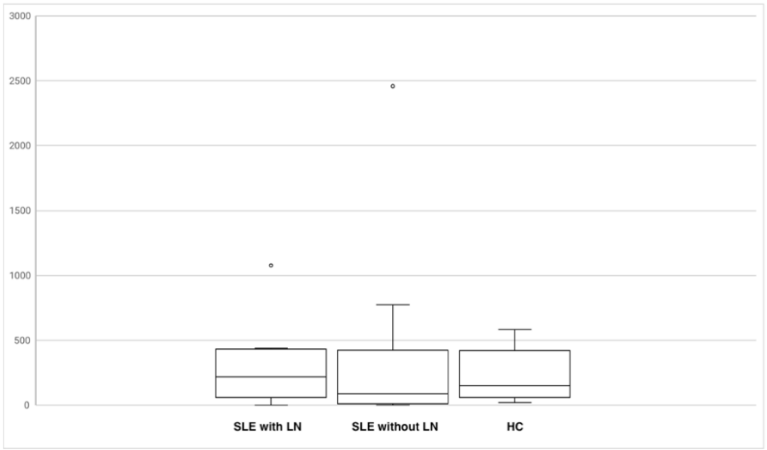


Figure 8.21. Observed reads for *Rikenellaceae* family in SLE patients with LN (n=10), SLE patients without LN (n=20), and HC (n=13) (p=0.0385). HC – healthy controls, LN – lupus nephritis, SLE – systemic lupus erythematosus. p-value significant < 0.05.

8.7. GLP-1

GLP-1 is an incretin hormone pointed out as a possible biomarker of dysbiosis, loss of intestinal barrier integrity, and inflammation (222)(223). GPL-1 is secreted in two forms, one of which is GLP-1-(7-36). After secretion GLP-1-(7-36) is rapidly degraded in GLP-1-(9-36), the most abundant form of GLP-1 in postprandial plasma (224)(225). Both GPL-1-(7-36) and (9-36) were assessed by ELISA.

Although SLE patients had higher GLP-1 concentrations than HC, the difference was not statistically significant (p=0.0739, Mann-Whitney, **Figure 8.22**). GLP-1 concentrations were not associated with the other variables studied (**Table S12.27**), including treatment (**Table S12.28, Figure S13.8**) and immunological status (**Table S12.29, Figure S13.9**).

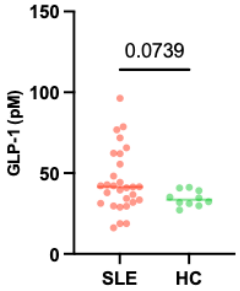


Figure 8.22. Glucagon-like peptide-1 (GLP-1) plasma concentrations between SLE patients (n=29) and HC (n=9). HC – healthy controls, SLE - systemic lupus erythematosus. p-value significant < 0.05.

8.7.1 Correlations between nutrients, body composition and SLEDAI-2K and GLP-1

Finally, it was assessed if any body composition parameter or dietary nutrient could be correlated with SLE disease activity or GLP-1 values, the latter as a surrogate of gut dysbiosis. The intake of zinc (r=-0.4650, p=0.0110, **Figure 8.23A**) and SFA 15:0 (r=-0.4123, p=0.0262, **Figure 8.23B**) were moderately inversely correlated with SLE disease activity (**Table S12.30**).

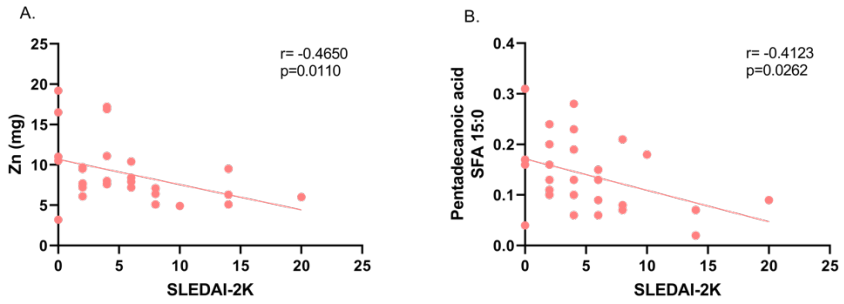


Figure 8.23. Correlation between disease activity (SLEDAI-2K) and (A) zinc, and (B) SFA 15:0. HC–healthy controls, SLE–systemic lupus erythematosus, r–Spearman correlation coefficient. p-value significant < 0.05.

Regarding GLP-1 values, there was a moderate inverse correlation with lactose (r= -0.3998, p=0.0173, **Figure 8.24A**), a strong positive correlation with n-6 PUFA (r= 0.5538, p=0.0006, **Figure 8.24B**) and a moderate positive correlation with linoleic acid (r= 0.4741, p=0.0040, **Figure 8.24C**)(**Table S12.31**), meaning that these dietary components may play a role in gut dysbiosis .

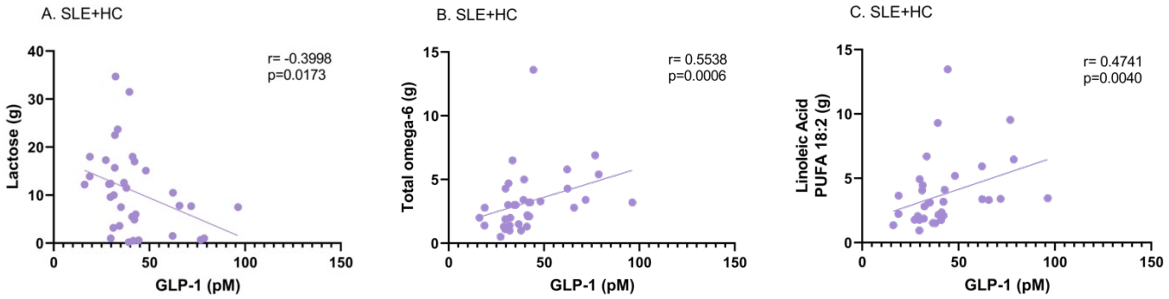


Figure 8.24. Correlation between glucagon-like peptide 1 (GLP-1) and dietary components. (A) Lactose. (B) Total n-6 PUFA. (C) Linoleic acid (PUFA 18:2). HC – healthy controls, PUFA – Polyunsaturated fatty acids, SLE – systemic lupus erythematosus, g- grams, r – Spearman correlation coefficient. p-value significant < 0.05.

A moderate to strong positive correlation was found between waist/hip ratio (r=0.5607, p=0.0044, **Figure 8.25A**), waist circumference (r=0.4241, p=0.0089, **Figure 8.25B**), and GLP-1, and a moderate correlation between this marker and the waist/height ratio (r=0.3501, p=0.0337, **Figure 8.25C**) (**Table S12.31**).

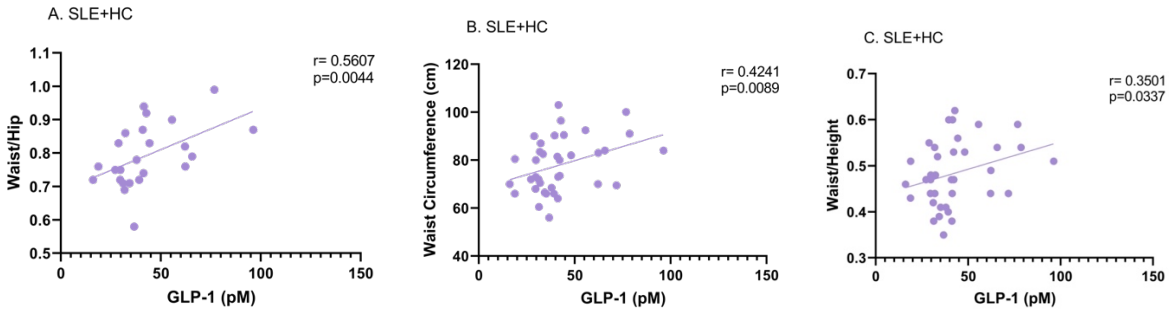


Figure 8.25. Correlation between glucagon-like peptide 1 (GLP-1) and (A) waist/hip ratio, (B) waist circumference, and (C) waist/height ratio. HC – healthy controls, SLE – systemic lupus erythematosus, r – Spearman correlation coefficient. p-value significant < 0.05.

Finally, although there was no correlation between disease activity and physical activity (r=-0.0932, p=0.6368) or sitting time (r=-0.1084, p=0.5828), there was a moderate positive correlation between GLP-1 concentrations and sitting time (r=0.4329, p=0.0094, **Figure 8.26A),** and a moderate inverse correlation with physical activity (r=-0.3720, p=0.0278, **Figure 8.26B**) (**Table S12.31**).

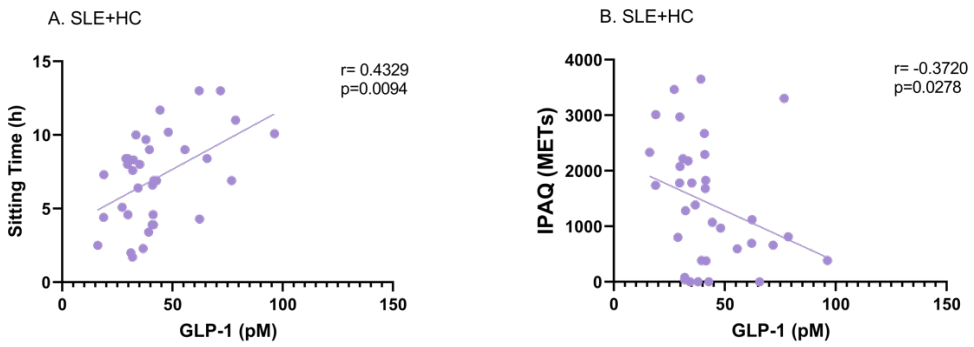


Figure 8.26. Correlation between GLP-1, as a surrogate for gut dysbiosis, and sitting time(A), and (B) physical activity. HC – healthy controls, SLE – systemic lupus erythematosus, r – Spearman correlation coefficient. p-value significant < 0.05.

In summary, these results show that SLE patients have a low adherence to the Mediterranean diet, a low intake of several macro and micronutrients, and a higher sedentary behavior than HC. Moreover, SLE patients have gut dysbiosis. These factors may contribute to SLE pathogenesis.

CHAPTER 9. Discussion

SLE is an immune-mediated disease that, despite several advances in treatment, is still one of the main causes of non-traumatic death in young women (10). The disease burden and prolonged treatment greatly impact the patient's quality of life, warranting better treatment strategies, ideally with fewer adverse effects.

The gut microbiota has been considered a potential contributor to SLE pathogenesis. Dysbiosis is frequently identified in SLE patients (292)(293)(310)(316)(319-323), which, together with an impaired gut barrier, may lead to the translocation of gut-microbiota-derived metabolites with local and systemic inflammatory effects.

This cross-sectional study assessed the gut microbiota of Portuguese SLE patients, also analyzing factors that may influence its composition, such as diet, body composition, and physical activity. The gut microbiota of SLE patients was less diverse than that of HC ($p=0.028$), reflecting a dysbiosis. These results are similar to those found by other authors in other populations (292)(293)(310)(316)(319-323).

Gut microbiota dysbiosis may exacerbate SLE manifestations (325)(334)(335). However, the broad spectrum of clinical manifestations and the small sample size limited the microbiota analysis in different sub-groups according to the profile of clinical manifestations.

There were no significant differences between the gut microbiota diversity of children and adults ($p=0.617$). Considering that, depending on the authors, the gut microbiota becomes stable between 5 years old and puberty (258)(259) and that most of our child population had already entered puberty, the lack of differences in the gut microbiota composition was expected.

Women and men had different gut microbiota diversity ($p=0.043$), which was also found by other authors (262)(268-270), and may be related to hormonal factors (269)(271) since estrogens regulate the gut microbiota diversity (274).

A sample imbalance was detected between men and women and between adults and children, which may be influencing the results. However, both were expected and reflected the SLE characteristics, considering its higher prevalence in women than in men (5)(6)(8)(10) and in adults than in children, being a rare disease in the latter age group (13).

Ethnicity did not influence gut microbiota diversity. Ethnicity has been considered a proxy for dietary and lifestyle habits (268), which may overrule ethnic background (283). These observations are aligned with our results once no differences were found in the adherence to the Mediterranean diet between ethnic groups.

The F/B ratio is considered a marker of gut homeostasis, and variations have been associated with several diseases. In SLE, both higher (309) and lower F/B ratios (308)(314) have been found, which may reflect differences between animal and human studies or even between populations. In this cohort, SLE patients had a considerably higher F/B ratio than HC, although it did not reach statistical significance ($p=0.4041$).

An altered taxonomical distribution of the gut microbiota also impacts gut homeostasis. The significant taxonomic distribution variation between samples did not allow the identification of a distinguishable taxonomic pattern between SLE and HC.

Rikenellaceae family was differentially present in the gut microbiota of SLE patients with LN, patients without LN, and HC ($p=0.0385$), being higher in patients with LN. *Rikenellaceae* are gram-negative bacteria that belong to the Bacteroidetes phylum which comprises *Alistipes* and *Rikenella* genera (544). These are mucin-degrading bacteria present in the gastrointestinal tract of several animals (544). In a mouse model of systemic autoimmunity, characterized by age-dependent splenomegaly and abnormal stimulation of activated T follicular helper cells, there was an increase in *Rikenellaceae* (334). A higher abundance of these bacteria was also found in SLE mouse models (545)(546) and an even

higher presence in severe glomerulonephritis (314), which supports our findings. Interestingly, in one study, the *Rikenellaceae* presence was reduced with N-acetylcysteine treatment, a known antioxidant (545), and in another, it was increased by retinol supplementation (546). However, despite the possible association between LN and *Rikenellaceae* abundance, these bacteria may have beneficial effects when present in the gut in adequate concentrations, which may explain the lower abundance of these bacteria in SLE patients without LN than in HC (**Figure 8.21**). Jaagura and colleagues reported an increase in *Rikenellaceae* abundance in patients with overweight or obesity after a decrease in BMI, especially when there was a low abundance of these bacteria before the dietary intervention (547). Moreover, *Rikenellaceae* seems to exist in lower concentrations in Crohn's disease and ulcerative colitis (548). It is also important to note that patients with LN may have genetic characteristics that differ from patients without LN, which could explain the differences in *Rikenellaceae* abundance. Future studies analyzing *Rikenellaceae* abundance in SLE and detailing the species variation will help shed more light on these bacteria's possible contribution to SLE and LN.

Dysbiosis of the gut microbiota can impair the gut barrier integrity by decreasing the mucus thickness (191) or modulating TJ proteins (218). In addition, the gut microbiota is involved in host defense by producing antimicrobial components that will prevent colonization by pathobionts (336), which means that changes in the gut microbiota composition may lead to increased pathogenic bacteria-inducing immune responses.

GLP-1 is a candidate biomarker of dysbiosis, inflammation (223)(227), and gut injury (223). In addition, intestinal fatty acid-binding protein (I-FABP) is released when intestinal mucosal damage occurs, and it is, therefore, a marker of epithelial cell loss (549). Interestingly, GLP-1 response can be obtained even in the presence of light epithelial injury and before I-FABP rise (223), justifying an exploratory analysis of its concentration in this cohort. SLE patients showed higher GLP-1 levels than HC, although without statistical significance (p=0.0739).

Curiously, when in this group of SLE patients, gut permeability was assessed by our group using the lactulose/mannitol test, the SLE patients showed higher gut permeability than HC (**Appendix 8**). Moreover, SLE patients also showed higher levels of zonulin, assessed by ELISA, and a higher concentration of soluble CD14 (**Appendix 8**), an indirect marker of endotoxemia (550). Furthermore, adult SLE patients had a higher level of I-FABP than adult HC (**Appendix 8**), suggesting epithelial damage and increased gut permeability. These results support the GLP-1 results in this cohort, showing that GLP-1 may be a good marker for gut permeability and inflammation. In addition, the increase in GLP-1 may be a compensatory mechanism of the colonocytes in response to a decrease in butyrate, a fundamental energy source for these cells (221), which is frequently decreased in the presence of dysbiosis. Nevertheless, these results may be influenced by the small sample size that may hinder these assumptions. Thus, it warrants further investigation in larger populations.

The current hypothesis supporting the influence of the gut microbiota in immune-mediated diseases' pathogenesis is that the impairment of the gut barrier results in the opening of the paracellular pathway and consequent passage of gut microorganisms and their antigens to the gut lamina propria (214). This translocation of microorganisms results in the activation of the host immune system, targeting tissues and organs and ultimately favoring the occurrence of immune-mediated diseases in genetically predisposed individuals (214). In fact, LPS-producing bacteria can enter circulation and have systemic effects by triggering immune-mediated responses in SLE patients (323). Furthermore, increased levels of LPS were identified in SLE patients by our group (337) and were associated with SLE progression (294), showing that Gram-negative bacteria may favor SLE pathogenesis (294).

Several lifestyle factors can influence the gut microbiota composition, particularly diet and physical activity. The Mediterranean diet is known for its favorable impact preventing multiple chronic diseases (551), thus warranting further investigation. SLE patients had lower adherence to the Mediterranean diet than HC (p=0.0222).

The dietary habits of the Portuguese population have moved away from the Mediterranean diet. The last national inquiry revealed that 84.3% of adult Portuguese do not have good compliance with this dietary pattern (392). In our study, 87.5% of the adult SLE patients had an insufficient adherence to the Mediterranean diet, slightly higher than the national results, and than HC (54%) (**Table S12.5**) (392). In a study that determined the influence of the Mediterranean diet on SLE, 51.1% had high adherence (552). These patients also showed better anthropometric profiles, lower triglycerides, and lower corticoid use than patients with lower adherence (552). Although in our cohort, it was not possible to analyze the association between the Mediterranean diet and disease activity, other authors found an association with the risk of having active disease and the presence of damage (552).

In our cohort, the Mediterranean diet influenced the gut microbiota diversity ($p=0.014$), showing an association between higher microbiota diversity and higher adherence to this dietary pattern. Other authors have shown that the Mediterranean diet improves the gut microbiota bacterial profile, increasing Bacteroidetes abundance while decreasing Firmicutes (339). The high fiber content of this dietary pattern also leads to a higher SCFA production (339), essential metabolites for maintaining gut homeostasis (302). Moreover, the high content of bioactive compounds of the Mediterranean diet that influences the metabolization of dietary polyphenols also increases *Faecalibacterium prausnitzii* concentrations (303), an SCFA-producing bacteria (303) that was found to be decreased in lupus mouse models (308) and SLE patients (253). The encouragement to follow a Mediterranean diet in SLE is even more relevant, considering its possible inverse association with the risk of having active disease (345) and that this dietary pattern is efficient in preventing cardiovascular diseases (553), which are leading causes of death in SLE (5)(9).

Although the beneficial outcomes of the Mediterranean diet are attributed to this dietary pattern as a whole (553), some nutrients are of particular interest. The n-3 polyunsaturated fatty acids EPA and DHA have known local and systemic anti-inflammatory properties (366). In addition, they can improve the gut barrier integrity and modulate the gut microbiota, increasing butyrate-producing bacteria and decreasing LPS-producing bacteria (366)(367). The gut microbiota modulatory action of EPA and DHA was confirmed in our cohort since these n-3 PUFA influenced the gut microbiota diversity ($p=0.0127$). We then assessed if there were differences between the dietary intake of n-3 PUFA of SLE patients and HC, and the results showed that SLE patients consumed less total n-3 PUFA ($p=0.0248$), ALA ($p<0.0001$), and EPA ($p=0.0077$) than HC. Furthermore, only 16.7% of SLE patients reached the EFSA recommendation. In comparison, 50% of HC reached the same recommendations ($p=0.0491$), confirming that SLE patients have a low intake of these PUFAs. Interestingly, other authors found an inverse correlation with anti-dsDNA antibodies (554).

