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"ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF THE KETOGENIC DIET WITH ENHANCEMENT OF THE USE OF ORGANIC FOOD PRODUCTS"

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1. INTRODUCTION

In recent years, numerous reviews and publications have been published regarding the constant and growing consumption of organically derived foods and their effect on individual health. On average, according to new research, organic foods could contain fewer traces of pesticides, synthetic residues, heavy metals, and a lower nitrate content but, at the same time, a greater content of compounds with an antioxidant and anti-inflammatory action, especially vitamins and antioxidants [1]. In fact, according to new research, the regular and frequent consumption of organic products could lead to positive effects on the individual's health and generally reduce the onset of certain pathologies and the risk of overweight and obesity [2]. At the same time, new research has highlighted how certain dietary regimes could positively and significantly influence individual health, especially in certain pathological contexts. In this regard, Ketogenic Diet (KD), a diet rich in fats, with a correct protein intake and a low level of carbohydrates, would seem to be a diet with possible positive effects, especially in some pathologies [3] such as obesity [4], diabetes [5], polycystic ovary syndrome (PCOS) [6], cancer [7] and other conditions [8]. In particular, KD has been recognized as a neuroprotective factor, especially in cases of brain lesions and neurodegenerative diseases [9,10], for example Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's (AD) and Huntington's disease (HD), which are closely linked to a progressive loss of neuronal material and function [11], and also due to a constant pro-inflammatory microglial activation [12], namely as macrophages resident in the central nervous system (CNS), which represent the first cellular defence for immune and host defence purposes [13]. If activated, microglia could be responsible to produce proinflammatory mediators, cytokines and reactive oxygen species (ROS) [14,15]. In this context, but also in other pathologies mentioned above, KD could exert protective effects by modulating numerous inflammatory patterns [16]. This

potential is presumed to be determined by the presence of secondary metabolites, produced in the liver mitochondria, such as Acetoacetate (AcAc) and, in greater quantities, β-hydroxybutyrate (BHB) [17], considered as the main ketone bodies and alternative energy substrates, consumed by the body, especially by the brain in the absence of glucose [18,19]. Among ketone bodies, BHB has a predominantly neuroprotective role, especially at microglial level [20], as it could modulate the immune response, inhibiting the activation of the NLRP3 inflammasome [21], reducing the levels of anti-inflammatory cytokines IL-1β and caspase-1 together with the decrease in ROS production and reduction of cell death observed in vitro and in vivo [22]. AcAc, on the other hand, one of the main ketone body which are common hallmarks of cancer metabolism, and its formulations, could currently present a new therapeutic frontier especially with regards to the treatment of lung cancer [7], as it presents positive and modulatory effects and various cellular processes that characterize cancer, particularly related to hindered tumor growth [23]; however these mechanisms still require further studies and confirmation. Although KD can be identified among the new therapeutic strategies that would bring benefits to microglia with consequent reduction in the onset of neurodegenerative diseases [24], however, KD could be characterized by a long-term imbalance in micronutrients, with a possible decrease or increase in serum levels of minerals and especially vitamins [25]. Among these, stands out Vitamin E, one of the major antioxidant compounds present in KD, with a possible antioxidant and anti-inflammatory effect especially in neurodegenerative diseases [26]. Vitamin E is a fat-soluble compound consisting of eight isoforms, four tocopherols and four tocotrienol derivatives. Each group is further renamed into four different isomers, namely α, β, δ and γ, due to the presence and position of one or more methyl groups in the side chain that characterizes them [27]. Vitamin E isoforms are naturally present in various food groups and specific foods, predominantly of plant origin, such as legumes, cereals, seeds, nuts and oil [28], as well as in some foods of animal origin

such as milk [29], dairy products [30], animal fats as butter [30], egg yolk [31] and also in some fish products and meat [32] [33]. Part of these food groups constitute the food sources belonging to the KD [34] and, according to current studies, Vitamin E content may vary based on the cultivation and breeding method, which could determine a change in the content of antioxidant compounds in foods, generally with an increase in natural methods compared to conventional ones [35]. However, the role of Vitamin E is still controversial, as there are numerous clinical studies and meta-analyses in the literature in which a probable pro-oxidant effect is also reported in case of high doses [36]. Therefore, considering the hypotheses and research perspectives mentioned, the aim of this doctoral project was to evaluate the antioxidant and anti- inflammatory effects of ketogenic diet enriched with organic food product on microglial BV2 cells and epithelial lung cancer A549 and PC9 cell lines. In particular, the objective was to evaluate the combined effect of formulations of ketone bodies, produced during ketogenic diet, such as BHB and AcAc, together with Vitamin E, fat-soluble micronutrient contained in KD and organically derived foods.

