Università di Foggia

PhD Thesis in

Biotechnology and Smart Practices for a sustainable Management of Natural Resources, Food and Agriculture

XXXVIII Cycle

Synergistic anti-inflammatory effects of β-hydroxybutyrate and Medium-Chain Triglycerides on Caco-2 cells mimicking the Ketogenic Diet

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Accademic year 2024/2025

TABLE OF CONTENTS

PART I: BACKGROUND

1. PREFACE

2. ULTRA-PROCESSED FOOD INTAKE AND INCREASED RISK OF OBESITY: A NARRATIVE REVIEW

2.1. Introduction

2.2. Understanding obesity

2.2.1. Definition and classification of obesity

2.2.2. Phenotype of obesity

2.2.3. Factors contributing to obesity epidemic

2.2.4. Health consequences of obesity: obesity and associated conditions

2.2.4.1. Neurodegenerative Disease

2.2.4.2. Diabetes

2.2.4.3. Apnea

2.2.4.4. Autoimmunity

2.2.4.5. Cardiovascular Disease

2.3. Ultra-Processed Food: Definition and Characteristics

2.4. Link between Ultra-Processed Food Consumption and Obesity

2.5. Ultra-Processed Food and Dietary Patterns

2.6. Public Health Implications

2.7. Challenges and Future Directions

2.8. Conclusions

3. THE KETOGENIC DIET, AN ADJUVANT STRATEGY TO IMPROVE CANCER MANAGEMENT: FOCUS ON PROSTATE CANCER

3.1. Introduction

3.2. Ketogenic diet

3.2.1. Mechanism of action of the ketosis process

3.3.Focus on cancer: a comprehensive view of the functioning of ketone bodies

3.3.1. Ketone bodies and Warburg effect

3.3.2. Ketogenic diet and oxidative stress

3.3.3. Ketogenic diet and systemic inflammation

3.3.4. Ketogenic diet and mitochondrial metabolism

3.4. Effectiveness of ketogenic diet on prostate cancer

3.4.1. Role of the ketogenic diet on the tumour microenvironment

3.4.2. Dietary implications in prostate cancer

3.4.3. The emerging role of Schwann cells in prostate cancer: potential links with the ketogenic diet

3.4.4. Clinical and translational implications

3.4.5. The role of the microbiota in the efficacy of ketogenic diet in cancer

3.4.6. Interindividual variability and potential biomarkers of response to ketogenic diets

3.4.7. Safety considerations and potential adverse effects

3.5. Conclusion

4. B-HYDROXYBUTYRATE AND MEDIUM-CHAIN TRIGLYCERIDES: BIOLOGICAL RATIONALE FOR IN VITRO INVESTIGATION IN INTESTINAL EPITHELIAL CELLS

4.1. β-hydroxybutyrate as a nutritional modulator of intestinal inflammation

4.2. Microbiota-driven immune dysregulation in inflammation: impact of ketogenic diet and β-hydroxybutyrate

4.3. Protective role of medium-chain triglycerides in intestinal inflammation and epithelial integrity

4.4. Rationale for investigating direct epithelial effects of BHB and MCTs

5. OBJECTIVE OF THE DOCTORAL THESIS PROPOSAL

PART II: EXPERIMENTAL INVESTIGATION USING CACO-2 CELLS

6. SYNERGISTIC ANTI-INFLAMMATORY EFFECTS OF B-HYDROXYBUTYRATE AND MEDIUM CHAIN TRIGLYCERIDES ON CACO-2 CELLS MIMICKING THE KETOGENIC DIET

6.1. MATERIALS AND METHODS

6.1.1. Caco-2 cell culture

6.1.2. Preparation of β-hydroxybutyrate (BHB) solution

6.1.3. Preparation of MCT oil nanoemulsion

6.1.4. Cell viability assay (MTT)

6.1.5. Cell morphology assay

6.1.6. Cell migration (wound closure assay)

6.1.7. Cytokine quantification by ELISA
6.1.8. Western blot analysis
6.1.9. Flow cytometry analysis

6.2.STATISTICAL ANALYSIS
6.3.RESULTS

6.3.1. Effects of β-hydroxybutyrate and MCT oil on Caco-2 cell viability
6.3.2. Effect of β-hydroxybutyrate and MCT oil on Caco-2 cell morphology
6.3.3. Effect of β-hydroxybutyrate and MCT oil on cell migration (wound closure assay)
6.3.4. Modulation of cytokine expression by β-hydroxybutyrate and MCT oil (ELISA analysis)
6.3.5. Effects of β-hydroxybutyrate and MCT oil on intestinal barrier–related adhesion proteins in Caco-2 cells
6.3.6. Effects of β-hydroxybutyrate and MCT oil on ZO-1 expression assessed by Flow Cytometry

6.4. DISCUSSION
6.5. LIMITATIONS OF THE STUDY
6.6. CONCLUSION

PART III: GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

LIST OF PUBLICATIONS
ACKNOWLEDGEMENT

PART I: BACKGROUND

PREFACE

In recent decades, nutrition has acquired a central role in understanding the pathogenic processes of major chronic noncommunicable diseases. Obesity and related diseases such as type 2 diabetes mellitus, cardiovascular disease, inflammatory bowel disease, as well as neurodegenerative diseases and cancers, share a common denominator: a state of chronic, low-grade inflammation, significantly influenced by lifestyle and, in particular, dietary habits.

Obesity, recognized by the World Health Organization as a major global health emergency, today represents the physiopathological precursor to numerous pathological conditions. It is not merely excess weight, but is a complex condition characterized by chronic, low-grade inflammation, alterations in body composition, adipose tissue dysfunction, and profound metabolic dysregulation. Over a billion people worldwide are affected by excess weight or obesity, with a significant impact in terms of morbidity, mortality, and socioeconomic costs.

Adipose tissue plays a central role: it is not simply an energy depot, but a true endocrine and immunomodulatory organ, capable of producing pro-inflammatory cytokines, altering metabolic homeostasis, and promoting insulin resistance, endothelial dysfunction, and tumor progression. Obesity is associated with an increased risk of neurodegenerative diseases, diabetes, autoimmune diseases, and cancers, including prostate cancer.

The rise in obesity is associated with a profound shift in current global dietary patterns. The increased consumption of ultra-processed foods (UPF), classified according to the NOVA classification system, has only exacerbated this "global epidemic," altering nutritional quality and moving away from Mediterranean diet patterns. UPFs, energy-dense foods rich in added sugars, saturated and trans fats, additives, and emulsifiers, as well as low in fiber and micronutrients, now represent a significant portion of daily caloric intake in Western countries, which are now defined as "obesogenic."

Scientific evidence demonstrates that excessive intake of these products is not only associated with weight gain, but also contributes to broader metabolic dysregulation: alteration of the gut microbiota, increased epithelial barrier permeability, activation of the innate immune system, and amplification of systemic inflammation. The gut microbiota emerges as a crucial mediator in the dialogue between diet and host, influencing carbohydrate and lipid metabolism, the immune response, and susceptibility to the development of metabolic and oncological diseases.

In contrast to this dietary pattern, numerous studies have highlighted the protective role of traditional dietary patterns, such as the Mediterranean diet, in reducing cardiometabolic risk. However, in recent years, growing interest has been directed at nutritional interventions for both preventive and therapeutic purposes, including the ketogenic diet.

The ketogenic diet is a dietary regimen characterized by a drastic reduction in caloric intake, specifically carbohydrates, a relative increase in fat content, and protein intake tailored to specific needs. This induces a metabolic state called nutritional ketosis. In this condition,

the liver is stimulated to produce ketone bodies, primarily β-hydroxybutyrate (BHB), acetoacetate, and acetone, which become alternative energy substrates to glucose.

The ketogenic diet, in addition to its well-established neurological effects, is currently being studied as an adjuvant strategy in the management of metabolic and oncological diseases.

In cancer, carbohydrate restriction promotes a metabolic environment unfavorable to tumor proliferation.

Neoplastic cells, in fact, exhibit impaired metabolic flexibility because they predominantly utilize aerobic glycolysis (Warburg effect).

In particular, in prostate cancer, one of the most common malignancies in the male population, nutritional modulation represents a promising integrated approach to conventional therapies, with potential effects on systemic inflammation, oxidative stress, and the tumor microenvironment.

In this context, β-hydroxybutyrate represents not only an alternative energy source, but a true signaling molecule with pre-iotropic action.

Its action as an epigenetic modulator, regulator of inflammation through inhibition of the NLRP3 inflammasome, and promoter of intestinal barrier integrity suggests a possible role in reducing intestinal permeability, modulating the immune response, and improving chronic inflammatory conditions, including inflammatory bowel disease (IBD).

Various ketogenic protocols have been described in the literature, differentiated by macronutrient composition, degree of carbohydrate restriction, and clinical purpose. The classic ketogenic protocol, originally developed for the treatment of drug-resistant epilepsy, involves a lipid-to-protein-carbohydrate ratio (ketogenic ratio) generally equal

to 4:1 or 3:1. The Modified Atkins Diet (MAD) represents a less restrictive variant of the classic ketogenic protocol. Unlike the 4:1 or 3:1 diet, the MAD does not require a rigorous calculation of the ketogenic ratio and allows for a higher protein intake while maintaining significant carbohydrate restriction. The Very Low-Calorie Ketogenic Diet (VLCKD) combines carbohydrate restriction and marked calorie reduction, finding application primarily in the treatment of obesity. Finally, the medium-chain triglyceride (MCT) ketogenic diet represents a variation of the traditional protocol, introduced with the aim of increasing the efficiency of ketone body production while maintaining greater flexibility in carbohydrate and protein intake. Medium-chain triglycerides, due to their unique chemical structure, are absorbed directly into the portal circulation and rapidly oxidized in the liver, promoting faster and more consistent production of ketone bodies compared to long-chain triglycerides.

This allows for a state of ketosis with a lower total lipid percentage than the traditional diet, allowing for greater carbohydrate and protein intake and, in some cases, improving palatability and treatment adherence. From a metabolic standpoint, the MCT diet is therefore characterized by greater ketogenic efficiency with the same carbohydrate restriction. In addition to promoting faster ketone body production, MCTs exhibit unique metabolic properties that may contribute to the modulation of inflammation and the maintenance of intestinal epithelial function.

In light of these considerations, this doctoral thesis aims to explore the role of nutrition as a prevention tool and a potential integrated therapeutic strategy for major chronic diseases associated with modern lifestyles.

Part I of the thesis includes two narrative reviews:

• The first analyzes the link between the consumption of ultra-processed foods and increased obesity risk, exploring definitions, obesity phenotypes, cardiometabolic implications, and public health impacts;

• The second examines the ketogenic diet as an adjuvant strategy in cancer management, with a particular focus on prostate cancer, illustrating its biochemical and molecular mechanisms, including its effects on mitochondrial metabolism, oxidative stress, systemic inflammation, and the tumor microenvironment.

Part II is dedicated to the *in vitro* experimental validation of the biological rationale outlined in the previous sections. Specifically, the combined effects of β-hydroxybutyrate and MCT oil were analyzed on Caco-2 intestinal epithelial cells, a widely used model for studying barrier function and intestinal inflammatory responses. Therefore, the study aims to evaluate the individual and combined effects of β-hydroxybutyrate and MCTs in the presence or absence of a proinflammatory stimulus (LPS) on key parameters of epithelial homeostasis, including barrier integrity and modulation of the inflammatory response.

The entire thesis develops along a coherent and progressive thread: from a critical analysis of dietary factors contributing to the obesity epidemic and metabolic dysregulation, to the evaluation of emerging nutritional models with potential therapeutic value, and to *in vitro* experimental studies aimed at validating their biological effects at the cellular level. From this perspective, nutrition is considered not solely as an environmental risk factor, but as an active biological determinant capable of directly modulating cellular and molecular processes involved in the pathogenesis of chronic diseases.

In conclusion, this work offers an integrated understanding, highlighting how nutritional modulation can represent a concrete and measurable biological lever in the prevention and therapeutic support of modern chronic diseases. This perspective paves the way for further investigations aimed at developing personalized nutritional strategies, grounded in solid molecular foundations and geared towards the biological modulation of key processes involved in the pathogenesis of chronic diseases.

2. ULTRA-PROCESSED FOOD INTAKE AND INCREASED RISK OF OBESITY: A NARRATIVE REVIEW (doi: 10.3390/foods13162627)

2.1.INTRODUCTION

Obesity has been identified as a complex multifactorial disease, the antechamber for the onset of pathological conditions such as diabetes, coronary heart disease, stroke, hypertension, and various tumors affecting the colon, breast, and prostate. Currently, it represents one of the main causes of preventable death: approximately 100,000–400,000 deaths have been estimated every year in the United States, and there has been an increase in health spending amounting to 140–215 billion dollars [1-3].The exponential increase in obesity over the last 30 years indicates that weight gain may depend on individual choices in response to the economic environment. The literature has identified several factors that potentially play a crucial role related to the increase in obesity. Prominent among these are higher income levels, the technological evolution of the food industry, the increased prevalence of fast-food restaurants and commercials inducing the population to purchase junk food, possible changes in time preferences, declining smoking rates, an increased prevalence in major depression, and changes in health insurance [4]. To identify dietary factors related to weight gain and obesity, researchers have focused on the choice of dietary patterns [5]. Several epidemiological studies indicate a strong inverse correlation between adherence to a healthy and balanced dietary protocol, such as the Mediterranean model, and an

increase in cardiometabolic risk [6-8]. Conversely, a diet characterized by highly processed foods is closely associated with obesity and related metabolic comorbidities [9,10]. An understanding of the impact of ultra-processed foods (UPFs) on health and as a risk factor for diet-related diseases, disorders, and conditions is rapidly emerging. In modern society, the role of UPFs proposed by the food industry contrasts with traditional dietary models that promoted the consumption of whole meal products and 0 km foods and emphasized the importance of the locality and seasonality of foods [11,12]. In recent years, the whole food production chain has been significantly influenced by the evolution of technological advances, which have led to the greater accessibility and commercialization of UPFs. The sophistication of production processes has structurally and sensorially modified the nutritional content of foods [13,14] in proportion to the degree of transformation. UPFs are therefore "empty" from a nutritional point of view, but full of added food substances such as sugars, salt, protein isolates, syrups with high fructose content, maltodextrins, and additives such as colorants, flavorings, artificial sweeteners, and thickeners used to enhance the taste of the product, making it palatable, easily usable, and economically accessible. Recently, the view that it is the processing of products that is closely associated with the rise of the obesity epidemic has been gaining more and more credence. This has been verified and supported by a review of prospective cohort studies conducted on more than one million subjects [4]. This led to the development of the NOVA food classification system according to the transformation process, which allows us to identify the dietary drivers related to the increased risk of obesity [15-17]. The NOVA classification system provides for a grouping of foods into four

categories depending on the degree and extent of processing used [18,19]: unprocessed or minimally processed foods; processed culinary ingredients; processed foods that are produced by adding salt, oil, sugar, or other culinary ingredients to minimally processed foods and processed foods; and finally, at the pinnacle of the processing spectrum, ultra-processed foods (UPFs) [15-17,20]. Several studies highlight that UPF consumption is increasing exponentially in recent years and characterizes more than half of the daily calories in US [16], Canadian [19], or British [21] diets. UPF abuse leads to a nutritionally unbalanced diet, rich in calorie-dense foods, saturated fats, sugar, and salt, but low in fiber and micronutrients [21], with a potentially high impact on health [22]. In light of this evidence, the purpose of this narrative review lies in researching the correlation between ultra-processed foods and the increased trend of obesity and related cardiometabolic outcomes.

2.2.UNDERSTANDING OBESITY

2.2.1. Definition and classification of obesity

Obesity is a public problem of considerable health importance throughout the world. It affects more than 1 billion people worldwide, of which 650 million are adults [23]. Obesity leads to the onset of metabolic syndrome [24], defined by the National Institutes of Health as a procession of closely related metabolic dysfunctions, including abdominal adiposity, hypercholesterolemia, hypertension, and impaired fasting glycemia [24]. The clinical picture presented is associated with an increased risk of mortality and many non-communicable diseases (NCDs) [24]. The World Health Organization (WHO) defines obesity as “a clinical condition characterized by excessive body

weight due to the accumulation of adipose tissue to an extent that negatively affects health" [4,25]. Recent surveys conducted by the WHO on a global scale have shown that obesity is seriously affecting the healthcare system, with risk factors that have led to premature deaths and disabilities (Millennium Burden of Disease Analysis). Therefore, obesity has been recognized as a rapidly expanding global epidemic, and its speed of spread is demonstrated by the prevalence of excess weight (overweight and obesity) doubling between 1980 and 2008 [26]. The late clinical recognition of obesity drastically reduces the possibility of intervention on a clinical, educational, and nutritional level. A system widely used to stratify the population, recognize the degree of severity of the disease, and be easy to use and non-invasive is the Body Mass Index (BMI), an easily calculable indicator defined as the ratio between body weight expressed in kilograms (kg) and height measured in square meters (m^2). According to the BMI, an underweight individual has a BMI < 18.5, normal weight $18.5 \leq BMI \leq 24.99$, overweight $25.0 \leq BMI \leq 29.99$, and obese $BMI \geq 30.0$. Obesity is subclassified into Class I obesity ($30.0 \leq BMI \leq 34.99$), Class II ($35.0 \leq BMI \leq 39.99$), and Class III or morbid obesity ($BMI \geq 40.0$). BMI is a less specific measure as it tends to overestimate obesity among subjects with greater muscle mass and underestimate obesity among older adults who have lost muscle mass [27]. To specifically identify obesity, it is necessary to analyze not only the BMI, but also other anthropometric measures: many epidemiological studies have shown that anthropometric measures to identify abdominal obesity such as waist circumference (WC) and waist–hip ratio (WHR) are strong predictors for non-communicable diseases such as type 2 diabetes mellitus [28] and cardiovascular disease (CVD) [29]. World

Health Organization (WHO) guidelines state that WC, WHR, and waist-to-height ratio (WHtR) have been shown to be superior predictors to BMI [4,25]. Despite these limitations, BMI continues to be the most commonly used indicator in empirical research on obesity. It is easily measured during a physical examination and cross-referenced with other anthropometric parameters to determine whether the examined subject has a tendency toward obesity. In fact, epidemiological and economic studies on the effects of excess weight, classified according to BMI, indicate that in 2016, obesity levels in the adult population in the United States were estimated at 36.2%, of which 67.9% were overweight, compared to data reported in 1975 where obesity rates did not exceed 11.9%. Even in the pediatric population the trend is rapidly increasing: the prevalence rate of obesity has reached 21.4%. In 2016, Italy recorded an obesity prevalence of 19.4%, with 58.5% of pre-obese individuals. Costa et al. [30] reported that the prevalence of overweight children in Italy is comparable to that in the United States. It has been estimated that the costs related to excess weight in Italy amount to 4% of the total annual health expenditure of the Italian National Health Service, approximately 4.5 million euros. However, the recorded costs are underestimated due to limitations linked to the classification based on BMI, which does not precisely estimate the prevalence of obesity in the population [31], due to the use of a single scale for both sexes and for all ages; it is therefore an imprecise method, because it may not correspond to the same percentage of body fat [32]. The quantification of adipose tissue with anthropometric measurements, plicometry, and ultrasound is influenced by the distribution of fat by sex and age and by operator-dependent error.

Diagnostic imaging, especially computed tomography, nuclear magnetic resonance, biohymoedenziometry, and dual X-ray absorptiometry (DEXA), are considered more accurate and reliable techniques. DEXA is the gold standard for detecting body composition, not only for the degree of accuracy of the results, but also because it is minimally invasive and has an excellent price/detection time ratio. DEXA, a very accurate diagnostic tool, defines the entire body or a segment in terms of the fat, lean, and bone mass [33]. Studies of the Italian population by sex and age indicate a fat mass percentage of 30% for women and 25% for men [34]. In clinical practice, additional techniques such as plethysmography and bio-impedance testing are used, but they cannot be considered diagnostic tools in the same way as DEXA, as they are not very accurate assessments.

Early screening and immediate intervention and prevention policies and strategies have proven to be effective tools to reduce the spread of obesity and related complications, and therefore health and economic spending, with the aim of increasing investments in prevention campaigns, medical training, and the promotion of a healthy and balanced lifestyle [35].

2.2.2. Phenotype of obesity

There are different obese phenotypes, and this distinction depends on a series of factors: the co-partitioning and diffusion of adipose tissue, genetic predisposition, body composition, the state of inflammation, and the altered metabolism of macronutrients. Therefore, based on the relationship between these factors and their clinical manifestations, four phenotypic typologies have been proposed: normal-weight obese

subjects (NWOs), metabolically unhealthy normal-weight obese subjects, metabolically healthy obese subjects, and metabolically unhealthy obese subjects. This new classification is not based on anthropometric measures such as BMI [36]; in fact, the NWO phenotype presents a normal BMI, but a strong reduction in lean mass, low-grade inflammation, a greater cardiovascular risk, hypertension, and metabolic alteration [37]. It might seem like a paradox to define a subject as obese, with a normal weight, but there is an explanation: the distribution of visceral and ectopic fat. In fact, metabolically unhealthy obese subjects have a central fat distribution and are therefore subjected to a greater cardiovascular risk [38]. It is now known that adipose tissue is not a simple, inert storage tissue, but is at the center of a complex molecular and hormonal crosstalk. It is to all intents and purposes an endocrine organ capable of modulating metabolism, hormones, and inflammation through the production of pro- and anti-inflammatory cytokines. Its spread and expansion at the subcutaneous level is followed by visceral and ectopic fat deposition, with a progressive increase in inflammation and infiltration [39]. Obesity therefore involves inflammation at a local level, which then extends systemically. The growth of adipose tissue also influences strength, muscle mass, and bone mass. Therefore, among the previously described phenotypes, for completeness, we must also include sarcopenic and osteosarcopenic obesity [40].

2.2.3. Factors contributing to obesity epidemic

Numerous studies state that excess weight is a very complex and rapidly expanding public health problem resulting from a combination of individual (genetics, family education) and environmental (unhealthy

eating habits, social class) factors [41]. According to the predictions of the World Health Organization for 2030, 30% of deaths in the world will be attributable to the so-called “diseases of abundance” or lifestyle-related diseases, and only early identification and adequate management of the factors of risk triggering the disease, and prevention and disease policies, will be able to reverse this trend. Low rates of physical activity and easy and chronic accessibility to junk food make obesity an “acquired” disease whose harmful causes are to be found in lifestyle and behavioral choices. In addition to biological and behavioral factors, the socioeconomic aspect should not be underestimated: Zhang et al. (2020), in fact, report that in specific geographical areas, the environmental impact, defined as “obesogenic”, strongly increases the probability of developing obesity [42].

Additionally, the cultural environment and race likely influence weight. One study highlighted that racial segregation in metropolitan areas was different between the two sexes: there was no obvious association of obesity rates among men, but a positive influence among some women. Specifically, racial segregation for black women was significantly associated with a 1.29 times higher prevalence of obesity (95% CI, 1.00–1.65), while moderately segregated areas had a higher prevalence, 1.35 times higher (95% CI, 1.07–1.70). In contrast, for Mexican-American women living in a highly segregated area, the trend was significantly lower (prevalence ratio, 0.54; 95% CI, 0.33–0.90) [43].

The urban environment also influences obesity rates; in fact, in the first phase of the National Longitudinal Study of Adolescent Health conducted on 20,745 adolescents, it was recorded that the proximity of specific facilities for physical activity in socioeconomically more

affluent residential areas showed a correlation with obesity and overweight rates among adolescents who practiced physical activity at least five times a week [44]. Movement is important in weight gain management programs, especially among adolescents, but in school and work environments, the idea that physical activity is not a priority still reigns.