SLE patients also had a higher n-6/n-3 PUFA ratio than HC ($p=0.0042$), with only 10% reaching the ideal ratio (**Table S12.12**) (365). These data are particularly relevant, because n-6 PUFA interferes with n-3 PUFA metabolism by competing for the same enzymes (361). Moreover, a high n-6/n-3 PUFA ratio is associated with endotoxemia (368). In contrast, a lower ratio (4:1) enables the endogenous conversion of n-6 PUFA to n-3 PUFA, reducing endotoxemia and inflammatory cytokines (368). Finally, an increased n-6/n-3 PUFA ratio is associated with higher LPS-producing bacteria and lower LPS-suppressing bacteria such as *Bifidobacterium*, *Akkermancia muciniphila*, and *Lactobacillus*, increasing endotoxemia and inflammation (368). However, these outcomes can be effectively changed with n-3 PUFA supplementation (368). Interestingly, our results show a positive correlation between total n-6 PUFA and GLP-1 concentration ($r= 0.5538$, $p=0.0006$), which may be driven by linoleic acid, since the same positive correlation was found between this marker and GLP-1 concentration ($r= 0.4741$, $p=0.0040$). Despite our analysis only considering n-6 PUFA intake, other authors reported that SLE patients had increased serum concentrations of linoleic acid and ALA than HC, the first were positively correlated with ANA antibodies and doses of steroids (554). These results show that a higher n-6 PUFA intake may increase gut permeability and inflammation.

The intake of the PUFA stearidonic acid (p=0.0171) was also lower in SLE patients than in HC. Stearidonic acid is an n-3 PUFA, which results from the desaturation of ALA (555). Its efficient conversion into EPA leads to a higher increase in tissue EPA concentrations when compared to ALA (556). Stearidonic acid-rich oils can decrease triacylglycerol levels, LDL, and oxidized LDL levels (557). Additionally, supplementation with these oils can increase EPA and DHA on erythrocytes' membrane (558) and decrease inflammatory markers (559). Interestingly, patients with atopic dermatitis consuming a stearidonic acid-rich oil for 20 weeks reduced skin dryness, itchiness, and the use of dermal medication (560). Thus, consuming stearidonic acid may be a good alternative to increase EPA concentrations, which are associated with several therapeutic benefits. Some of the best dietary sources of stearidonic acid are the oils of black currant (*Ribes nigrum*), hemp (*Cannabis sativa*), and primula (*Primula sikkimensis*) (555).

There were no differences in the intake of total SFAs between SLE patients and HC. When looking at the dietary SFA profile, SLE patients had a lower intake of myristic acid (p=0.0455), arachidic acid (p=0.0461), and behenic acid (p=0.0160**)** than HC. Even though some authors have linked long-chain saturated fatty acids such as myristic acid and arachidic acid to cardiovascular diseases and obesity (561), some conflicting results have been published (562)(563). Thus, not all SFA seem to have the same consequences (564), and the dietary background (high or low-fat diet) may influence the results (561). In an osteoarthritis rat model, a high-myristic acid diet induced a lower increase in inflammatory markers than a high-fat diet and increased anti-inflammatory markers, similar to controls, suggesting a lower inflammatory response to these SFA (565). Moreover, myristic acid was positively correlated with the absence of immune-mediated diseases and was considered a predictive marker for their occurrence (566). Furthermore, very long-chain saturated fatty acids, such as behenic acid, have been associated with decreased cardiovascular risk and higher physical activity (567). Finally, a moderate inverse correlation was found between SFA 15:0 (r=-0.4123, p=0.0262) intake and SLE disease activity. Interestingly, other authors reported that pentadecanoic acid (C15:0) concentrations strongly correlate with the absence of immune-mediated diseases (566). The altered SFAs are found in olive oil (myristic and arachidic acid), peanut oil (arachidic and behenic acid), sunflower oil (behenic acid) (568), and coconut oil (myristic acid) (569).

To have a complete picture of the participants' dietary lipid profile, we also assessed the dietary intake of MUFA. There were no differences between total MUFA intake in SLE patients and HC. Safflower, sesame, pumpkin seed, rice bran, rapeseed, olive oil and olives, and nuts are good dietary sources of these fatty acids (569). In this cohort, SLE patients consumed significantly fewer nuts than HC (p=0.0362). Some MUFA were significantly reduced in SLE patients: palmitoleic acid (p=0.0105), oleic acid (p=0.0461), one of the main components of olive oil, eicosenoic acid (p=0.0045), and docosenoic acid (p=0.0028). Palmitoleic acid, in particular, which can be found in macadamia nuts and oil, and fish oils, may improve cholesterol levels and glucose metabolism, decrease inflammation, and promote weight loss (570). MUFA have anti-inflammatory properties. Thus, a higher intake may be useful in SLE. In RA, the high intake of these fatty acids was considered an independent factor for remission (571). Furthermore, the intake of extra-virgin olive oil decreased inflammatory cytokines in a RA mouse model, while in a SLE mouse model, it shaped organ and articular manifestations as well as cytokine production (359). Interestingly, some of the beneficial properties of MUFA may derive from their influence on gut microbiota (569). These fatty acids can influence the F/B ratio and the abundance of Proteobacteria and Actinobacteria, decreasing BMI, hypertension, adipose tissue, and incidence of type 2 diabetes and inflammatory bowel diseases (569).

To complete the macronutrient dietary assessment, total protein and carbohydrates were assessed. There were no differences in total protein intake (p=0.3275) and carbohydrate intake (p=0.0818, **Table S12.6**) between SLE patients and HC. However, SLE patients consumed less glucose (p=0.0027**)** and fructose (p=0.0006**)** than HC, which may be related to a lower fruit intake. When looking

at PREDIMED and KIDMED answers, SLE patients more frequently did not reach the recommended fruit intake for a Mediterranean diet than HC (p=0.0145). High glucose and fructose intake are considered potential risk factors for non-communicable diseases (389), and can occur when these sugars are added to food products. However, they are important components of fruits and some vegetables. Thus, adequate consumption of these carbohydrates can be an indicator of dietary quality.

An interesting inverse correlation was found between lactose intake and GLP-1 concentration (r= -0.3998, p=0.0173), meaning that lactose may decrease gut permeability, probably by improving the gut barrier and positively modulating the gut microbiota. Lactose is the main carbohydrate in dairy products, such as milk and cheese. When not digested, it can be used as a prebiotic (substrate for fermentation) by the gut bacteria to produce SCFA and antimicrobial peptides, stimulating the growth of commensal bacteria that will compete with pathogens (572)(573). In fact, an *in vitro* study demonstrated that lactose can improve the production of SCFA in the gut (574)(575), which is extremely relevant for the maintenance of gut homeostasis (291). Lactose can also regulate GALTs' immune responses (243), and it has anti-inflammatory effects on epithelial and immune cells (292). Lactose also seems to increase *Bifidobacterium* and *Lactobacillus* (572)(574)(575). Moreover, lactose supplementation in pigs decreased diarrhea induced by Rotavirus and improved the crypts' jejunal mucosa morphology and the expression of TJ proteins (575).

SLE patients consumed less fiber than HC (p<0.0001), and the majority (96.7%) did not reach the DRI. Fibers are a broad category of carbohydrates resistant to digestion and absorption that comprehends different fiber types with various chemical compositions and metabolic and taxonomic outcomes (576)(577). Hence, diversified sources of fiber are essential to improve gut microbiota composition and diversity (578). The low intake of fiber in SLE patients may be related to the lower intake of fruit (p=0.0145), nuts (p=0.0362), vegetables (p<0.0001), and legumes (p=0.0276) seen in SLE patients when compared with HC. The fiber intake of SLE patients was also below the national average (12.8g *vs*. 17.2g), according to the *Inquérito Alimentar Nacional e de Atividade Física* 2015-2016 (392). A fiber intake below the recommendations was also identified in a Spanish population of SLE patients (552). Fiber is a substrate for SCFA production (348) by bacteria such as Bifidobacterium (349), which promote the differentiation and maintenance of Treg cells in the gut (176). Moreover, a low-fiber diet was associated with increased disease progression and immune dysregulation in a lupus-mouse model (401). In addition, resistant starch improved the gut barrier and prevented the translocation of *L. reuteri* (402), a bacteria that has been found to be altered in SLE.

Minerals and vitamins are essential components of a diversified diet. They can modulate immune functions, maintain antioxidant balance, influence gut microbiota diversity and composition, and maintain gut barrier integrity. In general, SLE patients have a lower intake of minerals than HC. The intake of potassium (p=0.0062) and magnesium (p=0.0004) were significantly lower when compared with HC and below the Portuguese national intake average (392). Moreover, most SLE patients did not meet the DRIs for these minerals. Other authors also identified insufficient intakes of potassium (579) and magnesium in SLE patients (443)(552). Potassium and magnesium contribute to blood pressure reduction. Thus, stimulating a higher intake of good dietary sources of these minerals may be beneficial to reduce cardiovascular risk (580), as well as to decrease inflammation markers (66) and gut dysbiosis (422)(424).

A decreased iron (p=0.0002) and manganese intake (p=0.0004) were also found in our cohort of SLE patients, significantly below the intake of HC. Similarly to what was observed previously, most SLE patients did not meet the DRIs for these minerals, and the iron intake was below the Portuguese average (392). An insufficient iron intake was also reported in Spanish SLE patients (552) and another cohort of children and adolescents with SLE (581). In addition, other groups found lower serum concentrations of iron in SLE patients than in HC (450). In our cohort, 80% of the SLE patients did not meet the DRI, leaving them susceptible to anemia. Iron deficiency alters immune-cell function

(413)(414), and it also promotes dysbiosis, decreasing butyrate and propionate-producing bacteria (432). Thus, iron homeostasis is relevant in SLE, as iron may have a protective role (448) when maintained in adequate concentrations.

Altered manganese levels may increase the risk for coronary artery disease, cardiovascular death, and inflammatory progression of osteoarthritis (427), also increasing the susceptibility to intestinal barrier disruption (426)(428). Despite the important functions of manganese, to our knowledge, no studies with SLE patients included its assessment. In a very interesting study, Nakata and colleagues demonstrated that, in mice, a manganese-deficient diet is sufficient to alter the gut mucus layer, compromising the gut barrier function (582). The gut barrier impairment exposes the host immune system to the gut microbiota, which, when perpetuated, leads to chronic intestinal inflammation, which itself can lead to manganese deficiency (582), showing that manganese may have an essential role in maintaining gut homeostasis. Considering that the detrimental effects of manganese deficiency may be masked by the compensatory mechanisms of organs and tissues and are only visible when these mechanisms are disrupted (582), monitoring dietary manganese intake is essential. Considering that, in children, manganese blood levels influenced specific gut microbiota taxa (583), assessing the taxonomic variations associated with manganese deficiency could bring more light on its role in SLE. Thus, evaluating this parameter in a bigger population will allow for a more detailed analysis and help clarify the influence of manganese on gut permeability and microbiota composition.

In our cohort, despite a tendency towards a lower zinc intake by SLE patients compared to HC, it did not reach statistical significance ($p=0.0547$). However, there was a moderate inverse correlation between zinc and SLE disease activity ($r=-0.4650$, $p=0.0110$), similar to what was found by other authors in SLE (441), and in other immune-mediated diseases (584) (585). In addition, 80% of SLE patients did not meet the DRI, and the median zinc intake was below the Portuguese average (392). Zinc is an essential nutrient for the optimal function of several enzymes (585) and immune cells (403)(408), which show impaired activity in a zinc-deficient environment (410)(411). Furthermore, zinc, copper, and manganese are essential for superoxide dismutase function, which is central in managing oxidative damage, as seen in SLE (586). Interestingly, in immune-mediated diseases, combining treatment with zinc supplementation may improve disease severity (584)(587)(588).

In general, SLE patients also had a lower intake of vitamins than HC, and most SLE patients did not reach the DRIs for vitamins B2, B5, B7, C, E, K1, and folate. However, only the intake of vitamin B6 ($p=0.0060$), folate ($p=0.0006$), and vitamin K1 ($p=0.0235$) were significantly different between SLE patients and HC. In addition, the intake of vitamins E, B2, B3, B6, C, and folate was below the Portuguese population average (392), while retinol and vitamin B12 intake were above. A lower intake of vitamins B1, B3, and E and a higher intake of vitamin B12 and retinol were also found in Spanish SLE patients compared to the national population intake (552). Other cohorts of SLE patients also did not meet the folate and vitamin E RDIs (502)(552).

Vitamins B12, B6 and folate, are homocysteine cofactors. Their deficiency may lead to hyperhomocysteinemia, a known cardiovascular risk factor (589)(590). Thus, the decreased intake of folate may lead to an imbalance between these vitamins, which may be aggravated if there are polymorphisms in the folate metabolizing enzyme methylene tetrahydrofolate reductase, leading to increased cardiovascular risk in SLE patients. In fact, adult (589)(591) and adolescent (342) SLE patients seem to have increased homocysteine levels, which may occur because of treatments, renal involvement, altered folate metabolism (589) or insufficient intake of vitamins B6, B12, and folate (590). Consistently with these observations, SLE patients also seem to have low intracellular levels of 5-methyltetrahydrofolate, the main biologically active form of folate (591), and increased metabolic risk associated with insufficient folate intake (579).

Comparably to what was observed in our work, Mexican SLE patients also had an insufficient intake of vitamin K (502). Vitamin K1 is synthesized by photosynthetic organisms such as green plants,

while vitamin K2 is synthesized by the gut microbiota (592). An altered gut microbiota and damaged mucosa will influence vitamin K levels by increasing its absorption (593). On the other hand, vitamin K deficiency can decrease the diversity of gut microbiota and bacteria concentrations with health-promoting characteristics (593), while increasing dietary vitamin K increases butyrate and propionate production (594). Furthermore, the immunoregulatory role of vitamin K in intestinal health has recently been highlighted (593). Vitamin K can decrease intestinal oxidative stress, inhibit LPS-induced inflammation (595), and the production of cytokines with pro-inflammatory characteristics (593).

The caloric intake between SLE patients and HC did not differ. Hence, the lower intake of minerals and vitamins is rather related to a worse dietary pattern, translated into a lower adherence to the Mediterranean Diet, than to differences in caloric intake. Fruits and vegetables are relevant dietary sources of vitamins and minerals. We have demonstrated that SLE patients have a lower intake of these foods than HC. Thus, encouraging a higher intake of fruits and vegetables will naturally increase mineral intake, which may be a good strategy to mitigate the detrimental effects of mineral deficiency.

Obesity is associated with several chronic inflammatory and immune-mediated diseases (493), and there seems to be a link between these diseases, obesity and the gut microbiota, which justifies the assessment of the anthropometric measures and body composition of the participants in this study. Apart from disease duration, obesity has been associated with a higher incidence of LN and organ damage, higher cumulative prednisolone dosage, and more frequent prescription of immunosuppressant drugs (503). In our analysis, despite 40% of SLE patients and 17% of HC excess weight or obesity, the differences were not significantly different ($p=0.2833$), which was also found by some authors (493)(538)(596) but not all (496)(502)(579). The diverse prevalence of overweight and obesity may be related to the mean age differences between populations, as age is an important factor influencing BMI. Our small sample size did not allow for a detailed characterization of the disease activity according to BMI. However, some authors found an association between excess weight and disease activity (502).

BMI is an easy and widely used tool to assess the prevalence of obesity, but it does not account for the fat mass and fat-free mass distribution (536). Hence, the body composition of SLE patients and HC was assessed by whole-body plethysmography. Adult SLE patients had a higher fat mass percentage than adult HC ($p=0.0118$). This may be explained by prolonged medication use, lower adherence to the Mediterranean diet, decreased physical activity, and increased sitting time. Even though obesity has been widely associated with decreased gut microbiota diversity and taxonomic alterations in its composition (499-501) we did not find differences in the gut microbiota diversity associated with fat mass or BMI. The small sample size may influence these results since stratification reduces the statistical power of the analysis.

Intra-abdominal fat mass increases the risk of unfavorable health outcomes, including cardiovascular disease. Considering that waist circumference provides a simple method to identify individuals at increased risk of obesity-associated illness due to abdominal fat deposition (523), the waist circumference of SLE patients and HC was assessed. SLE patients showed a higher risk for cardiovascular events than HC ($p=0.0143$). Other authors also found a higher prevalence of increased risk for cardiovascular events assessed by waist circumference (579). Moreover, a shorter event-free survival from cardiovascular events and a threefold increase in cardiovascular risk were associated with higher waist circumference, higher triglycerides levels, and the presence of diabetes mellitus in patients with mild SLE for longer than ten years (597). An increased waist circumference and BMI were also reported in adolescent SLE patients (342). This is particularly relevant, as together with increased fat mass, low adherence to the Mediterranean diet, and low intake of essential nutrients to maintain blood pressure homeostasis, it shows that SLE patients' lifestyle habits are not protective against cardiovascular events.

The waist/height ratio is an interesting index that can be used in clinical practice as a predictive marker of obesity (598). It is also a measure of central fat and metabolic risk (538) in adults and

adolescents (598). In addition, the waist/height ratio has been considered a predictive factor of hypertension (599)(600), glucose intolerance (600), and cardiovascular events (601). We found that patients had a higher waist/height ratio than HC ($p=0.0303$), and 48.5% of SLE patients were at high metabolic risk ($p=0.0168$). Other authors also found an increased waist/height ratio in SLE patients associated with cardiorespiratory fitness (602). A higher waist/height ratio has also been associated with inflammation markers such as IL-6 and C-reactive protein (598).

In SLE, physical activity is inversely associated with blood pressure, cardiovascular risk (513)(596), and fatigue (514). It improves physical and executive functions (515), LN (603), body weight and composition (602), sleep and vitality (604), and reduces inflammatory markers (605) and pain (506). On the other hand, sedentary time is associated with disease activity, depression, and anxiety (343). We have assessed the physical activity of SLE patients and HC with the IPAQ questionnaire. SLE patients had lower physical activity than HC ($p=0.0302$) and spent more time sitting than HC ($p=0.0479$). In the Portuguese population, 27.1% are active, while in our cohort, only 19.4% of SLE patients were active (392).

Other authors also found that SLE patients had lower physical activity (343)(512)(596)(604). However, most Danish SLE seem to be active (606). The different results may be related to the heterogeneity in assessment methods (607). Self-reported assessment methods are practical but prone to overestimation compared to objective measures (512). For example, in a population of RA patients, IPAQ was only modestly correlated with accelerometer measures (607). Moreover, differences in disease activity and clinical manifestations between populations may also explain the different results.

Physical activity can modulate the gut microbiota and improve the gut barrier integrity (508)(608), which may explain the moderate inverse correlation between GLP-1 and physical activity ($r=-0.3720$, $p=0.0278$) and the moderate positive correlation between GLP-1 and sitting time ($r=0.4329$, $p=0.0094$).

There are several objective reasons for the low physical activity and high sedentary time in SLE, including disease activity, pain, fatigue, mood disorders, altered sleep patterns, and reduced physical capacity. However, it is also possible that patients' perception of the disease-associated limitations may influence their proactivity. In a qualitative study, 78% of patients believed that SLE disallowed physical activity despite considering it beneficial in improving mobility and decreasing stiffness and muscle weakness (609). In addition, some patients believed physical activity could precipitate flares and increase pain. When asked about the main barriers to physical activity, patients highlighted lack of time due to family and work obligations, weather, comorbidities, and lack of motivation, sometimes associated with SLE manifestations and medication adverse effects (609). Considering the many benefits of physical activity and exercise and the fact that sedentary behavior may fuel a vicious circle in which lower physical capacity leads to lower physical activity that enhances physical impairment, physical activity must be encouraged in SLE patients.

This work has some limitations. The recruitment was delayed by the SARS-COV2 pandemic, which affected patients' in-person clinical visits. Moreover, the several assessments needed were time-consuming, which led to some participation refusals, and the healthy controls recruitment required tight criteria. Thus, these factors contributed to the small sample size and class imbalance that hindered some data analyses and stratification.

The kit used in the feces sample collection ensured there was no sample degradation after collection. However, it was a self-collection kit, which is subject to variability. Furthermore, the low sequencing depth of the bacterial DNA may have influenced the sensitivity of the gut microbiota analysis, limiting the detection of less abundant species and the gut microbiota characterization.

In the whole-body plethysmography, the total lung capacity and residual volume were estimated once its measurement procedure was not feasible by all the participants, particularly children. Although the estimation of this variable has comparable results to the measured ones, some variations may exist.

Self-reported questionnaires are susceptible to recall bias. While physical activity questionnaires are prone to over-reporting, dietary 24-hour recall is prone to under-reporting. Individual interviews by a trained professional and pictorial models were employed to minimize the recall bias on the dietary 24-hour recall. However, bias may still be present.