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2. ORGANIC FOOD

2.1 HISTORY OF ORGANIC FOOD

Over the centuries, agriculture and livestock have undergone notable and important changes, a true evolution in response to a series of external factors, including technological, demographic, social and economic changes [1]. Starting from the industrial revolution of 1750-1850 with the introduction of new technologies, mechanization and increased productivity [2], up to the current scientific developments, with the birth of fertilizers [3], drugs [4], pesticides and hybridization processes [5] in order to obtain greater resistance of crops to diseases, agriculture and livestock have been a key factor in these modernizing influences to improve and revolutionize food production. Naturally, as consequences of these processes, agricultural and livestock evolution has allowed a large-scale expansion of the sector, through the introduction of high-yielding crop varieties [6] and intensive animal farming [7], thus increasing the availability of food at global level. Despite the high food yield, these practices have simultaneously contributed to the increase in land exploitation which, combined with the widespread use of fertilizers and pesticides, has currently raised environmental and social concerns at a global level. Precisely for these reasons, since the 80s, attempts have been made to further develop the birth of new modern technologies such as precision agriculture [8], drones [9], genomics [10], to allow farmers and breeders to optimize production, but at the same time reducing waste and improving environmental well-being [11]. In this field, animal food production has also undergone changes over time, with a significant number of amended regulations aimed at improving animal welfare status, greater care of the environment, limited use of drugs and the production of a

healthy product without residues. such as pesticides or drugs [12]. In this context, therefore, the concept of "Sustainability" applied to global food policies becomes fundamental [13]. Sustainable food refers to food production derived from practices that seek to meet the needs of the present without compromising the ability of future generations with a sustainable approach covering various aspects, including agriculture, livestock, production, distribution and consumption [14]. In this field, Organic agriculture and consequently Organic farming, which follows its principles, during the last year, have received a lot of interest from the scientific and agricultural community, with a clearly increasing trend motivated by several reasons, including concerns for health, environment and sustainability [15]. The history of organic farming dates to different eras and has gone through different phases of development, starting from the Organic Movement of the 20th century, with the birth of the "Soil Association" in the United Kingdom, which was founded in 1946, with the aim of be among the first organizations to develop official standards for organic farming [16]. Subsequently, around the 60s and 70s, interest in organic agriculture grew more and more due to the Green Revolution and the subsequent Return to Agroecology, during which a greater intensification of agriculture developed with widespread use of fertilizers chemicals and pesticides, and the consequent increase in criticism and long-term unsustainability of these practices [17]. Due to these concerns, in recent years, there has been an increase in the development of agroecology and organic agriculture as more sustainable alternatives [18]. Precisely for these reasons, during the 80s and 90s there began to be the first international certifications and standards for agricultural practices to guarantee that products considered "organic" respected certain regulations [19]. Around this time, organic farming became a global movement with the emergence of organizations such as the International Federation of Organic Agriculture Movements (IFOAM) [20]. To date, since the 2000s, there is a growing popularity and consumer awareness of sustainability, food safety and animal welfare, which

has contributed to the success of organic products [21]. In fact, many countries have now developed policies and incentives to support organic farming, and the demand for these products is constantly increasing exponentially [22]. Indeed, since the 1990s, consumer demand has increased, thus providing US farmers and ranchers with market incentives for a wide range of products. Taking the USDA surveys of the National Agricultural Statistics Service for the 2011 and 2021 decades as a reference, the amount of certified organically cultivated land increased by approximately 79% and, in 2020, traditional grocery stores, club stores and supercentres represented 56% of the share of organic foods sold to consumers [23]. According to estimates and studies from the United States Department of Commerce, there has been an increase in sales in all organic food categories starting from 2011 through 2021. In fact, sales US organic food products in 2010 were approximately $26.9 billion and reached $52.0 billion in 2021, and among the best-selling organic foods, fresh fruits and vegetables were the leading category of organically grown foods, with retail sales amounting to approximately $19.2 billion in 2021, recording a steady upward trend over the past two decades [23]. In 2021, agricultural products accounted for approximately 40% of organic food sales in the United States, followed numerically by dairy and eggs (13%), beverages (12%), packaged/prepared foods (11%), bread/grains (9%), snacks (6%), condiments (5%) and meat/fish/poultry (4%) [23]. Phenomenon due to a market difference compared to conventional growers, as organic growers are more likely to sell their products directly to consumers through direct markets or mail orders, or thanks to consumer perception on the matter.