Lack of physical activity, individual behavior, and food choices drastically influence daily calorie intake. Furthermore, in addition to caloric intake, we must also focus on food quality: due to technological progress in food production processes, there has been a strong reduction in the consumption of healthy foods such as whole foods, fruit, vegetables, and fresh products and an increase in the consumption of canned, ultra-processed foods that are unhealthy, as they are rich in hidden ingredients such as fats, simple sugars, salt, and other ingredients that increase their palatability and shelf life, and harmful not only due to the greater caloric intake [45], but above all because they negatively influence the microbial communities that populate our intestine [46].

The microbiome, i.e., the set of our genetic heritage and the environmental interactions of the microorganisms that populate our body (internal and external), influence our metabolism. Emerging evidence demonstrates that the gut microbiota mediates the relationship between host and environment, feeding on the host's undigested food and in turn producing metabolites and cytokines that influence the host's metabolism. Nutrition therefore has a crucial impact on the composition of the microbiota, and intestinal microbial imbalance, so-called dysbiosis, is strongly associated with metabolic diseases such as obesity. As mentioned above, the intestinal microbiota influences

the host's metabolism through metabolically active substances produced by it that act on satiety centers, insulin resistance, epigenetic factors, bile acid metabolism, and metabolic signaling (Figure 1). This association proves to be a promising field of study at a therapeutic level to treat, or better yet prevent, obesity [46].

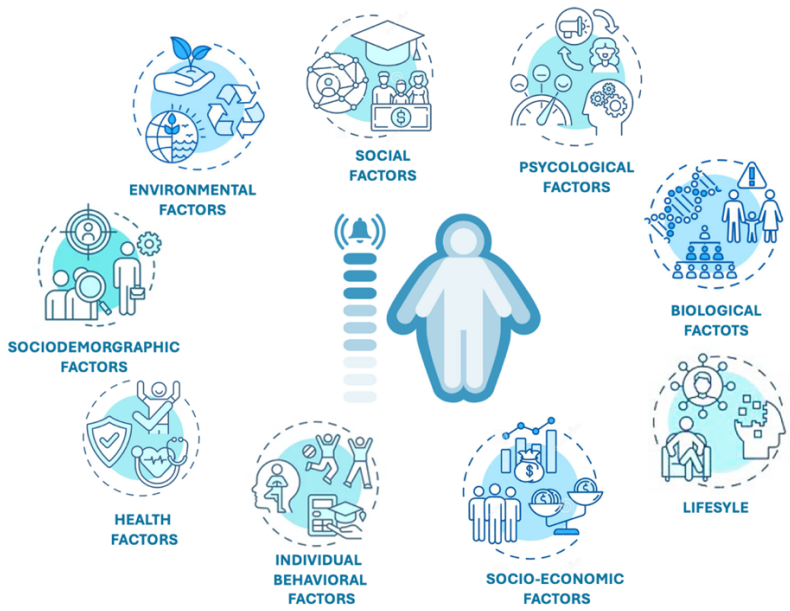


Figure 1: The principal factors leading to obesity.

2.2.4. Health consequences of obesity: obesity and associated conditions

Obesity is associated with pathological conditions of considerable clinical importance or, worse, pre-existing diseases such as coronary heart disease, neurodegenerative disease, hypertension, stroke, type 2 diabetes mellitus, sleep apnea, and osteoarthritis [47].

2.2.4.1. Neurodegenerative disease

As regards neurodegenerative diseases, or pathologies in which the central or peripheral nervous system undergoes progressive degeneration, a strong correlation has been highlighted between obesity and Alzheimer's disease (AD), Parkinson's disease (PD), and dementia in the elderly. A recent study [48] found a strong correlation between AD, PD, and metabolic changes induced by diet. Profenno et al. [49] confirm that obesity and diabetes in adults increase the risk of developing AD.

2.2.4.2. Diabetes

Researchers have estimated that type 2 diabetes mellitus is related to an increased BMI and excess weight in 90% of cases, representing the most associated comorbidity. Obesity and diabetes seem to go hand in hand in metabolically decompensated subjects, and the primum movens is represented by insulin resistance and low-grade inflammation [50,51,52].

2.2.4.3. Apnea

The respiratory system is greatly affected by excess weight. Respiratory diseases such as asthma and obstructive sleep apnea can arise or, if pre-existing, can worsen with weight gain and appear to be more widespread in overweight/obese children [53].

2.2.4.4. Autoimmunity

Recent studies record increased rates of diseases involving the immune system in those suffering from obesity. These conditions include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE),

inflammatory bowel disease (IBD), multiple sclerosis (MS), type 1 diabetes (T1D), and thyroid autoimmunity (TAI), in particular Hashimoto's thyroiditis (HT) [54].

2.2.4.5. Cardiovascular disease

Excess weight is associated with an increase in cardiovascular diseases (CVDs), but reconnecting with the discussion of the obesity phenotype, normal-weight obese patients may even present a worse prognosis. This phenomenon can represent a paradox. The explanation is to be found in the progressive loss of lean mass, such that obese subjects of normal weight, overweight, and with Class I obesity present a greater cardiovascular risk compared to more serious conditions (Class II, III, and above Class III) [55]. Undoubtedly, overweight and obesity increase the onset of heart failure as they influence the structure and function of the heart, ventricular and diastolic [24, 56]. Certainly, excess weight is related to the dysregulation of physiological parameters that lead to the development and worsening of cardiovascular disease, including dyslipidemia, elevated blood sugar, low-grade systemic inflammation, metabolic syndrome, and type 2 diabetes mellitus [24]. One study showed that 23% of heart disease in men and 15% in women was attributable to excess adipose tissue [57] Another study showed that the risk of death increased proportionally with the years of life lived with obesity. Khan et al., in a study with follow up from 1964 to 2015, confirmed that the cardiovascular event was greater in overweight/obese subjects [58].

2.3. ULTRA-PROCESSED FOOD: DEFINITION AND CHARACTERISTICS

The spread of obesity represents a great concern for health, as it is classified as the fifth cause of death worldwide [36]. The latest studies focus on a new perspective: the obesity epidemic seems to derive from a high intake of ultra-processed foods (UPFs). These data emerge from a review of prosthetic cohort studies that analyzed more than 1 million people. UPFs are industrial food formulations that undergo a processing and transformation process through the addition of various ingredients such as simple sugars, fats, salt, and other chemicals such as chemical additives, combined with whole or non-whole foods with the aim of increasing their level of palatability, prolonging their shelf life, and making them convenient and easily available [59,60].

In a recent systematic review, several prospective studies focused on the association between the consumption of ultra-processed foods and the incidence of obesity and cardiometabolic diseases in the adult population [61-66]. In most of the articles analyzed, there is an increasing trend of obesity and complications (such as dyslipidemia, hypertension, type 2 diabetes mellitus, and metabolic syndrome) linked to excessive caloric intake, characterized predominantly by packaged and ultra-processed foods to the detriment of a diet rich in whole meal products, fruit, vegetables, and fresh foods.

In 2010, a NOVA classification model was created, which divides foods into four groups based on the degree and type of food processing and transformation [19, 67, 68]. The first group includes foods that have not undergone a transformation process or have done so only

to a minimal extent: for example, processes aimed at ensuring the edibility, suitability for consumption and conservation, safety, and palatability of altered plants and animals (Figure 2).

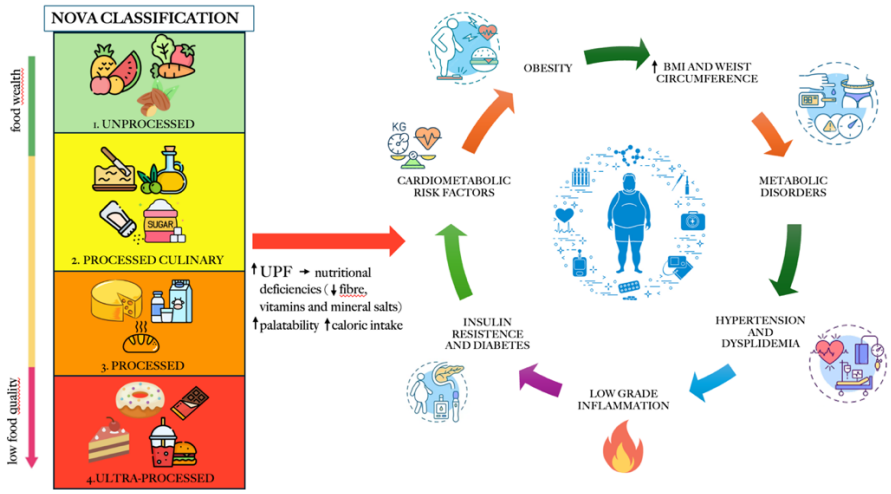


Figure 2: The relationship between UPF foods and obesity.

The second group includes processed ingredients such as butter, oils, salt, and sugar used to make dishes more palatable. The third group includes processed foods, i.e., products to which two/three ingredients from the first and second groups are added to prolong their shelf life and improve their organoleptic quality. Finally, the last classification includes ultra-processed foods (UPFs), i.e., food formulations characterized by the presence of five or more modified or unmodified substances extracted from other foods and combined with minimally whole and non-whole foods, and food additives that improve sensorial quality and prolong shelf life. These include packaged products,

breakfast cereals, sweet and savory snacks, packaged bread, margarine, reconstituted meat foods, ready-to-eat soups, ready-to-eat frozen foods, and carbonated and distilled alcoholic beverages. The consumption of UPF foods is spreading exponentially, resulting in nutritional deficiencies (fiber, vitamins, and mineral salts) and a high caloric intake. Several studies show a positive correlation between the consumption of UPF foods and increased rates of obesity and cardiometabolic risk among adult and child populations [61, 69, 70] (Table 1).

Topic	Details
Global Concern	Obesity is the fifth leading cause of death worldwide.
Impact on Quality of Life	Excess weight compromises quality of life and affects over 1 billion people globally, including 650 million adults.
Obesity and UPFs	Recent studies suggest the obesity epidemic may be driven by the high intake of ultra-processed foods (UPFs). UPFs are rich in calories but low in nutrients, contributing to metabolic disorders.
Nature of UPFs	Industrial food formulations processed with added sugars, fats, salt, and chemicals to increase palatability, shelf life, and convenience.
NOVA Classification Model:	Created in 2010, it divides foods into four groups based on processing degree and type. Unprocessed or minimally processed foods for edibility, consumption suitability, conservation, safety, and palatability (Group 1). Processed ingredients (e.g., butter, oils, salt, sugar) used to enhance palatability (Group 2). Processed foods with added ingredients from Groups 1 and 2 to prolong shelf life and improve organoleptic quality (Group 3). Ultra-processed foods (UPFs) with five or more ingredients, including additives for sensory quality and shelf life (Group 4).
Examples of UPFs	Packaged products, breakfast cereals, snacks, packaged bread, margarine, reconstituted meat foods, ready-to-eat soups and frozen foods, carbonated and distilled alcoholic beverages.
Nutritional Impact of UPFs	Lead to nutritional deficiencies (fiber, vitamins, minerals) and high caloric intake.
Health Correlation	Positive correlation between UPF consumption and higher rates of obesity and cardiometabolic risk in both adults and children.

Table 1: Key points summarizing the negative effect of UPFs on health and the close correlation between the excessive consumption of UPFs and increased obesity.

2.4. LINK BETWEEN ULTRA-PROCESSED FOOD CONSUMPTION AND OBESITY

The demand for UPF foods has increased over time, and the causes are to be found in the lifestyle of today's society: intense work rhythms, the lack of time, and quick meal management lead to a greater demand for foods that can be easily consumed during work activity [8, 71, 72]. This leads to an unconscious consumption that alters neural and digestive functions, which dysregulate hunger perception and satiety levels, which in turn leads to excessive consumption [73, 74]. Furthermore, it has been shown that these foods—precisely because they are poor from a nutritional point of view (in particular in fiber, micronutrients and bioactive compounds [21, 63] but rich in saturated and trans fatty acids, sodium, and refined sugars—reduce satiety levels and therefore increase the glycemic response, altering the insulin response [75] which leads to a greater search for food, favoring a greater energy intake [76] and establishing a serious vicious circle which over time leads to excess weight.

The picture presented determines cardiovascular, metabolic, and blood pressure outcomes that are risky for health. In most UPFs, the salt content is high, and this contributes to increasing the risk of developing hypertension [77]. As previously mentioned, the addition of simple sugars also alters fructose metabolism, in particular favoring insulin resistance first at the liver level and then at the systemic level, exacerbating low-grade inflammation. To overcome insulin resistance and restore glycemic levels, pancreatic β cells produce a greater amount of insulin through a negative feedback mechanism, leading to structural damage and reduced functionality [78]. Furthermore, the

excessive consumption of UPFs also leads to a modification of the lipid structure due to the high presence of saturated and trans fatty acids, compromising the catabolism of very-low-density lipoproteins (VLDL-C) and increasing triglycerides in the blood and reducing HDL cholesterol [79]. Finally, the presence of chemical additives, preservatives, and synthetic antioxidants further worsens the clinical picture [80, 81]. The packaging used for UPF foods is characterized by releasing chemicals such as bisphenol A, which is harmful to the endocrine system (Figure 2) [82-84].

2.5. ULTRA-PROCESSED FOOD AND DIETARY PATTERNS

UPF foods differ from non-UPF foods not only in their composition and type of production, but also in their destination. As previously reported, the aim is to obtain artificial, ready-made, quick, easy-to-take, highly palatable foods produced with cheap ingredients [21, 67, 68]. The production of UPF products contrasts with the recommendations of the dietary model proposed by the Mediterranean diet, which limits the consumption of packaged, processed, and ultra-processed foods. From a nutritional point of view, UPFs appear very unbalanced, composed mostly of ingredients that should be limited, such as added sugars, salt, and saturated and trans fats, and poor in fiber, vitamins, and mineral salts [19]. To paraphrase Paracelsus, it is the dose that makes the poison. In the Mediterranean model, in fact, the consumption of these foods is not demonized, but their intake is limited. Numerous studies conducted on a national scale have recorded a close co-occurrence between the consumption of UPF and the nutritional inadequacy of diets [85]. The appearance of pathological conditions

and metabolic complications does not depend only on an excessive daily intake, but on the entirety of the serious nutritional imbalance that derives from the excessive use of these foods. In fact, manufacturing processes generate an inevitable loss of important nutrients for our body and transform others into harmful substances; this happens, for example, with the hydrogenation of polyunsaturated fats. The risk linked to the manipulation and transformation of the food matrix is not limited only to the addition of ingredients such as sugars, vegetable oils, protein isolates, milk powder, additives, emulsifiers etc., but to the formation and release of harmful substances from synthetic packaging [86]. The physico-chemical profile of UPF foods is very complex, so it is reductive to focus on the analysis of a small group of nutrients, which can overlook the evaluation of the substances that are released by the type of ultra-process used. In fact, the analysis often hides the modification at the molecular level to which these products are subjected. Research is moving towards the identification not only of the nutritional profile, but also of composition, chemical structure, and unclear mechanisms that impact health [87]. How does the new formation of toxic substances, chemical hydrogenation, and the presence of synthetic substances influence our biological systems? What are the consequences? At this point, we need to introduce the term “reconstitution”: ingredients used in the manufacturing process are reconstituted through various operations such as the hydrogenation of fats, the extrusion of cereals, and the mechanical extraction of meat which modify the chemical–physical structure of the food matrix, generating new substances [75,76]. The list of ingredients present in UPF foods is very long, among which we also find colorants, artificial sweeteners such as aspartame, saccharin, and acesulfame K, and

emulsifiers [88, 89]. The use of these substances is not accidental; in fact, they make the product very tasty; they create addiction, leading to greater consumption [65]. They are mistakenly called “foods”, but in reality they are anything but nutritious compared to minimally processed or fresh/whole foods which maintain their own nutritional characteristics. It would be more appropriate to define them as non-nutrient in terms of the substances that are lost and degraded during the processing operations. When the diet is particularly rich in these non-nutrients that replace unprocessed foods, the impact on health is harmful. Ultimately, it is possible to distinguish two dietary models: the first includes minimally processed products, i.e., products or dishes prepared at home or artisanally in restaurants with few processed ingredients or with the addition of processed foods, while the second model includes UPF foods, i.e., drinks and ultra-processed foods such as ready meals and fast food distributed on a large scale [90]. Supermarket shelves are full of this wide range of UPF foods, sweet and savory, and drinks of all kinds, accompanied by claims that attract the attention of the consumer, who is less and less attentive to food quality and consumes his meal quickly during his lunch break. Therefore, the background into which UPFs are inserted contrasts with the model of the Mediterranean diet, which on the contrary encourages consuming meals as a family, around a well-laid table that offers healthy and tasty foods, prepared together. Marketing strategies prove to be very effective in promoting these products and in their combination, such as hamburgers, chips, and carbonated drinks rich in added sugars or snacks and fruit juices, which contain everything other than fruit. The damage that these products generate in the long

term on the health of those who feed themselves totally or almost entirely with these products is worrying: a randomized and controlled study recorded that subjects who consumed 80% of their calories from these non-nutrients had a high caloric intake, an increase in adipose tissue, and ultimately severe malnutrition (Table). In fact, some studies have revealed a positive correlation between the ultra-processed dietary model and the nutritional scarcity of the diet [92]. Finally, further perspectives are shown in meta-analyses and cohort studies which demonstrate what was previously asserted: dose–response models show in fact that the UPF model can potentially cause damage and become dangerous, leading to the onset of chronic non-communicable diseases and therefore to an increase in mortality when the consumption of these foods becomes dominant.

Topic	Details
UPF Production and Composition	UPFs are made with cheap ingredients, aiming to be ready-made, quick, easy-to-take, and highly palatable.
Nutritional Imbalance of UPFs	High in added sugars, salt, saturated, and trans fats; low in fiber, vitamins, and minerals.
Mediterranean Diet Contrast	Limits consumption of packaged, processed, and ultra-processed foods. Promotes fresh, minimally processed foods.
Nutritional Inadequacy	UPFs linked to unbalanced diets and pathological conditions due to excessive intake and nutritional imbalance.
Nutrient Loss and Harmful Substances	Manufacturing processes of UPFs cause nutrient loss and creation of harmful substances (e.g., hydrogenation of fats).
Risks from Packaging	Harmful substances can be released from synthetic packaging used for UPFs.
Complexity of UPFs	Physico-chemical profile is complex, often hiding harmful modifications at the molecular level.
Reconstitution in UPFs	Ingredients are reconstituted through processes like hydrogenation, extrusion, and mechanical extraction, altering the food matrix.
Additives in UPFs	Includes colorants, artificial sweeteners (aspartame, saccharin, acesulfame K), and emulsifiers. These enhance taste and create addiction.
Health Impact	UPF-rich diets lead to high caloric intake, increased adipose tissue, severe malnutrition, and chronic non-communicable diseases.
Regulation Needs	Urgent need for stricter regulation on UPFs, evaluating their nutritional composition and public health impact.
Prevention Strategies	Reduce UPF consumption, especially in countries with high intake. Define safe cut-offs for sensitive age groups.

Table 2: Main characteristics which summarize the chemical and nutritional composition of UPFs.

In conclusion, the aspect that should not be underestimated is the market strategy implemented by companies that produce UPF foods, which use advertising policies aimed at promoting and legitimizing their products, regardless of their nutria-chemical composition and therefore quality. Tighter regulation of these foods is necessary and urgent, in terms of evaluating the nutritional composition and the impact that this dietary model produces on public health. UPF dietary patterns should be reduced, especially in countries that consume them widely and with a view to primary and secondary prevention, especially in the most sensitive age groups, defining safe cut-offs for health [93] (Table 2).

2.6. PUBLIC HEALTH IMPLICATIONS

Addressing the global epidemic of obesity requires multifaceted approaches, and public health policies play a pivotal role in shaping individuals' dietary habits. One critical aspect is curbing the consumption of ultra-processed foods, which are laden with unhealthy additives, sugars, and fats. Governments can implement policies aimed at reducing the availability and accessibility of ultra-processed foods. This includes imposing taxes on sugary beverages and snacks, restricting marketing to children, and regulating portion sizes in food outlets. In addition, establishing clear nutritional guidelines can help consumers make informed choices [48]. Governments can collaborate with health experts to develop guidelines that prioritize whole, minimally processed foods, while discouraging the consumption of ultra-processed options. Educating the public about the health risks associated with ultra-processed foods is essential. Public health campaigns

can highlight the adverse effects of excessive sugar, salt, and trans fats on overall health, particularly in relation to obesity, cardiovascular diseases, and diabetes. Also, improving food labeling is crucial for empowering consumers to make healthier choices. Clear and concise labeling that indicates the presence of additives, excessive sugars, and unhealthy fats can help individuals identify ultra-processed products and opt for healthier alternatives. Encouraging the consumption of whole foods, such as fruits, vegetables, whole grains, and lean proteins, is fundamental [46]. Initiatives that make these foods more affordable, accessible, and appealing can shift dietary preferences away from ultra-processed options. It is important, also, to have school-based interventions; indeed, schools play a significant role in shaping children's dietary habits. Implementing nutrition education programs and ensuring that school meals adhere to health guidelines can promote healthier eating behaviors from a young age [91]. Furthermore, engaging communities through initiatives like farmers' markets, community gardens, and cooking classes can foster a culture of healthier eating. These interventions not only provide access to fresh, whole foods but also empower individuals with the knowledge and skills to prepare nutritious meals at home. Holding food manufacturers accountable for the nutritional quality of their products is essential. Encouraging reformulation to reduce the levels of added sugars, sodium, and unhealthy fats in processed foods can contribute to improving their overall dietary quality. Combating obesity and reducing the consumption of ultra-processed foods requires a comprehensive approach that encompasses policy changes, education, community involve-

ment, and industry cooperation. By implementing these strategies collectively, societies can promote healthier food environments and mitigate the burden of obesity-related diseases [92].

2.7. CHALLENGES AND FUTURE DIRECTIONS

Addressing the complex issues of obesity and ultra-processed food consumption presents numerous challenges, but also opportunities for innovative research and policy development. One of the greatest challenges is the unequal distribution of resources and access to healthy foods. Low-income communities often have limited access to fresh, nutritious foods and may rely heavily on cheap, ultra-processed options [90]. Addressing these disparities requires multifaceted approaches that consider social, economic, and environmental factors. In addition, the powerful influence of the food industry presents a significant barrier to promoting healthier food environments. Marketing tactics, product placement, and lobbying efforts can perpetuate the consumption of ultra-processed foods, making it challenging to implement effective policies. Future interventions must navigate this landscape, while advocating for public health [91]. Changing dietary behaviors is inherently challenging, requiring sustained motivation, support, and access to resources. Many individuals face barriers such as cultural preferences, lack of cooking skills, and ingrained habits that make it difficult to adopt healthier eating patterns. Future interventions should incorporate behavioral science principles to facilitate lasting change [84]. Ultra-processed foods often have a higher environmental footprint compared to whole, minimally processed alternatives. Addressing obesity while promoting environmental sustainabil-

ity requires a balance between health outcomes and ecological considerations. Future research should explore strategies for promoting sustainable diets that benefit both human health and the planet. In addition, individual genetic predispositions and biological factors can influence susceptibility to obesity and metabolic disorders [92, 93]. Understanding the interplay between genetics, metabolism, and dietary factors is essential for personalized approaches to obesity prevention and treatment. Emerging research areas such as nutrigenomics offer insights into how genetic variation affects responses to dietary interventions. Evaluating the effectiveness of policy interventions is crucial for refining strategies and maximizing their impact. Robust evaluation methods, including longitudinal studies and randomized controlled trials, are needed to assess the outcomes of public health policies targeting obesity and ultra-processed food consumption. Additionally, translating research findings into actionable policies requires collaboration between policymakers, researchers, and stakeholders. Also, the proliferation of digital health technologies offers new avenues for addressing obesity and promoting healthier food choices. Mobile apps, wearable devices, and online platforms can facilitate behavior tracking, provide personalized feedback, and deliver educational content to support individuals in managing their weight and making healthier dietary choices.