Finally, we conducted an observational study in humans, so these results do not reflect a cause-effect relationship but solely associations between variables, which is the result of the study design. The interplay between diet, gut microbiota, and gut permeability is complex, and certainly, several variables will influence each other, so it is difficult to isolate the unique effect of each variable. Nevertheless, our goal was to better characterize, globally, diet, physical activity, body composition, and gut microbiota, and this goal was reached.

CHAPTER 10. Conclusion

This work shows, for the first time, that the gut microbiota of Portuguese adult and children SLE patients has decreased diversity compared to healthy controls. It also shows that SLE patients with LN have increased bacterial concentrations of the *Rikenellaceae* family than patients without LN.

We have also demonstrated that higher compliance with the Mediterranean diet was associated with a more diverse gut microbiota, pointing out that a healthy dietary pattern is beneficial.

SLE patients had a low intake of ALA and EPA fatty acids, a high n-6/n-3 PUFA ratio, and a low intake of some SFA and MUFA fatty acids with anti-inflammatory potential. Furthermore, we have shown that SLE patients have low compliance with the Mediterranean diet, with a particularly low intake of fruits, vegetables, pulses, and nuts. Glucose and fructose were also less consumed, probably due to a low intake of fruits and vegetables, which may also explain the low intake of minerals such as potassium, magnesium, iron, manganese, and vitamins like B6, K1, and folate.

The dietary pattern, low physical activity, and high sedentary time of SLE patients may explain their higher body fat percentage when compared with HC. SLE patients also showed a higher cardiovascular and metabolic risk and high visceral fat, assessed by waist circumference and waist/height ratio.

Taken together, our results point out that diet affects the gut microbiota diversity, and, together with physical activity, may have a role in the risk of comorbidities in this population. Dysbiosis of the gut microbiota may play a role in SLE pathogenesis by impairing the gut barrier integrity, favoring endotoxemia, and, through this mechanism, perpetuating the chronic activation of the immune system. However, future studies with a larger sample will be necessary to validate our findings. Moreover, these results do not reflect casualty but merely associations between variables. Nevertheless, this study establishes strong foundations for the development of strategies to improve diet and physical activity, reestablish microbiota equilibrium, and increase the integrity of the gut barrier in SLE. These strategies will undoubtedly decrease the cardiovascular risk in these patients, improve fatigue, and may also be responsible for better disease control.

FINAL NOTE

I had the opportunity to be involved in each step of this translational research work, which included: submit the project for approval from the Ethics committee of *Centro Académico de Medicina de Lisboa* / *Hospital de Santa Maria*; write the informed consents; recruit patients and healthy controls; select the variables to be studied; plan the best methods to collect the samples; assess the body composition and diet; extract DNA and assess GLP-1 concentrations and all the lab work and statistical analysis. In addition, I also had the opportunity to participate in the submission of the project to additional funding opportunities.

The preliminary results of this work were presented in several scientific meetings:

- iMed Conference. Innovate Competition, Lisbon, Portugal, 2021 (**Appendix 8**)
- XXVII Jornadas de Pediatria do Hospital de Santa Maria, Lisbon, Portugal, 2022: oral communication (**Appendix 9**)
- 22.ºCongresso Nacional de Pediatria, Porto, Portugal, 2022:oral communication (**Appendix 10**)
- European Congress of Rheumatology EULAR 2023, Milan, Italy, 2023: poster (**Appendix 11**)
- XXV Congresso da Associação Portuguesa de Nutrição Entérica e Parentérica (APNEP), Lisbon, Portugal, 2023: Oral presentation; Table: *Exploring the Gutsy World of Microbiota in Disease*. (**Appendix 12**)
- VII Congresso Hispano-Português de Nefrologia Pediátrica, Lisbon, Portugal, 2023: long oral communication; awarded Best Portuguese Communication (**Appendix 13**)

Moreover, I also wrote the review paper *Let's review the gut microbiota in Systemic Lupus Erythematosus*, which was published in the journal *Exploration of Medicine* (Explor Med. 2022;3:540–560 DOI: https://doi.org/10.37349/emed.2022.00112).

Finally, the following papers have been submitted for publication:

Reviewing the Evidence on Diet and Systemic Lupus Erythematosus, a review paper on the effect of nutrients in SLE, and on how diet may contribute to its management. Submitted to the scientific journal Nutrients.

GUT-LUPUS: connecting the dots between gut microbiota, gut permeability, and the pathogenesis of systemic lupus erythematosus, a paper that summarizes this thesis work. Submitted to the scientific journal Annals of the Rheumatic Diseases.

Considering that I will pursue a Ph.D. in the near future to broaden my scientific knowledge and experience, I have also recently integrated a molecular biology project that will study whether abnormal X-chromosome inactivation confers an increased risk for developing systemic lupus erythematosus in women.

Finally, since the results presented in this dissertation are the result of an exploratory research project, besides the need to increase our sample, several new research paths developed, such as the study of the fungi of the gut microbiota and the analysis of the metabolomic profile of the SLE patients. Thus, this project was the perfect beginning for an exciting research path.

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SUPPLEMENTAL TABLES

Table S12.1. Main functions of B vitamins and their main bacterial producers in the gut

Vitamin	Name	Functions	Deficiency	Vitamin-producing bacteria	References
B1	Thiamin	Antioxidant; inhibition of stimulation of NF-kB; gut immune regulatory functions	Neuroinflammation; T cell infiltration; overexpression of pro-inflammatory cytokines (IL-1, IL-6, TNF-a); reduction of Payer's patches and decrease of B-cell follicles size.	90% of the gut Bacteroides; Prevotella and Desulfovibrio	Mitra S, et al. 2022; Hossain K, et al. 2022
B2	Riboflavin	Enzymatic cofactor; energy metabolism; antioxidant; phagocytic activity modulation; postnatal development of the gastrointestinal tract; modulate gut mucosal immunity	Macrophage activity and survival impairement.Alterations in the crypts morphology (gut); loss of proliferative potentials in intestinal cells.	Baceteroidetes and Fusobacteria; ~92% of Proteobacteria; ~50% of Firmicutes	Mitra S, et al. 2022; Hossain K, et al. 2022 Magnúsdóttir et al. 2015
B3	Niacin	Inflammation control by vascular permeability inhibition in intestinal tissues; protection of colonic epithelial cells against the dextran-sulfate-sodium-induced apoptosis; cell proliferation stimulation; maintenance of intestinal epithelium barrier.	Lower plasma levels in patients with Crohn's disease.	Class: Bacilli (4 strains); Clostridia (44 strains); Proteobacteria (29 strains). Bacteroides fragilis. Bacillus and Clostridium; *Prevotella copri; Ruminococcus lactaris*	Mitra S, et al. 2022; Hossain K, et al. 2022
B5	Pantothenic Acid	Precursor of coenzyme A; acyl-carrier protein; cell growth, neurotransmitter synthesis, and fatty acid oxidation.		Nearly all Bacteroidetes and Proteobacteria; *Escherichia coli; Salmonella typhimurium; Corynebacterium glutamicum.*	Mitra S, et al. 2022; Hossain K, et al. 2022
B6	Pyridoxine	Amino acid biosynthesis and catabolism; anti-inflammatory properties; fatty acid and neurotransmitter biosynthesis; IL-1, IL-18, and LPS-primed macrophages inibition; protection from fatal endotoxins; Increased T-cell responses; Supplementation increased number of M cells in the appendix.	Suppression of T-cell proliferation; decreased IL-2; Increased IL-4; decrease in anti-inflammatory cytokines (TGF-b, IL-4, IL-10, IL-11, IL-13); increase in pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IL-15, IL-17); reduction of β-diversity; alteration of intestinal metabolites; increased symptoms in irritable bowel disease.	*Bacteroides fragilis* and *Prevotella copri* (Bacteroidetes), *Bifidobacterium longum* and *Collinsella aerofaciens* (Actinobacteria), and *Helicobacter pylori* (Proteobacteria)	Mitra S, et al. 2022; Hossain K, et al. 2022
B7	Biotin	Coenzyme for glycolysis, cell signalling, and epigenetic regulation; modulation of IL-1 production.	Higher IL-1 mRNA.	*Bacteroides fragilis, Prevotella copri, Fusobacterium varium,* and *Campylobacter coli*	Mitra S, et al. 2022; Hossain K, et al. 2022
B9	Folate	DNA methylation; T lymphocyte proliferation; delayed hypersensitive responses; increased phagocytosis; upregulated immunoglobulin production; Treg maintenance; reduction of NK cell cytotoxicity; Regeneration of the intestine lining.	Abnormal DNA and RNA synthesis and methyl metabolism; Decreased Treg cells in the small intestine; increase in the duodenal and jejunal mucosa crypt depth; reduced villus/crypt ratio; alteration of the intestinal cell morphology; increased occurrence of intestinal carcinogenesis.	Nearly all Bacteroidetes, and most Fusobacteria and Proteobacteria; *Prevotella copri, Clostridium difficile, Lactobacillus plantarum, L. reuteri, L. sakei, L. delbrueckii, L. fermentum, Streptococcus thermophilus, Bifidobacterium* spp. (some species such as *B. bifidum, B. infantis, B. adolescentis, B. dentium* and *B. longum*), *Fusobacterium varium*, *Salmonella enterica.*	Mitra S, et al. 2022; Hossain K, et al. 2022 Magnúsdóttir et al. 2015
B12	Cobalamin	Methyl donor synthesis; nucleic acid synthesis; protein and lipid metabolism; cofactor for methionine synthase in sulphur amino acid metabolism; central nervous system homeostasis; erythrocytes synthesis; cytotoxic immune responses mediated by NK and CD8+ T cells.	Lymphocite downregulation; Reduced NK cell activity; Decreased CD8+ T-cells and higher CD4+ T-cells; High CD4+/CD8+ ratio.	Fusobacteria, *Pseudomonas denitrificans, Bacillus megaterium, Propionibacterium freudenreichi, Bacteroides fragilis, Prevotella copri, Clostridium difficile, Faecalibacterium prausnitzii, Ruminococcus lactaris, Bifidobacterium animalis, B.infantis, B.longum, Fusobacterium varium, Lactobacillus* spp, *L. reuteri*, *Listeria monocytogenes*	Mitra S, et al. 2022; Hossain K, et al. 2022 Magnúsdóttir et al. 2015

Table S12.2. Clinical manifestations of SLE patients (adult and children) at diagnosis. (A) Manifestations included in the American College of Rheumatology (ACR) 1997 and Systemic Lupus International Collaborating Clinics (SLICC) 2012 criteria. (B) Manifestations included in the 2019 European League Against Rheumatism/ ACR classification criteria (EULAR/ACR 2019). ANA- Antinuclear antibodies, anti-ds-DNA- Antibodies to double-stranded DNA, SLE- systemic lupus erythematosus, n – total number of individuals, y- years. p-value significant < 0.05.

ACR 1997						**SLICC 2012** (items not included in ACR 1997)				
		n total (<18y / ≥18y)	**% total**	**p-value***** (<18y vs. ≥18y)				**n total** (<18y / ≥18y)	**% total**	**p-value***** (<18y vs. ≥18y)
Malar rash	Yes	24 (9/15)	72.7%	0.68		Acute / subacute cutaneous lupus	Yes	24 (9/15)	72.7%	0.68
	No	9 (2/7)	27.3%				No	9 (2/7)	27.3%	
Discoid rash	Yes	4 (0/4)	12.1%	0.28		Chronic cutaneous lupus	Yes	3 (0/3)	9.1%	0.53
	No	29 (11/18)	87.9%				No	30 (11/19)	90.9%	
Photosensitivity	Yes	23 (9/14)	69.7%	0.43		Alopecia	Yes	15 (6/9)	45.5%	0.49
	No	10 (2/8)	30.3%				No	18 (5/13)	54.5%	
Oral/nasal ulcers*	Yes	11 (6/5)	34.4%	0.12		Neurological Disease	Yes	1 (0/1)	3.0%	>0.99
	No	21 (5/16)	65.6%				No	32 (11/21)	97.0%	
Arthritis	Yes	28 (10/18)	84.8%	0.64		Hemolytic anemia	Yes	10 (7/3)	30.3%	**0.006**
	No	5 (1/4)	15.2%				No	23 (4/19)	69.7%	
Serositis*	Yes	6 (3/3)	18.8%	0.39		Leukopenia (<4000/mm³) *	Yes	20 (10/10)	62.5%	**0.023**
	No	26 (8/18)	81.3%				No	12 (1/11)	37.5%	
Lupus Nephritis	Yes	22 (7/15)	66.7%	>0.99		Thrombocytopenia (<100,000/mm³)*	Yes	11 (2/9)	34.4%	0.25
	No	11 (4/7)	33.3%				No	21 (9/12)	65.6%	
Neurological Disease	Yes	1 (0/1)	3.0%	>0.99		Low complement (C3 and/or C4)	Yes	27 (11/16)	81.8%	0.08
	No	32 (11/21)	97%				No	6 (0/6)	18.2%	
Hematological disease	Yes	25 (11/14)	75.8%	**0.03**		Positive Direct Coombs test**	Yes	9 (6/3)	69.2%	0.27
	No	8 (0/8)	24.2%				No	4 (1/3)	30.8%	
Immunological disease*	Yes	33 (11/22)	100%	NA		Lupus nephritis + ANA / anti-dsDNA positivity	Yes	18 (7/11)	54.5%	0.71
	No						No	15 (4/11)	45.5%	
ANA positivity	Yes	33 (11/22)	100%	NA						

EULAR/ACR 2019 (items not included in ACR1997 nor SLICC2012)				
		n total (<18y / ≥18y)	**% total**	**p-value***** (<18y vs. ≥18y)
Constitutional manifestations	Yes	24 (11/13)	75.0%	**0.03**
	No	8 (0/8)	25.0%	
Neuropsychiatric	Yes	1 (0/1)	3.0%	>0.99
	No	32 (11/21)	97.0%	
Mucocutaneous	Yes	28 (9/19)	84.8%	>0.99
	No	5 (2/3)	15.2%	
Low complement (C3 and C4)*	Yes	21 (10/11)	65.6%	0.05
	No	11 (1/10)	34.4%	
Low complement (C3 or C4)*	Yes	25 (11/14)	78.1%	0.07
	No	7 (0/7)	21.9%	

*n=32; **n=13; ***Fisher's exact test

Table S12.3. Treatment strategies of SLE patients (n=33). ACE - Angiotensin-converting enzyme; ARB - Angiotensin receptor blocker; IvIg – Intravenous immunoglobulin; SLE- systemic lupus erythematosus; n – total number of individuals.

Treatment		n (%)
Hydroxychloroquine		
	Used in the past	4 (12.1%)
	Current Use	29 (87.9%)
	No	0
Prednisolone		
	Used in the past	3 (9.1%)
	Current Use	29 (87.9%)
	No	1 (3%)
Mycophenolate mofetil*		
	Used in the past	0
	Current Use	19 (59.4%)
	No	13 (40.6%)
Azathioprine*		
	Used in the past	6 (18.8%)
	Current Use	5 (15.6%)
	No	21 (65.6%)
Methotrexate		
	Used in the past	6 (18.2)
	Current Use	4 (12.1%)
	No	23 (69.7%)
Belimumab		
	Used in the past	0
	Current Use	6 (18.2%)
	No	27 (81.8%)
Rituximab*		
	Used in the past	3 (9.4%)
	Current Use	1 (3.1%)
	No	28 (87.5%)

Treatment		n (%)
Cyclophosphamide*		
	Used in the past	6 (18.8%)
	Current Use	0
	No	26 (81.2%)
IvIg*		
	Used in the past	3 (9.4%)
	Current Use	0
	No	29 (90.6%)
Tacrolimus*		
	Used in the past	1 (3.1%)
	Current Use	2 (6.3%)
	No	29 (90.6%)
Cyclosporine*		
	Used in the past	1 (3.1%)
	Current Use	0
	No	31 (96.9%)
ACE inhibitor*		
	Used in the past	2 (6.3%)
	Current Use	12 (37.5%)
	No	18 (56.3%)
ARB*		
	Used in the past	1 (3.1%)
	Current Use	2 (6.3%)
	No	29 (90.6%)

* n=32

Table S12.4. Analyzed parameters for the adherence to the Mediterranean Diet, assessed with the PREDIMED (adults), and KIDMED (children) questionnaires. The questionnaires scores were categorized in "good adherence to the Mediterranean Diet" and "Insufficient adherence to the Mediterranean Diet". HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.

Adherence to the Mediterranean Diet (2 categories)						
		n	**Good Adherence** (n/%)	**Insufficient Adherence** (n/%)	**Test**	**p-value**
Disease Status	SLE	32	4 (12.5%)	28 (87.5%)	Fisher's exact test	**0.0222**
	HC	13	6 (46.2%)	7 (53.8%)		
Age (years) 2 categories	SLE <18	10	4 (40.0%)	6 (60.0%)	Fisher's exact test	>0.9999
	HC < 18	1	0 (0%)	1 (100%)		
	SLE ≥18	22	0 (0%)	22 (100%)	Fisher's exact test	**0.0007**
	HC ≥ 18	12	6 (50.0%)	6 (50.0%)		
Age at diagnosis (years) 2 categories	<18	14	4 (28.6%)	10 (71.4%)	Fisher's exact test	**0.0278**
	≥18	18	0 (0%)	18 (100%)		
Disease duration (years) 2 categories	<2	5	3 (60%)	2 (40%)	Fisher's exact test	**0.0076**
	≥2	27	26 (96.3%)	1 (3.7%)		
Lupus Nephritis	With LN	21	3	18	Fisher's exact test	>0.9999
	Without LN	11	1	10		

Table S12.5. Intake of macro and micronutrients, adherence to the Mediterranean diet, and level of physical activity of SLE patients and healthy controls (HC) compared with the Portuguese population. IAN-AF- *Inquérito Alimentar Nacional e de Atividade Física* 2015-2016. SLE - systemic lupus erythematosus, mg- milligrams, ug- micrograms, TEI – Total Energy Intake, *- mean.