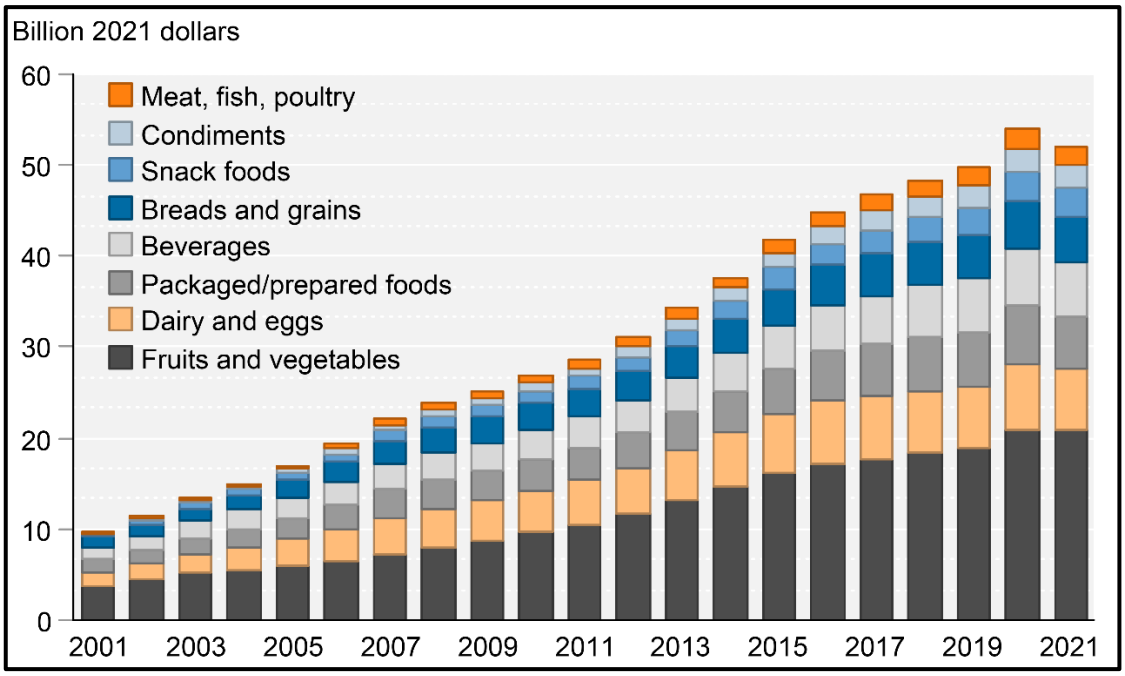

Figure 1. US Organic Food Retail by category 2001-2021 [23].

Despite the Covid-19 pandemic, and consequently the limitations resulting from it which have had consequences on trade and agri-food production, the trend of organic products turns out to be positive with an increase expected for 2023. In fact, a recent report by The Business Research Company expects the global organic food market to grow from $259.06 billion in 2022 to $294.54 billion in 2023 at a compound annual growth rate (CAGR) of approximately 13.7%. In fact, it is estimated that organic food will be increasingly widespread and important in world trade and will reach $512.01 billion in 2027 with a CAGR of 14.8% [24].

2.2 ORGANIC FOOD: DEFINITION, POLICIES AND IMPACTS

Organic food is currently defined by the Department for Agriculture and Rural Affairs (DEFRA) as:

"Organic food is the product of an agricultural system that avoids the use of artificial fertilizers and pesticides, growth regulators and livestock feed additives. The irradiation and use of genetically modified organisms (GMOs) or products obtained from or by GMOs are generally prohibited by organic legislation."