In the coming years, research should continue to address these challenges, while exploring innovative approaches to promoting healthier food environments and reducing the burden of obesity-related diseases. Collaboration across disciplines, sectors, and stakeholders will be essential for developing comprehensive solutions that improve public health outcomes on a global scale.

2.8. CONCLUSIONS

In conclusion, the link between ultra-processed food consumption and obesity is undeniable, with a mounting body of evidence highlighting the detrimental effects of these foods on public health. The proliferation of ultra-processed foods in modern diets, characterized by their convenience, affordability, and palatability, has contributed to the global obesity epidemic. These foods, laden with unhealthy additives, sugars, and fats, disrupt normal hunger and satiety cues, leading to excessive calorie consumption and metabolic dysregulation. Behavioral science principles should be integrated into interventions to facilitate lasting behavior change, while efforts to promote sustainability must balance health outcomes with environmental considerations. Personalized approaches to obesity prevention and treatment, informed by genetics and metabolism, offer promising avenues for future research. Robust evaluation methods are needed to assess the effectiveness of policy interventions, and digital health technologies can enhance efforts to promote healthier food environments and empower individuals to make informed choices. In the face of these challenges, continued collaboration and innovation are essential for developing comprehensive solutions that improve public health outcomes and reduce the burden of obesity-related diseases on a global scale.

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3. THE KETOGENIC DIET, AN ADJUVANT STRATEGY TO IMPROVE CANCER MANAGEMENT: FOCUS ON PROSTATE CANCER (under review)

3.1. INTRODUCTION

Cancer, often referred to as the disease of the century, is insidious because it frequently develops silently and goes unnoticed. The disease is further complicated by the ability of some tumour types to evade the body's defence mechanisms, resulting in resistance to conventional oncological and pharmacological therapies. Despite numerous medical efforts to combat cancer, it remains one of the leading causes of death today. Recent estimates from the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) suggest that 19.98 million new cancer cases were diagnosed worldwide in 2022. Future projections suggest a dramatic increase in incidence; it is estimated that this figure will double by 2050 compared to 2022 [1].

In cancer research, efforts have focused extensively on approaches such as surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy to mitigate disease progression, metastasis and therapeutic resistance [2]. Therapeutic resistance arises from the complex cellular network of the tumour microenvironment (TME), which includes cancer-associated fibroblasts (CAFs), immune cells (e.g. T lymphocytes, macrophages, and dendritic cells), endothelial cells (ECs), and pro-inflammatory cytokines, chemokines, and growth factors (e.g. vascular endothelial growth factor (VEGF)). The TME's primary function is to modulate inflammation, angiogenesis, and proliferation. One of the pathophysiological mechanisms that is currently

of considerable relevance within this cellular complexity is endothelial-mesenchymal transition (EndoMT). This predominantly influences tumour progression by promoting fibrosis and immunosuppression, as well as facilitating metastatic dissemination. During EndoMT, endothelial cells lose their phenotypic characteristics and acquire mesenchymal traits. This process promotes extracellular matrix remodelling and fosters a pro-tumourigenic microenvironment and high resistance to therapies [3].

Recent scientific evidence has identified glial-derived cells, particularly Schwann cells (SCs), as potential promoters of TME remodelling. In response to tumour stimuli, SCs can acquire a pro-tumourigenic phenotype by producing neurotrophic factors, pro-inflammatory cytokines and matrix-remodelling substances. This leads to increased tumour invasiveness and dissemination. The mediation between neural and tumour cells contributes to the creation of an immunosuppressive microenvironment and metabolic reprogramming that support cell survival and resistance to therapy [4, 5]. Given the crucial role of TME in tumor progression, including neural components such as SCs, their involvement in prostate cancer metabolism has gained increasing attention. In this context, the metabolic reprogramming induced by ketogenic diets may interact with Schwann cell–tumor crosstalk, potentially offering further insight into mechanisms linking ketogenic metabolism to prostate cancer biology. This association could therefore represent an important step forward in research exploring SCs as potential therapeutic targets.

Conversely, tumour cells have evolved to meet their increased energy demands in order to survive and spread, remodelling their metabolism in the process. Following the discovery of the Warburg effect in the

1920s, the theory that tumour cells follow different metabolic pathways to healthy cells became widely accepted. Metabolically, tumour cells use a process called aerobic glycolysis, or fermentation, even in the presence of oxygen [6–8]. In terms of energy yield, this is not ideal as glycolysis produces fewer energy units than cellular respiration. However, this process enables the sudden biosynthesis of components necessary for the proliferation and growth of tumour cells [9, 10]. Research is increasingly focusing on dietary approaches such as fasting, caloric restriction and, in particular, the ketogenic diet (KD), which are potentially effective strategies for disrupting the energy metabolism of tumour cells [11–14].

The KD is characterised by a high fat content, an adequate protein intake tailored to individual needs, and a remarkably low carbohydrate intake of less than 50 g per day. The classic ketogenic diet (cKD) was the first dietary protocol developed to complement drug treatments for drug-resistant childhood epilepsy, with fat, carbohydrate and protein ratios of 3:1 or 4:1 [15]. Since then, new variations have been developed to improve palatability and long-term compliance. These include the medium-chain triglyceride ketogenic diet (MCTKD), the low-glycaemic index treatment (LGIT) and the modified Atkins diet (MAD) [16]. Following glycogen depletion, low carbohydrate availability stimulates hepatic mitochondria to produce ketone bodies (KBs), including acetoacetate (AcAc), β-hydroxybutyrate (BHB) and acetone, via fatty acid oxidation. In the absence of glucose, KBs become the primary energy source for cells. However, their role extends beyond mere energy function; they exert a range of biological effects that may be beneficial, particularly in the context of cancer.

Following lung cancer, prostate cancer (PCa) is one of the most commonly diagnosed cancers and the leading cause of cancer-related death in men worldwide. Although standard treatments such as androgen deprivation therapy (ADT), radiotherapy, immunotherapy, chemotherapy and androgen receptor axis-targeted therapy (ARAT) are effective, the disease can evolve into resistant forms such as castration-resistant prostate cancer (CRPC). This presents a significant clinical challenge [17, 18]. Therapeutic resistance has multifactorial causes involving interconnected mechanisms, such as metabolic reprogramming, alterations in androgen receptor signalling and transmembrane changes.

In this context, a new approach is needed that works alongside anticancer therapies by targeting energy metabolism to slow tumour progression. This review examines the mechanisms of action of KBs, focusing on BHB, which has demonstrated anti-tumour and anti-inflammatory effects. It also explores the pathways that modulate TME) modifications associated with resistance to hormone therapy in prostate cancer (PCa), when combined with standard anti-cancer treatments. The review also summarises relevant preclinical and clinical studies. This narrative review aims to summarize the recent scientific evidence supporting the ketogenic diet as an adjuvant approach to current cancer therapies for prostate cancer. The first part describes variants of the ketogenic diet, their potential effects on glucose metabolism, insulin/IGF-1 signaling, and the tumor microenvironment, and how these processes may influence tumor biology. Next, the implications of the ketogenic diet in prostate cancer are discussed, with a focus on the tumor microenvironment and metabolic alterations associated with disease progression, including key pathways involved in tumor

metabolism. Finally, the available preclinical and clinical evidence is reviewed and potential therapeutic implications and future research directions are discussed.

3.2. KETOGENIC DIET

The ketogenic diet was born in the 1920s as a dietary treatment for drug-resistant epilepsy [15]. For decades, the classic ketogenic diet (cKD) was the only ketogenic protocol used clinically. However, due to the severe carbohydrate restriction patients were subjected to and poor adherence, other types of ketogenic diet protocols have since been developed (Figure 1) to make them more sustainable while achieving the same metabolic effects as the classic protocol [16]. Therefore, the ketogenic diet has evolved into four main therapeutic protocols: 1) the cKD with a ketogenic ratio of 3:1 or 4:1 (i.e., 87% or 90% of energy comes from fat and 7% from protein + 6% from carbohydrates respectively or 7% from protein + 3% from carbohydrates respectively); 2) the MAD diet which provides 64% fat, 30% protein and 6% carbohydrates [19,20]; 3) the MCTKD diet which provides 70% fat of which 30-60% of energy comes from medium chain triglycerides (MCTs) such as caproic (C6), caprylic (C8), capric (C10) and lauric (C12) acids, which have a higher ketogenic value than LCTs, 10% protein and 20% carbohydrates [21]; and 4) the LGIT diet, which provides 60% fat, 30% protein, and 10% low-glycemic index carbohydrates [22,23].

Finally, these protocols include the very-low-calorie ketogenic diet (VLCKD), which involves consuming less than 800 kcal daily and significantly reducing carbohydrate intake, ideally between 20 and 30

grams per day or under 50 grams. A protein intake of 1.2-1.4 g/kg of ideal body weight is recommended, with the rest of the calories derived mainly from fat, such as extra virgin olive oil [24,25]. Different ketogenic diet protocols vary in macronutrient distribution, metabolic effects, and long-term feasibility. These factors must be carefully evaluated when considering their use in cancer treatment, including tolerability and adherence in clinical or preclinical settings. Although several KD protocols are known, there is currently no consensus on the comparative efficacy of these variants with respect to oncological outcomes. Specifically, no specific KD protocol has been identified as more suitable for patients with prostate cancer. The available studies are highly heterogeneous in terms of dietary composition, duration, and study design, making direct comparisons difficult. Therefore, further well-designed clinical studies are needed to clarify the potential role of specific KD approaches in this setting.

An overview of the main ketogenic diet variants and their key features is provided in Table 1. In cases where direct evidence in cancer is lacking, references to studies in other conditions (e.g., obesity, epilepsy) or preclinical models are included to demonstrate potential metabolic effects.

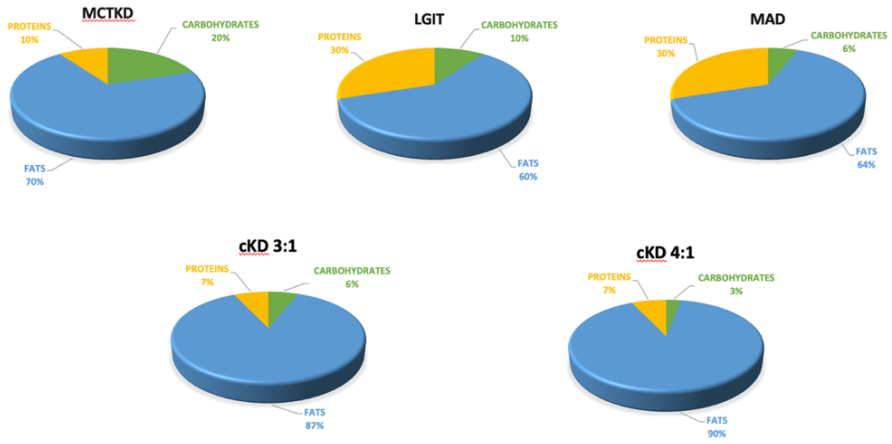


Figure 1: Distribution of macronutrients (fats, proteins and carbohydrates) for the four main types of ketogenic diet, expressed as a percentage of total energy requirements (calories). MCTKD: medium-chain triglyceride ketogenic diet (70% fat, 10% protein and 20% carbohydrate); LGIT: low glycemic index treatment (60% fat, 30% protein and 20% carbohydrate); MAD: modified Atkins diet (64% fat, 30% protein and 6% carbohydrate); cKD 3:1: classic ketogenic diet with a 3:1 ratio (87% fat, 7% protein and 6% carbohydrate); cKD 4:1: classic ketogenic diet with a 4:1 ratio (90% fat, 7% protein and 3% carbohydrate) [16].

KETOGENIC DIET TYPE	MACRONUTRIENT COMPOSITION	FEASIBILITY/ADHERENCE	METABOLIC IMPACT	POTENTIAL CLINICAL APPLICABILITY	REF
CLASSIC KETOGENIC DIET (CKD)	Very high fat (≈87–90%), very low carbohydrates	Low adherence due to strict carbohydrate restriction	Strong ketosis induction	Mainly used in controlled clinical settings	(16)
MODIFIED ATKINS DIET (MAD)	High fat, moderate protein, low carbohydrates	Higher adherence compared with cKD	Moderate ketosis	Potentially suitable for long-term dietary interventions	(9,20)
MEDIUM-CHAIN TRIGLYCERIDE KD (MCTKD)	High fat with significant proportion of MCTs	Better flexibility in carbohydrate intake	Efficient ketone production due to MCT metabolism	May improve tolerability and compliance	(21)
LOW GLYCAEMIC INDEX TREATMENT (LGIT)	Moderate fat, controlled low-GI carbohydrates	High feasibility	Mild ketosis and improved glycaemic control	Potential option for patients requiring less restrictive diets	(22,23)
VERY-LOW-CALORIE KETOGENIC DIET (VLCKD)	Severe carbohydrate restriction with caloric deficit	Short-term adherence possible under medical supervision	Rapid ketosis and weight loss	Often used in obesity or metabolic interventions	(24,25)

Table 1: Main variants of ketogenic diets and their clinical characteristics.

3.2.1. Mechanism of action of the ketosis process

As previously mentioned, carbohydrate restriction, fasting, extended physical activity, and a ketogenic diet elevate serum levels of KBs, which supply the brain and other organs (heart, skeletal muscle, etc.) with an alternative energy source to glucose. However, the role of KBs is pleiotropic, meaning they are not solely involved in energy production; they are also linked to various biological systems, including signaling metabolites that affect gene expression, protein post-translational modifications (PTMs), inflammation, and oxidative stress [26].

The quantity and quality of food influence metabolic body functions [27,28]. During prolonged fasting or a significant calorie deficit involving carbohydrates and proteins, acetyl-CoA, which is derived from free fatty acids and, to a lesser extent, ketogenic amino acids such as phenylalanine, tyrosine, lysine, tryptophan, leucine, isoleucine, and threonine, stimulates the production of KBs in hepatic mitochondria. Once produced in the liver, KBs are released into the bloodstream and absorbed by extrahepatic tissues *via* monocarboxylate transporter 1 (MCT1; SLC16A1). Then, they are metabolized as an alternative energy substrate to glucose [29,30]. Furthermore, KBs are involved in ketolysis, which occurs in the heart, brain and skeletal muscle. This process mobilizes NAD^+, enabling the conversion of (R)-β-hydroxybutyric acid into acetoacetic acid. This intermediate can then enter the Krebs cycle directly to be used as an energy substrate [31]. Insulin and glucagon skilfully orchestrate this complex process (Figure 2).

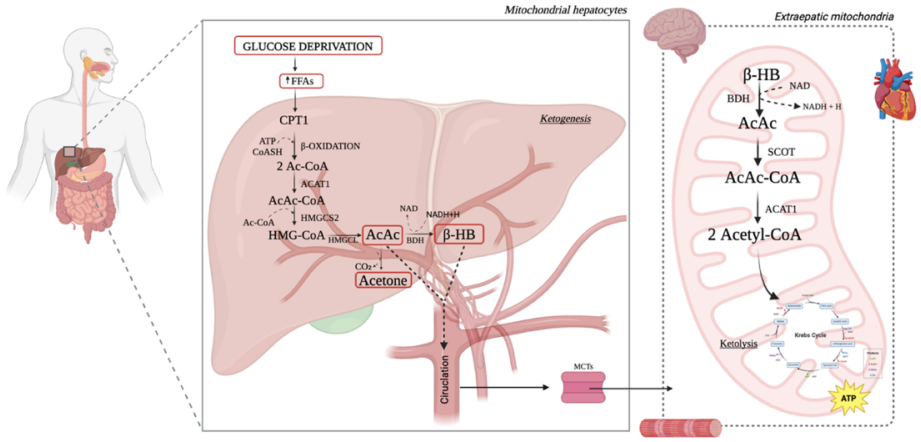


Figure 2: Metabolism of intracellular ketone bodies (KB): hepatic formation versus peripheral utilization. Factors such as a ketogenic diet (KD), fasting, and fasting-mimicking diets (FMD) increase the availability of free fatty acids (FFAs). In hepatic mitochondria, ketone bodies (KB) are synthesized primarily from acetyl-coenzyme A (Ac-CoA), which in turn results from the breakdown of FFAs. In extrahepatic tissues such as the brain, muscle, and heart, ketones are metabolized via ketolysis, producing acetyl-CoA. This then enters the citric acid cycle (TCA) for ATP synthesis. ACAT1: acetoacetyl-CoA thiolase; Ac-CoA: acetyl-CoA; AcAc-CoA: acetoacetyl-CoA; BDH: β-hydroxybutyrate dehydrogenase; BHB: β-hydroxybutyrate; CPT1: carnitine palmitoyltransferase 1; HMGCL: HMG-CoA lyase; HMGCS2: HMG-CoA synthase; MCT: monocarboxylate transporter 1; SCOT: succinyl-CoA:3-ketoacid-CoA transferase.

3.3. FOCUS ON CANCER: A COMPREHENSIVE VIEW OF THE FUNCTIONING OF KETONE BODIES

The effectiveness of the ketogenic diet lies in its ability to remodulate cellular metabolism. This explains its widespread application in epilepsy, neurological diseases, cardiovascular diseases, obesity, type 2 diabetes, and cancer [32].

In oncology, there is growing interest in studying new therapeutic interventions that can modify tumour cell metabolic pathways and TME, thereby mediating the response to cancer treatments and making them more effective. The use of KBs has demonstrated significant modulation of tumour proliferation and expansion, reducing inflammation and "protecting" healthy cells from the damage caused by cancer therapies [33,34]. Nonetheless, this topic is widely discussed and controversial.

The effects of caloric [35,36], carbohydrate [37,38], and protein [39,40] restriction on tumour cell metabolism result in significant genetic, hormonal, metabolic, and immune changes that alter tumour activity [41,42].

3.3.1. Ketone bodies and Warburg effect

To understand how ketones starve cancer cells, it is necessary to consider cellular energy metabolism. Typically, in the presence of oxygen, healthy cells convert acetyl-CoA, derived from the breakdown of carbohydrates, proteins, and fats, into CO_2 *via* the Krebs cycle. This process occurs in the mitochondria and produces nicotinamide adenine dinucleotide hydrogen (NADH). NADH is then used in the oxidative phosphorylation step to produce adenosine triphosphate (ATP)

with minimal lactate production. However, in the absence of oxygen, cells use anaerobic fermentation to produce large amounts of lactate. Otto Warburg first described this phenomenon in 1956 [43], observing that many cancer cells preferred anaerobic glycolysis even when sufficient oxygen was available for cellular respiration [44]. This process allows tumour cells to strategically choose anaerobic glycolysis not for greater energy efficiency, but rather for rapid proliferation, expansion, survival in hostile environments, and evasion of defense systems. Consequently, tumour cells have different metabolic needs than healthy cells, which are regulated by signaling pathways involving recognized oncogenes and tumour suppressor genes [45-47].

This premise can be exploited to target tumour cells selectively. Glucose restriction and KB production result in reduced levels of insulin and insulin-like growth factor-1 (IGF-1). This process downregulates the phosphatidylinositol 3-kinase (PI3K)-Akt and mammalian target of rapamycin (mTOR) signaling pathways, which are overexpressed in various tumours. Consequently, energy efficiency is compromised. Furthermore, KBs are not efficiently metabolized by tumour cells. High glucose levels have been shown to increase tumour proliferation in both animals and humans [48-51].

Hyperglycemia, in particular, consequently leads to increased insulin levels, which in turn stimulate the overexpression of insulin receptors (IR). This activates a metabolic pathway that promotes tumour cell proliferation, the RAS and MAPK cascades. Furthermore, the PI3K, Akt, and mTOR pathways are activated, as NF-κB, which regulates apoptotic and pro-inflammatory pathways [51]. Insulin increases the

expression of vascular endothelial growth factor (VEGF), thus activating angiogenesis and promoting the formation of new blood vessels that support tumour growth and dissemination [52,53].

3.3.2. Ketogenic diet and oxidative stress

In addition to anaerobic glycolysis, tumour cells use the pentose phosphate pathway to produce energy, obtaining nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate from glucose. The goal is to protect themselves from oxidative damage by using NADPH to reduce hydroperoxides. The accelerated energy metabolism of tumour cells subjects them to high levels of reactive oxygen species (ROS), which damage DNA, proteins, and membranes [54]. Although tumour cells can protect themselves from oxidative stress, some therapies exploit this process to induce apoptosis [55,56]. Strategies aimed at enhancing mitochondrial oxidative metabolism and reducing glucose utilization can synergistically and selectively undermine cancer spread. A clinical approach that exploits this metabolic mechanism is the KD: it restricts carbohydrates, thereby inhibiting gluconeogenesis and the production of glucose-6-phosphate [57]. This results in a reduced ability to defend against oxidative stress. Other studies highlight the antioxidant potential of the KD, leading to reduced ROS levels [58,59].

Recent studies have demonstrated that KD can induce tumour ferroptosis in mouse models of pancreatic and colorectal cancer. Specifically, the KD resulted in increased lipid peroxidation and reduced tumour cell redox defense systems, leading to cell death due to the accumulation of toxic peroxides. However, although the KD had antitumour effects, these were undermined by systemic redox imbalance.

For instance, NADPH depletion in the adrenal glands impaired cortisol synthesis, making the animals more vulnerable to cachexia [60]. Another study by Zhang et al. (2020) found that KD increased intratumoural oxidative stress in mouse models of colon cancer by suppressing matrix metalloproteinase-9 (MMP-9) expression [61]. Therefore, recent scientific evidence underscores the importance of the KD as a metabolic adjuvant and its ability to utilize oxidative stress to make tumour cells more sensitive to oncological therapies.

Furthermore, it has been discovered that BHB plays an essential role as an epigenetic regulator. At physiological concentrations (approximately 10 mM), BHB inhibits class I histone deacetylases (HDAC1 and HDAC2). This leads to the hyperacetylation of histones at the promoters of genes implicated in oxidative stress resistance, including FOXO3a and MnSOD (SOD2), thereby enhancing their transcription. An increased expression of antioxidant enzymes and neutralization of ROS are observed, in both *in vivo* and *in vitro* models. In an oncological context, this mechanism represents a considerable benefit: BHB increases antioxidant defenses, protecting tissues from oxidative damage induced by cancer therapies. Through epigenetic modulation, it also prevents tumour cells from proliferating and surviving, making them susceptible to therapies [62]. BHB acts as a metabolic epigenetic signal that remodels redox balance, a central vulnerability of tumour cells.

3.3.3. Ketogenic diet and systemic inflammation

Systemic inflammation is a well-recognized fundamental component of cancer, acting as a predisposing factor and promoter of tumour

dissemination [63]. It plays a key role in supporting the growth, spread, and resistance of cancer to therapies [64,65].

The NLRP3 inflammasome is one of many mediators of inflammation and plays a key role in orchestrating the innate inflammatory response. This intracellular multiprotein structure activates inflammatory processes in response to infections, chronic inflammatory diseases, autoimmunity, and even cancer [66]. Its activity is impacted by the KD. Furthermore, Youm et al. reported that BHB inhibits the assembly of NLRP3 inflammasome components, thereby reducing the production of proinflammatory cytokines [67].