		Portuguese population (IAN-AF)	SLE	HC
Mediterranean Diet	Insufficient adherence (adults)	84.3%	87.5%	53.8%
Physical Activity	Active	27.1%	19.4%	30.8%
	Moderately Active	30.3%	38.7%	53.8%
	Sedentary	42.6%	41.9%	15.4%
Fiber (mean)	Fiber	17.2 g	12.8 g	22.6 g
Fatty Acids (median)	Saturated	10.2% TEI	13% TEI	10.1% TEI
	Monounsaturated	12.5% TEI	11.6% TEI	14.3% TEI
	Polyunsaturated	5% TEI	4.6% TEI	5.2% TEI
Minerals (median/mean*)	Potassium*	3055 mg	2420 mg	3224 mg
	Magnesium	269 mg	219.2 mg	325.9 mg
	Iron	11 mg	9.3 mg	13 mg
	Zinc	9.7 mg	7.7 mg	9.9 mg
	Calcium*	774 mg	793.8 mg	1003 mg
	Phosphorus	1198 mg	1178 mg	1245 mg
	Sodium*	2872 mg	1899 mg	2250 mg
Vitamins (median/mean*)	A	706 ug	881.5 ug	1512 ug
	B1*	1.3 mg	1.3 mg	1.7 mg
	B2	1.5 mg	1.2 mg	1.5 mg
	B3	34.3 mg	18.7 mg	24.1 mg
	B6	1.8 mg	1.45 mg	2.15 mg
	Folate	221 ug	173.8 ug	265.3 ug
	B12	3.9 mg	4.2 mg	6.35 mg
	C*	109 mg	62.3 mg	87.3 mg
	E	8.7 mg	5.75 mg	7.75 mg

Table S12.6. Dietary intake of macronutrients. g-grams, HC- healthy controls, kcal- kilocalories, MUFA- monounsaturated fatty acids, n-3 PUFA- omega-3, n-6 PUFA- omega-6, PUFA – polyunsaturated fatty acids, SFA- saturated fatty acids, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Kcal	SLE	30	1403	883.8-3598.1	Mann-Whitney test	0.0557
	HC	12	1638	941.4-2787.2		
Protein (g)	SLE	30	80.58	40.1-137.8	t-test	0.3275
	HC	12	89.73	39.6-147		
Lipids (g)	SLE	30	52.65	28.7-101.7	Mann-Whitney test	0.2976
	HC	12	64.10	31.4-125.7		
Carbohydrates (g)	SLE	30	177.1	96.1-580.4	Mann-Whitney test	0.0818
	HC	12	212.9	133.1-284.8		
Total Sugars (g)	SLE	30	53.85	9.5-145.6	Mann-Whitney test	0.4532
	HC	12	62.40	37.3-130.3		
Glucose	SLE	30	7.76	1.4-21.2	t-test	**0.0027**

(g)	HC	12	14.56	4.8-36.2		
Fructose (g)	SLE	30	8.857	1.7-17.7	t-test	**0.0006**
	HC	12	15.82	7.4-30.8		
Sucrose (g)	SLE	30	19.50	1.5-89.6	Mann-Whitney test	0.7151
	HC	12	20.85	6.6-47.2		
Maltose (g)	SLE	30	3.30	0.4-10.4	Mann-Whitney test	0.5045
	HC	12	3.95	0.3-10.6		
Lactose (g)	SLE	30	10.65	0.5-31.5	t-test	0.8951
	HC	12	11.03	0.2-34.7		
SFA (g)	SLE	30	18.3	9.4-42.7	Mann-Whitney test	0.3668
	HC	12	20.55	9.7-41.1		
MUFA (g)	SLE	30	18.12	11.2-41.6	Mann-Whitney test	0.1360
	HC	12	26.10	11-58		
PUFA (g)	SLE	30	7.2	2.5-27.6	Mann-Whitney test	0.0805
	HC	12	9.45	4.9-17.7		
Trans Fatty Acids (g)	SLE	30	0.90	0.2-2.9	Mann-Whitney test	0.4760
	HC	12	1.00	0.4-4.9		
Total n-3 PUFA (g)	SLE	30	0.3	0.1-1.5	Mann-Whitney test	**0.0248**
	HC	12	0.6	0.1-2.4		
Total n-6 PUFA (g)	SLE	30	3.1	1-13.6	Mann-Whitney test	0.7571
	HC	12	3.2	0.5-6.1		
n-6/n-3	SLE	30	9.55	0.9-29	Mann-Whitney test	**0.0042**
	HC	12	6.35	1.1-12.9		
Fiber (g)	SLE	30	12.80	4.8-23.1	t-test	**<0.0001**
	HC	12	22.56	11.1-35		
Water (ml) Not included in food	SLE	30	1167	417-3333	Mann-Whitney test	0.7465
	HC	12	1042	397-2333		

Table S12.7. Dietary intake of saturated fatty acids (SFA). g-grams, HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Butyric acid SFA 4:0 (g)	SLE	30	0.2547	0.03-0.64	t-test	0.4795
	HC	12	0.2992	0-0.63		
Caproic acid SFA 6:0 (g)	SLE	30	0.1577	0.02-0.4	t-test	0.3324
	HC	12	0.1933	0-0.38		
Caprylic acid SFA 8:0 (g)	SLE	30	0.110	0.02-0.42	Mann-Whitney test	0.3099
	HC	12	0.185	0-0.33		
Capric acid SFA 10:0 (g)	SLE	30	0.2273	0.03-0.53	t-test	0.1816
	HC	12	0.3000	0-0.57		
Lauric acid SFA 12:0 (g)	SLE	30	0.340	0.06-3.01	Mann-Whitney test	0.1358
	HC	12	0.525	0.02-1.23		
Myristic Acid SFA 14:0 (g)	SLE	30	1.067	0.16-2.22	t-test	**0.0455**
	HC	12	1.535	0.12-3.2		
Pentadecanoic acid SFA 15:0 (g)	SLE	30	0.1380	0.02-0.31	t-test	0.1696
	HC	12	0.1842	0-0.5		

Palmitic Acid SFA 16:0 (g)	SLE	30	6.665	2.54-19.53	Mann-Whitney test	0.1320
	HC	12	8.585	3.54-22.1		
Margaric acid SFA 17:0 (g)	SLE	30	0.120	0.03-0.28	Mann-Whitney test	0.3230
	HC	12	0.145	0.07-0.49		
Stearic Acid SFA 18:0 (g)	SLE	30	2.955	0.80-9.08	Mann-Whitney test	0.5136
	HC	12	3.480	1.28-7.56		
Arachidic acid SFA 20:0 (g)	SLE	30	0.080	0.03-0.24	Mann-Whitney test	**0.0461**
	HC	12	0.125	0.06-0.27		
Behenic acid SFA 22:0 (g)	SLE	30	0.020	0.01-0.29	Mann-Whitney test	**0.0160**
	HC	12	0.035	0.02-0.23		
Lignoceric acid SFA 24:0 (g)	SLE	30	0.03	0-0.10	Mann-Whitney test	0.7310
	HC	12	0.04	0-0.06		

Table S12.8. Dietary intake of monounsaturated fatty acids (MUFA). g-grams, HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Caproleic acid MUFA 10:1 (g)	SLE	30	0.01	0-0.03	Mann-Whitney test	0.7171
	HC	12	0.01	0-0.03		
MUFA 12:1 (g)	SLE	30	0.000	0-0.01	Mann-Whitney test	>0.9999
	HC	12	0.000	0-0.01		
Myristoleic acid MUFA 14:1 (g)	SLE	30	0.050	0-0.16	Mann-Whitney test	0.4680
	HC	12	0.035	0-0.18		
MUFA 15:1 (g)	SLE	30	0.000	0-0.06	Mann-Whitney test	0.5913
	HC	12	0.000	0-0.07		
Palmitoleic Acid MUFA 16:1 (g)	SLE	30	0.53	0.18-1.23	Mann-Whitney test	**0.0105**
	HC	12	0.72	0.25-3.64		
MUFA 17:1 (g)	SLE	30	0.050	0.01-0.13	Mann-Whitney test	0.5081
	HC	12	0.055	0.02-0.12		
Oleic Acid MUFA 18:1 (g)	SLE	30	11.76	5.65-29.6	Mann-Whitney test	**0.0461**
	HC	12	18.87	6.76-31.62		
Eicosenoic acid MUFA 20:1 (g)	SLE	30	0.08	0.03-1.28	Mann-Whitney test	**0.0045**
	HC	12	0.20	0.05-6.07		
Erucic acid MUFA 22:1 (g)	SLE	30	0.010	0-1.82	Mann-Whitney test	**0.0028**
	HC	12	0.045	0-6.31		

Table S12.9. Dietary intake of amino acids. HC- healthy controls, mg-milligrams, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Isoleucine (mg)	SLE	30	1286	343.4-3189.9	Mann-Whitney test	0.8584
	HC	12	1227	520.4-3497.8		
Leucine (mg)	SLE	30	2288	614.8-5247.2	t-test	0.5606
	HC	12	2548	946.1-5705.2		
Lysine (mg)	SLE	30	2074	427.2-5185.8	Mann-Whitney test	0.9017
	HC	12	1590	558-5798.6		
Methionine	SLE	30	681.2	170.6-1665.6	Mann-Whitney test	0.8800

(mg)	HC	12	620.3	244.1-1904.3		
Cystine (mg)	SLE	30	363.3	80.5-951.3	Mann-Whitney test	0.4659
	HC	12	356.1	174.7-912.6		
Phenylalanine (mg)	SLE	30	1322	345.3-3183.3	t-test	0.5818
	HC	12	1464	602.6-3106.2		
Tyrosine (mg)	SLE	30	978.2	272.3-2419.7	Mann-Whitney test	0.6904
	HC	12	988.4	409-2588.9		
Threonine (mg)	SLE	30	1245	279.7-2812.6	t-test	0.7270
	HC	12	1333	453.8-3145		
Tryptophan (mg)	SLE	30	329.4	106.4-905.6	Mann-Whitney test	0.4829
	HC	12	372.9	172.2-1008.3		
Valine (mg)	SLE	30	1595	397.6-3526.6	t-test	0.5492
	HC	12	1780	637.1-3929.6		
Arginine (mg)	SLE	30	1654	271.3-3862.8	t-test	0.6469
	HC	12	1816	615.7-4084.9		
Histidine (mg)	SLE	30	774.5	214.1-2271.5	Mann-Whitney test	0.8369
	HC	12	834.8	291.6-2249.5		
Alanine (mg)	SLE	30	1589	354.3-3766.9	t-test	0.5920
	HC	12	1771	558.1-4230		
Aspartic acid (mg)	SLE	30	2483	596.7-6715.6	Mann-Whitney test	0.7108
	HC	12	2556	1080.7-6964.7		
Glutamic acid (mg)	SLE	30	4436	1317.6-12297.3	Mann-Whitney test	0.8369
	HC	12	3700	2503.1-12624.9		
Glycine (mg)	SLE	30	1286	222.8-3306.8	Mann-Whitney test	0.8584
	HC	12	1068	435.3-3451.8		
Proline (mg)	SLE	30	1415	455.2-3429.1	Mann-Whitney test	0.6904
	HC	12	1298	789.9-3772.8		
Serine (mg)	SLE	30	1236	310.5-3008.8	Mann-Whitney test	0.9453
	HC	12	1042	619.8-3311.8		

Table S12.10. Dietary intake of minerals. HC- healthy controls, mg-milligrams, n – total number of individuals, SLE- systemic lupus erythematosus, ug- micrograms. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Sodium (Na) (mg)	SLE	30	1899	864.1-3493.2	t-test	0.1256
	HC	12	2250	1379.6-4043.4		
Potassium (K) (mg)	SLE	30	2420	1140.8-4718.4	t-test	**0.0062**
	HC	12	3224	2105.8-5608.6		
Calcium (Ca) (mg)	SLE	30	793.8	394.6-1415	t-test	0.0681
	HC	12	1003	402.3-1970.6		
Magnesium (Mg) (mg)	SLE	30	219.2	116-535.7	Mann-Whitney test	**0.0004**
	HC	12	325.9	184-890		
Phosphorus (P)	SLE	30	1178	462.2-1743.5		0.1794

(mg)	HC	12	1245	881.4-2736.6	Mann-Whitney test	
Iron (Fe) (mg)	SLE	30	9.30	4.9-22.4	Mann-Whitney test	**0.0002**
	HC	12	12.95	11.1-26.6		
Copper (Cu) (mg)	SLE	30	0.90	0.5-16.6	Mann-Whitney test	0.0657
	HC	12	1.10	0.6-3.7		
Zinc (Zn) (mg)	SLE	30	7.70	3.2-19.2	Mann-Whitney test	0.0547
	HC	12	9.90	4.2-40.5		
Selenium (Se) (ug)	SLE	30	55.80	27.1-204.9	Mann-Whitney test	0.3674
	HC	12	62.95	24.9-122.6		
Manganese (Mn) (mg)	SLE	30	2.0	1-4-04	Mann-Whitney test	**0.0004**
	HC	12	3.4	1.9-4.8		
Iodine (I) (mg)	SLE	30	140.4	50.3-301.95	Mann-Whitney test	0.5544
	HC	12	189.6	63.5-278.2		

Table S12.11. Dietary intake of vitamins. HC- healthy controls, mg-milligrams, n – total number of individuals, SLE- systemic lupus erythematosus, ug- micrograms. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

		n	Median/Mean*	Min - Max	Test	p-value
Thiamin (B1) (mg)	SLE	30	1.317	0.5-2.8	t-test	0.771
	HC	12	1.650	0.8-2.7		
Riboflavin (B2) (mg)	SLE	30	1.20	0.5-3.6	Mann-Whitney test	0.0926
	HC	12	1.50	0.9-3.0		
Niacin (B3) (mg)	SLE	30	18.70	7.4-49.9	Mann-Whitney test	0.1397
	HC	12	24.05	8-39.1		
Pantothenic acid (B5) (mg)	SLE	30	4.25	1.7-10.1	Mann-Whitney test	0.2105
	HC	12	5.10	2.4-12.5		
Pyridoxine (B6) (mg)	SLE	30	1.45	0.6-2.8	Mann-Whitney test	**0.0060**
	HC	12	2.15	1.1-21.6		
Biotin (B7) (ug)	SLE	30	25.80	9.3-80.4	Mann-Whitney test	0.2488
	HC	12	35.10	15-80.1		
Folate (B9) (ug)	SLE	30	173.8	48.4-702.5	Mann-Whitney test	**0.0006**
	HC	12	265.3	160.2-687.3		
Cobalamin (B12) (mg)	SLE	30	4.15	1.3-508.1	Mann-Whitney test	0.2544
	HC	12	6.35	1.6-304.1		
Ascorbic Acid (C) (mg)	SLE	30	62.26	8.5-153.9	t-test	0.1015
	HC	12	87.30	21-193.7		
Calciferol (D) (ug)	SLE	30	1.90	0.2-51.8	Mann-Whitney test	0.3102
	HC	12	2.15	0.6-18		
Tocopherol (E) (mg)	SLE	30	5.75	2.2-23.8	Mann-Whitney test	0.0781
	HC	12	7.75	5.7-17.3		
Phytonadione (K1) (ug)	SLE	30	43.15	2.2-519.9	Mann-Whitney test	**0.0235**
	HC	12	126.4	19.4-682		
Retinol (A) (ug)	SLE	30	881.5	283.8-34839.2	Mann-Whitney test	0.2293
	HC	12	1512	358.7-2637.8		
Carotenoids	SLE	30	2082	120.4-10044.8	Mann-Whitney test	0.1535

(ug)	HC	12	4001	457.5-11746		
Alpha-carotene (ug)	SLE	30	429.9	0-2045	Mann-Whitney test	0.2610
	HC	12	941.6	4.1-1504.8		
Beta-carotene (ug)	SLE	30	1455	27-5993.9	Mann-Whitney test	0.1887
	HC	12	2940	269.2-5822.5		

Table S12.12. Met needs of the dietary reference intake (DRI) of macro and micronutrients. HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. *Population reference intake (EFSA), **Adequate intake (EFSA), *** Reference intake (EFSA), [+]Biomedicine & Pharmacotherapy. 2002 Oct;56(8):365–79. p-value significant < 0.05.

Dietary Reference Intake (DRI)								
	Meet DRI? (SLE)	n	%	Meet DRI? (HC)	n	%	Test	p-value
Carbohydrates***	Yes	19	63.3%	Yes	6	50%	Fisher's exact test	0.4982
	No	11	36.7%	No	6	50%		
Fiber**	Yes	1	3.3%	Yes	5	41.7%	Fisher's exact test	**0.0047**
	No	29	96.7%	No	7	58.3%		
EPA+DHA** (n-3 PUFA)	Yes	5	16.7%	Yes	6	50%	Fisher's exact test	**0.0491**
	No	25	83.3%	No	6	50%		
n6/n3[+] (4:1)	Yes	3	10%	Yes	4	33.3%	Fisher's exact test	0.0883
	No	27	90%	No	8	66.7%		
Potassium** (K)	Yes	3	10%	Yes	5	41.7%	Fisher's exact test	**0.0309**
	No	27	90%	No	7	58.3%		
Magnesium** (Mg)	Yes	8	26.7%	Yes	9	75%	Fisher's exact test	**0.0061**
	No	22	73.3%	No	3	25%		
Phosphorus** (P)	Yes	29	97%	Yes	12	100%	Fisher's exact test	>0.9999
	No	1	3%	No	0	0%		
Iron* (Fe)	Yes	6	20%	Yes	4	33.3%	Fisher's exact test	0.4331
	No	24	80%	No	8	66.7%		
Zinc* (Zn)	Yes	6	20%	Yes	5	41.7%	Fisher's exact test	0.2432
	No	24	80%	No	7	58.3%		
Manganese** (Mn)	Yes	6	20%	Yes	9	75%	Fisher's exact test	**0.0014**
	No	24	80%	No	3	25%		
Retinol* (A)	Yes	22	73.3%	Yes	9	75%	Fisher's exact test	>0.9999
	No	8	26.7%	No	3	25%		
Thiamin* (B1)	Yes	30	100%	Yes	12	100%	Fisher's exact test	>0.9999
	No	0	0%	No	0	0%		
Riboflavin (B2)*	Yes	7	23.3%	Yes	5	41.7%	Fisher's exact test	0.2740
	No	23	76.7%	No	7	58.3%		
Niacin* (B3)	Yes	26	86.7%	Yes	12	100%	Fisher's exact test	0.3082
	No	4	13.3%	No	0	0%		
Pantothenic Acid** (B5)	Yes	13	43.3%	Yes	7	58.3%	Fisher's exact test	0.4994
	No	17	56.7%	No	5	41.7%		
Pyridoxine* (B6)	Yes	16	53.3%	Yes	10	83.3%	Fisher's exact test	0.09
	No	14	46.7%	No	2	16.7%		
Biotin** (B7)	Yes	6	20%	Yes	5	41.7%	Fisher's exact test	0.2432
	No	24	80%	No	7	58.3%		

Folate* (B9)	Yes	2	6.7%	Yes	5	41.7%	Fisher's exact test	**0.0138**
	No	28	93.3%	No	7	58.3%		
Cobalamin (B12)**	Yes	16	53.3%	Yes	8	66.7%	Fisher's exact test	0.5059
	No	14	46.7%	No	4	33.3%		
Ascorbic Acid* (C)	Yes	8	26.7%	Yes	5	41.7%	Fisher's exact test	0.4635
	No	22	73.3%	No	7	58.3%		
Tocopherol** (E)	Yes	8	26.7%	Yes	4	33.3%	Fisher's exact test	0.7154
	No	22	73.3%	No	8	66.7%		
Phytonadione** (K1)	Yes	12	40%	Yes	9	75%	Fisher's exact test	0.0855
	No	18	60%	No	3	25%		

Table S12.13. Analyzed parameters for physical activity assessed with the International Physical Activity Questionnaire (IPAQ), validated for individuals with ages ≥ 14 years old. HC- healthy controls, HCQ- hydroxychloroquine n – total number of individuals, METs- metabolic equivalents of task, SLE- systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

IPAQ (METs) (≥ 14 years)						
		n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Disease Status	SLE	30	748	0-3306	Mann-Whitney test	**0.0302**
	HC	13	1780	40-3652		
Gender	SLE Female	26	678	0-3012	Mann-Whitney test	**0.0371**
	HC Female	11	1780	40-3652		
	SLE Male	4	1553	0-3306	Mann-Whitney test	>0.9999
	HC Male	2	1565	1280-1849.5		
	SLE+HC Female	37	967.5	0-3652	Mann-Whitney test	0.4170
	SLE+HC Male	6	1565	0-3306		
Age (years) 2 categories	SLE <18	8	888.8	80-3012	Mann Whitney test	0.3667
	SLE ≥ 18	22	645	0-3306		
	HC ≥ 18	12	1765	40-3652	Mann Whitney test (SLE vs HC ≥ 18)	**0.0366**
	HC < 18	1	1780			
Age (years) 5 categories	SLE <13	NA				
	SLE 13-18	8	888.75	80-3012	Kruskal-Wallis test	0.3524
	SLE ≥18-50	21	594	0-3306		
	SLE ≥50-65	1	1829.5	NA		
	SLE ≥65	0				
Age at diagnosis (years) 2 categories	<18	12	735	0-3012	Mann Whitney test	0.8183
	≥18	18	748	0-3306		
Disease duration (years) 2 categories	<2	3	696	80-967.5	Mann Whitney test	0.6951
	≥2	27	800	0-3306		
Disease duration (years) 5 categories	<1	2	523.75	80-967.7	Kruskal-Wallis test	0.9153
	≥1-2	1	696	NA		
	≥2-5	9	810	0-3012		
	≥5-10	3	385	0-2296.5		
	≥10	15	594	0-3306		
SLEDAI-2K at sample collection	>6	6	537	0-1386	Kruskal-Wallis test	0.6036
	≥3 and ≤6	10	730	0-3306		
	<3	12	965	0-2330		

	<3, HQC only	1	198			
Lupus Nephritis	With	20	805	0-3306	Mann Whitney test	0.4734
	Without	10	522	0-2970		

Table S12.14. Physical activity according to the patients' treatments. The physical activity was assessed with the International Physical Activity Questionnaire (IPAQ), validated for individuals with ages ≥ 14 years old. ACE- Angiotensin-converting enzyme inhibitors, IvIg- Intravenous immunoglobulin, METs- metabolic equivalents of task, MMF- Mycophenolate Mofetil, n – total number of individuals. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