As already mentioned previously, agriculture and livestock, during the times before industrialization, were fundamentally "Organic". The modern term "Organic", over the years, has emerged in response to a continuous and constant mass industrialization concerning the food system, which has led to a large offer of food products, which certainly cannot be considered organic [25]. In fact, according to modern organic standards. products defined as "organic" can be considered organic at a percentage of 100% if they strictly contain only up to 5% of inorganic inclusions, which are permitted for the processing of food products as drawn up by Regulation (EC) no. 834/2007 of the Council, of 28 June 2007, and by the Safe Food for Canadian Regulations (SFCR) in 2022. Organic production, therefore, is a global system of farm management and agri-food production based on interaction and combination of best environmental practices, a high level of biodiversity, the protection of natural resources, the application of rigorous animal welfare criteria and production suited to the preferences of certain consumers for products obtained with natural substances and processes [26-27]. The first documents that regulated the production and labelling of organic products in the EU were published, initially, in 1991 [28]. Subsequently in 2023, the European Commission regulations n. 834/2007 and n. 889/2008 and also a new EU organic regulation 2018/848, have been implemented and revised

[29]. These cited regulations describe and establish the basic requirements for food production and for the labelling of organic products in Europe, with terms "bio" or "eco", alone or in combination (Regulation (EU) 2018/ 848, 2018). Currently organic products, which are recognized by European Union standards, are marked and recognizable by an organic label often referred to as "Euro-leaf" (Figure 2) [30].

Figure 2. EU Organic label

The rules regarding organic farming may vary, based on the country in which it operates and on the certification. However, all EU and non-EU rules are united by common principles and do not differ much from each other regarding organic food production [29]. The principles they refer to include the promotion of ecological balance, the renewal and cyclicality of resources, the conservation of biodiversity, the restriction of chemical pesticides, toxic herbicides, synthetic fertilizers and additives, the increase in the use of organic matter derived from animal farming for organic animal production, the prohibition of genetically modified organisms, and guarantee crop rotation and intercropping of plants, in order to improve soil fertility and water quality [31] (Regulation (EU) 2018/848, 2018). Furthermore, concerning organic farming, the regulations establish that it is appropriate for animals to have access, whenever possible, to open air spaces or pastures, respecting animal welfare and satisfying the specific behavioural needs of the animals according to the species by encouraging disease prevention, paying particular attention to housing conditions, zootechnical practices and animal density (Regulation (EU) 2018/848, 2018). It appears clear, therefore, that

organic agriculture is currently considered as a valid alternative much closer to the concept of "Sustainability" compared to conventional food production methods. Indeed, from an environmental point of view, the incessant use of fertilizers, food overproduction and the large-scale use of pesticides in conventional food production methods have caused, and are currently causing, enormous damage, especially to local ecosystems, the health of the soil, the biodiversity of flora and fauna, the healthiness of groundwater and so on [32-35]. Currently, according to some studies, organic agriculture or livestock farming could reduce a certain negative environmental impact compared to conventional methods. Naturally, the extent of the reduction is difficult to quantify, as further studies are needed, and varies depending on the cultivation methods implemented by farmers or specifically by consumers [35]. Despite the need to delve deeper into the topic through greater research attention, studies state that organic production positively influences various environmental sectors (Figure 3).