Evidence from human studies indicates that KD reduces tumour growth by modulating systemic inflammation [68]. More precisely, *in vitro* and *in vivo* studies have reported that KBs reverse the NLRP3 inflammasome's action *via* anti-inflammatory, anti-angiogenic, and anti-invasive mechanisms [69]. KBs have also been shown to reduce the activation of inflammatory mediators in the brain, in colorectal cancer, and in glioma [70-72], as well as to contain the influence of the tumour microenvironment on tumour dissemination and progression [73,74].

3.3.4. Ketogenic diet and mitochondrial metabolism

Another challenge in oncology is studying mitochondrial metabolism in tumour cells. Suppose the tumour cell can survive in the presence of high levels of ROS that it releases, as well as in a hypoxic microenvironment. In that case, its mitochondrial metabolism is altered. DNA sequencing studies have revealed that tumour cells harbor mitochondrial mutations, leading to abnormal mitochondrial protein ex-

pression. The speed at which tumours grow requires efficient and effective mitochondrial function to support the increased energy expenditure. For this reason, it has been hypothesized that oncogenic lesions modify mitochondrial metabolism [75].

Mutations in oncogenes such as K-Ras and Myc can increase glucose and glutamine metabolism in tumour cells, thereby significantly influencing mitochondrial metabolism. Although glutamine is a non-essential amino acid, in cancer, it becomes indispensable for the proliferation and survival of malignant cells. In some “reprogrammed” metabolically active cells, glutamine is essential for supporting biosynthesis, NADPH production, redox homeostasis, and rapid growth [76]. Numerous studies emphasized the importance of these mutations in increasing ROS production [57, 75, 77-79]. As previously discussed, KD drastically reduces glucose availability, forcing both healthy and tumour cells to utilize ketones as their primary energy source.

In a 2024 study, Hajihassani et al. investigated the effects of a ketogenic diet (KD) on mouse models of pancreatic cancer. The results revealed increased levels of citric acid cycle intermediates and glutamine, suggesting that tumour cells adapt to reduced glucose availability by relying more heavily on glutamine metabolism. Interestingly, when the KD was combined with inhibitors of glutamine metabolism, tumour cells became more vulnerable due to the simultaneous restriction of primary and alternative energy substrates. However, the KD alone may not be sufficient to completely deplete glutamine in the tumour microenvironment. Consequently, combining KD with glutamine-targeting agents emerges as a promising strategy for exploiting tumour cells' metabolic dependencies, which could enhance

the efficacy of cancer therapies and increase oxidative stress within tumour cells [80].

Tumour cells can survive in a hypoxic environment, which is more prevalent in solid cancers. Newly formed malignant cells receive less oxygen and adapt to survive through the action of hypoxia-inducible factor 1 (HIF-1). Overexpression of HIF-1 is essential for angiogenesis and the survival, adhesion, migration, and dissemination of tumour cells [81]. HIF-1 enables cancer cells to convert pyruvate into lactate, thereby blocking mitochondrial acetyl-CoA metabolism and reducing oxygen consumption [82,83]. A study by Wolf et al. investigating the effects of KD in a mouse model of malignant glioma showed that KBs significantly reduced the expression of proteins involved in hypoxia signaling, thereby supporting tumour survival and progression by downregulating HIF-1 expression. By lowering HIF-1 levels, the KD reduces glycolytic dependence and partially restores mitochondrial substrate flow. Tumour cells that are metabolically adapted to hypoxia are inefficient at using KBs. Consequently, tumour cells experience metabolic and oxidative stress, rendering them more vulnerable and increasing their risk of death. Thus, the KD exploits the metabolic inflexibility of tumour mitochondria, highlighting a mechanistic link between ketone metabolism, HIF-1 modulation, and mitochondrial dysfunction [84].

3.4. EFFECTIVENESS OF KETOGENIC DIET ON PROSTATE CANCER

3.4.1. Roles of the ketogenic diet on the tumour microenvironment

Carcinogenesis is a highly complex process involving numerous interactions between tumour cells and their surrounding microenvironment. In recent years, many studies have investigated the impact of tumour microenvironment (TME) cells on tumour growth and response to cancer treatments. The TME includes cancer-associated fibroblasts (CAFs), immune cells (including T lymphocytes, macrophages, and dendritic cells), endothelial cells (ECs), nervous, and glial cells. The TME also produces pro-inflammatory cytokines, chemokines, and growth factors, such as vascular endothelial growth factor (VEGF). This complex environment regulates fundamental tumour processes, including inflammation, angiogenesis, and cell proliferation [85,86].

Prostate cancer was likely first described in detail in 1853 by surgeon J. Adams from the London Hospital, who at the time considered it a scarce condition [87]. Then, the aging population and the growing adoption of a Western lifestyle have contributed to making it the second most common cancer among men globally. This disease is characterized by androgen dependence, a class of steroid hormones responsible for the development and maintenance of male sexual characteristics [88]. Therefore, new therapeutic strategies are based on endocrine therapies aimed at reducing androgen production or targeting the androgen receptor (AR) [88,89]. Physiologically, when testosterone, and its derivative dihydrotestosterone (DHT) bind to AR, a ligand-gated nuclear transcription factor, transcription of genes involved in: (1) prostate development and maturation, (2) secretion of

proteins such as PSA (prostate-specific antigen), (3) development of sexual characteristics, (4) increased muscle protein synthesis, and (5) stimulation of libido occurs. In pathological conditions such as prostate cancer, binding of AR to its ligand activates several genes involved in cell proliferation, survival, uncontrolled differentiation, and prostatic secretion [88, 90].

Despite new therapeutic strategies, androgen deprivation (ADT) remains the standard of care to treat hormone-dependent PCa. However, disease can evolve into a lethal hormone-independent tumour or castration-resistant prostate cancer (CRPC), which can activate the androgen receptor independently of the presence of androgens associated with treatments resistance. Therefore, androgen receptor (AR) signaling is hypothesized to play a crucial role in tumour growth and progression to resistant and uncontrolled forms [91].

The ketogenic diet is considered a promising nutritional approach in the fight against cancer, particularly drug-resistant cancer. The presence of ketones appears to be metabolically unfavorable for the growth, spread, and survival of cancer cells, as well as for the TME, as they can inhibit several pro-inflammatory signaling pathways. Thus, an increase in tumour resistance related to tumour reprogramming has been reported [92]. This metabolic reprogramming is largely associated with the Warburg effect, whereby tumor cells preferentially utilize aerobic glycolysis even in the presence of oxygen [93]. As discussed previously, this metabolic phenotype represents a potential therapeutic vulnerability that can be exploited by low-carbohydrate or ketogenic diets, which limit glucose availability to tumor cells [94].

In an emerging *in vitro* study, Murphy et al. (2024) reported that the tumour immunology of murine prostate adenocarcinoma cells resistant to immune checkpoint blockade (ICB) can be significantly altered when treated with a cyclic ketogenic diet or an oral BHB-based diet combined with ICB. Specifically, BHB acts as an HDAC inhibitor, increasing primary histocompatibility complex class I expression and making the tumour more recognizable and interceptable by CD8+ T lymphocytes [95]. Specific inhibition of class I HDACs by different concentrations of BHB was also observed in human embryonic kidney 293 (HEK293) cells in a dose-dependent manner. This resulted in changes in gene transcription, increasing the expression of genes encoding resistance to oxidative stress [96]. This demonstrates that glucose restriction or prolonged fasting influences gene expression, where BHB acting as an epigenetic cofactor regulating histone post-translational modifications.

KBs, when used alongside ICB therapy, sensitized immunotherapy-resistant prostate tumours through epigenetic and immunomodulatory actions, thereby enhancing tumour antigen expression. Another remodeling effect mediated by ketosis in combination with ICB therapy was the rebalancing of macrophages from an immunosuppressive M2 phenotype to an antitumour M1 phenotype. This shift decreases the number of tumor-infiltrating dendritic cells that produce tumour necrosis factor-alpha (TNF-α) and iNOS, which promote tumour immunosuppression, angiogenesis, and metastasis [95,97]. These promising findings encourage further investigations into the potential use of KBs in immunotherapy. However, unlike other types of cancer, immunotherapy with immune checkpoint inhibitors requires further studies to confirm its efficacy in prostate cancer.

As previously reported by our team, EndoMT is a crucial process in tumour growth and spread that is regulated by oxidative stress [3, 98]. This transition process seems to develop gradually, initially characterized by a partial downregulation of endothelial markers (VE-cadherin, CD31, Tie-1, Tie-2 and vWF) and up-regulation of some early mesenchymal markers (a-SMA,SM22a and FSP-1) followed by a total reduction of endothelial markers and a predominance of early mesenchymal markers together with CD44, N-cadherin, vimentin, COL I/III and (S100A4) [3] (Figure 3).

As suggested by Yu et al. (2024), since prostate cancer often metastasises to bone early on, cellular transdifferentiation may prepare the metastatic 'niche' by releasing signals that attract and enable tumour cell migration [99]. While these studies do not directly connect KD to prostate cancer cell migration to bone, the well-documented anti-inflammatory and anti-tumour effects of KD imply an indirect link. KD reduces oxidative stress by activating Nrf2 and inhibiting NF-κB signalling, thereby decreasing TNF-α and IL-1β levels [100]. BHB inhibits the NLRP3 inflammasome, directly decreasing pro-inflammatory cytokine release [67]. BHB also influences HDACs, thereby increasing the acetylation of H3K9/14, and supporting the epigenetic expression of endothelial markers such as VEGFR, VE-cadherin, Claudin-5, and CD31. This stabilises the endothelial phenotype [101, 102]. Furthermore, KD reduces MMP-9, which degrades the extracellular matrix and promotes angiogenesis, and influences macrophage polarization [61]. These combined effects create a microenvironment that is less conducive to EndoMT, thereby inhibiting new vessel formation and tumour growth. Other researches have shown that the KD enhances immune responses by increasing CD8+ and CD4+ T cells

while decreasing FOXP3+/CD4+ Treg cells, which are involved in angiogenesis and immunosuppression. The diet also decreases VEGF-A, p-STAT3, and HIF-1α expression, preventing epithelial-to-mesenchymal transition (EMT) by increasing E-cadherin, N-cadherin, and vimentin. Furthermore, the KD influences apoptosis by decreasing STAT1 and p-STAT1, while increasing the tumour suppressors p53, Bax/Bcl-2, and caspase-3 [103,104]. Finally, KD reduces the expression of immune checkpoints, such as PD-L1 and CTLA4, in tumour cells and the tumour microenvironment.

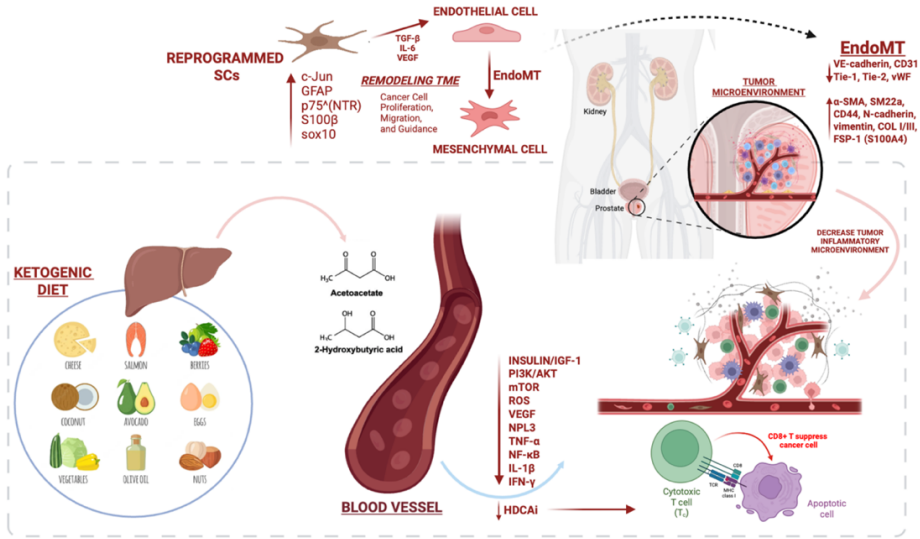


Figure 3: Figure 3. Effects of the ketogenic diet on the tumor microenvironment (TME). A ketogenic diet (high fat, adequate protein, low carbohydrates) promotes hepatic production of ketone bodies, mainly acetoacetate and β-hydroxybutyrate (15). These metabolites enter the circulation and modulate tumor-related metabolic and inflammatory pathways, including reduced insulin/IGF-1 signaling and downregulation of PI3K/AKT/mTOR, VEGF, and inflammatory mediators such as TNF-α, IL-1β, and NF-κB (51). BHB may also act

as a histone deacetylase inhibitor, increasing MHC-I expression and potentially enhancing $CD8^+$ T-cell–mediated tumor recognition. These effects are associated with a reduction in tumor inflammatory activity and with modulation of EndoMT, characterized by decreased endothelial markers (VE-cadherin, CD31, Tie-1/2, vWF) and increased mesenchymal markers (α-SMA, SM22α, CD44, N-cadherin, vimentin, collagen I/III, FSP-1/S100A4) (3). Emerging evidence suggests that reprogrammed Schwann cell expressing S100B, SOX10, c-Jun, GFAP, and p75^NTR, may contribute to TME remodeling and therapy resistance (125).

In summary, these findings highlight the potential role of low-carbohydrate dietary interventions in modulating the tumor microenvironment through metabolic, immunological, and epigenetic mechanisms. However, current studies stem from different levels of investigation, ranging from in vitro studies and preclinical animal models to translational and clinical research. To better contextualize the current state of evidence, the hierarchy of available evidence on ketogenic or low-carbohydrate diets in prostate cancer is summarized in Table 2.

EVIDENCE LEVEL	MODEL/SYSTEM	KEY FINDINGS	IMPLICATIONS FOR PROSTATE CANCER	REF
• **IN VITRO**	Prostate cancer cell lines	Ketone bodies and glucose restriction influence cancer cell metabolism and proliferation	Suggests metabolic vulnerabilities that may be targeted through dietary interventions	(105)

		depending on metabolic context		
• **PRECLINICAL**	Murine prostate adenocarcinoma models resistant to immune checkpoint blockade	Cyclic ketogenic diet or oral BHB supplementation combined with immunotherapy increased MHC-I expression and enhanced $CD8^+$ T-cell recognition of tumor cells	Indicates that ketogenic metabolism may sensitize prostate tumors to immunotherapy	(95)
• **PRECLINICAL/ MECHANISTIC**	Endothelial and inflammatory signaling pathways	Ketone bodies reduce oxidative stress, inhibit NF-κB signaling, suppress NLRP3 inflammasome activation, and stabilize endothelial phenotype, potentially limiting EndoMT and angiogenesis	Indicates potential effects of ketogenic diets on tumor vascular remodeling and metastatic processes	(67, 99, 100, 102)
• **PRECLINICAL**	Mouse xenograft model of prostate cancer	Low-carbohydrate ketogenic diets slowed tumor growth and improved survival compared with Western diets	Indicates that metabolic restriction may suppress tumor progression	(106)

• **PRECLINICAL**	Transgenic adenocarcinoma of mouse prostate (TRAMP) model	Carbohydrate restriction reduced circulating insulin levels and tumor growth	Supports the role of insulin signaling in prostate cancer progression	(107)
• **CLINICAL**	Pilot dietary interventions in prostate cancer patients	Low-carbohydrate diets associated with weight loss and improved metabolic parameters	Clinical relevance remains under investigation; larger trials needed	(108)

Table 2: Hierarchy of experimental and clinical evidence on ketogenic or carbohydrate-restricted diets in prostate cancer.

3.4.2. Dietary implications in prostate cancer

Another particularly relevant factor in the onset and prognosis of prostate cancer is lifestyle and dietary habits [109]. Weight gain, a high body mass index (BMI), insulin resistance, obesity, and diabetes are key characteristics of metabolic syndrome which generates chronic low-grade inflammation that promotes tumour development and proliferation [110]. Insulin resistance (as estimated by HOMA-IR), hyperinsulinaemia, and the glucose-to-insulin ratio have been linked to an increased risk of PCa [111-115]. Advanced tumours exhibit increased numbers IR, indicating a poor disease prognosis for individuals with type 2 diabetes [116]. Periprostatic fat volume (PPFV) is another key indicator associated with an increased risk of advanced prostate cancer. A study of 932 Dutch patients undergoing radiotherapy found that elevated PPFV was associated with cancer having a Gleason

score of 8-10 and with initial PSA levels greater than 10 ng/mL [117]. In another study, PPFV was a significant predictor of response to ADT; notably, it was considerably higher in subjects with CRPC [112]. This phenomenon of resistance is explained by the ability of periprostatic adipocytes to promote extraprostatic tumour spread via the chemokine CCL7. This suggests a clear association between excess weight and worsening disease prognosis [118].

In an emerging meta-analysis, elevated insulin levels are associated with an increased risk of prostate cancer, supporting previous studies that linked obesity with a high Gleason score at diagnosis and disease recurrence with a worse prognosis. [119-121]. Another study suggests that excess weight plays a crucial role in the progression of prostate cancer for every 5-point increase in BMI over 25 kg/m^2 [122]. A prospective study demonstrated that high levels of hypercholesterolemia are associated with a higher incidence of prostate cancer (>240 mg/dl) in subjects with high BMI. Overall, calorie restriction, particularly carbohydrate restriction, reduces obesity and disrupts insulin signaling, both of which are crucial factors in the etiopathogenesis of prostate cancer [123,124]. As discussed previously, reducing insulin/IGF-1 signaling may contribute to the antitumor effects of ketogenic diets. Fokidis et al. (2015) reported a reduction in prostate cancer in castrated mice fed a low-carbohydrate diet compared to those fed a Western diet, which had significantly higher serum glucose levels and IR in tumour tissue [125]. Although further clinical studies in this field are needed to refine its clinical application as an adjuvant therapy, ketogenic diets are currently being implemented in prostate cancer patients undergoing active surveillance (NCT02194516).

The Carbohydrate and Prostate Study 2 (CAPS2), a recent investigation into the link between a low-carbohydrate diet (LCD) and its potential as a treatment for patients with biochemically recurrent (BCR) PCa, showed significant decreases in weight, triglycerides, and HbA1c after 6 months, along with an extended PSA doubling time (PSADT). A metabolomic analysis found higher serum levels of BHB, glycine, and hydroxyisocaproic acid, and reduced levels of alanine, cytidine, asymmetric dimethylarginine (ADMA), and 2-oxobutanoate, which are involved in cardiovascular disease. Elevated BHB was linked to a longer PSADT, indicating slower prostate cancer growth [126].

3.4.3. The emerging role of Schwann cells in prostate cancer: potential liinks with the ketogenic diet

An intriguing link involves Schwann cells (SCs) and their influence on TME, a topic that has recently attracted increased scientific interest. Generally, in healthy tissue, SC, the primary glial cells in the peripheral nervous system (PNS), participate in immune surveillance, turn off inflammation, conduct nerve impulses along axons, support trophic functions, protect and repair damaged nerves, contribute structurally to the extracellular matrix, and can present antigens to immune cells [127,128]. In tumour tissue, SCs activity appears protumourigenic [129,130]. Emerging data link the action of activated SCs (expressing markers such as S100B, sox10, c-Jun, GFAP, p75^(NTR)) to TME reprogramming through the EndoMT process [129]. These markers reveal the plastic nature of SCs, suggesting their role in stromal remodeling in the TME.

An *in vitro* study by Stroka et al. (2015) found that treating prostate cancer cells with SC-conditioned culture medium increased tumour cell invasion. This effect depended on integrin α6, a cell-surface protein that binds to laminin, a component of the extracellular matrix, indicating a highly efficient mechanism by which tumour cells can invade nerves and adjacent tissues [131-132].

The interaction of prostate cancer with the PNS represents a fascinating emerging field of research that could explain tumour resistance. Schwann cells have been shown to promote epithelial–mesenchymal transition (EMT) and induce Schwann-like differentiation of tumor cells through activation of the BDNF/TrkB signaling axis, as demonstrated in salivary adenoid cystic carcinoma models. These findings support the role of tumor–nerve interactions in cancer progression by contributing to the formation of a pro-metastatic microenvironment [133]. Highly innervated organs such as the prostate are prone to perineural invasion (PNI), a process of local tumour invasion in which tumour cells infiltrate the space surrounding nerves (the perineural space) or penetrate the nerve sheath. PNI can be found in up to 75% of prostate cancer cases [134,135]. Furthermore, in a mouse model of prostate cancer metastasis, in which tumour cells were injected into the bone, a significant increase in local nerve fiber density was observed, suggesting neurogenesis within the metastatic microenvironment [136-139].

Interestingly, SCs have been shown to promote macrophage polarization toward the M2 phenotype, thereby supporting tumour progression. In this context, several studies have been conducted on other types of cancers: in lung cancer models, SCs secrete cytokines such

as CCL2, CXCL5, CXCL12, and CXCL8, which induce M2 polarization and encourage tumour cell proliferation [140-142]. Moreover, a single-cell transcriptomic study of head and neck rhabdomyosarcoma found that high SC infiltration correlated with an increased number of M2-like macrophages, linked via receptor–ligand interactions such as MIF-CD74 and PTN-SDC3 [143]. Therefore, we suggest that stem cells act as intermediaries in the TME response to oncological therapy.

Based on this data, KD could promote M1 polarization and immune activation in prostate cancer. It might also effectively counteract the SC-induced M2 phenotype and the immunosuppressive effects of EndoMT. This would shift the tumour microenvironment back toward a more anti-tumour state, driven by M1 macrophages and enhanced immune surveillance. A recent study shows that BHB inhibits the proliferative and migratory capacity of PCa cells by inducing β-hydroxybutyrylation of indolethylamine N-methyltransferase, thereby promoting PCa cell stemness and inhibiting proliferation and invasiveness [144].

Although the action of KBs on metabolic, immune and epigenetic modulation in prostate cancer is recognized (HDAC inhibition, oxidative stress, mTOR/AMPK signaling, insulin/IGF-1 reduction), this is still an unknown field of research that, however, opens the way to new research hypotheses that bring to the fore the action of glucose deprivation also in the modulation of SCs in promoting a pro-tumourogenic and metastatic environment. Remodeling the TME can hinder angiogenesis, epithelial-mesenchymal transition (EMT), and tumour reprogramming, thereby reducing tumour growth, invasion, and immune

evasion. These multifaceted effects suggest that the KD could serve as an additional strategy in cancer therapy (Figure 3).

3.4.4. Clinical and translational implications

Applying ketogenic strategies in prostate cancer treatment involves several practical considerations. Patient selection is crucial since metabolic interventions could be more effective for those with metabolic issues like obesity, insulin resistance, or when managing early-stage prostate cancer through active surveillance. Early clinical evidence suggests these approaches are feasible. For instance, a pilot study with overweight and obese men on active surveillance found that a short-term ketogenic diet was well tolerated and led to notable weight loss, with no negative impact on PSA levels or inflammatory markers [145]. However, maintaining a ketogenic diet long-term may be difficult due to the strict carbohydrate restrictions, highlighting the need for careful nutritional guidance and metabolic monitoring to ensure safety and enhance adherence. Future research should focus on combining ketogenic strategies with standard treatments and assessing their long-term effects on metabolism and cancer outcomes.

3.4.5. *The role of the microbiota in the efficacy of ketogenic diet in cancer*

Emerging evidence highlights the role of the gut microbiota in mediating the metabolic and immunological effects of ketogenic diets.