IPAQ (METs) (≥ 14 years) **vs Treatments**					
	n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Current Use Hydroxychloroquine	26	748	0-3306	Mann Whitney test	0.6585
No Current Use Hydroxychloroquine	4	1107	99-2296.5		
Current Use Prednisolone	26	805	0-3306	Mann Whitney test	0.2439
No Current Use Prednisolone	4	129	0-1829.5		
Current Use MMF	17	810	0-3306	Mann Whitney test	0.1645
No Current Use MMF	12	381.5	0-2178		
Current Use Azathioprine	4	752.5	0-1737	Mann Whitney test	0.8786
No Current Use Azathioprine	25	696	0-3306		
Current Use Methotrexate	4	330	0-2178	Mann Whitney test	0.3268
No Current Use Methotrexate	26	805	0-3306		
Current Use Belimumab	5	1120	594-3012	Mann Whitney test	0.1209
No Current Use Belimumab	25	660	0-3306		
Current Use Rituximab	1	967.5	NA		
No Current Use Rituximab	28	678	0-3306		
Current Use Cyclophosphamide	0				
No Current Use Cyclophosphamide	29	696	0-3306		
Current Use IvIg	0				
No Current Use IvIg	29	696	0-3306		
Current Use Tacrolimus	2	1841	1386-2296.5	Mann Whitney test	0.1773
No Current Use Tacrolimus	27	660	0-3306		
Current Use Cyclosporine	0				
No Current Use Cyclosporine	29	696	0-3306		

Table S12.15. Other analyzed parameters. HC- healthy controls, n – total number of individuals, SLE- systemic lupus erythematosus. p-value significant < 0.05. *Median: non-normal distribution; Mean: normal distribution.

		n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Time Sitting (≥ 14 years old)	SLE	30	8.263	2.30-14.00	t-test	**0.0479**
	HC	13	6.215	1.70-10.90		
Fasting (Hours)	SLE	31	11.750	6.00-16.00	t-test	0.7971
	HC	13	11.590	9.50-13.67		
Sleep (Hours)	SLE	31	8.220	6.83-12.25	Mann-Whitney test	0.0956
	HC	13	7.670	6.92-9.67		
Bristol Scale	SLE	32	3		Mann-Whitney test	0.2135
	HC	13	4			

Table S12.16. Body mass index analyzed parameters. HC- healthy controls, kg/m^2- kilogram per meter square, n – total number of individuals, SLE- systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

Body Mass Index (kg/m2)						
		n	Median/Mean*	Min - Max	Test	p-value
Disease Status (Adults)	SLE	22	24.77	18.70-31.00	t-test	0.0899
	HC	11	22.33	18.20-27.70		
Disease Status (2 categories BMI)	SLE Normal weight	20 (60%)			Fisher's exact test	0.2833
	SLE Overweight/Obesity	13 (40%)				
	HC Normal weight	10 (83%)				
	HC Overweight/Obesity	2 (17%)				
Gender (Adults)	SLE Female	19	24.90	18.70-31.00	t-test	0.1441
	HC Female	8	22.43	18.20-27.70		
	SLE Male	3	22.00	20.80-29.00	Mann-Whitney test	0.8000
	HC Male	2	23.90	23.30-24.50		
	SLE+HC Female	27	24.17	18.20-31.00	t-test	0.8972
	SLE+HC Male	5	23.92	20.80-29.00		
Disease duration (years) 2 categories (adults)	<2	1	18.70	NA	Small sample	
	≥2	21	25.06	18.80-31.00		
SLEDAI-2K at sample collection (Adults)	>6	5	26.90	18.70-30.80	Kruskal-Wallis test	**0.0048**
	≥3 and ≤6	5	27.28	22.00-29.90		
	<3	10	22.70	18.80-27.20		
	<3, HQC only	1	31.00	NA		
Lupus Nephritis (Adults)	With	15	24.67	18.70-30.80	t-test	0.8771
	Without	7	24.97	19.30-31.00		

Table S12.17. Analyzed parameters for % of fat mass. HC-healthy controls, n – total number of individuals, SLE-systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05. *Median: non-normal distribution; Mean: normal distribution.

% Fat Mass (BodPod)						
		n	Median/Mean*	Min - Max	Test	p-value
Disease Status (Adults)	SLE	19	36.16	18.10-47.80	t-test	**0.0118**
	HC	11	27.22	13.40-38.80		
Gender	SLE Female	27	33.48	14.05-47.80	t-test	0.1172
	HC Female	10	28.01	17.30-38.08		
	SLE Male	2	30.45	24.50-36.40	Mann-Whitney test	0.3333
	HC Male	2	18.30	13.40-23.20		
	SLE+HC Female	37	32.00	14.50-47.80	t-test	0.1313
	SLE+HC Male	4	24.38	13.40-36.40		
Age (years) 5 categories	SLE <13	2	32.70	31.20-34.20	Kruskal-Wallis test	0.1057
	SLE 13-18	9	24.50	14.50-42.50		
	SLE ≥18-50	17	38.00	18.10-46.50		
	SLE ≥50-65	1	28.10	NA		
	SLE ≥65	0				
Age at diagnosis (years) 2 categories	<18	15	30.87	14.50-46.50	t-test	0.2538
	≥18	11	35.12	18.10-46.00		
Age at diagnosis (years)	<13	8	33.73	21.30-46.50	One-way ANOVA	0.3030
	13-18	6	27.52	14.50-36.70		

5 categories	≥18-50	12	34.53	18.10-46.00		
	≥50-65	0				
	≥65	0				
Disease duration (years) 2 categories	<2	6	31.90	19.90-34.20	Mann-Whitney test	0.2185
	≥2	20	36.55	14.50-46.50		
SLEDAI-2K at sample collection	>6	7	31.20	19.90-46.50	Kruskal-Wallis test	0.6228
	≥3 and ≤6	8	34.70	14.50-42.50		
	<3	12	32.80	18.10-45.30		
	<3, HQC only	1	45.20	NA		
Lupus Nephritis	With	19	31.40	14.50-46.50	t-test	0.2693
	Without	8	35.80	23.80-45.30		

Table S12.18. Analyzed parameters for waist circumference. CDV- cardiovascular, HC- healthy controls, HCQ- hydroxychloroquine n – total number of individuals, SLE- systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

Waist Circumference (cm)						
		n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Disease Status (Adults)	SLE	22	81.12	56.0-103.0	t-test	0.1516
	HC	11	74.78	64.0-87.0		
Disease Status (2 categories of waist circumference)	SLE no CDV risk	16 (48.5%)			Fisher's exact test	**0.0143**
	SLE with CDV risk	17 (51.5%)				
	HC no CDV risk	11 (91.7%)				
	HC with CDV risk	1 (8.3%)				
Gender (Adults)	SLE Female	19	80.87	56.0-103.0	t-test	0.0959
	HC Female	9	72.83	64.0-83.5		
	SLE Male	3	81.50	66.5-100.1	Mann-Whitney test	0.8000
	HC Male	2	78.85	76.3-81.4		
	SLE+HC Female	28	78.29	56.0-103.0	t-test	0.6238
	SLE+HC Male	5	81.16	66.5-100.1		
Disease duration (years) 2 categories (Adults)	<2	1	70.00	NA	Small sample	
	≥2	21	81.65	56.0-103.0		
SLEDAI-2K at sample collection (Adults)	>6	5	84.00	56.0-103.0	Kruskal-Wallis test	0.1429
	≥3 and ≤6	5	87.92	66.5-100.1		
	<3	10	73.50	66.0-90.0		
	<3, HQC only	1	102.00	NA		
Lupus Nephritis (Adults)	With	15	81.34	56.0-103.0	t-test	0.9117
	Without	7	80.64	66.0-102.0		

Table S12.19. Cardiovascular risk assessed by waist circumference according to patients' treatments. ACE- Angiotensin-converting enzyme inhibitors, CDV- cardiovascular, MMF- Mycophenolate Mofetil, n – total number of individuals. *total n=32. p-value significant < 0.05.

Waist Circumference vs Treatments					
	No Risk (n)	**High CDV Risk (n)**	**Very high CDV Risk (n)**	**Test**	**p-value**
Current Use Hydroxychloroquine	13	7	9	Chi-square test for trend	0.1740
No Current Use Hydroxychloroquine	3	1	0		
Current Use Prednisolone	14	8	7	Chi-square test for trend	0.5919

No Current Use Prednisolone	2	0	2		
Current Use MMF*	10	4	5	Chi-square test for trend	0.5406
No Current Use MMF*	5	4	4		
Current Use Azathioprine*	1	3	1	Chi-square test for trend	0.5893
No Current Use Azathioprine*	14	5	8		
Current Use Methotrexate*	2	1	1	Chi-square test for trend	0.9237
No Current Use Methotrexate*	14	7	8		
Current Use Belimumab	3	1	2	Chi-square test for trend	0.8841
No Current Use Belimumab	13	7	7		
Current Use Rituximab*	0	1	0	Chi-square test for trend	0.8217
No Current Use Rituximab*	15	7	9		
Cyclophosphamide*	0	0	0	Small sample	
No Cyclophosphamide*	15	8	9		
Current Use Intravenous immunoglobulin*	0	0	0	Small sample	
No Current Use Intravenous immunoglobulin*	15	8	9		
Current Use Tacrolimus*	2	0	0	Chi-square test for trend	0.1604
No Current Use Tacrolimus*	13	8	9		
Current Use Cyclosporine*	0	0	0	Small sample	
No Current Use Cyclosporine*	15	8	9		

Table S12.20. Cardiovascular risk assessed by waist circumference according to the patients' immunological status. (+) positive, (-) negative, Anti-ds-DNA- Anti-double stranded DNA, Anti-RNP- antinuclear ribonucleoprotein, Anti-Sm- Anti-Smith, CDV- cardiovascular, n – total number of individuals, *total n=32, ** total n=31. p-value significant < 0.05.

Waist Circumference vs Immunological Status					
	No Risk (n)	High CDV Risk (n)	Very high CDV Risk (n)	Test	p-value
Anti-dsDNA (+) *	11	8	8	Chi-square test for trend	0.2350
Anti-dsDNA (-) *	4	0	1		
Anti-Sm (+) *	7	0	5	Chi-square test for trend	0.9140
Anti-Sm (-) *	8	8	4		
Anti-Ro (+) **	5	1	4	Chi-square test for trend	0.5609
Anti-Ro (-) **	10	7	4		
Anti-La (+) **	0	0	1	Chi-square test for trend	0.1339
Anti-La (-) **	15	8	7		
Anti-RNP (+) **	5	1	6	Chi-square test for trend	0.0998
Anti-RNP (-) **	10	7	2		
Lupus anticoagulant (+) *	7	6	5	Chi-square test for trend	0.5622
Lupus anticoagulant (-) *	8	2	4		
Anticardiolipin (+) *	4	3	5	Chi-square test for trend	0.1604
Anticardiolipin (-) *	11	5	4		
Anti-B2-glycoprotein (+) *	4	3	5	Chi-square test for trend	0.1604
Anti-B2-glycoprotein (-) *	11	5	4		

Table S12.21. Analyzed parameter for waist/hip ratio (only assessed for adults according to the World Health Organization recommendations). HC-healthy controls, HCQ-hydroxychloroquine n–total number of individuals, SLE-systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05. *Median: non-normal distribution; Mean: normal distribution.

Waist/Hip (≥ 18 years)						
		N	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Disease Status	SLE	22	0.80	0.58-0.99	t-test	0.0890
	HC	11	0.75	0.69-0.86		
Disease Status (2 categories of waist/hip)	SLE no metabolic risk	15 (68%)			Fisher's exact test	0.0674
	SLE with metabolic risk	7 (32%)				
	HC no metabolic risk	11				
	HC with metabolic risk	0				
Gender	SLE Female	19	0.79	0.58-0.94	t-test	0.0704
	HC Female	9	0.73	0.69-0.75		
	SLE Male	3	0.87	0.71-0.99	Mann-Whitney test	0.80
	HC Male	2	0.84	0.82-0.86		
	SLE+HC Female	28	0.78	0.58-0.94	t-test	0.0905
	SLE+HC Male	5	0.85	0.71-0.99		
Disease duration (years) 2 categories	<2	1	0.82	NA	Small sample	
	≥2	21	0.80	0.58-0.99		
SLEDAI-2K at sample collection	>6	5	0.82	0.58-0.94	Kruskal-Wallis test	0.4704
	≥3 and ≤6	5	0.85	0.71-0.99		
	<3	10	0.78	0.62-0.87		
	<3, HQC only	1	0.87	NA		
Lupus Nephritis	With	15	0.81	0.58-0.99	t-test	0.5292
	Without	7	0.78	0.71-0.87		

Table S12.22. Analyzed parameters for waist/height ratio. HC- healthy controls, HCQ- hydroxychloroquine n – total number of individuals, SLE- systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

Waist/Height						
		N	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Disease Status	SLE	33	0.496	0.350-0.620	t-test	**0.0303**
	HC	12	0.445	0.380-0.540		
Disease Status (2 categories of waist/height)	SLE no metabolic risk	17 (51.5%)			Fisher's exact test	**0.0168**
	SLE with metabolic risk	16 (48.5%)				
	HC no metabolic risk	11 (92%)				
	HC with metabolic risk	1 (8%)				
Gender	SLE Female	29	0.496	0.350-0.620	t-test	**0.0381**
	HC Female	10	0.442	0.380-0.540		
	SLE Male	4	0.505	0.390-0.590	Mann-Whitney test	0.8000
	HC Male	2	0.460	0.440-0.480		
	SLE+HC Female	39	0.482	0.350-0.620	t-test	0.9323
	SLE+HC Male	6	0.485	0.390-0.590		
Age (years) 2 categories	SLE <18	11	0.491	0.380-0.600	t-test	0.7705
	SLE ≥ 18	22	0.499	0.350-0.620		
	HC ≥ 18	11	0.448	0.380-0.540	t-test (SLE vs HC ≥ 18)	0.0626
	HC < 18	1	0.410			

Disease duration (years) 2 categories	<2	6	0.465	0.380-0.530	t-test	0.2587
	≥2	27	0.503	0.350-0.620		
SLEDAI-2K at sample collection	>6	8	0.500	0.350-0.620	Kruskal-Wallis test	0.3373
	≥3 and ≤6	10	0.510	0.390-0.600		
	<3	13	0.470	0.410-0.590		
	<3, HQC only	1	0.610	NA		
Lupus Nephritis	With	22	0.4895	0.350-0.620	t-test	0.4641
	Without	11	0.5100	0.390-0.610		

Table S12.23. Pairwise permanova test between genders. p-value significant < 0.05.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value
Female	Male	44	999	1.6903323982280825	0.043

Table S12.24. ANOVA single factor test for the Firmicutes/Bacteroidetes ratio between SLE patients and HC. HC – healthy controls, SLE – systemic lupus erythematosus. p-value significant < 0.05.

Groups	*Count*	***Average***	*Variance*			
HC	13	**17.46693452**	742.861771			
SLE	31	**126.1157196**	212803.1285			
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	108119.0245	1	108119.0245	0.7103070871	**0.4041160129**	4.072653663
Within Groups	6393008.197	42	152214.4809			
Total	6501127.221	43				

Table S12.25. Average of the Firmicutes/Bacteriodetes ratio for the different disease activity categories. SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.

Groups	*Count*	***Average***	*Variance*			
SLEDAI-2K >6	8	**268.9144618**	555284.6814			
SLEDAI-2K ≥ 3 and ≤6	8	**5.595906092**	18.2396384			
SLEDAI-2K <3	14	**120.8941116**	169708.2736			
SLEDAI-2K 4 <3, HCQ only	1	**20.98680352**	NA			
ANOVA						
Source of Variation	*SS*	*Df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	290765.8514	3	96921.95048	0.4294685369	**0.7335370558**	2.960351351
Within Groups	6093328.004	27	225678.815			
Total	6384093.856	30				

Table S12.26. ANOVA single factor test for the presence of *Rikenellaceae* family in SLE patients with LN, SLE patients without LN, and HC. HC–healthy controls, LN–lupus nephritis, SLE–systemic lupus erythematosus. p-value significant < 0.05.

Groups	*Count*	*Sum*	***Average***	*Variance*		
Without LN	10	2107	**210.7**	30026.01111		
With LN	20	6426	**321.3**	319491.1684		
HC	13	3774	**290.31**	93797.39744		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1720243.113	3	573414.3709	3.072081421		

Within Groups	7466135.069	40	186653.3767		**0.03852327887**	2.838745406
Total	9186378.182	43				

Table S12.27. Glucagon-like peptide-1 (GLP-1) analyzed parameters. HC – healthy controls, HCQ – hydroxychloroquine, n – total number of individuals, SLE- systemic lupus erythematosus, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

GLP-1						
		n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Disease Status	SLE	29	41.38	16.13-96.25	Mann-Whitney test	0.0739
	HC	9	33.55	27.24-41.19		
Gender	SLE Female	25	41.40	16.13-96.25	t-test	0.2217
	HC Female	8	33.49	27.24-41.19		
	SLE Male	4	58.90	34.40-78.60	Small sample	
	HC Male	1	32.26	32.26-32.26		
	Female SLE vs Male SLE				t-test	0.1534
	SLE+HC Female	33	39.2	16.1-96.3	Mann-Whitney test	0.29
	SLE+HC Male	5	41	32.26-76.80		
Ethnicity	SLE Caucasian	23	41	16.13-96.25	Mann-Whitney test	0.1977
	HC Caucasian	9	32.26	27.24-41.19		
	SLE Asian	1	62.09	NA	Small sample	
	SLE Black	2	36.15	34.35-37.95		
	SLE Mixed	1	71.8	NA		
	SLE Not Given	2	53.3	44.37-62.26		
Age (years) 2 categories	SLE <18	11	42.52	18.87-78.64	t-test	0.6226
	SLE ≥ 18	18	46.24	16.13-96.25		
	HC ≥ 18	8	34.19	27.24-41.19	t-test (SLE vs HC ≥ 18)	0.1155
	HC < 18	1	35.13			
Age at diagnosis (years) 2 categories	<18	13	42.68	18.87-78.64	t-test	0.5976
	≥18	16	46.57	16.13-96.25		
Age at diagnosis (years) 5 categories	<13	8	40.86	29.62-44.37	One-way ANOVA	0.4752
	13-18	8	39.76	18.87-78.64		
	≥18-50	13	48.97	16.13-96.25		
	≥50-65	0				
	≥65	0				
Disease duration (years) 2 categories	<2	6	42.98	31.35-62.09	t-test	0.7971
	≥2	23	45.31	16.13-96.25		
Disease duration (years) 5 categories	<1	4	42.18	31.96-48.13	Kruskal-Wallis test	0.8420
	≥1-2	2	46.72	31.35-62.09		
	≥2-5	9	36.69	18.87-78.64		
	≥5-10	3	41	34.35-96.25		
	≥10	11	42.78	16.13-76.79		
SLEDAI-2K at sample collection	>6	8	46.32	31.35-65.71	One-way ANOVA	0.9112
	≥3 and ≤6	10	43.18	18.87-76.79		
	<3	10	46.79	16.13-96.25		
	<3, HQC only	0				

Lupus Nephritis	With	20	45.11	16.13-96.25	t-test	0.9078
	Without	9	45.25	18.76-71.80		

Table S12.28. GLP-1 values according to the patients' treatments. ACE- Angiotensin-converting enzyme inhibitors, n – total number of individuals. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

GLP-1 vs Treatments					
	n	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Current use Hydroxychloroquine	26	40.58	16.13-78.64	Mann-Whitney	0.3886
No current use Hydroxychloroquine	3	41.38	40.99-96.25		
Current use Prednisolone	28	44.95	16.13-96.25		
No current use Prednisolone	1	41.38			
Current Use Mycophenolate Mofetil	17	42.63	16-13-78.64	t-test	0.3625
No current use Mycophenolate Mofetil	11	49.60	18.76-96.25		
Current use Azathioprine	5	62.26	18.76-96.25	t-test	0.2386
No Current use Azathioprine	23	40.99	16.13-78.64		
Current use Methotrexate	3	47.73	33.43-71.80	t-test	0.7886
No current use Methotrexate	26	44.49	16.13-96.25		
Current use Belimumab	6	30.54	18.87-62.26	t-test	0.2118
No current use Belimumab	22	41.57	16.13-96.25		
Current use Rituximab	1	48.13			
No current Rituximab	27	45.42	16.13-96.25		
Current use Cyclophosphamide	0				
No current use Cyclophosphamide	28	45.37	16.13-96.25		
Current use Intravenous immunoglobulin	0				
No current use Intravenous immunoglobulin	28	45.37	16.13-96.25		
Current use Tacrolimus	2	38.84	36.69-40.99	Mann-Whitney test	0.6402
No current use Tacrolimus	26	41.85	16.13-96.25		
Current use Cyclosporine	0				
No current Cyclosporine	28	45.37	16.13-96.25		
Current use ACE	11	51.80	18.87-96.25	t-test	0.1617
No current use ACE	17	41.21	16.13-71.80		
Current use ARB	2	41.85	33.43-71.80	Mann-Whitney test	0.6984
No current use ARB	26	46.53	16.13-96.25		

Table S12.29. GLP-1 values according to the patients' immunological status. (+) positive, (-) negative, Anti-ds-DNA- Anti-double stranded DNA, Anti-RNP- antinuclear ribonucleoprotein, Anti-Sm- Anti-Smith, n – total number of individuals. p-value significant < 0.05.*Median: non-normal distribution; Mean: normal distribution.