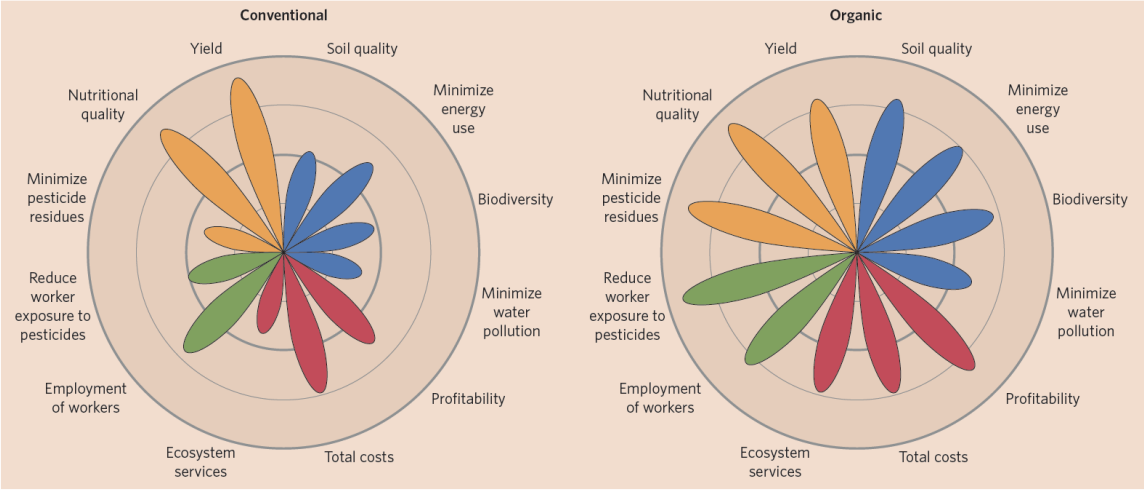

Figure 3. Impacts and sustainability compared: Organic farming versus conventional agriculture [36].

- Biodiversity on organic farms: Biodiversity is a fundamental factor for the stability and "health" of agroecosystems [37] and, consequently, for a stable food supply. Evaluating biodiversity on organic farms and conventional farms, it reveals a 30% higher species diversity and a 50%

higher abundance of flora and fauna in organic fields [38-40], with a clear difference in several taxonomic groups, including microorganisms, earthworms, weeds, wildflowers, insects, mammals, and birds [39-43]. In particular, in regions where the number of organic farms has increased over time, the diversity and numerical abundance of bees has increased considerably, contributing to the pollination of crops and wild plants over larger areas [44]. This increased in biodiversity could primarily be the effect of the ban on quick-release pesticides, herbicides and fertilizers. Furthermore, diversified crop rotation or mechanical weeding could have a positive effect on species diversity on organic farms [39]. As regards organisms defined as potential parasites, for example species of harmful butterflies, aphids, herbivorous insects and nematodes that feed on plants, no significant effect of the organic cultivation system has been found in the various studies in the literature [45], although current studies regarding the size of the negative effect suggest that conventional farms actually support higher abundances of potential pests [38]. Despite the evidence, the number of studies present in the literature is still low to give a concrete answer to the phenomenon.

- Stable soil stability and reduced erosion: The presence of fertile soils with stable physical properties are the top priority for sustainable agriculture [46]. Currently, there is abundant evidence that organic farms and organic soil management lead to good fertility. In fact, compared to soils managed with conventional methods, soils managed with biological methods show a higher content of organic matter, greater biomass, greater enzymatic activity of microorganisms, better stability of aggregates (such as large populations of bacteria, fungi, insects and earthworms), improved water infiltration and retention capacity and lower susceptibility to water and wind erosion [47-50]. Furthermore, traditional agriculture's high

dependence on chemical fertilizers, herbicides and pesticides has caused considerable environmental damage. Precisely for this reason, in organic farms, since there is a ban on chemical fertilizers, approximately 35-65% less nitrogen escapes from arable fields, improving the quality of the soil and drinking water [51]. Furthermore, some nutritional elements, such as potassium and phosphorus, were not found in excessive quantities in soil managed with organic methods, resulting in better and more efficient use. [47]. Furthermore, on organic farms, thanks to the absence of conventional pesticides, it is likely that "run-off" and "leaching" effects, which also cause soil erosive processes, will not occur [52]. Currently, the only pesticides used in organic farming that release residues into the soil are copper fungicides, used in horticultural crops such as potatoes, wine, hops and some vegetables at annual doses of 3-4 kg of copper/ha [53]. The replacement of cupric fungicides with the selection of disease-resistant varieties and botanicals that are easily degradable presents a high priority in biological research both at national and European level [53].