Dietary interventions are known to significantly alter microbial composition, and KD has been associated with changes in gut microbial communities and metabolic activity. Specifically, changes in micro-

bial taxa such as Akkermansia muciniphila and variations in the Firmicutes/Bacteroidetes ratio have been highlighted following dietary interventions with low-carbohydrate diets, which can potentially influence host metabolism, immune regulation, and systemic inflammation [146].

Alterations in the gut microbiota can lead to the production of bioactive microbial metabolites that directly influence tumor activity. For example, experimental studies have found that KD-induced modulation of the gut microbiota can increase the production of a long-chain fatty acid, stearic acid, a metabolite capable of promoting tumor cell apoptosis and reducing proinflammatory Th17 responses in colorectal cancer models [147]. In parallel, recent studies in preclinical tumor models, including glioma, show that enrichment of bacteria producing a short-chain fatty acid, butyrate, induced by carbohydrate restriction, can enhance immune system responses by promoting immune cell activation and modulating inflammatory pathways. Specifically, the antitumor effects of KD were significantly reduced in animals with a depleted microbiota, highlighting the key role of the gut microbiota in mediating these effects [146, 148].

Although most of the available recent evidence comes from preclinical models or tumor types other than prostate cancer, these findings highlight the potential role of the gut microbiota as an important mediator of the metabolic and immunological effects of ketogenic dietary interventions. Further studies are needed to clarify the link between the influence of ketogenic diets on gut microbiota selection and tumor activity, particularly in the context of prostate cancer.

3.3.6 Interindividual variability and potential biomarkers of response to ketogenic diets

Another aspect that should not be underestimated in the management of prostate cancer patients undergoing a ketogenic regimen is the significant interindividual variability in metabolic responses, which can influence the efficacy of treatment. Genetic variability influences ketone body metabolism and may be a potential determinant of response. For example, differences in monocarboxylate transporters (MCT1), which regulate cellular uptake of ketone bodies, may alter how tumor cells utilize ketone substrates [149]. Similarly, alterations in enzymes involved in ketone metabolism, such as β-hydroxybutyrate dehydrogenase (BDH) and succinyl-CoA:3-oxoacid CoA transferase (OXCT1/SCOT), can alter the ability of tumor cells to metabolize ketone bodies [149,151].

Alterations in key enzymes involved in ketone body metabolism, including acetyl-CoA acetyltransferase (ACAT1), BDH, and OXCT1, have been found during the progression of castration-resistant prostate cancer (CRPC). Increased expression of these ketolytic enzymes suggests that advanced prostate cancer cells may acquire the ability to utilize ketone bodies as an alternative metabolic fuel. This metabolic adaptability highlights the complexity of tumor metabolism and suggests that the response to ketogenic metabolic interventions may vary depending on the tumor's metabolic phenotype [152, 153].

Beyond metabolic enzymes, ketone bodies also influence biological processes through epigenetic mechanisms. Recent studies show that BHB can regulate gene expression by targeting epigenetic factors. For instance, a recent study found that administering a ketone ester drink

improved therapeutic outcomes in prostate cancer models by affecting the epigenetic regulator EZH2 (enhancer of zeste homolog 2). BHB inhibited EZH2 activity, resulting in decreased tumor cell growth and increased responsiveness to anti-cancer treatments. These results imply that ketogenic metabolic strategies might impact prostate cancer development not only via metabolic reprogramming but also through epigenetic regulation of oncogenic pathways [154]. Consequently, the effectiveness of ketogenic therapy may vary depending on the tumor type. Evidence indicates that some tumors can adapt to ketone bodies, creating metabolic diversity due to tumor plasticity driven by metabolic reprogramming in highly aggressive and resistant cancers. Recognizing this could be important for disease management and for identifying biomarkers predictive of therapy response.

In addition to tumor-intrinsic factors, a patient's metabolic status can also pose a crucial obstacle to therapy response. Components of metabolic syndrome, including insulin resistance, obesity, and dyslipidemia, are frequently found in patients with prostate cancer, influencing its progression and consequently altering the metabolic and hormonal response to carbohydrate restriction. Because ketogenic diets can reduce circulating glucose, insulin, and insulin-like growth factor 1 (IGF-1) signaling, patients with greater underlying metabolic dysregulation may exhibit different responses than metabolically healthier individuals. In this field, too, the identification of predictive metabolic and molecular biomarkers associated with these metabolic pathways could help predict which patients would respond positively to KD [155, 156].

3.3.7. Safety considerations and potential adverse effects

Although keto diets have been widely studied for their metabolic and neurological benefits, safety concerns and adverse effects, especially in cancer patients, remain important. These high- fat, low- carb diets can cause side effects such as gastrointestinal issues, nausea, diarrhea, vomiting, constipation, abdominal pain, fatigue, headache, dehydration, and electrolyte imbalances, often called "ketogenic flu". In the long term, adherence may lead to dyslipidemia, micronutrient shortages, fatty liver, kidney stones, hypoglycemia, acidosis, pancreatitis, and elevated cholesterol, triglycerides, uric acid, or low magnesium and sodium levels [157-160]. Without proper balance and supplementation with vitamins, minerals, and omega-3s, deficiencies may develop, but these risks can be mitigated by maintaining a balanced diet and using sugar-free B vitamins, omega-3, selenium, zinc, calcium, and carnitine supplements [159-163]. Ketone production suppresses appetite hormonally and neurologically, but meal monotony might reinforce food aversion, reducing adherence, especially in the classic ketogenic diet with severe carbohydrate restriction [164, 165]. This effect is problematic for malnourished patients, such as those with cancer or neurodegenerative conditions [157, 158, 165]. In cancer care, concerns include unintentional weight loss, muscle wasting, and reduced nutritional intake, potentially worsening outcomes in patients at risk for cachexia. Evidence indicates that a well- designed ketogenic diet, emphasizing balanced intake of proteins, monounsaturated and saturated fats, can lower triglycerides, LDL cholesterol, body fat, and inflammation markers, while raising HDL cholesterol [166]. However, data on the safety of ketogenic diets in cancer patients are limited, underscoring the need for rigorous clinical trials to assess

long- term safety and effectiveness. Currently, few studies have explored ketogenic diets in prostate cancer patients; ongoing trials are evaluating such diets in patients under active surveillance (NCT 02194516). Systematic reviews suggest high- fat diets do not increase prostate cancer risk or progression [167]. A preclinical mouse study of prostate cancer showed that a high- fat diet increased metastasis risk and weight gain, but these mice derived about 21% of their calories from carbohydrate, much higher than what's permitted in standard ketogenic diets [57]. Human studies typically show weight loss with ketogenic diets, often outperforming low- fat diets in this regard [168].

Therefore, the implementation of ketogenic dietary interventions in prostate cancer patients should be implemented in carefully selected subjects and monitored with periodic follow-ups, and preferably conducted under medical and nutritional supervision to ensure safety and adequate nutrient intake.

3.5. CONCLUSION

Prostate cancer is particularly vulnerable to metabolic reprogramming, especially in forms resistant to conventional oncological and pharmacological treatments. The ketogenic diet (KD) is emerging as a promising dietary approach that can complement standard therapies by altering tumor cell metabolism and modulating the tumor microenvironment. Preclinical studies and early clinical trials suggest that ketone bodies can influence inflammation, oxidative stress, and key signaling pathways involved in prostate cancer progression. However, current evidence remains limited and sometimes inconsistent. Future

research should therefore focus on several key priorities: (i) biomarker-driven clinical trials to identify patients most likely to benefit from ketogenic interventions, including metabolic and molecular markers such as circulating β-hydroxybutyrate levels, insulin/IGF-1 signaling, PSA kinetics, and markers of tumor metabolism or immune activation; (ii) evaluating KD in combination with standard therapies such as androgen deprivation therapy, immunotherapy, or other metabolic-targeting strategies; and (iii) long-term clinical trials evaluating safety, tolerability, and metabolic effects in prostate cancer patients. Furthermore, further investigation is needed to clarify how glucose restriction may influence SCs's activity and EndoMT processes involved in tumor microenvironmental remodeling and metastatic progression. Addressing these priorities will help determine whether ketogenic strategies can be effectively integrated into prostate cancer management.

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4. β-HYDROXYBUTYRATE AND MEDIUM-CHAIN TRIGLYCERIDES: BIOLOGICAL RATIONALE FOR IN VITRO INVESTIGATION IN INTESTINAL EPITHELIAL CELLS

4.1. β-hydroxybutyrate as a nutritional modulator of intestinal inflammation

As previously discussed, the ketogenic diet, and specifically the most abundant ketone body in mammals, the BHB, beyond its role as an alternative energy substrate, is increasingly recognized as a signaling molecule capable of modulating cellular metabolism, inflammatory responses, carcinogenesis, and gene expression. This section introduces the role of nutrition, with particular reference to the ketogenic diet, in another area of research: intestinal inflammation. Specifically, the effects of β-hydroxybutyrate (BHB), both in isolation and in synergy with other molecules, such as coconut oil, in modulating and downregulating intestinal inflammatory processes will be examined. These processes, if not adequately controlled, can evolve into chronic conditions, ranging from irritable bowel syndrome (IBS) to chronic inflammatory bowel disease (IBD).

Among IBDs, two main clinical phenotypes are recognized: ulcerative colitis (UC) and Crohn's disease (CD). Both conditions are characterized by chronic intestinal inflammation, which frequently results in impaired nutrient absorption [1]. Crohn's disease is distinguished by a transmural and often discontinuous inflammatory process that leads to the formation of lesions, strictures, and fistulas, and can affect any segment of the gastrointestinal tract, from the oral cavity to the anus, with a predominant involvement of the small intestine and colon

[2]. In contrast, ulcerative colitis is characterized by a continuous inflammatory process limited to the superficial mucosal layer of the colon, resulting in chronic inflammation of the large intestine, disruption of epithelial barrier integrity, and, in severe cases, an increased risk of systemic infection [3]. The gastrointestinal tract, in addition to its usual functions of digestion and nutrient absorption, plays a fundamental and complex role in maintaining the homeostasis of microbial communities. This efficiency relies on the ability to modulate the immune response against commensal microflora, while simultaneously ensuring effective protection against pathogenic microorganisms [4–7]. The intestinal barrier is a selectively permeable interface that allows the diffusion of water, electrolytes, and dietary nutrients, while simultaneously providing immune defense and maintaining structural integrity against external agents, pathogens, and toxins, as illustrated in the figure below [8–11].

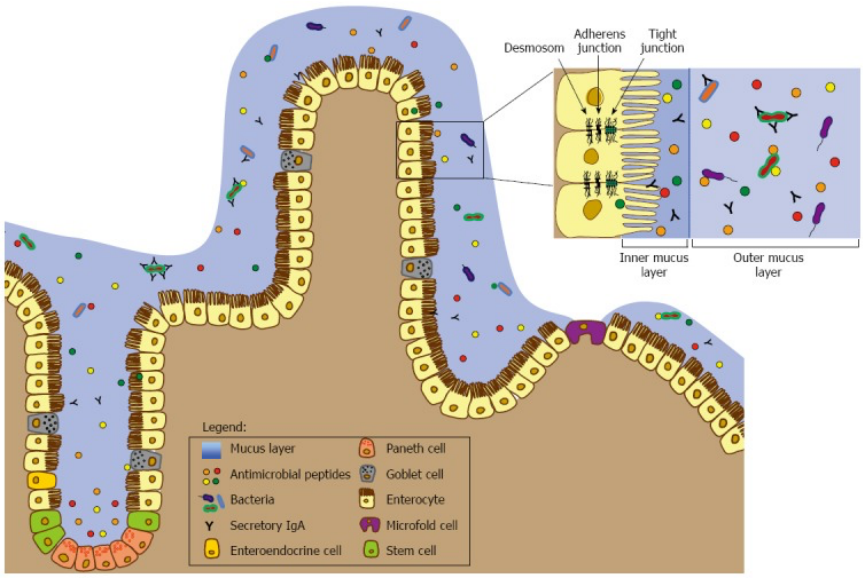


Figure 1: Defense mechanisms of the healthy intestinal mucosal barrier. (©2014 Baishideng Publishing Group Co., Limited. All rights reserved. World J Gastroenterol. 2014 Feb 7;20(5):1165–1179. doi: 10.3748/wjg.v20.i5.1165)

The intestinal epithelium lines the luminal surface of the intestine and is composed of tightly packed cells that form a physical barrier between the luminal contents and the interior of the body [5,7,12]. The protein complexes that enable cell-cell interconnection are tight junctions, adherens junctions, and desmosomes, which stabilize cohesion between epithelial cells, define the boundary between the apical and basolateral membranes, and regulate intestinal permeability.

Epithelial homeostasis is orchestrated by multiple factors that regulate the balance between cell proliferation and apoptosis. Transforming growth factor-α (TGF-α) is one of the main mediators of epithelial proliferation, while TGF-β exerts an inhibitory effect on cell growth.

The Wnt/β-catenin signaling pathway plays a key role in modulating epithelial proliferation; in particular, a deficiency of β-catenin impairs epithelial homeostasis [13,14]. Furthermore, pattern recognition receptors (PRRs) are involved in the recognition of specific microbial structures associated with Gram-negative microorganisms, such as lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, and flagellin. The activation of these receptors triggers a cascade of immune responses that leads, downstream, to the activation of molecules such as nuclear factor κB (NF-κB), pro-inflammatory proteins, chemokines, and antimicrobial peptides [7,9,15].

Among the most studied PRRs are the intracellular nucleotide-binding oligomerization domain-like receptors (NLRs), RIG-like receptors (RLRs), C-type lectin receptors (CLRs) and the membrane-based Toll-like receptors (TLRs) which are capable of recognizing a broad range of pathogen-associated molecular patterns (PAMPs) [16,17].

IBD is characterized by alterations in several protective mechanisms, including PRRs, impaired mucus layer production, impaired autophagy and antimicrobial peptide (AMP) production, and increased intestinal permeability. This leads to uncontrolled passage of bacteria, which, upon contact with the epithelium, trigger an immune response that perpetuates itself and leads to chronic intestinal inflammation. IBD predominantly affects intestinal regions characterized by a high microbial density, such as the ileum and the distal colon, strongly suggesting a role for the intestinal microbiota in the etiopathogenesis of the disease [18].Despite extensive research efforts, no single pathogenic microorganism has been definitively identified as the causative agent of IBD [19].

Toll-like receptor 4 (TLR4), the first Toll-like receptor to be described, is a key component of the innate immune system and specifically recognizes LPS. By interacting with its co-receptors CD14 and MD-2, TLR4 can activate both MyD88-dependent and MyD88-independent signaling pathways, leading to the nuclear translocation of NF-κB or IRF3, respectively, and the subsequent production of cytokines and inflammatory mediators [20]. Although TLR4-mediated signaling is essential for immune cell recruitment and tissue repair, overactivation or deregulation represents a double-edged sword, contributing to pathological processes such as impaired wound healing, organ fibrosis, and chronic inflammation. Given the localization of the colonic microbiota, which is closely related to the intestinal epithelium, TLR4 expression must be maintained at low levels in intestinal epithelial cells. Protection against LPS is regulated by other immune signals, such as interferon-α [15,21].

In IBD patients, TLR4 expression is significantly upregulated in intestinal biopsies, suggesting a critical role for TLR4-mediated signaling in disease-related inflammation. Furthermore, recent studies suggest that enterocyte membrane expression and the location of TLR4 and CD14 are involved in regulating intestinal tight junction permeability [22].

Frosali et al. (2015) suggest that gastrointestinal pathology is evidently orchestrated by a close correlation between TRL4 activation, microbiota and intestinal immunity. It is still unclear whether the alteration of the intestinal microbiota in IBD patients is a determining factor in the disease or a consequence of the dysfunction itself. Stephens et al. (2019) sought an answer using TLR4-transfected HEK293

cells and the Caco-2 intestinal epithelial permeability model, through which it was demonstrated that different LPS species induce distinct NF-κB activation profiles and differential effects on epithelial barrier integrity, supporting the concept of serotype-specific epithelial damage and altered barrier function in the context of a dysbiotic microbiome. Overall, these findings provide new insights into how LPS derived from altered microbiota contributes to intestinal epithelial dysfunction and chronic inflammation in IBD [22].

Therefore, this study suggests that different bacterial species could have a different efficacy in activating TLR4, consequently producing an alteration of the intestinal epithelial barrier, cellular activity and localized species-specific chronic inflammation. In fact, LPS treatment of different bacterial species modulates the tight junctions of the Caco-2 monolayer differently. For example, *S. marcescens* shows a significant reduction of the ZO-1 tight junction, increasing the permeability of the epithelial barrier and influencing the production of pro-inflammatory cytokines [23]. Therefore, the composition of the intestinal microbiota and the different production of LPS has a notable influence on the inflammatory balance, cell death and intestinal permeability.

The LPS used in this study are derived from bacterial species capable of colonizing the gastrointestinal (GI) tract and involved in modulating intestinal permeability, a cause of chronic inflammation such as IBD. Complement-induced opsonization processes could lead to bacterial lysis and subsequent release of endotoxins into the intestinal lumen, producing a heightened TRL4-induced inflammatory response that increases intestinal permeability. This leads to an amplification of

the immune response linked to facilitated bacterial translocation into the peritoneum and periphery, or into the mesenteric lymphatics that flow into the mesenteric lymph node.

In animal models of IBD, impaired mesenteric lymph flow has been observed, and this alteration is an early and consistent sign of intestinal inflammation [24,24]. Whether lymphatic dysfunction is a cause or consequence of bacterial translocation is still a matter of debate. However, evidence indicates that the lymphatic system is a key component in the progression of the disease.

In the context of intestinal inflammation, several studies highlight the anti-inflammatory potential of BHB, however, their role in regulating the innate immune response is unknown. BHB has demonstrated effects on reducing the production of pro-inflammatory cytokines IL-1β and IL-18 mediated by the NLRP3 inflammasome in human monocytes. In vivo, BHB attenuated the activation of caspase 1, involved in the release of proinflammatory cytokines such as IL-1β and IL-18, in mouse models of NLRP3-mediated diseases [25].

Recent studies have investigated the effect of β-hydroxybutyrate in the context of intestinal inflammation, showing its efficacy in modulating the innate immune response by acting on macrophage polarization [26]. This is involved in the inflammatory processes that sustain the disease, so much so that it is a target as a potential therapeutic strategy for IBD [27–29]. Macrophages can differentiate into different phenotypes depending on the signals arriving from the tissue microenvironment. In particular, M1 macrophages are activated in response to stimuli from lipopolysaccharide (LPS) and interferon-γ (IFN-γ), promoting a Th1-type immune response and the production of pro-

inflammatory cytokines involved in host defense mechanisms against infections [2,30,31]. In contrast, M2-type macrophages are mainly activated by stimuli from interleukin-4 (IL-4) and interleukin-13 (IL-13) and play a central role in tissue remodeling, wound healing, and immune response regulation through the activation of the IL-4 receptor α chain (IL-4Rα) [32–34]. Aberrant macrophage polarization occurs when the normal ability to balance pro-inflammatory and anti-inflammatory responses is lost due to abnormal stimuli and has been observed during the development and progression of inflammatory bowel disease (IBD), contributing to the maintenance of a chronic inflammatory state. BHB has been shown to significantly ameliorate colitis in murine models of dextran sulfate sodium (DSS)-induced acute colitis by promoting macrophage polarization from a proinflammatory M1 to an anti-inflammatory M2 phenotype. This effect is regulated by activation of the STAT6 signaling pathway, resulting in increased expression of M2 anti-inflammatory markers and reduced production of pro-inflammatory cytokines. Furthermore, BHB significantly upregulated the expression of M2 macrophage-associated genes, including IL-4Ra, IL-10, arginase 1 (Arg-1), and chitinase-like protein 3, following DSS exposure, suggesting increased M2 macrophage disinclination in vivo. Furthermore, BHB was found to enhance M2 macrophage-induced mucosal repair by promoting intestinal epithelial proliferation. In this study, BHB concentrations were significantly reduced in the colonic mucosa of IBD patients. Exogenous BHB administration reduced the severity of acute experimental colitis, associated with less weight loss, a marker of disease activity, colonic shortening, and histological scores, and a reduction in crypt loss

and epithelial damage compared to the control group [26]. These results suggest that BHB acts as an immunometabolic modulator capable of rebalancing the intestinal inflammatory response, contributing to the resolution of inflammation and the maintenance of mucosal homeostasis.

4.2. Microbiota-driven immune dysregulation in inflammation: impact of ketogenic diet and β-hydroxybutyrate

The incidence of IBD has increased significantly worldwide, so much so that it has been defined as a global disease of the 21st century [35,36]. As previously mentioned, the pathophysiology is still unclear, but it is known that there is an interconnection between endogenous factors (genetic predisposition) and exogenous factors (environmental factors such as stress, diet, and medications). Among these factors, the intestinal microbiota plays a central role, 10-100 trillion microorganisms, including bacteria, viruses, fungi, and protozoa that live in the gastrointestinal tract in a perfect balance called eubiosis [37–39], which perform crucial activities such as nutrient metabolism, immune modulation, metabolism, and defense against foreign agents [40]. Dysbiosis, or a qualitative and quantitative alteration of the intestinal microbiota, can lead to intestinal inflammation and damage [41]. Indeed, in subjects with IBD, the microbial composition is altered compared to healthy individuals.

Growing evidence indicates that genetic variants and predispositions, together with environmental factors and the characteristics of the intestinal microbiota, contribute significantly to the pathogenesis of

IBD, through alterations in immune mechanisms and the composition of the microbial community [42].

As previously described, dysbiosis can also cause damage to the intestinal epithelial barrier, which acts as a mechanical, chemical, immune, and microbiological "sorter". Colitis-associated pathogens such as Veillonella spp. and Clostridium spp. can lead to the activation of the NLRX1 (oligomeric nucleotide-binding domain, leucine-rich repeat X1) receptor, which activates the SIRT1 signaling pathway, which in turn triggers dysbiosis and inflammation [43,44]. Increased permeability is a crucial factor in the pathogenesis of IBD [45]. In particular, in CD, 50% of patients present intestinal colonization with invasive-adherent E. coli (AIEC) [46], which adheres to epithelial cells and activates inflammatory factors such as TNF-α [47].
Furthermore, Klebsiella pneumoniae invades intestinal epithelial cells, interacting with macrophages and inducing the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) [48]. Similarly, Fusobacterium nucleatum has been shown to modulate colonic epithelial cell activity through the activation of intracellular NOD2 receptors, resulting in upregulation of caspase recruitment domain 3 (CARD3). This process triggers the IL-17F/NF-κB signaling pathway, promoting the establishment and maintenance of intestinal inflammation [49].

Another factor worth highlighting is the role of the intestinal microbiota in the differentiation of T cells into Th17 cells and regulatory T cells (Treg). Physiologically, Th17 cells activate an immune response against pathogens, but if the response is overactivated, it can lead,

conversely, to the development of intestinal inflammation, as in the case of IBD [38,50]. In subjects with IBD, an imbalance between Th17 and Tregs occurs, with an increase in Th17 and a decrease in Tregs, which promotes an uncontrolled immune response that results in intestinal mucosal lesions [51].

As previously mentioned, the composition of the intestinal microbiota is highly influenced by nutrition [52] taking part in the conduction of the host's metabolism and immunity [53,54]. In fact, in a hospital crossover study conducted by Ang et al. (2021) on 17 overweight or class I obese adult subjects, after 4 weeks of intake of a basal diet (BD) (50% CHO, 15% proteins, 35% fats) followed by another 4 weeks of ketogenic diet (KD) (5% CHO, 15% proteins, 80% fats), significant changes in the intestinal microbiota between the two diets were observed through 16S rRNA gene sequencing (16S-seq). Metagenomic sequencing of stool samples confirmed the reduction of several bifidobacterial species in subjects who followed the ketogenic diet.