GLP-1 vs Immunological Status					
Antibodies	**n**	**Median/Mean***	**Min - Max**	**Test**	**p-value**
Anti-dsDNA (+)	25	41.38	18.76-96.25	Mann-Whitney test	0.9444
Anti-dsDNA (-)	3	44.37	16.13-62.09		
Anti-Sm (+)	11	40.82	18.76-71.8	t-test	0.3272
Anti-Sm (-)	17	48.82	16.13-96.25		
Anti-Ro (+)	9	36.84	18.76-55.6	t-test	0.1125
Anti-Ro (-)	18	49.69	16.13-96.25		
Anti-La (+)	0				
Anti-La (-)	27	45.40	16.13-96.25		
Anti-RNP (+)	11	43.45	18.76-71.8	t-test	0.6789
Anti-RNP (-)	16	46.75	16.13-96.25		
Lupus anticoagulant (+)	17	47.35	18.76-96.25	t-test	0.5103
Lupus anticoagulant (-)	11	42.30	16.13-78.64		
Anticardiolipin (+)	11	50.28	28.9-96.25	t-test	0.2886
Anticardiolipin (-)	17	42.19	16.13-78.64		
Anti-B2-glycoprotein (+)	11	41.57	28.9-96.25	Mann-Whitney test	0.9263
Anti-B2-glycoprotein (-)	17	41.38	16.13-78.64		

Table S12.30. Correlations with the disease activity (SLEDAI-2K). IPAQ-International Physical Activity Questionnaire, METs- metabolic equivalents of task, SFA- saturated fatty acids, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000, Zn-zinc. p-value significant < 0.05.

	Spearman r	95% confidence interval	p-value
SLEDAI-2K vs Dietary Zn (mg)	-0.4650	-0.7160 to -0.1075	**0.0110**
SLEDAI-2K vs Dietary SFA 15:0 (g)	-0.4123	-0.6827 to -0.04265	**0.0262**

Table S12.31. Correlations with glucagon-like peptide 1 (GLP-1) as a surrogate for gut dysbiosis. g- grams, IPAQ-International Physical Activity Questionnaire, METs- metabolic equivalents of task, n-6- omega-6, PUFA- polysaturated fatty acids, SLEDAI-2K - Systemic Lupus Erythematosus Disease Activity Index 2000, Zn-zinc. p-value significant < 0.05.

SLE PATIENTS + HC			
	Spearman r	95% confidence interval	p-value
GLP-1 vs IPAQ (METs)	-0.3720	-0.6336 to -0.0340	**0.0278**
GLP-1 vs Sitting Time	0.4329	0.1063 to 0.6752	**0.0094**
GLP-1 vs Waist Circumference	0.4241	0.1062 to 0.6634	**0.0089**
GLP-1 vs Waist/Hip	0.5607	0.1911 to 0.7910	**0.0044**
GLP-1 vs Waist/Height	0.3501	0.0195 to 0.6117	**0.0337**

GLP-1 vs Dietary Lactose (g)	-0.3998	-0.6528 to -0.0666	**0.0173**
GLP-1 vs Dietary n-6 PUFA (g)	0.5538	0.2610 to 0.7533	**0.0006**
GLP-1 vs Dietary linoleic acid (PUFA 18:2) (g)	0.4741	0.1573 to 0.7024	**0.0040**

SUPPLEMENTAL FIGURES

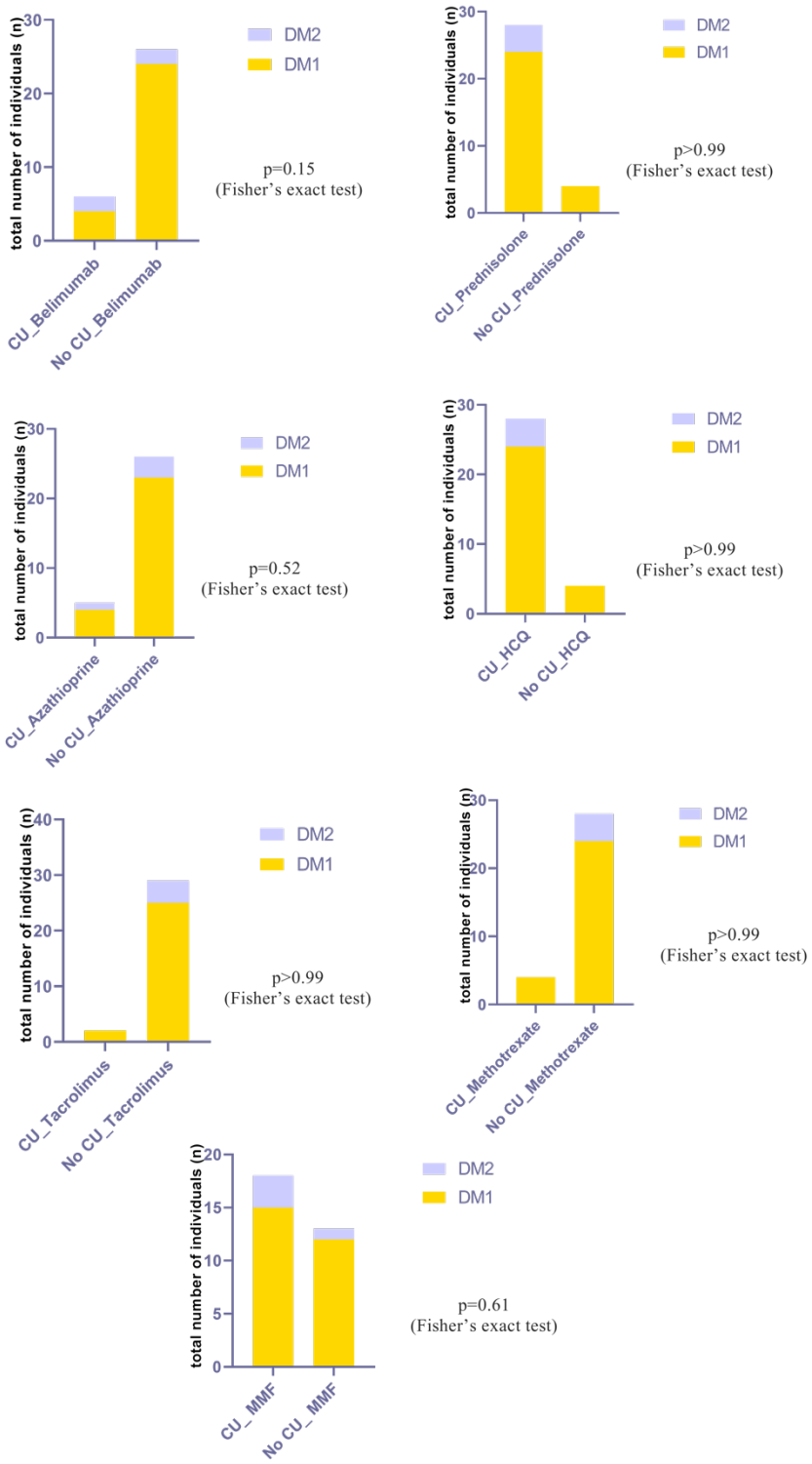

Figure S13.1. Adherence to the Mediterranean Diet (MD) according to the patients' treatments. The MD was assessed by the PREDIMED (adults), and KIDMED (children) questionnaires. The questionnaires scores were categorized in "good adherence to the Mediterranean Diet" (DM2) and "Insufficient adherence to the Mediterranean Diet" (DM1). No patients were currently being treated with Cyclophosphamide, Intravenous immunoglobulin, and Cyclosporine. HCQ – hydroxychloroquine, DM1- insufficient adherence to the Mediterranean Diet, DM2- good adherence to the Mediterranean Diet, MMF- mycophenolate mofetil.

Figure S13.2. Physical activity assessed with the International Physical Activity Questionnaire (IPAQ), validated for individuals with ages ≥ 14 years old, according to patients' therapeutics. Only one patient was currently being treated with Rituximab. No patients were currently being treated with Cyclophosphamide, Intravenous immunoglobulin, and Cyclosporine. ACE- Angiotensin-converting enzyme inhibitors, CU – Current Use, HCQ – hydroxychloroquine, METs- metabolic equivalents of task.

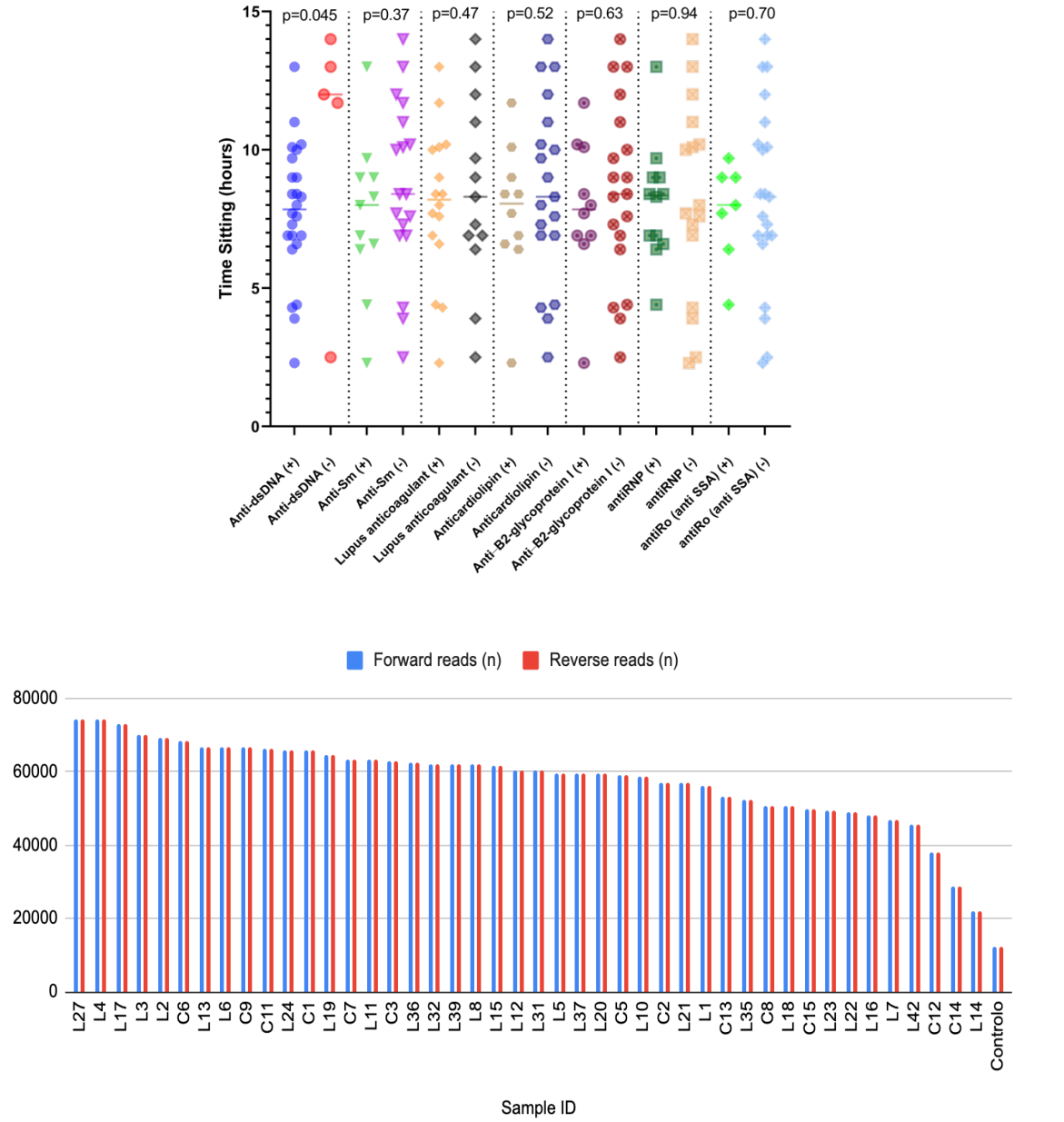

Figure S13.3. Number of reads per sample. Three samples had half the median value of reads (C12, C14, L14). C – healthy controls, Controlo – negative control, L- SLE patients, n - total number of reads.

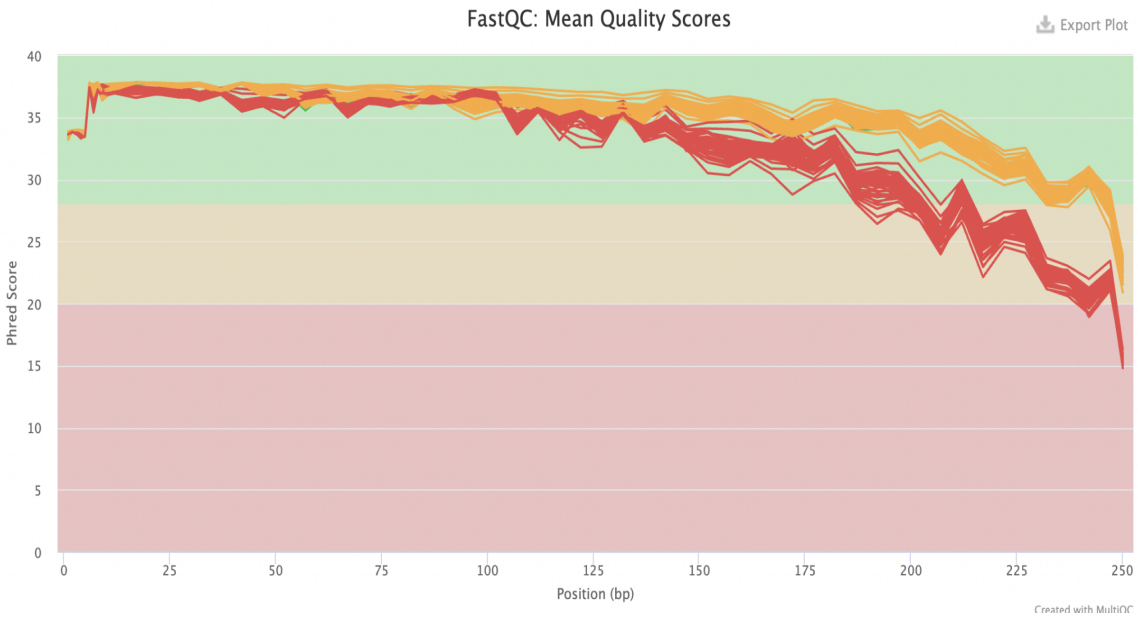

Figure S13.4. Mean quality score plot. Forward reads are identified in yellow and reverse reads in red. bp- base pairs.

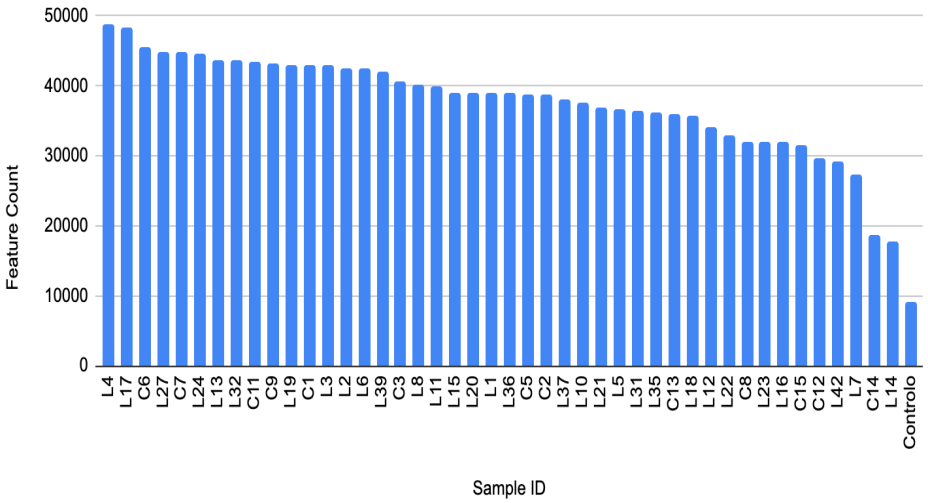

Figure S13.5. Number of Operational Taxonomic Unit in each sample. C – healthy controls, Controlo– negative control, L- SLE patients.

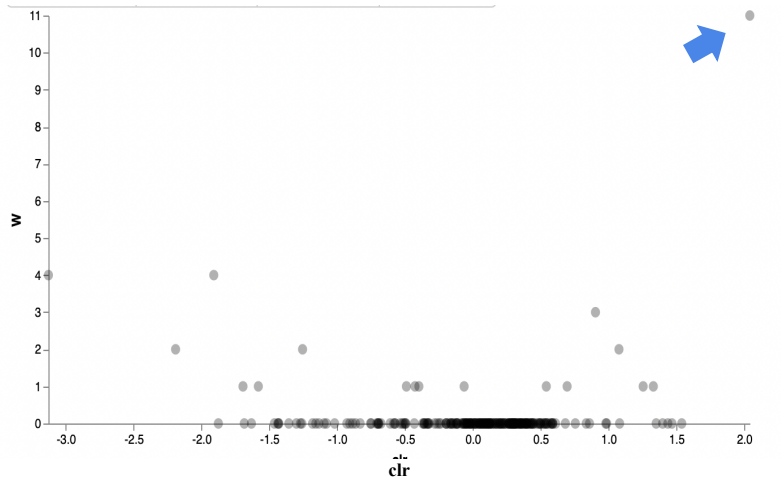

Figure S13.6. ANCOM volcano plot for the genus taxa differently observed in SLE patients and HC. Non reached statistical significance. The blue arrow marks the *Streptococcus* genus. *W* - count of the number of sub-hypotheses that have passed for a given taxon. The ANCOM test calculates the metric W which is used to reject the null hypothesis of no difference in the absolute abundance of a taxon in two groups. The W value is essentially a count of the number of sub-hypotheses that have passed for a given taxon. The results are summarized in a volcano plot, with the W statistic on the y-axis, and the clr on the x-axis. The clr is a transformation representing a log-fold change relative to the average. Therefore, the x-axis is summarizing the effect size difference of the given taxa between the groups, and the y-axis is the strength of the ANCOM test statistic.

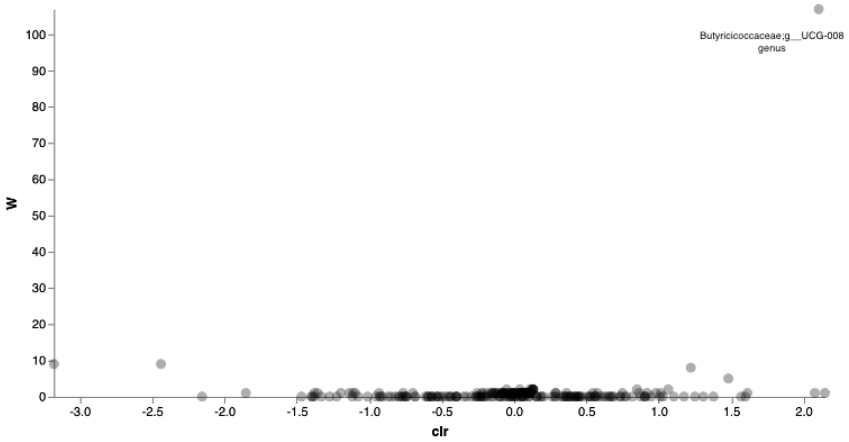

Figure S13.7. ANCOM volcano plot for the genus taxa differently observed between male and female SLE patients. Genus *Butyricicoccaceae*;g UCG-008 appears separated from the other genus. *W* - count of the number of sub-hypotheses that have passed for a given taxon, clr - transformation representing a log-fold change relative to the average.