- Carbon sequestration: Soil carbon sequestration (SOC) is a process in which CO2 is transferred from the atmosphere to the soil in the form of organic carbon. This process begins with photosynthesis, in which plants convert atmospheric carbon dioxide into organic compounds, which are subsequently incorporated into the soil via plant residues [54]. SOC is the carbon fraction of soil organic matter that derives not only from plants but also from animals and microorganisms, but also from the by-products of animal and microbial activity [55]. Farmers who use the organic method implement various techniques to increase soil fertility, including fertilization using animal manure but also the use of composted crop residues and the use of legumes as main and intermediate crops [56]. Furthermore, the introduction of grass and clover leaves as feed suitable

for ruminants, the rotation and diversification of cropping sequences, as well as the reduction of the depth and frequency of plowing [57]. All these techniques determine an increase in carbon sequestration in organic fields. A scientific meta-analysis of 74 long-term field trials affirmed significant carbon gains in organically managed plots, whereas, in conventional or integrated plots, soil organic matter became exposed to losses due to mineralization [58], or that process by which carbon, nitrogen, phosphorus and organic sulfur underwent conversion into mineral forms [59]. In this meta-analysis, the average difference in annual carbon sequestration rate between organic and conventional methods was approximately 450 kg atmospheric C/ha for one year, with an average difference in soil carbon stocks of 3.5 tonnes (t)/ha per year [58]. In conclusion, the combination of organic farming together with reduced tillage practices is probably among the best strategies for increasing carbon sequestration [60]. Unfortunately, although this technique is very positive for carbon sequestration, it is unfortunately not yet fully adopted by organic farmers due to the difficulty of managing weeds.

- Nitrogen efficiency, greenhouse gas reduction and emissions in organic farms: The use of fertilizers, mainly nitrogen, and synthetic pesticides has led to an increase in crop productivity over the years [61]. However, the studies conducted so far analyse the controversial effect of the use of these compounds in soil and systemically. According to a meta-analysis conducted by Erisman et al. in the USA, only 17% relating to the 100 Mt of industrial nitrogen that are applied annually in conventional agricultural companies can be absorbed by crops, the remaining, consequently is dispersed into the environment causing environmental damage [62]. Precisely for this reason, careful and efficient management of fertilizers is necessary [63], as high levels of reactive nitrogen (NH_4, NO_3) in the soil

could contribute to the emission of nitrogen oxides, which represent one of the main sources of agricultural emissions [64]. Some meta-analyses relating to long-term field studies confirm how the total nitrogen input in fields with organic methods was equal to approximately 64% of fields cultivated with conventional methods and furthermore the total biological yields, considered in the same period, were equal to approximately 83% to conventional ones [65]. Therefore, it can be deduced that organic farms use nitrogen in a more efficient and with low-polluting methods [47], also reducing nitrous oxide emissions on a territorial scale [65]. This highlights the importance of addressing yield stability with innovative and less polluting methods, given the importance of context in global greenhouse gas emissions, and closely linked to total food production.

Although the organic method is associated with environmental benefits, it has also been shown to influence other human factors and sectors positively or negatively. Therefore, even if the organic method can be considered as a more sustainable approach than the traditional method, it is important to consider its impacts on broad spectrums and different aspects:

- Yield gap: Over the years organic farming has often been the subject of discussion due to the quantity of food production. Indeed, the rapid growth of the human population raises the question of whether agriculture can satisfy the constant and growing demand for food globally [66]. Organic yields appear lower, which is why they are often the main reason it is questioned. In this regard, there are two meta-analyses in the literature, which illustrate these aspects. In fact, it is estimated that the overall food yield gaps in organic food production are only 25% lower [67] compared to conventional production [68]. The reason may be due to organic crop rotations, which may be limited in nitrogen availability.

Despite the studies present in the literature regarding a lower yield of mainly agricultural production in organic farming, another meta-analysis, which mainly collected data from a case study in Africa [69], indicating how the organic farms are more resilient in water-limited sites affected by drought and would therefore likely be more productive than conventional farms under the same conditions [69]. Therefore, the main factors that positively influence the productivity of organic farms are the improvement of soil fertility and the increase in biodiversity of the flora and fauna in the field.