In short, the study results suggest that KD induces profound changes in metabolites and microbiota composition that translate functionally into the host immune system, particularly Th17 release. The impact of KD appears to be different from that of high-fat diets, and this is attributable to the caloric deficit and the production of the ketone body, β-hydroxybutyrate (βHB). Ketone bodies select for the growth of certain bacterial taxa, inhibiting Bifidobacterium, whose reduction is associated with a low proliferation of Th17 cells in the intestine and adipose tissue (Figure 2) [55].

The role of Th17 cells in obesity and the promotion of low-grade inflammation is recognized [56], and therefore, their reduction may be beneficial at the metabolic level, including improved glycemic control [57] and reduced fat mass [58].

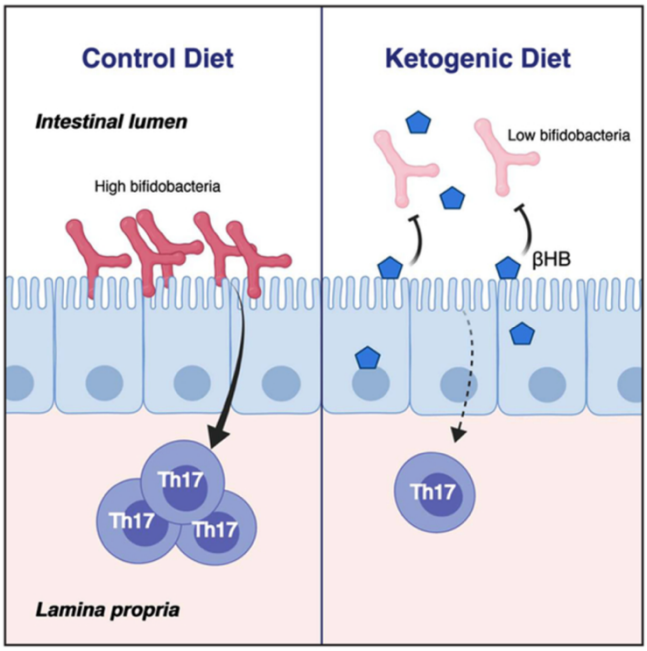


Figure 2: Proposed model illustrating the effects of KD on gut microbiota composition and Th17 cell responses.
(All rights reserved. Cell. 2020 May 20;181(6):1263–1275.e16. doi: 10.1016/j.cell.2020.04.027)

Fecal microbiota transplant experiments demonstrate that the microbiota of donors undergoing KD reproduced the reduction of intestinal Th17 cells. Among the bacteria involved, Bifidobacterium emerges as a key regulator of the Th17 response, consistent with previous studies that associate it with the differentiation of these cells. However, KD

also produces changes in other bacterial taxa that could have side effects, highlighting the complexity of KD's action on the intestinal bacterial ecosystem [55].

In conclusion, the results of this study suggest the strong impact of the KD in causally modulating the microbiota, reducing bacterial species responsible for chronic inflammation, such as in inflammatory bowel disease, and regulating host immune responses. This opens new frontiers of research in the field of personalized prevention and treatment of metabolic diseases.

4.3. Protective role of medium-chain triglycerides in intestinal inflammation and epithelial Integrity

Medium-chain triglycerides (MCTs) and β-hydroxybutyrate (BHB) exert distinct yet complementary biological effects on intestinal epithelial cells. Importantly, these two compounds, different in composition, structure, and utilization, are physiologically interconnected, as the energy metabolism of MCTs promotes endogenous ketogenesis and BHB production. This metabolic link provides a strong rationale for their combined use.

To better understand their biological effects, it is important to first consider their chemical structure. Fatty acids are important biomolecules involved in many biological processes, ranging from energy production and storage [59], cell membrane function [60] to gene activation [61] and modulation of signaling pathways [62]. Based on the length of their carbon chain, they are classified as short-chain fatty

acids (SCFA) with fewer than 6 carbon atoms, medium-chain fatty acids (MCFA) with 6-12 carbon atoms, and finally, long-chain fatty acids (LCFA) with 12 carbon atoms or more. Triglycerides, instead, are characterized by three fatty acids with carbon chains of different lengths linked to a glycerol molecule [63]. Therfore, medium-chain triglycerides (MCTs) are molecules esterified with glycerol molecules linked to MCFAs. They are found primarily in coconut oil, palm kernel oil, and milk [64].

MCT fatty acids include dodecanoic (12:0), octanoic (8:0), hexanoic (6:0), and decanoic (10:0) acids. Among these, lauric acid (C:12) is a medium-chain saturated fatty acid with 6 to 12 carbon atoms and is found primarily in coconut oil and palm kernel oil (approximately 45 g/100 g of edible portion) [65], vegetable oils, fruits, seeds, and breast milk [66,67]. Caprylic acid, a saturated fatty acid, is also found primarily and naturally in the milk of various mammals and to a lesser extent in coconut oil and palm kernel oil.

MCTs also include caproic acid (C6) and capric acid (C10), which, along with caprylic acid, constitute 15% of the fatty acids present in goat's milk fat [63].

What significantly links the use of MCT with BHB is that these fatty acids, due to their smaller size, reach the liver quickly and directly, bypassing pancreatic lipase, compared to long-chain fatty acids. In fact, medium-chain fatty acids do not use membrane transporters for absorption and reach the mitochondrial intermembrane space directly, without the carnitine shuttle [68]. In the liver, the entry of acetyl-CoA into the citric acid cycle is limited by the subtraction of oxaloacetate and malate from gluconeogenesis and by the allosteric inhibition induced by the high $NADH/NAD^+$ ratio. This metabolic structure biases

the metabolism of medium-chain fatty acids toward the formation of ketone bodies [69] (Figure 3).

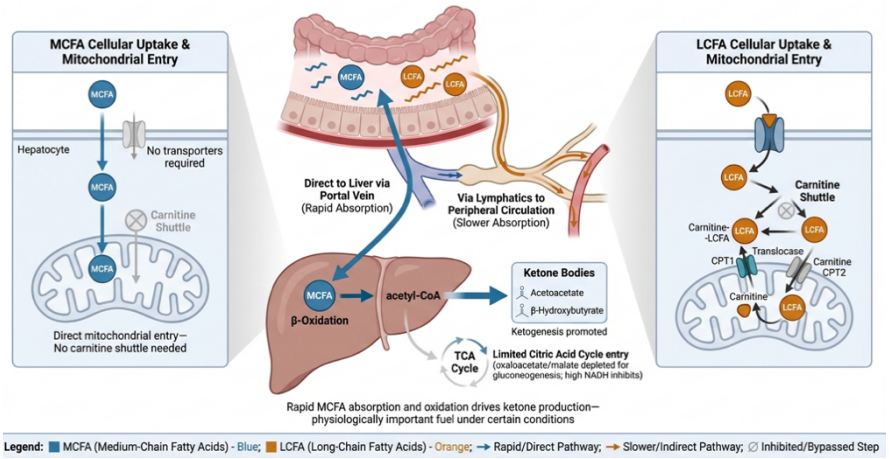


Figure 3: Comparative Metabolism and Absorption of Medium-Chain vs Long-Chain Fatty Acids.

Recent evidence suggests that medium-chain triglycerides improve and protect intestinal integrity and function under inflammatory conditions. MCTs were used as dietary enrichment in the presence of LPS in nursery pigs (an animal model for human nutrition research), used to induce intestinal injury. It was hypothesized that MCT use could reduce the production of intestinal proinflammatory cytokines by modulating intestinal signaling pathways and protecting against necroptotic damage to enterocytes.

MCT supplementation produced significant improvements in jejunal villous height compared to pigs fed the corn oil diet. The same beneficial effect was also observed for sucrase and maltase activities in the

jejunum and ileum, an indicator of intestinal digestive function, compared to pigs fed the corn oil diet. Plasma concentrations of the proinflammatory cytokine TNF-α were downregulated in the presence of MCTs, compared to pigs fed corn oil diets, among LPS-treated pigs. MCT supplementation increased the expression of heat shock protein 70 (HSP70) in the jejunum ($P<0.05$) and claudin-1 protein in both the jejunum and ileum, a result associated with improved epithelial barrier function.

Current evidence suggests that activation of the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) signaling pathways is associated with inflammatory bowel disease [70,71]. Activation of these signaling pathways is further associated with increased proinflammatory cytokines such as TNF-α and thus motivates the expression of anti-inflammatory cytokines such as HSP70 [72]. In this study, the expression of the genes TLR4, TRAF6, and NF-κB in the TLR4 signaling pathway, as well as NOD2 and RIP2 in the NOD signaling pathway, was reduced in pigs fed MCT compared to the non-MCT group.

In conclusion, LPS stimulation increased the expression of RIP1, RIP3, and MLKL, key mediators of the necroptotic process (programmed cell death, similar to necrosis, recognized by the release of immunogenic substances that in turn activate inflammatory signals such as TLRs), associated with intestinal epithelial damage. MCTs reduced the expression of these proteins, suggesting a possible protective effect.

In summary, dietary MCT supplementation protected intestinal morphology, disaccharidase activity, and barrier function in the presence of LPS. The beneficial effects of MCT on the intestine are primarily related to the downregulation of intestinal proinflammatory cytokine expression mediated by the upstream inhibition of TLR4 and NOD signaling pathways, and also to the reduction of enterocyte necroptosis mediated by the inhibition of RIP3 and MLKL mRNA expression [73].

4.4. Rationale for investigating direct epithelial effects of BHB and MCTs

The studies highlighted in the previous sections demonstrate the emerging role of β-hydroxybutyrate (BHB) as an immunometabolic modulator and the protective potential of medium-chain triglycerides (MCTs) in intestinal inflammation. BHB has been shown to attenuate inflammatory responses, modulate immune cell polarization, and influence cytokine production both in vivo and in immune cell-based models. Similarly, MCT supplementation has demonstrated beneficial effects on intestinal morphology, barrier integrity, and inflammatory tone.

However, several significant knowledge gaps persist.

First, much of the available evidence comes from animal models or immune cell systems, where systemic metabolic changes and immune cell interactions can confound the interpretation of direct epithelial effects. The intestinal epithelium is not merely a passive barrier, but

an active regulator of inflammatory responses and barrier permeability. However, the direct impact of BHB on intestinal epithelial cells under inflammatory conditions remains insufficiently explored.

Second, although MCTs are metabolically linked to endogenous ketone production, it is unclear whether their protective effects on the intestine are mediated exclusively by increased BHB availability or whether MCTs exert independent epithelial actions. The potential complementary or additive effects of BHB and MCTs at the epithelial level have not been clearly defined.

Third, while lipopolysaccharide (LPS)-induced inflammation is widely used as a model of epithelial barrier dysfunction associated with dysbiosis and intestinal inflammatory conditions, limited data are available on the ability of BHB to modulate intestinal inflammatory responses and barrier-related parameters in a controlled *in vitro* setting.

Therefore, an *in vitro* model using isolated intestinal epithelial cells stimulated by LPS represents a necessary intermediate step between systemic animal models and clinical application. This approach allows for the isolation of specific epithelial responses and the direct assessment of metabolic modulation in the absence of cell-mediated immune confounders.

The primary objective of this study is to investigate the effects of BHB, alone and in combination with MCTs, on inflammatory responses and epithelial integrity in LPS-stimulated intestinal epithelial cells.

Working Hypothesis:

BHB attenuates LPS-induced inflammatory responses at the epithelial level. BHB helps maintain the integrity of the epithelial barrier under conditions of inflammatory stress. MCTs exert protective effects on epithelial cells and may enhance or complement the action of BHB.

Overview of Experimental Design:

An *in vitro* intestinal epithelial cell model was stimulated with LPS to induce an inflammatory state. Cells were treated with:

- BHB alone,
- MCTs alone,
- BHB + MCT combination treatment.

Experimental results focused on markers of inflammatory response, epithelial integrity, and cell viability to determine whether metabolic modulation through ketone body and/or MCT supplementation can directly influence epithelial behavior under proinflammatory stimulation.

In summary, while previous studies support the anti-inflammatory and barrier-protective potential of BHB and MCTs, their direct epithelial effects in a controlled inflammatory context remain incomplete. Filling this gap provides a mechanistic basis for understanding how interventions related to ketogenic metabolism can influence intestinal epithelial homeostasis and supports the rationale for the present experimental investigation.

5. OBJECTIVE OF THE DOCTORAL THESIS PROPOSAL

In light of current scientific evidence, the present thesis aims to investigate and highlight the anti-inflammatory effects of β-hydroxybutyrate (BHB) and medium-chain triglycerides (MCTs), both individually and in combination, in the presence or absence of a pro-inflammatory stimulus, using the human colorectal adenocarcinoma cell line Caco-2. The study aims to evaluate their potential synergistic action on intestinal epithelial cells and explore their potential applicability in inflammatory bowel disease (IBD), suggesting a possible role for BHB and MCT oil as adjunctive agents capable of supporting current therapeutic strategies aimed at reducing intestinal inflammation.

PART II: EXPERIMENTAL INVESTIGATION USING CACO-2 CELLS

4. SYNERGISTIC ANTI-INFLAMMATORY EFFECTS OF B-HYDROXYBUTYRATE AND MEDIUM CHAIN TRIGLYCERIDES ON CACO-2 CELLS MIMICKING THE KETOGENIC DIET (under review)

5.2. MATERIALS AND METHODS

5.2.1. Caco-2 cell culture

The human epithelial cell line Caco-2 (purchased from ATCC HTB-37), obtained from colorectal adenocarcinoma, was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 in tissue culture flasks (75 cm2; Greiner Bio-One GmbH, Germany), and the culture medium was changed every two days. Prior to experimentation, Caco-2 cells were trypsinized using Trypsin-EDTA (2X in PBS), and the density was assessed microscopically using a Bürker counting chamber. Subsequently, cells were plated at an appropriate number and density for subsequent *in vitro* experimental trials.

5.2.2. Preparation of β-hydroxybutyrate (BHB) solution

β-hydroxybutyrate (BHB) (DL-β-hydroxybutyric acid sodium salt, ~98%; Sigma-Aldrich) was initially dissolved in sterile phosphate-

buffered saline (PBS) (Dulbecco's Phosphate-Buffered Saline without calcium; Euroclone, Milan, Italy) to obtain a 1 M stock solution. Final working concentrations were prepared by diluting the stock solution in DMEM, the same medium used for cell culture. Cells were pre-treated with BHB for 24 and 48 h prior to each experimental treatment.

5.2.3. Preparation of MCT oil nanoemulsion

Medium-chain triglyceride (MCT) oil, obtained from analytical-grade coconut oil (Sigma-Aldrich), was initially dissolved in absolute ethanol (EMSURE®, Sigma-Aldrich) to obtain a 1 M solution of MCT oil. A nanoemulsion was prepared using asymmetric dual centrifugation (DAC) with the SpeedMixer™ DAC 150 SP (Hauschild, Germany), preheating the lipophilic phase to 55 °C and subsequently mixing with the aqueous phase at 14,000 rpm for 10 minutes. A final homogenization step was performed using a two-stage high-pressure homogenizer (Stanste Fluid Power Ltd.) to ensure particle size uniformity.

5.2.4. Cell viability assay (MTT test)

For the cell viability assay, Caco-2 cells were plated in 96-well plates at a density of 1.5×10^4 cells and incubated at 37°C with 5% CO2 for 24 hours with initial concentrations of β-hydroxybutyrate of 5 mM, 10 mM, and 20 mM and concentrations of MCT oil of 0.5 mM, 1.5 mM, and 3 mM to study the cytotoxicity of these substances. An additional MTT assay was subsequently performed with concentrations of 5 mM β-hydroxybutyrate and 0.5 mM MCT oil, in the presence or

absence of LPS at a concentration of 1 μg/mL for 24 hours. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich). The assay is based on the reduction of MTT by mitochondrial dehydrogenase in intact cells to produce purple formazan. A stock solution of MTT (5 mg/mL) was prepared in PBS and subsequently diluted to a final working concentration of 1 mg/mL. The cells were then incubated in the dark with 200 μL of MTT for 4 hours at 37°C in a humidified atmosphere with 5% CO2. After incubation, the MTT solution was carefully removed by aspiration and 200 μL of DMSO was added to solubilize the formazan crystals. To read the absorbance, a spectrophotometer (Filter Max F5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA 95134, USA) with a wavelength of 595 nm was used. The results were expressed as the percentage of viable cells (%) compared to untreated controls, using the following formula: (absorbance of the treated sample/absorbance of the control) × 100. Six analytical replicates were performed for each sample, and each experiment was repeated three times.

5.2.5. Cell morphology assay

Cell morphology was analyzed using a morphological imaging assay to evaluate the effects of β-hydroxybutyrate (5 μM), MCT oil (0.5 mM), and their combination, both in the absence and presence of the proinflammatory stimulus LPS (1 μg/mL). Approximately 5×10^5 cells were seeded in 6-well plates. All experiments were performed in triplicate, and morphological measurements are reported as mean cell area. Images were acquired using a Leica DM IRB microscope (Leica

Microsystems GmbH, Wetzlar, Germany) at 10× and 20× magnifications. Cell surface areas (μm²) were quantified using ImageJ software.

5.2.6. Cell migration (wound closure assay)

Cell migration was analyzed using a wound closure assay. Caco-2 cells were plated at a density of 1×10^6 cells/well in 6-well plates. Once confluence was reached, a linear scratch was created using a sterile cell scraper. After washing with PBS and changing DMEM, the cells were incubated for 24 with 5 mM β-hydroxybutyrate and 0.5 mM MCT oil alone or in combination with or without LPS (1 μg/mL). Each condition was tested in triplicate. Wound closure was assessed at 24 using ImageJ software to quantify the percentage of wound area covered compared to baseline (T0).

5.2.7. Cytokine quantification by ELISA

After 24 of treatment with β-hydroxybutyrate (5 μM), MCT oil (0.5 mM), alone or in combination, with or without LPS (1 μg/mL), the supernatants were collected to quantify the levels of the cytokines TNF-α, IL-17, and IL-10 using commercially available ELISA kits (Quantikine™, R&D Systems). Experiments were conducted in triplicate. Cytokine concentrations (pg/mL) were calculated based on standard curves obtained with known amounts of the cytokines under study, according to the manufacturer's protocol.

5.2.8. Protein extraction and Western Blot analysis

Caco-2 cells were grown in a 6-well plate at 2x106 cells/well. After treatment cells were washed with 1X PBS and proteins are extracted

using RIPA cell lysis buffer supplemented with protease and phosphatase inhibitors (Complete Mini Protease Inhibitor Tablet, Roche, Germany). After 15 minutes on ice and 15 minutes of centrifugation at 14,000 rpm at 4°C, the protein-containing supernatant is collected. Samples are then stored at -20°C.

Protein quantification was performed using the Pierce BCATM Assay Kit (Thermofischer Scientific). Samples were then prepared and heated at 95°C for 5 min in Laemmli 4X + 5% 2-mercaptoethanol, before being loaded onto 5-20% polyacrylamide gel (Bio-Rad®) for SDS-PAGE migration in 1X migration buffer for approximately 1.5 hours at 110V. After electrophoresis, the proteins were transferred to a semi-dry nitrocelluose membrane using the Trans-Blot® TurboTM system (Bio-Rad, USA). To reduce non-specific antibody binding, the membranes were saturated in 5% (w/v) BSA diluted in 1X Tris/NaCl/Tween20 (TNT) buffer for 1 hour with shaking at room temperature. The primary antibodies (Table 1) diluted in the 5% BSA solution were then incubated overnight at 4°C on the membranes.
The following day, the membranes were rinsed three times in 1X TNT before being incubated with the secondary antibody coupled to a fluorochrome (Table 1) diluted 1/2000 in 1X TNT, with agitation for one hour. After another three-wash session, detection was observed with the LI-COR Odyssey® CLx Imager imaging system. GAPDH (CST2118) was used as a loading control.

	Antibody	Molecular Weights	Spécies	Origin	Concentration
Primary	GAPDH	38 kDa	Rabbit	CST2118 Cell Signaling Technology®	1:1000
	E-cadherin	135 kDa	Rabbit	CST3195 Cell Signaling Technology®	1:1000
	ZO-1	225 kDa	Rabbit	61-7300 Invitrogen, Thermofisher®	0.01-2 µg/mL
	Connexin 43	43 kDa	Rabbit	C6219 MilliporeSigma®	1 : 8000
Secondary	Anti-IgG de souris (DyeLight™ 680 Conjugate)	-	Goat	CST5470 Cell Signaling Technology®	1 : 15000
	Anti-IgG de lapin (DyeLight™ 800 4X PEG Conjugate)	-	Goat	CST5151 Cell Signaling Technology®	1 : 15000

Table 1: Antibodies used for western blot analysis.

5.2.9. Flow cytometry analysis

Cells were grown in 6-well plates at 2 x 106 cells/well. After treatment, the cells are recovered by trypsin detachment and all samples are collected.

The cells are then rinsed twice with a wash buffer (1X PBS + 0.1% BSA) before being resuspended in cold fixation buffer (wash buffer + 2% PFA) and left on ice for 15 minutes. Since the markers being studied are intracellular (ZO-1), a permeabilization step is required. Therefore, after two further washes with buffer, the cells are resuspended in permeabilization buffer composed of the wash buffer + 0.1% Tween20 and left on ice for 15 min. Finally, after 2 more

washes, the antibodies can be incubated according to the concentrations and instructions of the supplier (30 min on ice, protected from light), and the cells recovered in the wash buffer can then be passed to the flow cytometer.

Cells were incubated with ZO-1 antibody (Invitrogen) diluted 1:100. An isotype control was used to analyze the nonspecificity of the antibodies used.

Reading was performed with the BD FACSCanto™ II Clinical Flow Cytometry System equipment (Waters™ Biosciences). Analysis was performed with the BD Biosciences software.

5.3. STATISTICAL ANALYSIS

All data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's post hoc test for multiple group comparisons, as this method provides strong control over Type I error across all pairwise comparisons, using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

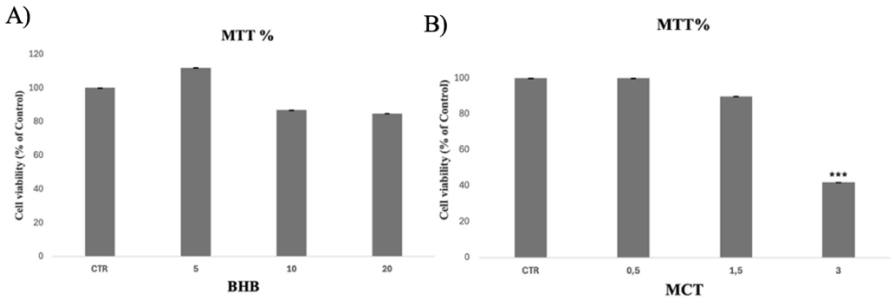
5.4. RESULTS

5.4.1. Effects of β-hydroxybutyrate and MCT oil on caco-2 cell viability

As shown in Figure 1, the effects of increasing concentrations of β-hydroxybutyrate (BHB; 5, 10, and 20 mM) and medium-chain triglyceride (MCT) oil (0.5, 1.5, and 3 mM) on Caco-2 cell viability were

assessed using the MTT assay. As shown in Panels A and B, concentrations of 5 mM BHB and 0.5 mM MCT did not significantly reduce cell viability compared to untreated controls and were therefore selected for subsequent experiments. At concentrations higher than 5 mM BHB and 0.5 mM MCT, a decrease in cell viability was observed, suggesting a concentration-dependent cytotoxicity threshold.