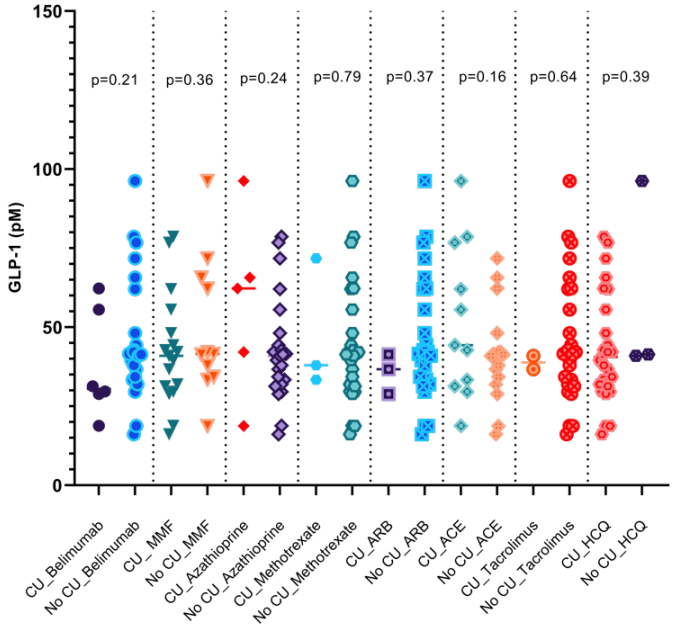

Figure S13.8. Glucagon-like peptide 1 (GLP-1) values according to the patients' treatments. No patients were currently being treated with Cyclophosphamide, Intravenous immunoglobulin, and Cyclosporine. Only one patient was being treated with Rituximab, and only one patient was not being treated with prednisolone. ACE-Angiotensin-converting enzyme inhibitors, ARB - Angiotensin receptor blocker, CU- Current Use, MMF - mycophenolate mofetil, HCQ – hydroxychloroquine.

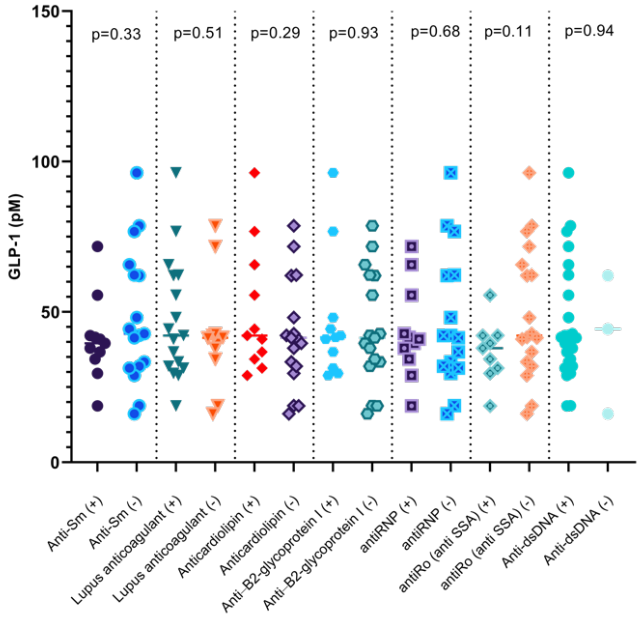

Figure S13.9. Glucagon-like peptide 1 (GLP-1) values according to the patients' immune status. No patients were positive for anti-La antibodies. (+) positive, (-) negative, Anti-RNP-antinuclear ribonucleoprotein, Anti-Sm-anti-Smith.

APPENDICES

Appendix 1

Definitions 2019 European League Against Rheumatism/ American College of Rheumatology classification criteria for SLE (21).

Criteria	Definition
Antinuclear antibodies (ANA)	ANA at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test at least once. Testing by immunofluorescence on HEp-2 cells or a solid phase ANA screening immunoassay with at least equivalent performance is highly recommended
Fever	Temperature >38.3°C
Leucopenia	White blood cell count <4.0×10^9/l
Thrombocytopenia	Platelet count <100×10^9/l
Autoimmune hemolysis	Evidence of hemolysis, such as reticulocytosis, low haptoglobin, elevated indirect bilirubin, elevated lactate dehydrogenase (LDH) AND positive Coomb's (direct antiglobulin) test.
Delirium	Characterized by (1) change in consciousness or level of arousal with reduced ability to focus, (2) symptom development over hours to <2 days, (3) symptom fluctuation throughout the day, (4) either (4a) acute/subacute change in cognition (e.g., memory deficit or disorientation), or (4b) change in behaviour, mood, or affect (e.g., restlessness, reversal of sleep/wake cycle)
Psychosis	Characterized by (1) delusions and/or hallucinations without insight and (2) absence of delirium
Seizure	Primary generalized seizure or partial/focal seizure
Non-scarring alopecia	Non-scarring alopecia observed by a clinician*
Oral ulcers	Oral ulcers observed by a clinician*
Subacute cutaneous or discoid lupus	Subacute cutaneous lupus erythematosus observed by a clinician*: Annular or papulosquamous (psoriasiform) cutaneous eruption, usually photodistributed Discoid lupus erythematosus observed by a clinician*: Erythematous-violaceous cutaneous lesions with secondary changes of atrophic scarring, dyspigmentation, often follicular hyperkeratosis/hematological(scalp), leading to scarring alopecia on the scalp If skin biopsy is performed, typical changes must be present. Subacute cutaneous lupus: interface vacuolar dermatitis consisting of a perivascular lymphohistiocytic infiltrate, often with dermal mucin noted. Discoid lupus: interface vacuolar dermatitis consisting of a perivascular and/or periappendageal lymphohistiocytic infiltrate. In the scalp, follicular keratin plugs may be seen. In longstanding lesions, mucin deposition and basement membrane thickening may be noted
Acute cutaneous lupus	Malar rash or generalized maculopapular rash observed by a clinician If skin biopsy is performed, typical changes must be present: interface vacuolar dermatitis consisting of a perivascular lymphohistiocytic infiltrate, often with dermal mucin noted. Perivascular neutrophilic infiltrate may be present early in the course
Pleural or pericardial effusion	Imaging evidence (such as ultrasound, X-ray, CT scan, MRI) of pleural or pericardial effusion, or both
Acute pericarditis	≥2 of (1) pericardial chest pain (typically sharp, worse with inspiration, improved by leaning forward), (2) pericardial rub, (3) electrocardiogram (EKG) with new widespread ST-elevation or PR depression, (4) new or worsened pericardial effusion on imaging (such as ultrasound, X-ray, CT scan, MRI)
Joint involvement	EITHER (1) synovitis involving two or more joints characterized by swelling or effusion OR (2) tenderness in two or more joints and at least 30 min of morning stiffness
Proteinuria >0.5 g/24 hours	Proteinuria >0.5 g/24 hours by 24 hours urine or equivalent spot urine protein-to-creatinine ratio
Class II or V lupus nephritis on renal biopsy according to ISN/RPS 2003 classification	Class II: mesangial proliferative lupus nephritis: purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposit. A few isolated subepithelial or subendothelial deposits may be visible by immune-fluorescence or electron microscopy, but not by light microscopy Class V: membranous lupus nephritis: global or segmental subepithelial immune deposits or their morphological sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations
Class III or IV lupus nephritis on renal biopsy according to International Society of Nephrology/ Renal Pathology Society (ISN/RPS) 2003	Class III: focal lupus nephritis: active or inactive focal, segmental or global endocapillary or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations Class IV: diffuse lupus nephritis: active or inactive diffuse, segmental or global endocapillary or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Positive antiphospholipid antibodies	Anticardiolipin antibodies (IgA, IgG, or IgM) at medium or high titer (>40 A phospholipids (APL), GPL or MPL units, or >the 99th percentile) or positive anti-β2GP1 antibodies (IgA, IgG, or IgM) or positive lupus anticoagulant
Low C3 OR low C4	C3 OR C4 below the lower limit of normal
Low C3 AND low C4	Both C3 AND C4 below their lower limits of normal
Anti-dsDNA antibodies OR anti- Smith (Sm) antibodies.	Anti-dsDNA antibodies in an immunoassay with demonstrated ≥90% specificity for SLE against relevant disease controls OR anti-Sm antibodies

Entry criterion

Antinuclear antibodies (ANA) at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test (ever)

↓

If absent, do not classify as SLE
If present, apply additive criteria

↓

Additive criteria

Do not count a criterion if there is a more likely explanation than SLE.
Occurrence of a criterion on at least one occasion is sufficient.
SLE classification requires at least one clinical criterion and ≥10 points.
Criteria need not occur simultaneously.
Within each domain, only the highest weighted criterion is counted toward the total score§.

Clinical domains and criteria	Weight
Constitutional	
Fever	2
Hematologic	
Leukopenia	3
Thrombocytopenia	4
Autoimmune hemolysis	4
Neuropsychiatric	
Delirium	2
Psychosis	3
Seizure	5
Mucocutaneous	
Non-scarring alopecia	2
Oral ulcers	2
Subacute cutaneous OR discoid lupus	4
Acute cutaneous lupus	6
Serosal	
Pleural or pericardial effusion	5
Acute pericarditis	6
Musculoskeletal	
Joint involvement	6
Renal	
Proteinuria >0.5g/24h	4
Renal biopsy Class II or V lupus nephritis	8
Renal biopsy Class III or IV lupus nephritis	10

Immunology domains and criteria	Weight
Antiphospholipid antibodies	
Anti-cardiolipin antibodies OR	
Anti-β2GP1 antibodies OR	
Lupus anticoagulant	2
Complement proteins	
Low C3 OR low C4	3
Low C3 AND low C4	4
SLE-specific antibodies	
Anti-dsDNA antibody* OR	
Anti-Smith antibody	6

Total score:

↓

Classify as Systemic Lupus Erythematosus with a score of 10 or more if entry criterion fulfilled.

Appendix 2

Clinical and Immunologic Criteria Used in the SLICC Classification Criteria (56).

Clinical Criteria	
1. Acute cutaneous lupus	including lupus malar rash (do not count if malar discoid): bullous lupus; toxic epidermal necrolysis variant of SLE; maculopapular lupus rash; photosensitive lupus rash (in the absence of dermatomyositis) or subacute cutaneous lupus (nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias).
2. Chronic cutaneous lupus	including classical discoid rash (localized (above the neck), generalized (above and below the neck)), hypertrophic (verrucous) lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chillblains lupus, discoid lupus/lichen planus overlap.
3. Oral ulcers: palate	Buccal, tongue or nasal ulcers; in the absence of other causes, such as vasculitis, Behcet's disease, infection (herpes), inflammatory bowel disease, reactive arthritis, and acidic foods.
4. Nonscarring alopecia (diffuse thinning or hair fragility with visible broken hairs)	in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia.
5. Synovitis involving two or more joints, characterized by swelling or effusion OR tenderness in 2 or more joints and thirty minutes or more of morning stiffness.	
6. Serositis	typical pleurisy for more than 1 day or pleural effusions or pleural rub; typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day or pericardial effusion or pericardial rub or pericarditis by EKG; in the absence of other causes, such as infection, uremia, and Dressler's pericarditis.
7. Renal	Urine protein/creatinine (or 24 hr urine protein) representing 500 mg of protein/24 hr or Red blood cell casts.
8. Neurologic	Seizures, psychosis, mononeuritis multiplex in the absence of other known causes such as primary vasculitis; myelitis, peripheral or cranial neuropathy in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus; acute confusional state in the absence of other causes, including toxic-metabolic, uremia, drugs.
9. Hemolytic anemia	
10. Leukopenia (< 4000/mm^3 at least once)	in the absence of other known causes such as Felty's, drugs, and portal hypertension OR Lymphopenia (< 1000/mm^3 at least once) in the absence of other known causes such as corticosteroids, drugs and infection.
11. Thrombocytopenia (<100,000/mm^3) at least once	in the absence of other known causes such as drugs, portal hypertension, and TTP.
Immunological criteria	
1. ANA above laboratory reference range	
2. Anti-dsDNA above laboratory reference range, except ELISA: twice above laboratory reference range	
3. Anti-Sm	
4. Antiphospholipid antibody: any of the following lupus anticoagulant, false-positive RPR, medium or high titer anticardiolipin (IgA, IgG, IgM), anti-β_2 glycoprotein I (IgA, IgG or IgM).	
5. Low complement: low C3, low C4, low CH50.	
6. Direct Coombs test in the absence of hemolytic anemia	

Appendix 3

SLEDAI-2K score (57).

SLEDAI-2K score	Descriptor	Definition
8	Seizure	Recent onset, exclude metabolic, infectious or drug causes.
8	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality.
8	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function.
8	Visual disturbance	Retinal changes.
8	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	Lupus headache	Severe, persistent headache which may be migrainous, but must be non-responsive to narcotic analgesia.
8	Cerebrovascular accident	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infection, spliter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	Arthritis	>=2 joints with pain and signs of inflammation
4	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or biopsy showing myositis.
4	Urinary casts	Heme granular or red blood cell casts.
4	Hematuria	>5 red blood cells/high power field. Exclude stone, infection, or other causes.
4	Proteinuria	>0.5g/24hours.
4	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	Rash	Inflammatory type rash
2	Alopecia	Abnormal, patchy, or diffuse loss of hair.
2	Mucosal ulcers	Oral or nasal ulcerations.
2	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2	Low complement	Decrease in CH50, C3 or C4.
2	Increased DNA binding	Increased DNA binding by Farr assay.
1	Fever	>38ºC. Exclude infectious cause.
1	Thrombocytopenia	<100 000 platelets / x10^9/L, exclude drug causes.
1	Leukopenia	< 3000 white blood cells / x10^9/L, exclude drug causes.

Appendix 4

CENTRO ACADÉMICO DE MEDICINA DE LISBOA

CENTRO HOSPITALAR LISBOA NORTE, EPE

HOSPITAL DE SANTA MARIA

Hospital Pulido Valente

LISBOA

FACULDADE DE MEDICINA LISBOA

MM

Instituto de Medicina Molecular

João Lobo Antunes

Exma. Senhora

Dra. Patrícia Costa Reis

Serviço de Pediatria

Centro Hospitalar Universitário Lisboa Norte, E.P.E

Lisboa, 26 de Novembro de 2020

Nossa Refª. Nº 468/19

Assunto: Projeto de Investigação **"Estudo da Permeabilidade Intestinal e do Microbioma em Doentes com Lúpus Eritematoso Sistémico"**

Relator - *Profª Doutora Ana Isabel Lopes*

Pela presente se informa que o projeto citado em epígrafe obteve, em Outubro de 2020, parecer favorável da Comissão de Ética, após o cumprimento de alguns requisitos, devidamente sancionados, considerando-se observados os imperativos que fundeiam as Boas práticas clínicas, os preceitos internacionalmente reconhecidos de qualidade ética e científica que devem ser respeitados na conceção e na realização dos estudos clínicos que envolvam a participação de seres humanos.

No uso das competências próprias constantes do disposto no Decreto-Lei. N.º 97/95 de 10 de Maio, e no exercício das suas funções em observância do deliberado na Lei n.º 21/2014 de 16 de Abril, que aprova a lei da investigação clínica, revista pelo Decreto-Lei n.º 80/2018 (DR n.º 198-2018, Série I de 2018/10/15) que reforça o papel das comissões de ética no contexto da instituição em que se integram nas diversas vertentes relevantes, nomeadamente, assistencial, institucional de investigação e de formação, e ainda em cumprimento dos regulamentos internos do CHULN, dos códigos deontológicos, das convenções, declarações e diretrizes internacionais, a Comissão de Ética avaliou o projeto, que considera obedecer aos requisitos éticos fundamentais que devem ser respeitados, refletindo o primado da dignidade e da integridade humanas.

Desta apreciação apuram-se no plano de investigação, as seguintes informações

Trata-se de um estudo prospetivo, observacional e não interventivo cujo objetivo geral é estudar a complexa interação entre o microbioma, a barreira intestinal e a atividade de doença no Lúpus Eritematoso Sistémico. O objetivo final é conhecer mais sobre a patogénese desta doença autoimune e estabelecer os alicerces para desenvolver novas estratégias terapêuticas para o seu controlo, com menos efeitos adversos.

O lúpus eritematoso sistémico é uma doença autoimune complexa, caracterizada por uma grande diversidade de manifestações clínicas, sendo a atividade desta doença e os efeitos adversos dos fármacos com indicação para o seu tratamento responsáveis por uma elevada taxa de morbilidade e mortalidade

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O impacto desta doença é particularmente importante nas crianças uma vez que crescem com o ónus de uma doença crónica e incapacitante, que se expressa através de fadiga, depressão, dor crónica, originando múltiplas deslocações ao hospital e a necessidade frequente de internamento. É uma doença que igualmente introduz uma carga psicológica considerável interferindo na vida escolar e social destas crianças promovendo o seu isolamento e assim comprometendo a integração social, fragilizando o estabelecimento das ligações emocionais e afetivas presentes e futuras, essenciais para o seu desenvolvimento saudável com especial enfoque no período conturbado como o da adolescência, quando a importância do grupo e a aceitação pelos seus pares assume uma dimensão marcante e poderosa. Esta conjuntura traduz-se na frequente e elevada prevalência de insucesso escolar e de desemprego neste grupo de doentes, consistindo o presente estudo uma tentativa de resolução destes fatores disruptivos, ao propor-se investigar a patogénese desta doença perspetivando a descoberta de novas estratégias para o seu tratamento.

Estudos recentes realizados num modelo animal de lúpus eritematoso sistémico mostraram que a translocação crónica de patobiontes pode contribuir para o descontrolo imunológico e para o aumento do interferão tipo I detetado nesta doença. Neste modelo foi confirmado que a manipulação do microbioma levava a uma melhoria significativa dos sintomas, permitindo assim vislumbrar a conceção de novos protocolos terapêuticos, reformulação ou adaptação dos existentes para esta e outras doenças autoimunes. Todavia, o modelo animal é redutor em relação à complexidade da interação entre dieta, microbioma e imunidade, daí a relevância da execução de estudos em humanos, que constituirão uma ferramenta fundamental para melhor gerir a doença e obter prognósticos mais favoráveis para que sofre desta patologia.

Encontra-se assegurado o direito à integridade moral e física do participante, cumpre as precauções essenciais, cujo desígnio visa minimizar eventuais danos para os seus direitos de personalidade, bem como o direito à privacidade e à proteção dos dados pessoais que lhe dizem respeito, em harmonia com o respetivo regime jurídico.

Com os melhores cumprimentos,

Presidente da Comissão de Ética do CHULN e CAML

Prof. Doutor José Luís P. Duda Soares

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Projects approval by the Ethics Committee of *Centro Académico de Medicina de Lisboa*.

Appendix 5

QUESTIONÁRIO DE AVALIAÇÃO DE ADESÃO À DIETA MEDITERRÂNICA
PREDIMED (PREvencio´n con DIeta MEDiterra´nea)

1 Utiliza azeite como principal gordura culinária?	Sim ☐	Não ☐
2 Que quantidade em colheres (c) de azeite consome num dia (incluindo o usado para fritar, temperar saladas, refeições fora de casa, etc.)?	< 4 c de sopa ☐	≥ 4 c de sopa ☐
3 Quantas porções (p) de produtos hortícolas consome por dia? (1 porção: 200 g; considere acompanhamentos como metade de uma porção)	< 2 p cozinhadas ☐	≥ 2 p cozinhadas ☐
4 Quantas peças de fruta (incluindo sumos de fruta natural) consome por dia?	< 3 peças por dia ☐	≥ 3 peças por dia ☐
5 Quantas porções de carne vermelha, hambúrguer ou produtos cárneos (presunto, salsicha, etc.) consome por dia? (1 porção: 100-150 g)	< 1 porção por dia ☐	> 1 porção por dia ☐
6 Quantas porções de manteiga, margarina, ou natas consome por dia? (1 porção: 12 g)	< 1 porção por dia ☐	> 1 porção por dia ☐
7 Quantas bebidas açucaradas ou gaseificadas bebe por dia?	< 1 porção por dia ☐	> 1 porção por dia ☐
8 Quantos copos de vinho bebe por semana?	< 7 copos por semana ☐	≥ 7 copos por semana ☐
9 Quantas porções de leguminosas consome por semana? (1 porção: 150 g)	< 3 porções por semana ☐	≥ 3 porções por semana ☐
10 Quantas porções de peixe ou marisco consome por semana? (1 porção: 100-150 g de peixe ou 4-5 unidades ou 200 g de marisco)	< 3 porções por semana ☐	≥ 3 porções por semana ☐
11 Quantas vezes por semana consome produtos de pastelaria ou doces comerciais (não caseiros), como bolos, bolachas, biscoitos?	< 3 vezes por semana ☐	≥ 3 vezes por semana ☐
12 Quantas porções de oleaginosas (nozes, amêndoas, incluindo amendoins) consome por semana? (1 porção 30 g)	< 3 porções por semana ☐	≥ 3 porções por semana ☐
13 Consome preferencialmente frango, peru ou coelho em vez de vaca, porco, hambúrguer ou salsicha?	Sim ☐	Não ☐
14 Quantas vezes por semana consome hortícolas, massa, arroz ou outros pratos confecionados com um refogado (molho à base de tomate, cebola, alho-francês ou alho e azeite)?	< 2 vezes por semana ☐	≥ 2 vezes por semana ☐

Adaptado de "Revista Factores de Risco Nº31 Jan-Mar 2014 Pág. 48-55.Direção Geral da Saúde.