- Economic factors and costs: Organic farming still has higher potential costs due to lower yields and higher labour costs, which lead to higher consumer prices [35] despite rapidly growing demand [70]. Currently, in fact, organic products cost approximately 10% to 50% more than products similarly derived from the conventional method [71]. This is due to many economic factors that influence the total and final price of the product, such as the costs of obtaining and maintaining certification or the intensity of labour which tends to be higher in organic companies, in fact, in the absence of use of synthetic pesticides and herbicides, some activities such as weeding, pest control and soil fertility management could result from manual labour, thus increasing production costs [72]. Furthermore, considering that the organic method uses organic inputs, such as natural fertilizers and pest control methods, they are usually more expensive than the synthetic counterparts used in conventional methods [73]. In addition, the infrastructures, and technologies that organic agriculture uses also influence the increase in costs and may require further investments; in fact, conventional agriculture is often based on advanced technologies and infrastructures that may not be directly applicable to the biological methods, thus determining higher costs for the transition [74]. Finally,

market supply and demand are important to influence the economic aspect. Although the demand for organic products is increasing, it could still be influenced by the dynamics of the market itself, in fact, if the supply of organic products is limited, prices could be higher [75].

- Employment Opportunities and Social Impact: Among the Sustainable Development Goals (SDGs) inherent to organic agriculture there is also the increase in the use of labour and social improvement [76]. Organic methods are often based on fundamentally traditional and less mechanized methods, consequently, these actions may require more manpower, thus offering further work and employment opportunities [77]. Differences in management between organic and conventional methods, for example include greater and different control of weeds and parasites, development of soil fertility, tillage and livestock management as critical factors contributing to labour needs [78]. According to some studies, it has been found that the organic sector employs 35% more manpower per company compared to conventional companies, a factor however to be considered and compared based on the hectares and size of the company [77]. Therefore, considering that organic has generally been associated with a greater demand for labour compared to the conventional method, it could be assumed that policies that encourage conversion to organic will consequently have positive effects on rural employment [79-80]. However, studies in the sector are still needed as only very few have addressed the topic.

- Health: Another key factor associated with organic farming is Health. Recent studies have shown that it can be hypothesized that exposure to pesticides is lower in organic farms than in conventional ones, probably due to the lower use of pesticides, representing a fundamental advantage

for the health of workers (especially for crops such as fruit and vegetables, which are often associated with typically high pesticide application rates) [35]. In fact, as stated by Costa et al. the results obtained from the study confirmed a greater presence of DNA-related damage especially in farmers exposed to pesticides (also considering exposure conditions and genetic background as influencing factors), compared to organic farmers who did not present any genetic or immunological damage evident to the DNA, showing a clear difference in terms of health between the two methods [81]. Furthermore, it is not only workers' exposure to pesticides that is affected, but also their mental health; according to some studies [82-84], it is indicated that there are higher levels of satisfaction among organic farmers compared to conventional ones. A study by Cross et al. [85], through assessments using the "Short Depression Happiness Scale", suggested that workers on organic farms would be happier than those employed on conventional farms, due to the greater variety of tasks performed every day. However, using other mental health assessment texts such as Short Form 36, Euro-Qol EQ-5D and Visual Analogue Scale, no clear significant differences emerged between organic and conventional workers, suggesting that overall, the mental health of agricultural workers could therefore be influenced by many additional external factors [86].

However, it is essential to consider that the environmental and social impacts related to the use of the organic method can vary based on factors such as the scale of production, the local and territorial context, but also on the specific practices used. Furthermore, currently, the "organic" field from a sustainability perspective is still a challenge at a global level, therefore, it requires constant evaluations of both environmental and social impacts.

2.3 ORGANIC FOOD AND HEALTH

As previously stated, organic food trade and sales have increased exponentially in the last decade, with sales from $259.06 billion in 2022 to approximately $294.54 billion in 2023 and an annual growth rate compound (CAGR) of approximately 13.7% [24]. Consumer perception, certainly, also influences the growth in sales of organic products. In fact, there is widespread public opinion that organic food is safer, more nutritious and tastier than conventional food [87]. This consumer perception, of course, has largely contributed to a greater development of organic food culture, pushing consumers to purchase organic foods for several reasons, including concerns about the effects of conventional agricultural practices on the environment, human health, and animal welfare [73][12]. Although there may be some differences, as reported by some studies, in the nutrient and antinutrient content of organically produced foods compared to conventionally produced foods, the variability in production, shipping, storage but also handling of foods makes it difficult to generalize and give certain results [88]. In fact, current research on the role of consumption of organic foods for human health is currently low, compared to other topics of nutritional epidemiology, despite the growing literature aiming to characterize lifestyles, motivations and eating patterns of the individual to the consumption of organic foods [89-90]. Today's research is focusing on studies that aim to analyse the composition of organic foods and their impact on human health; existing systematic reviews currently agree on the idea that the concentration of macronutrients, vitamins and minerals in crops is not influenced or is influenced slightly by the production system. Despite these statements, numerous studies find substantial differences, especially in terms of the quality of the nutrients and compounds present in some products of organic origin. For example, one of the key components that received maximum attention in this context was ascorbic