Cells treated individually with the anti-inflammatory substances, BHB and MCT, showed a slight increase in viability after 24 hours (Panel C). Under inflammatory conditions induced by LPS exposure (1 µg/mL), both BHB and MCT individually attenuated the reduction in cell viability observed in the LPS-only group, reversing its action. Notably, co-treatment with BHB and MCT further improved cell viability after 24 hours of treatment, indicating an additive or potentially synergistic effect, although confirmation requires further mechanistic analyses (Panel C). Although cell viability provides initial information, we integrated this with wound healing and cytokine profiles to better assess epithelial function under conditions of inflammatory stress.



C)

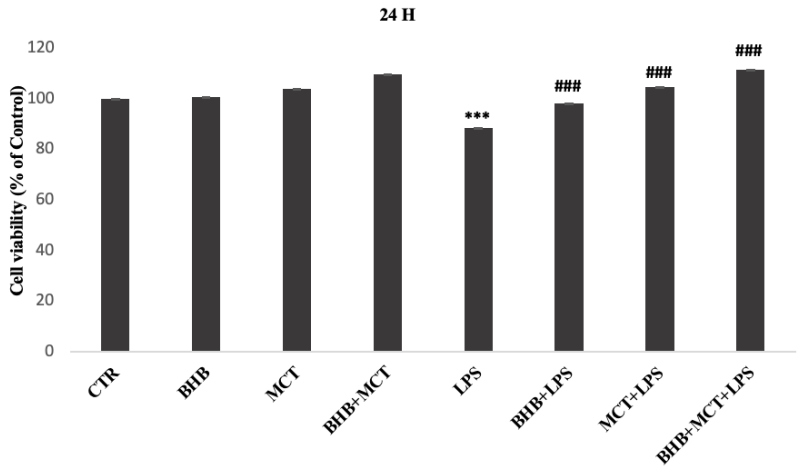
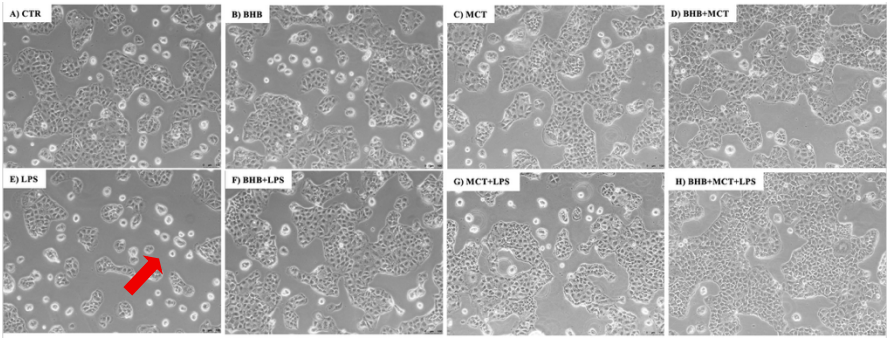


Figure 1: Cell viability assay of BHB and MCT. Dose-response curve for concentrations from 5 mM to 20 mM BHB assessed by MTT assay on Caco-2 cells (A). Dose-response curve for concentrations from 0.5 mM to 3 mM of MCT assessed by MTT assay on Caco-2 cells (B). Respectively, the concentrations of 5 mM BHB and 0.5 mM MCT were used to test the cell viability of cells in the absence or presence of LPS (1 µg/mL) at 24 hours (C). The results are expressed as means ± SD of three independent experiments performed in triplicate percentages compared to the control. *$p < 0.05$ compared to the same time points for CTR. # $p < 0.05$ compared to LPS time points.

5.4.2. Effect of β-hydroxybutyrate and MCT oil on Caco-2 cell morphology

The results of the morphological analysis of Caco-2 cells after administration of BHB, MCTs, alone and in combination, in the presence or absence of LPS (1 µg/mL) are shown in Figure 2 below.

1)



2) I)

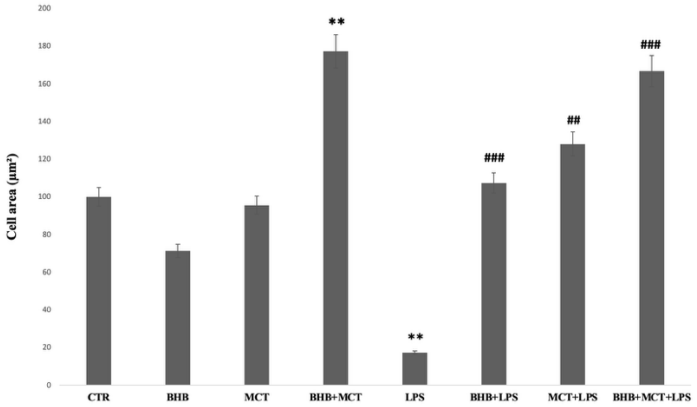


Figure 2: 1) Morphological assay after administration of BHB, MCT, BHB + MCT, with or without LPS. Morphological assay of Caco-2 cells in different conditions: control (A), 5 mM BHB (B), 0,5 mM MCT (C), BHB+MCT (D), 1 µg/mL LPS (E), BHB with 1 µg/mL LPS (F), MCT with 1 µg/mL LPS (G), BHB+MCT with 1 µg/mL LPS (H). Bar: 75 µm (20× objective). The arrows indicate the cells that underwent morphological change. **2) Cell areas expressed in µm² were quantified using ImageJ software (Panel I).** Data are ex-

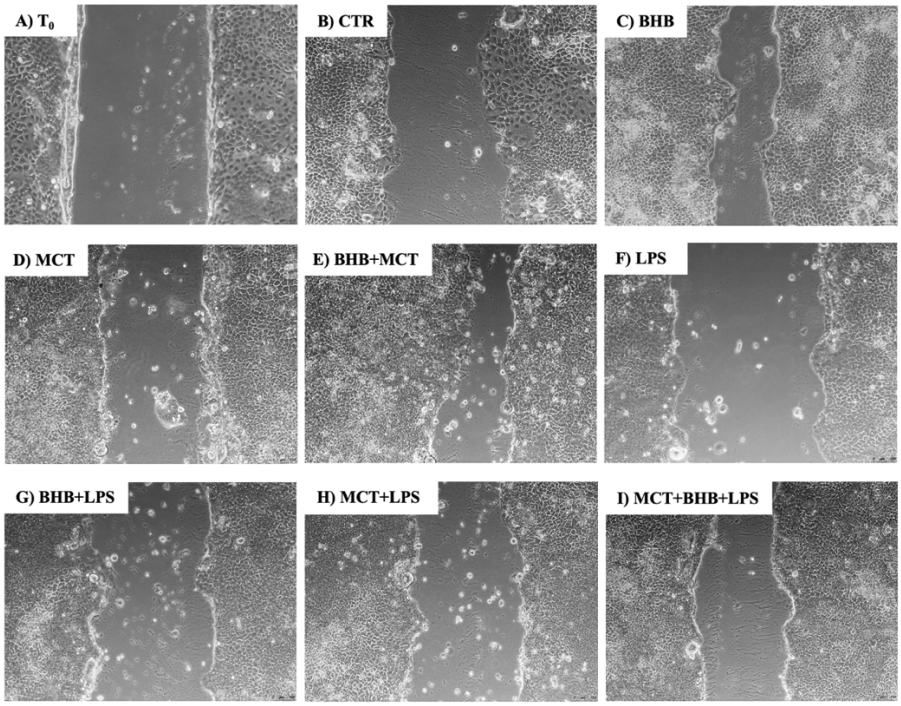
pressed as the means of three cell areas calculated from three independent experiments performed in triplicate ± SDs from three independent experiments. * $p < 0.05$ compared to CTR. # $p < 0.05$ compared to LPS.

As shown in Figure 2, in the untreated control (Panel A), Caco-2 cells were typically organized in clusters, while treatment with the proinflammatory stimulus (Panel E) significantly reduced both cell number and the typical aggregation of cell colonies (as indicated by the arrow). In the presence of costimulation with BHB (5 mM) and MCT (0.5 mM), as shown in Panel D, the cells showed enhanced proliferation compared to the control and the presence of each stimulus alone, thus confirming the results of the cell viability mentioned above. The results were further confirmed by morphological analysis of the cell area (Panel I): the synergistic anti-inflammatory action of the two substances was able to significantly counteract the effects of LPS, resulting in a significant increase in colony growth compared to the LPS-treated condition.

5.4.3. Effect of β-hydroxybutyrate and MCT oil on cell migration (wound closure assay)

The results of the cell wound assay for Caco-2 cells after administration of BHB and MCTs alone or in combination, in the presence or absence of LPS (1 μg/mL) at 24 h, are shown in Figure 3 below.

1)



2) L)

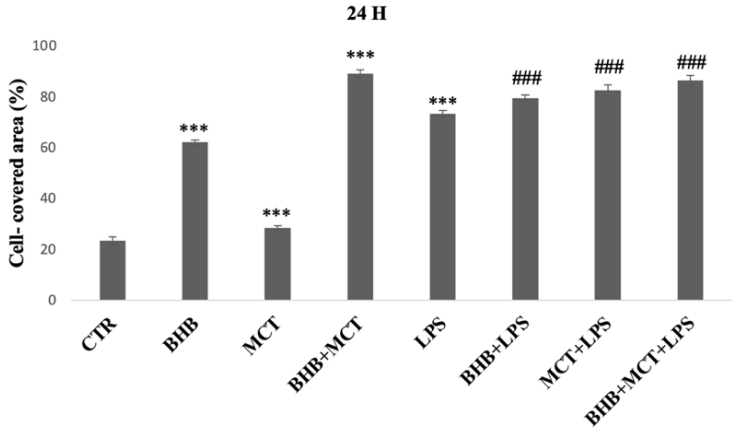


Figure 3: 1) Results of the wound-closure assay in Caco-2 cells after administration of BHB, MCT, or BHB + MCT, with or without LPS at 24 h. Cuts in the Caco-2 cell monolayer were assessed at

24 hours after treatment under the following conditions: Caco-2 cells at T_0 (A), control (B), 5 mM BHB (C), 0,5 mM MCT (D), BHB + MCT (E), 1 µg/mL LPS (F), BHB with 1 µg/mL LPS (G), MCT with 1 µg/mL LPS (H), and BHB + MCT with 1 µg/mL LPS (I). **2) Results are expressed as means ± SD of wound-closure percentages relative to the time-0 condition (L), using ImageJ software.** * $p < 0.05$ compared to CTR. # $p < 0.05$ compared to LPS.

The impact of BHB (5 mM) and MCT (0.5 mM) on epithelial regeneration was assessed using a wound closure (scratch) assay. As shown in Figure 3, Caco-2 cells exhibit increased migratory capacity in the presence of BHB (Panel C) and MCT (Panel D), compared to T_0 (Panel A) and the untreated condition (Control, Panel B) 24 hours after treatment. The cell area covered by the two substances, BHB+MCT, as shown in Panel E, significantly increased, demonstrating their synergistic action on intestinal cells. Pretreatment of cells with BHB, MCT, and BHB+MCT before the addition of LPS significantly increased the migratory capacity of Caco-2 cells compared to the LPS condition, reversing its action (Panel E). The two substances co-administered in the presence of LPS exhibited a greater protective effect after 24 hours. The wound closure assay is consistent with the cell viability and morphology data, supporting the hypothesis that both compounds contribute to enhanced epithelial regeneration.

5.4.4. Modulation of cytokine expression by β-hydroxybutyrate and MCT oil (ELISA analysis)

To assess the immunomodulatory effects of BHB and MCT, cytokine levels in the supernatant (TNF-α, IL-17, and IL-10) were quantified

by ELISA after 24 hours of treatment (Figure 5). As shown in Panels A and C, exposure to BHB or MCT alone significantly reduced the secretion of the pro-inflammatory cytokines TNF-α and IL-17 compared to LPS stimulation alone. Notably, the combined treatment produced the most pronounced reduction, suggesting a potential additive or synergistic effect in attenuating the inflammatory response.

Concomitantly, IL-10 levels increased, particularly under inflammatory conditions, with the greatest induction observed in the co-treatment group. Given the central role of IL-10 in limiting excessive immune activation and preserving epithelial integrity, this shift toward an anti-inflammatory cytokine profile suggests a rebalancing of the inflammatory environment rather than a mere suppression of cytokine production.

TNF-α and IL-17 are key mediators of intestinal inflammation, contributing to epithelial barrier disruption, immune cell recruitment, and amplification of inflammatory signaling pathways. Their downregulation, along with the upregulation of IL-10, suggests that BHB and MCTs may promote a more controlled inflammatory response, potentially supporting epithelial homeostasis. Overall, these findings indicate that BHB and MCTs may directly influence cytokine secretion in intestinal epithelial cells.

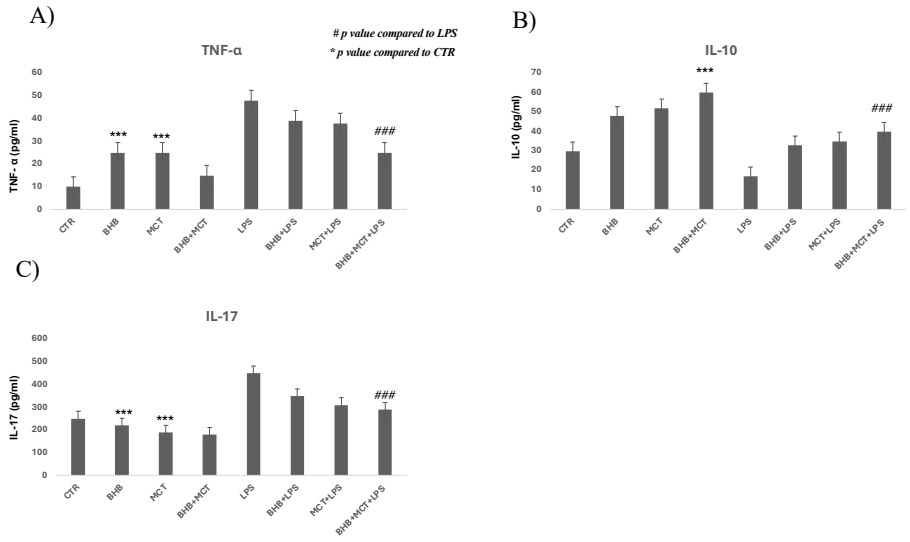


Figure 5: Effects of BHB and MCT on cytokine expression. Analysis of cytokine expression, performed by ELISA assay, of TNF-α (A), IL-17 (B) and IL-10 (C) with stimulation of 5 mM of BHB and 0,5 mM of MCT, in the presence or absence of LPS (1 µg/mL) at 24h of incubation. Experimental data are expressed as the means (pg/mL) ± SDs of three independent experiments. * $p < 0.05$ compared to the CTR. # $p < 0.05$ with respect to the LPS condition.

5.4.5. Effects of β-hydroxybutyrate and MCT oil on intestinal barrier–related adhesion proteins in Caco-2 cells

Expression of the gap junction protein Connexin-43 (Cx-43) was assessed by Western blotting and normalized to GAPDH after 24 hours. As shown in Figure 6 below, LPS treatment resulted in a significant reduction in Cx-43 expression compared to the control condition ($p < 0.001$).

BHB + MCT co-treatment showed a significant decrease in Cx-43 levels compared to the control ($p < 0.001$), while treatments with BHB or MCT alone did not induce significant changes compared to the control condition.

MCT pre-treatment resulted in a significant increase in Cx-43 expression compared to the LPS condition ($p < 0.05$), suggesting a possible protective effect of MCT on inflammation-induced Cx-43 loss. The BHB+LPS and BHB+MCT+LPS treatments showed a partial recovery of Cx-43 levels compared to the LPS condition, although these changes were not statistically significant.

Data are expressed as mean ± SD of independent experiments.

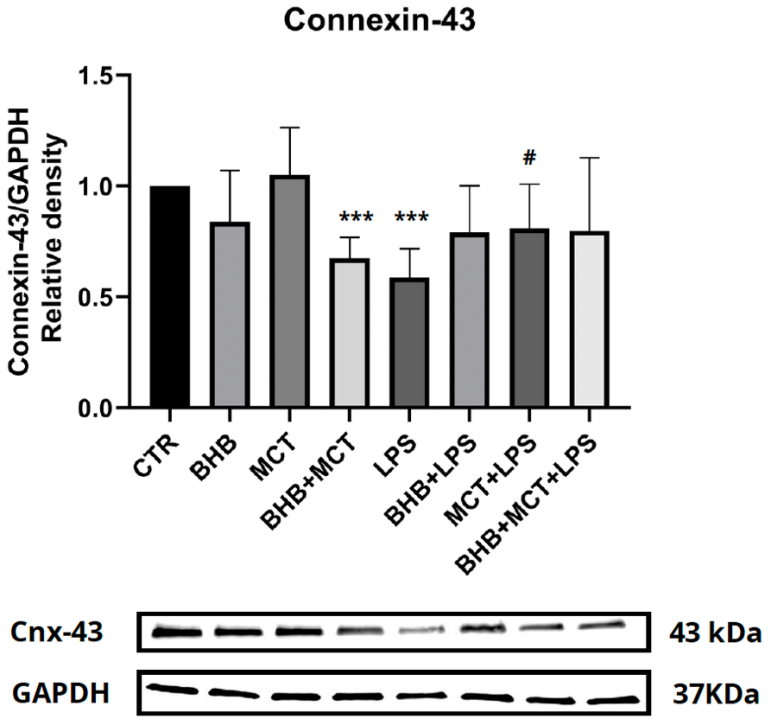


Figure 6: Western blotting of expression of adhesion proteins Connexin-43. The protein expression values are expressed in arbitrary units after normalization against GAPDH. The data are expressed as means ± SDs. (*** p< 0.001 compared to the control condition; # p<0,05 compared to the condition of LPSstimulated Caco-2).

Figure 7 below shows the expression of the adhesion protein E-cadherin analyzed by Western blotting and normalized to GAPDH.
In this case, none of the experimental treatments resulted in statistically significant changes in E-cadherin levels compared to the control condition.
However, an increasing trend in protein expression was observed following treatments with BHB, MCT, and, in particular, the BHB+MCT combination, compared to the control condition. Stimulation with a pro-inflammatory stimulus did not induce a significant reduction in E-cadherin levels, while the combined treatments BHB+LPS, MCT+LPS, and BHB+MCT+LPS showed values comparable to or slightly higher than the untreated condition. Overall, the data suggest that the tested treatments do not significantly alter E-cadherin expression in Caco-2 cells.

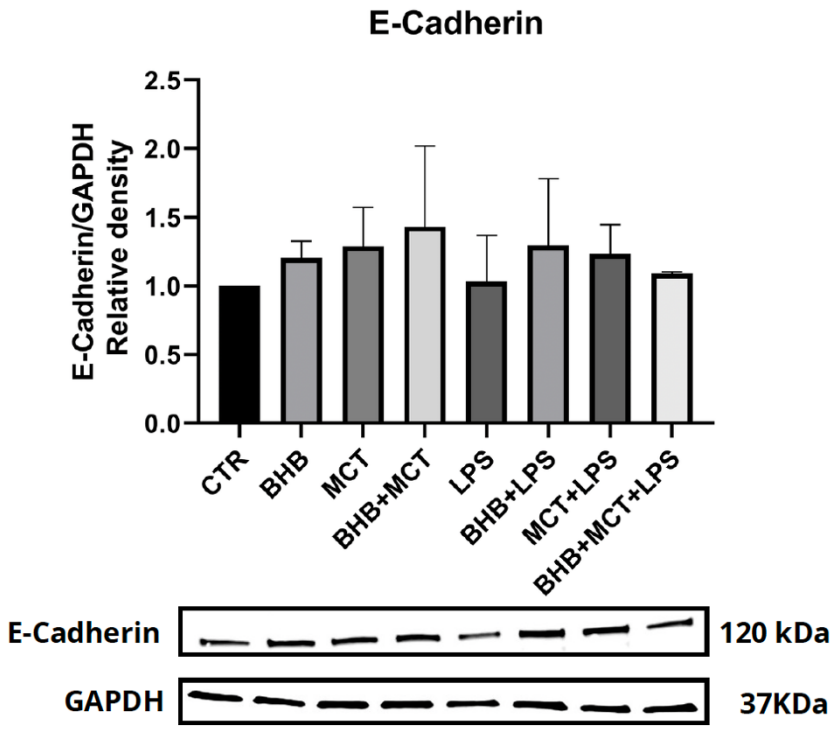


Figure 7: Western blotting of expression of adhesion proteins E-Cadherin. The protein expression values are expressed in arbitrary units after normalization against GAPDH. The data are expressed as means ± SDs. (*** $p < 0.001$ compared to the control condition; # $p < 0,05$ compared to the condition of LPS stimulated Caco-2)

Figure 8 below analyzes the expression of the tight junction protein ZO-1 by Western blotting and normalized to GAPDH. These results are contradictory to expectations: all treatments show an increase in ZO-1 levels compared to the control condition; however, these changes are not statistically significant.

Specifically, treatments with BHB, MCT, and BHB+MCT show an increase in ZO-1 expression compared to the control, however, treatment with LPS also induced an increase in ZO-1 protein levels.

In the groups treated with BHB+LPS, MCT+LPS, and BHB+MCT+LPS, ZO-1 expression is increased compared to the control, suggesting that these treatments do not compromise ZO-1 expression.