PREDIMED questionnaire (519).

Appendix 6

Quadro 1 – Tradução final do índice KIDMED para a língua portuguesa

Índice KIDMED	
+1	Consomes uma fruta ou sumo de fruta todos os dias
+1	Consomes uma segunda fruta todos os dias
+1	Consomes produtos hortícolas frescos ou cozinhados regularmente, pelo menos uma vez por dia
+1	Consomes produtos hortícolas frescos ou cozinhados regularmente, mais de uma vez por dia
+1	Consomes peixe regularmente (pelo menos, 2 a 3 vezes por semana)
−1	Frequentas, mais de uma vez por semana, restaurantes de "fast-food" (hambúrguer)
+1	Consomes leguminosas, mais de uma vez por semana
+1	Consomes massa ou arroz, quase todos os dias (5 ou mais vezes por semana)
+1	Consomes cereais ou produtos derivados de cereais (pão, etc) ao pequeno-almoço
+1	Consomes frutos oleaginosos (nozes, amêndoas, etc) regularmente (pelo menos, 2 a 3 vezes por semana)
+1	Usas azeite em casa
−1	Costumas tomar o pequeno-almoço
+1	Consomes lacticínios (leite, iogurte, etc) ao pequeno-almoço
−1	Consome produtos confecionados ou pastelaria ao pequeno-almoço
+1	Consomes 2 iogurtes e/ou queijo (40g) diariamente
−1	Consomes doces ou guloseimas várias vezes por dia

Adaptado de Serra-Majem *et al.* (2004)

KIDMED questionnaire (520).

Appendix 7

Estamos interessados em conhecer os níveis de actividade física habitual dos Portugueses. As suas respostas vão ajudar-nos a compreender o quanto activos somos.
As questões referem-se ao tempo que dispende na actividade física numa semana. Este questionário inclui questões acerca de actividades que faz no trabalho, para se deslocar de um lado para outro, actividades referentes à casa ou ao jardim e actividades que efectua no seu tempo livre para entretenimento, exercício ou desporto.
As suas respostas são importantes. Por favor responda a todas as questões mesmo que não se considere uma pessoa activa.

Obrigado pela sua participação

Ao responder às seguintes questões considere o seguinte:

***Actividade física vigorosa** refere-se a actividades que requerem muito esforço físico e tornam a respiração muito mais intensa que o normal.*
***Actividade física moderada** refere-se a actividades que requerem esforço físico moderado e torna a respiração um pouco mais intensa que o normal.*

Ao responder às questões considere apenas as actividades físicas que realize durante pelo menos 10 minutos seguidos.

1a Habitualmente, por semana, quantos dias faz actividades físicas **vigorosas** como levantar e/ou transportar objectos pesados, cavar, ginástica aeróbica ou andar de bicicleta a uma velocidade acelerada?

___ dias por semana
___ Nenhum (passe para a questão **2a**)

1b Quanto tempo costuma fazer actividade física vigorosa por dia?

____ horas ___ minutos

2a Normalmente, por semana, quantos dias faz actividade física **moderada** como levantar e/ou transportar objectos leves, andar de bicicleta a uma velocidade moderada ou jogar ténis? Não inclua o andar/caminhar.

_____ dias por semana
_____ Nenhum (passe para a questão **3a**)

2b Quanto tempo costuma fazer actividade física moderada por dia?

____ horas ___ minutos

3a Habitualmente, por semana, quantos dias **caminha** durante pelo menos 10 minutos seguidos? Inclua caminhadas para o trabalho e para casa, para se deslocar de um lado para outro e qualquer outra caminhada que possa fazer somente para recreação, desporto ou lazer.

____ dias por semana
____ Nenhum (passe para a questão **4a**)

3b Quanto tempo costuma caminhar por dia?

____ horas ___ minutos

3c A que passo costuma caminhar?

_____ Passo **vigoroso**, que torna a sua respiração muito mais intensa que o normal;
_____ Passo **moderado**, que torna a sua respiração um pouco mais intensa que o normal;
_____ Passo **lento**, que não causa qualquer alteração na sua respiração;

As últimas questões referem-se ao tempo que está sentado diariamente no trabalho, em casa, no percurso para o trabalho e durante os tempos livres. Estas questões incluem o tempo em que está sentado numa secretária, a visitar amigos, a ler ou sentado/deitado a ver televisão.

4a Quanto tempo costuma estar sentado num **dia de semana**?

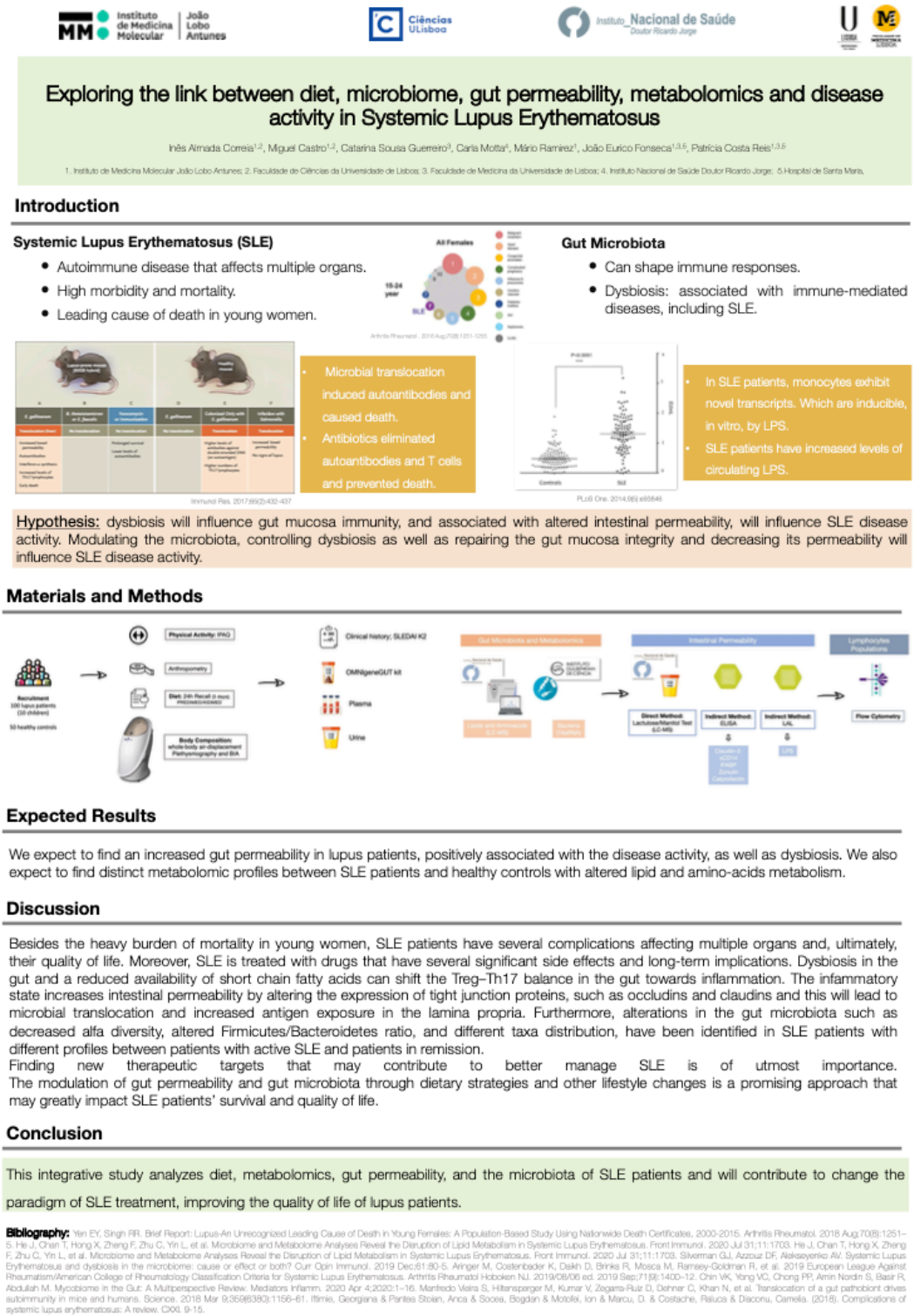
____ horas ___ minutos

4b Quanto tempo costuma estar sentado num **dia de fim-de-semana**?

____ horas ___ minutos

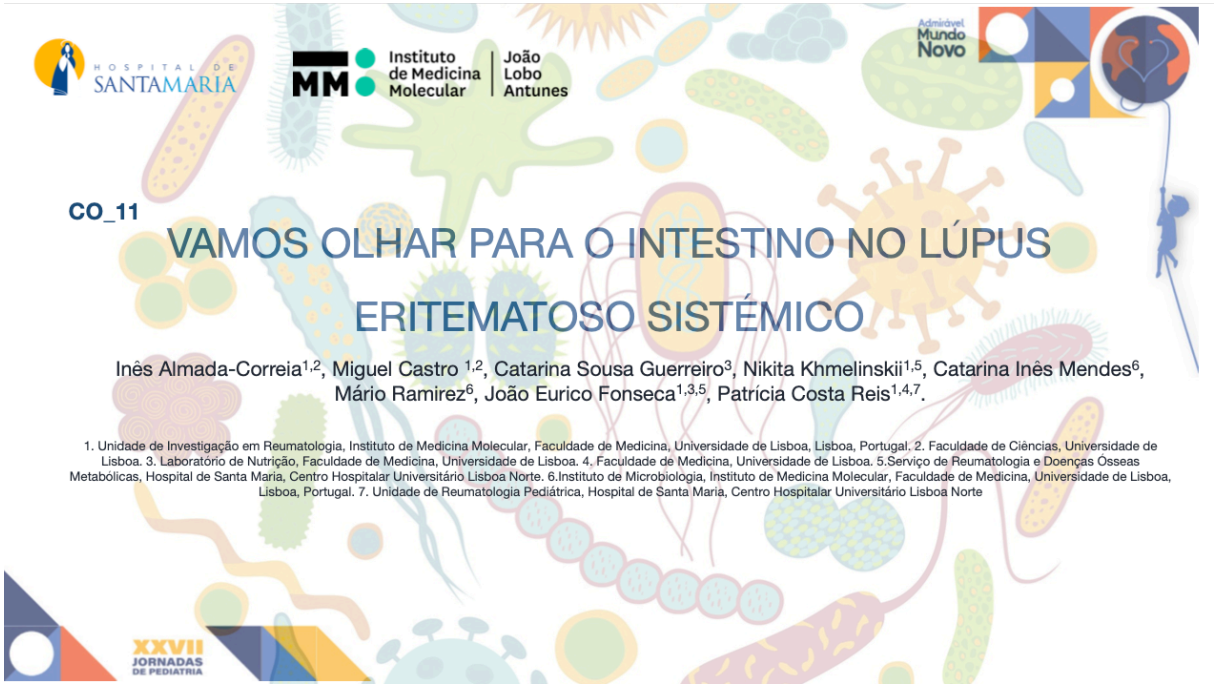
Physical Activity Questionnaire (IPAQ) short form (529).

Appendix 8



Exploring the link between diet, microbiome, gut permeability, metabolomics, and disease activity in Systemic Lupus Erythematosus (Poster). Almada-Correia I, Castro M, Sousa-Guerreiro C, Motta C, Ramirez M, Fonseca JE, Costa-Reis P. iMed Conference. Innovate Competition, Lisbon, Portugal, Oct. 7 (2021).

Appendix 9

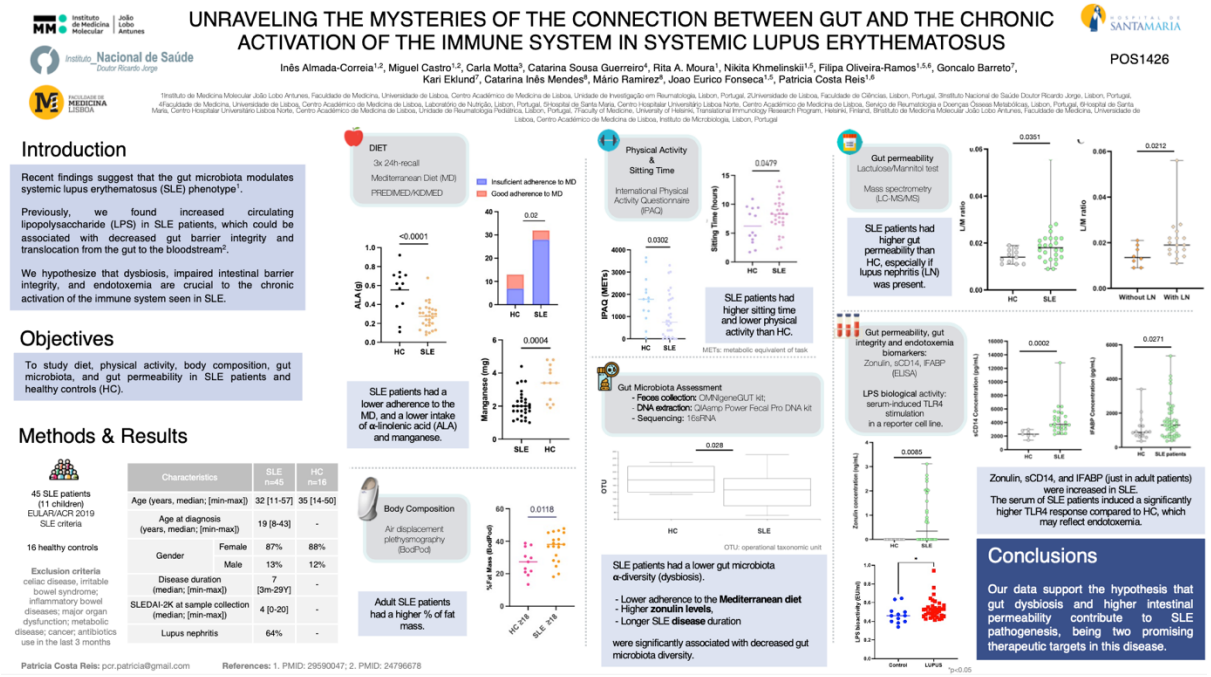


Vamos olhar para o intestino no lúpus eritematoso sistémico (Oral 11). Almada-Correia I, Castro M, Sousa-Guerreiro C, Khmelinskii N, Mendes CI, Ramirez M, Fonseca JE, Costa-Reis P. 2022. XXVII Jornadas de Pediatria do Hospital de Santa Maria, Lisbon, Portugal, February 23 - 25.

Appendix 10

Almada-Correia I, Castro M, Motta C, Sousa-Guerreiro C, Moura R, Khmelinskii N, Mendes CI, Ramirez M, Fonseca JE, Costa-Reis P. 2022. DIET, GUT MICROBIOTA, AND INTESTINAL PERMEABILITY IN SYSTEMIC LUPUS ERYTHEMATOSUS (Oral CO-030). 22º Congresso Nacional de Pediatria, Porto, Portugal, October 26 - 28.

Appendix 11

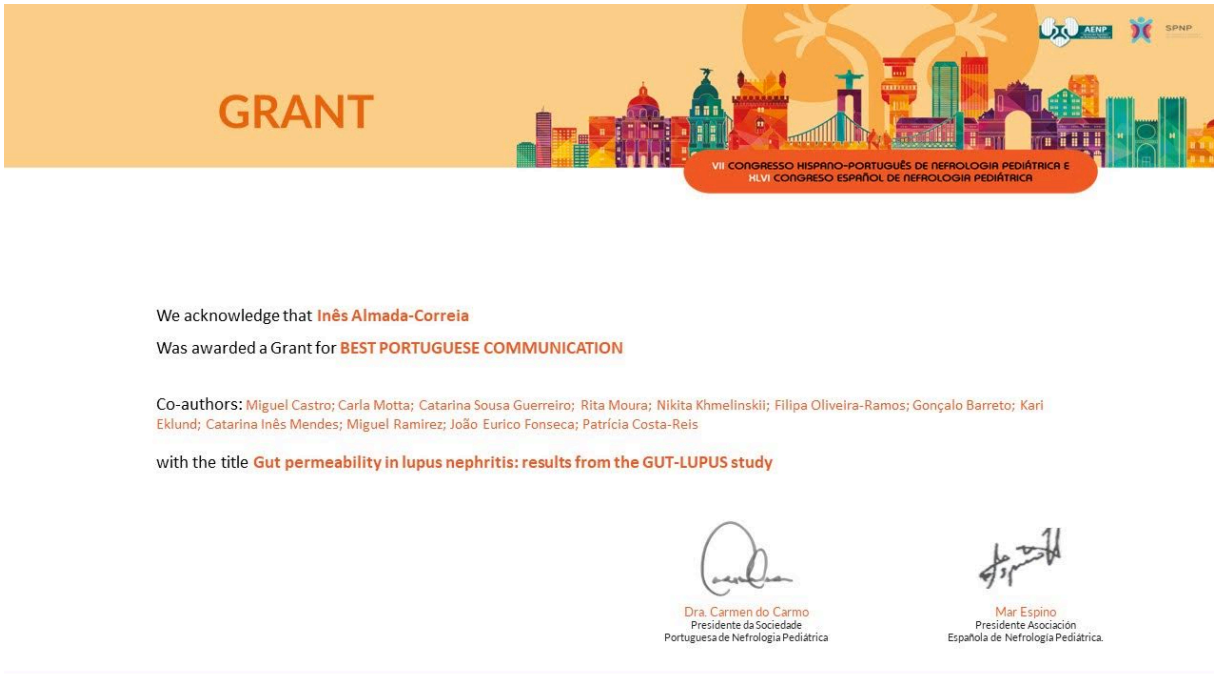


Almada-Correia I, Castro M, Motta C, Sousa-Guerreiro C, Moura R, Khmelinskii N, Oliveira-Ramos F, Mendes CI, Ramirez M, Fonseca JE, Costa-Reis P. 2023. Unraveling the mysteries of the connection between gut and the chronic activation of the immune system in Systemic Lupus Erythematosus (POS1426). European Congress of Rheumatology EULAR 2023, Milan, Italy, May 31 - June 03.

Appendix 12

Almada-Correia I, Castro M, Motta C, Sousa-Guerreiro C, Moura R, Khmelinskii N, Oliveira-Ramos F, Mendes CI, Ramirez M, Fonseca JE, Costa-Reis P. 2023. Com os olhos no intestino: Microbiota, Permeabilidade Intestinal e Dieta no Lúpus (Oral presentation; Table: Exploring the Gutsy World of Microbiota in Disease). XXV Congresso da Associação Portuguesa de Nutrição Entérica e Parentérica (APNEP), Lisbon, Portugal, April 15-18

Appendix 13



Almada-Correia I, Castro M, Motta C, Sousa-Guerreiro C, Moura R, Khmelinskii N, Oliveira-Ramos F, Mendes CI, Ramirez M, Fonseca JE, Costa-Reis P. 2023. Gut permeability in lupus nephritis: results from the GUT-LUPUS study (COL17). VII Congresso Hispano-Português de Nefrologia Pediátrica 2023 and XLVI Congreso Español de Nefrologia Pediátrica 2023, Lisbon, Portugal, May 18 - 19.