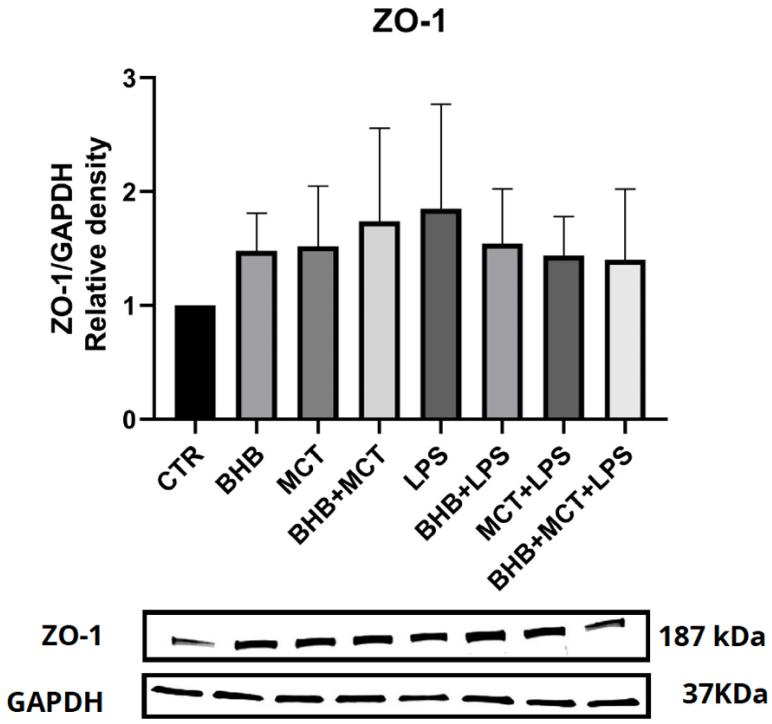
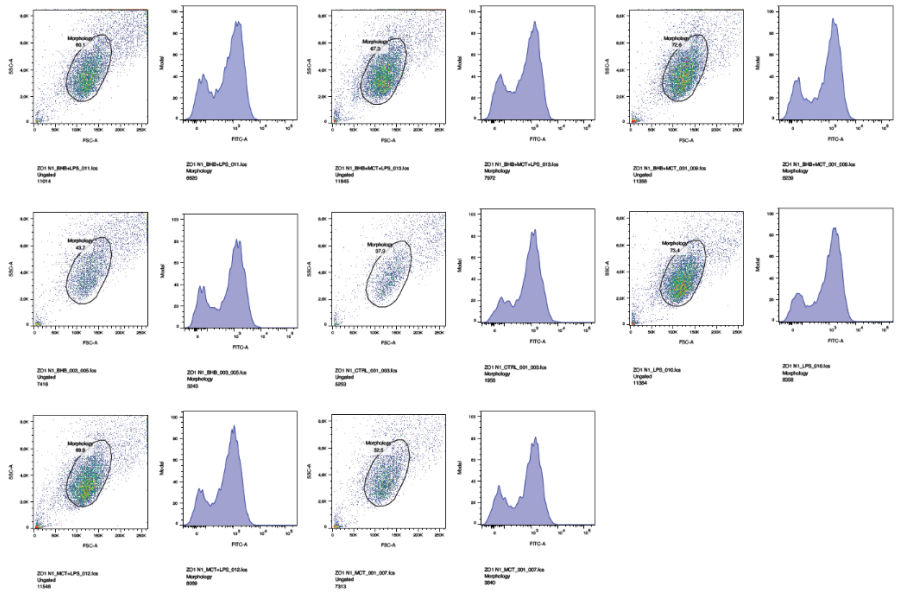


Figure 8: Western blotting of expression of adhesion protein ZO-1. The protein expression values are expressed in arbitrary units after normalization against GAPDH. The data are expressed as means ± SDs. (*** $p< 0.001$ compared to the control condition; # $p<0,05$ compared to the condition of LPS-stimulated Caco-2)

6.3.6 Effects of β-hydroxybutyrate and MCT Oil on ZO-1 expression assessed by flow cytometry

A)



B)

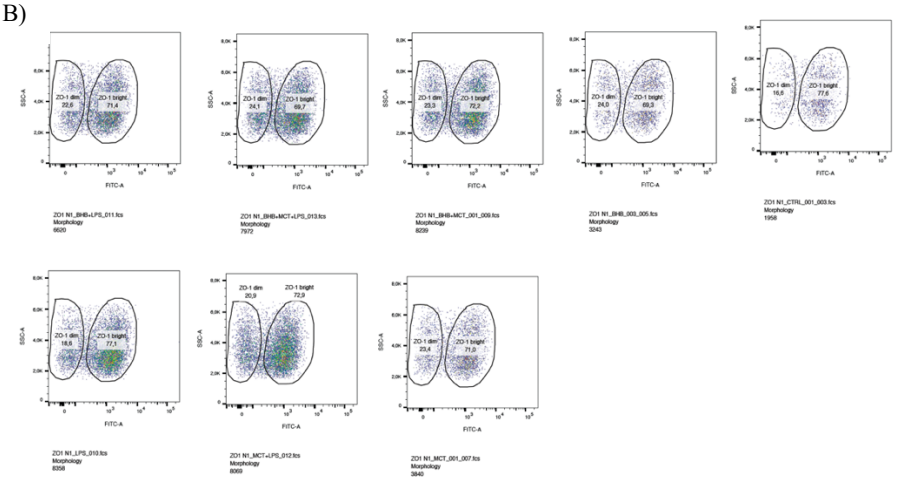


Figure 9: Detection of ZO-1 in human Caco-2 cells using the BD FACSCanto™ II Clinical Flow Cytometry System equipment (Waters™ Biosciences). Cells were treated with BHB, MCT,

BHB+MCT, LPS, BHB+LPS, MCT+LPS, and BHB+MCT+LPS. Numbers in each quadrant reflect the percentage of cells (population). Graphs represent the average expression of the marker in the population (A). Gate on the percentage of the population with low/high ZO-1 expression (B).

Flow cytometry was used to quantify ZO-1 expression at the single-cell level by measuring fluorescence intensity. This approach allowed for the assessment of mean fluorescence intensity (MFI) and the percentage of cells with low or high ZO-1 expression.

As shown in Figure 9, LPS treatment altered ZO-1 expression levels, while BHB and MCT treatments preserved or increased the percentage of ZO-1-positive cells, compared to the control. The combined BHB+MCT treatment showed the highest overall fluorescence signal, suggesting the maintenance of ZO-1 expression under inflammatory conditions, confirming the Western blotting data.

5.5. DISCUSSION

The findings of the present study are consistent with a growing body of evidence highlighting the role of ketone bodies and medium-chain lipids in the modulation of intestinal inflammation. Previous studies have demonstrated that β-hydroxybutyrate exerts anti-inflammatory effects through inhibition of the NLRP3 inflammasome and modulation of cytokine expression. Similarly, medium-chain triglycerides have been associated with beneficial effects on intestinal barrier function and cellular energy metabolism. The present results are in line

with these observations, further supporting the role of these compounds in maintaining epithelial homeostasis.

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and adequate-protein nutritional regimen that induces a metabolic state known as ketosis. In this state, hepatic metabolism shifts toward the production of ketone bodies, primarily acetoacetate (AcAc) and β-hydroxybutyrate (BHB), as alternative energy substrates to glucose [74]. This metabolic adaptation is particularly relevant when carbohydrate availability is significantly restricted, promoting the use of fatty acids for ATP generation [75,76].

Beyond its well-established applications in weight management and neurological conditions such as epilepsy, recent studies have highlighted the potential of ketogenic metabolism in modulating gastrointestinal (GI) health. Colonocytes, the epithelial cells lining the colon, exhibit a preferential utilization of ketone bodies over glucose, thereby supporting mucosal integrity, energy homeostasis, and tissue repair [77]. Unlike glucose metabolism, which is associated with rapid fluctuations in energy availability and pro-inflammatory signaling, ketone metabolism provides a stable and efficient energy source that may reinforce epithelial function and barrier competence.

The intestinal mucosa serves as a critical physical and immunological barrier, protecting the host from pathogens, toxins, and environmental insults [78]. Disruption of this barrier, as observed in “leaky gut” syndrome and inflammatory bowel diseases (IBD), is associated with chronic inflammation, dysbiosis, and systemic immune activation. BHB exerts anti-inflammatory effects through inhibition of the

NLRP3 inflammasome and suppression of pro-inflammatory cytokines, including IL-1β and TNF-α [79,80]. These mechanisms suggest a therapeutic potential for ketone bodies in restoring gut homeostasis and mitigating intestinal inflammation.

Additionally, the role of ketone metabolism in oncological prevention has gained increasing attention. Glucose-restrictive metabolic states, such as those induced by KDs, may interfere with tumor growth. Certain cancer cell types, including colon adenocarcinoma lines such as Caco-2, are known to preferentially utilize glucose as their main energy substrate [81]. The anti-cancer properties of BHB, coupled with its anti-inflammatory and antioxidant actions, underscore its multifaceted role in maintaining intestinal health.

From a dietary standpoint, medium-chain triglyceride (MCT) oil and coconut oil are critical exogenous sources of ketone precursors. Rich in medium-chain fatty acids (MCFAs), these lipids bypass conventional fat digestion and are directly transported to the liver, where they are rapidly converted into ketones [82,83]. MCT oil is known for its high ketogenic efficiency and is widely used in clinical and dietary interventions to maintain a state of nutritional ketosis [84,85].

Despite the growing body of literature on ketogenic metabolism and intestinal health, few studies have systematically investigated the combined effects of BHB and MCT oil on human intestinal epithelial cells. Considering the independent anti-inflammatory properties of both agents, it is plausible that their co-administration may exert a synergistic effect in promoting epithelial integrity and reducing inflammatory signaling [86].

From a mechanistic perspective, the observed effects may be explained by multiple biological pathways. β-hydroxybutyrate acts as a

signaling molecule capable of modulating gene expression through epigenetic mechanisms, including the inhibition of histone deacetylases. In addition, its ability to suppress NLRP3 inflammasome activation may contribute to the reduction of pro-inflammatory cytokine production. At the same time, medium-chain triglycerides, due to their rapid hepatic oxidation and efficient ketone body production, may support cellular energy metabolism and contribute to the maintenance of intestinal barrier integrity.

Therefore, the present study aims to evaluate the individual and combined effects of β-hydroxybutyrate and MCT oil on markers of inflammation and barrier function in the human colorectal adenocarcinoma CaCo-2 cell line. This model, widely used as a proxy for intestinal epithelium, offers a relevant system for exploring the molecular and cellular mechanisms underlying intestinal health. Through this approach, we seek to clarify the potential of ketogenic-derived metabolites as modulators of intestinal inflammation and to contribute to the development of novel dietary strategies for gut health optimization.

The present study provides compelling evidence that medium-chain triglyceride (MCT) oil and β-hydroxybutyrate (BHB) exert significant anti-inflammatory and cytoprotective effects on intestinal epithelial cells, as demonstrated in the Caco-2 cell model. Specifically, our findings show that both agents enhance cell viability, promote wound healing, and modulate cytokine expression by decreasing pro-inflammatory mediators (TNF-α and IL-17) and increasing the anti-inflammatory cytokine IL-10. Notably, the co-treatment with BHB and MCT elicited the most potent biological effects, indicating a synergistic interaction that exceeds the individual impact of each compound alone.

The reduction in cell viability at higher concentrations may reflect the cytotoxic effects of supraphysiological levels of ketones or lipids, possibly related to mitochondrial overload or ROS generation in transformed epithelial cells. A particularly relevant finding of this study is the potential synergistic effect between β-hydroxybutyrate and medium-chain triglycerides. This interaction suggests that the efficacy of ketogenic interventions may not rely solely on ketone body production, but rather on the combined action of multiple nutritional components. These results support the hypothesis that complex dietary approaches may exert stronger biological effects compared to single-compound interventions. In addition, this synergy is likely attributable to the complementary metabolic roles of MCTs and BHB. MCTs, once ingested, are rapidly absorbed and converted by the liver into ketone bodies, particularly BHB, which serves not only as an energy substrate but also as a signaling molecule that regulates inflammatory pathways and cellular homeostasis [87,88]. The dual availability of exogenous BHB and its endogenous production via MCT metabolism may amplify the anti-inflammatory signaling cascade and contribute to epithelial regeneration. MCTs may exert direct protective and regulatory effects on intestinal epithelial cells through multiple mechanisms. One key pathway involves the stabilization of tight junctions, which are critical for maintaining epithelial barrier integrity and preventing paracellular permeability. Studies have shown that MCTs can enhance the expression and organization of tight junction proteins such as claudins and occludin, thereby strengthening the intestinal barrier and reducing susceptibility to inflammatory stimuli. Additionally, MCTs are rapidly metabolized within cells and can enhance mitochondrial function by serving as a readily available energy

source, supporting ATP production and cellular resilience under stress conditions.

To support the functional data on barrier integrity and epithelial restitution, we then assessed the expression of key junctional proteins by Western blotting, including Connexin-43 (Cx-43), E-cadherin, and ZO-1, which play complementary roles in cell-to-cell communication, adherens junctions, and tight junction organization.
Exposure to LPS resulted in a significant reduction in Cx-43 expression, consistent with previous studies demonstrating that inflammatory stimuli disrupt communication between gap junctions and impair epithelial coordination. Specifically, MCT treatment partially restored Cx-43 levels in LPS-stimulated cells, suggesting a protective role against inflammation-induced loss of intercellular coupling.
In contrast, the expression of E-cadherin and ZO-1 was not significantly reduced after LPS stimulation. This observation is consistent with evidence demonstrating that LPS-induced barrier dysfunction is not necessarily associated with reduced tight junction protein expression, but rather with their redistribution and functional reorganization. Specifically, this finding was observed by Al-Sadi et al., who demonstrated that LPS-induced increased epithelial permeability is not necessarily associated with reduced tight junction protein expression, but rather with altered localization and subcellular organization. LPS-induced activation of Toll-like receptor 4 (TLR4) leads to recruitment of the MyD88-dependent signaling pathway, resulting in increased expression and activity of myosin light chain kinase (MLCK). MLCK activation promotes myosin light chain phosphorylation, resulting in

contraction of the perijunctional actomyosin ring and increased paracellular permeability, without a quantitative reduction in total ZO-1 levels. Therefore, epithelial barrier integrity is compromised primarily at the functional and structural level, rather than through downregulation of protein expression. The maintenance of E-cadherin and ZO-1 expression in our model suggests that BHB and MCTs primarily preserve epithelial barrier integrity by preventing inflammatory disruption of the junctional architecture rather than by inducing overexpression of junctional proteins. These findings are consistent with previous studies demonstrating that MCTs stabilize tight junction organization and attenuate LPS-induced epithelial damage [89]. These observations were further supported by flow cytometric analysis, which allowed quantitative assessment of ZO-1 expression at the single-cell level. Consistent with Western blot results, no marked reduction in overall ZO-1 fluorescence intensity was detected after LPS stimulation, confirming that total protein levels remained largely preserved across the entire cell population. Notably, treatments with BHB and MCT, especially in combination, maintained or slightly increased ZO-1 expression levels, strengthening the idea that these compounds protect epithelial integrity.

Together, these results indicate that LPS-induced barrier dysfunction in our model is unlikely to result from a loss of tight junction proteins, but rather presumably from functional or structural alterations in junctional organization, while BHB and MCT contribute to the maintenance of junctional homeostasis.

Furthermore, MCTs can engage G-protein coupled receptors, particularly GPR84, which is activated by medium-chain fatty acids and involved in modulating immune responses. While GPR84 has been traditionally associated with pro-inflammatory signaling, its role in intestinal epithelial cells may be more nuanced, contributing to immune homeostasis and epithelial repair processes. The combined effects on cellular metabolism, barrier function, and immune signaling may explain the immunomodulatory and pro-regenerative properties observed in our study. These mechanisms highlight the therapeutic potential of MCTs in maintaining gut epithelial health, particularly in conditions characterized by inflammation and barrier dysfunction [90]. Medium-chain TAG improves intestinal integrity by suppressing toll-like receptor 4, nucleotide-binding oligomerisation domain proteins, and necroptosis signalling in weanling piglets challenged with lipopolysaccharide. Shows how MCTs support barrier function and reduce inflammation in epithelial models [91].

While metabolic parameters such as ATP production or oxygen consumption were not directly measured, BHB and MCT are known to modulate energy metabolism and oxidative stress responses. Future work will explore these mechanisms.

Our results align with previous reports indicating that BHB may be inhibit the NLRP3 inflammasome, a central regulator of inflammation in intestinal and systemic diseases [90-92]. Additionally, BHB has been shown to act as a class I histone deacetylase (HDAC) inhibitor, thereby modulating epigenetic transcriptional programs in favor of anti-inflammatory gene expression [92-93]. These mechanisms likely underlie the increased IL-10 secretion and suppression of TNF-α and IL-17 observed at 24 in our ELISA assays.

Parallel to BHB, MCT oil itself has been shown to possess intrinsic immunomodulatory properties. Its capacity to stabilize epithelial tight junctions, improve mucosal barrier integrity, and influence immune cell polarization contributes to a reduction in intestinal permeability and systemic inflammation [94,95]. These findings underscore the potential of MCTs to prevent or mitigate pathologies characterized by epithelial disruption, such as inflammatory bowel disease (IBD), leaky gut syndrome, and colorectal inflammation [96,97]. Although Caco-2 cells are not immune cells, they can secrete cytokines in response to LPS. mRNA validation will be pursued in future studies to confirm and validate our results.

The observed increase in cell migration and wound closure, especially following co-treatment with BHB and MCT, highlights their reparative potential in epithelial restitution. Such effects are crucial in the context of chronic intestinal inflammation, where impaired epithelial healing exacerbates disease severity and recurrence [98-101]. Our findings therefore suggest that these agents could play a supportive role in promoting mucosal healing alongside conventional anti-inflammatory therapies.

The use of complementary assays in this study, namely, cell viability, migration (wound healing), and cytokine profiling, allowed for a more comprehensive evaluation of the biological effects of BHB and MCT on intestinal epithelial cells. While the MTT assay provided insight into the cytoprotective effects of these compounds by assessing metabolic activity and cell survival under inflammatory stress, the wound healing assay further demonstrated their capacity to enhance epithelial regeneration and motility, key processes in the maintenance and repair of mucosal integrity. In parallel, the cytokine profiling (TNF-α,

IL-17, IL-10) revealed a shift toward an anti-inflammatory milieu, supporting the immunomodulatory role of BHB and MCT.

Taken together, these findings illustrate a coherent and synergistic effect, where improved cell viability and enhanced migration are accompanied by reduced pro-inflammatory signaling and increased production of regulatory cytokines. This integrative approach provides functional evidence that BHB and MCT not only support epithelial survival but also actively contribute to the restoration of homeostasis in an inflamed epithelial environment.

Moreover, this study supports the broader therapeutic implications of the ketogenic diet. The metabolic shift induced by such a diet, favoring ketone production, mimics the cellular milieu we replicated *in vitro*, where BHB and MCT modulate inflammation and promote cell survival. Preclinical and clinical data have highlighted the anti-inflammatory potential of ketogenic states across various systems, including neurological and gastrointestinal [101-106]. Our results provide mechanistic insight into how this effect may be mediated at the intestinal level through modulation of epithelial and immune cell function.

In light of the growing interest in nutritional therapeutics, the combination of MCT oil and exogenous BHB shows potential as a non-pharmacological approach to support intestinal health and modulate inflammation. However, while our *in vitro* findings indicate synergistic anti-inflammatory and regenerative effects, these results remain preliminary. Further in vivo and clinical studies are needed to validate these observations, determine optimal dosing strategies, and assess long-term safety and efficacy. Additionally, elucidating the molecular mechanisms underlying BHB-MCT interaction, possibly involving

AMPK activation, oxidative stress pathways, or microbiota modulation, will be essential for translating these findings into therapeutic applications. Since Caco-2 cells are not capable of ketogenesis, the observed effects of MCTs are likely independent of local BHB synthesis and reflect direct cellular actions of MCTs on epithelial barrier integrity and inflammation.

From a translational perspective, these findings suggest that nutritional strategies based on the modulation of ketosis may represent a promising approach for the management of intestinal inflammation and the preservation of epithelial barrier function. However, the clinical applicability of these results requires further validation through in vivo studies and controlled clinical trials, in order to define appropriate dosages, safety, and long-term sustainability.

5.6. LIMITATIONS OF THE STUDY

This study presents several limitations that should be carefully considered when interpreting the findings. First, the experimental component is based on an in vitro model (Caco-2 intestinal epithelial cells), which, although widely used and well-established for studying intestinal barrier function and inflammatory responses, cannot fully replicate the complexity of the in vivo human physiological environment. In particular, the absence of systemic interactions, immune system components, hormonal regulation, and microbiota dynamics limits the direct translation of these results to clinical settings. Second, the experimental conditions, including the concentrations of β-hydroxybutyrate and medium-chain triglycerides, may not fully reflect physiological levels achievable through dietary interventions. Therefore,

caution is warranted when extrapolating these findings to real-life nutritional scenarios. Third, the study focuses on a limited number of biological endpoints, primarily related to inflammation and epithelial barrier integrity. While these are highly relevant, other important pathways, such as metabolic signaling, oxidative stress modulation, and long-term cellular adaptations, were not investigated and may provide additional insights into the mechanisms involved. Furthermore, the use of a single cell line model does not account for inter-individual variability, which represents a key factor in nutritional and metabolic responses. Genetic background, microbiota composition, and lifestyle-related factors may significantly influence the biological effects observed in vivo.

From a broader perspective, the narrative reviews included in this thesis, although comprehensive, may be subject to inherent limitations related to the selection and interpretation of the literature, including potential publication bias and the lack of systematic methodology. Finally, the absence of in vivo or clinical validation represents a major limitation. Future studies involving animal models or human subjects are necessary to confirm the biological relevance and clinical applicability of the observed effects.

For these reasons, future research should aim to validate these findings in more complex biological systems, including co-culture models incorporating immune cells and microbiota components, as well as in vivo models. Moreover, clinical studies are needed to evaluate the real-world applicability of ketogenic-based interventions and to identify potential biomarkers of response, taking into account interindividual variability.

5.7. CONCLUSION

In conclusion, this study provides preliminary evidence that MCT oil and BHB, particularly in combination, may exert beneficial effects on intestinal epithelial cells under inflammatory stress *in vitro*. The treatment enhanced cell viability, cell morphology, promoted wound healing, modulated cytokine profiles toward an anti-inflammatory state and induced protein expression changes as demonstrated by Western blot analysis. While these results support further investigation into ketogenic-based nutritional strategies for intestinal inflammation, they should be interpreted cautiously until confirmed by comprehensive *in vivo* and clinical research.

PART III: GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The literature reviews presented in the first part highlighted how the current nutritional landscape is characterized by a dual dynamic: on the one hand, the increased consumption of ultra-processed foods is associated with rising obesity and related comorbidities characterized by chronic low-grade inflammation, intestinal barrier dysfunction, and metabolic risk [107]; on the other, the growing interest in nutritional models, such as the ketogenic diet, capable of modulating energy metabolism and potentially influencing inflammatory processes [108]. However, within the ketogenic diet, despite extensive clinical and systemic research, a limited understanding of the direct cellular mechanisms through which ketosis metabolites might exert effects on the intestinal epithelium remains.

In this context, the combined use of molecules such as β-hydroxybutyrate (BHB) and medium-chain triglycerides (MCTs) has been guided by a specific metabolic rationale. BHB is the main circulating ketone body during a ketogenic diet and is recognized not only as an alternative energy substrate but also as a signaling molecule with potential epigenetic and immunomodulatory effects [109]. MCTs, thanks to their rapid absorption and preferential hepatic oxidation, are a highly ketogenic substrate, capable of driving, sustaining, and amplifying endogenous ketone body production [110]. The combination of the two compounds allowed us to reproduce *in vitro* a metabolic scenario closer to the physiology of nutritional ketosis, integrating the

direct effect of the active metabolite with that resulting from increased ketone availability.

The experimental model adopted in this thesis therefore lies midway between clinical observation and molecular mechanism, with the aim of verifying whether this metabolic modulation can translate into measurable effects at the level of the intestinal epithelium. The results obtained provide preliminary evidence supporting this hypothesis, demonstrating that exposure to BHB and MCTs, individually and in combination, is associated with a significant modulation of the cytokine profile under conditions of inflammatory stimulation.

Overall, the data suggest that the metabolic modulation induced by BHB and MCTs may directly influence the inflammatory response of the intestinal epithelium under conditions of pro-inflammatory stimulation. The reduction in TNF-α and IL-17, associated with the increase in IL-10, indicates a rebalancing of the cytokine profile consistent with a more immunologically regulated epithelial environment. This evidence is particularly relevant in the context of metabolic, chronic intestinal inflammatory, and oncological diseases, in which intestinal barrier dysfunction and systemic inflammation are key elements [111].

Compared to the gaps highlighted in the reviews, this study provides a direct experimental contribution, demonstrating that ketosis-associated metabolites can exert cellular effects independent of weight loss or complex systemic adaptations, suggesting a possible local action at the epithelial level.

However, it remains necessary to interpret the results in light of the limitations of the *in vitro* model, which lacks the complexities of the interaction with the microbiota and immune system. Future studies will therefore need to validate these observations in more complex systems and delve deeper into the molecular mechanisms involved, including the regulation of the inflammasome, oxidative stress, and epigenetic pathways mediated by β-hydroxybutyrate and medium-chain triglycerides.

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<u>OTHER ACTIVITIES</u>

- Participation in the project “Nuove strategie per la promozione della salute sui luoghi *di* lavoro per SanitaService - Foggia" con

valutazione dello stato nutrizionale di ogni singolo dipendente in collaboration with SANITASERVICE ASL FG SRL – Foggia.