acid, as Vitamin C, that is a water-soluble compound that plays a fundamental role as a powerful antioxidant, therefore a reducing agent [91-93]. Antioxidants are important as they are compounds that bind to oxygen free radicals (ROS) to neutralize their activity, thus protecting cells from damage and oxidative stress [94]. A possible imbalance of ROS with respect to antioxidant protective activity could be the cause of many diseases, including cancer and Alzheimer's disease, and has been shown to have a negative impact on the body's aging process [95]. Some studies show how the highest concentrations of Vitamin C were recorded especially from fruits and fruit juices from organic farming, in fact, a study by Huber et al. demonstrated how Vitamin C level was significantly higher in organically produced plant foods than in conventional ones [96]. Similar results obtained from a study by de Oliveira et al. in which organic passion fruits also had a lower total phenolic content but the activities of Vitamin C and antioxidant enzymes were increased compared to conventionally grown ones [97]. Furthermore, in a study by Mulukutla et al. organic orange fruits had more Vitamin C than non-organic oranges, but the others organic and non-organic fruits had no significant difference in Vitamin C content, increasingly evaluating the idea that the difference and the response depended on both the species and the cultivar [98]. Similar results were observed in some vegetables such as tomatoes, i.e. organic tomatoes had a higher content of Vitamin C and also of carotenoids, higher when evaluated on the fresh substance than on the dried substance [99]. β-carotene can also be considered as a powerful antioxidant and a single oxygen scavenger, also defined as a provitamin A having the ability to be converted into retinol [100]. Even the carotenoid content, according to some studies, in some organically grown products was higher, especially in crops such as peppers, yellow plums, tomatoes and carrots [96]. Results also confirmed by some studies by Leclerc et al. who reported higher concentrations of beta carotene especially in organic carrots [101]. Naturally the results appear different and contrasting in the literature, due to the great variability between samples.

Among the vitamins with an antioxidant effect, Vitamin E shows a key role in this field, which has a positive antioxidant effect especially on the immune system [102,103]. In some studies, it is denoted as Vitamin E, in addition to being naturally present in some foods such as oil, seeds, cereals, fruit and vegetables [104], Vitamin E is involved in maintaining the oxidative stability of some products such as milk and dairy products [105], eggs [106] or meat [107]. For example, the use of silage composed of grass and legumes has a higher content of Vitamin E and β-carotene than traditional hay; these organic practices are subsequently reflected in the concentrations of Vitamin E and β-carotene present in milk and dairy products. cows fed with this type of forage [108,109]. Furthermore, ruminal destruction of vitamin A, according to some studies, decreases as the proportion of forage in the diet increases [103]. However, concentrations of Vitamin E (RRR-α-tocopherol) and β-carotene in silage are highly variable and very often unstable [110,111]. Even in meat, dietary supplementation of Vitamin E determines an increase in α-tocopherol in plasma and muscles and a decrease in the susceptibility of muscle tissue to the oxidation of lipids and oxymyoglobin, leading to differences in the activity of glutathione muscular peroxidase [112], probably also during cooking processes, preventing the formation of oxysterols [107]. As cited, the quality of eggs could also be influenced by the levels of Vitamin E administered in the maternal diet, in fact Vitamin E could increase hatchability by decreasing early embryonic mortality and increasing the antioxidant status of egg yolk, as well as the preservation of eggs [113]. Fat stability also influences the presence of omega-3 fatty acids. Omega-3 fatty acids are long-chain polyunsaturated fatty acids and are precursors for physiologically important longer-chain PUFA [114]. The presence of feed fatty acids and vitamins is a strong determinant of the fatty acid composition of milk, eggs or meat [115,116]. In fact, the fodder used in the organic method, in addition to containing a greater quantity of vitamins, also presents 30% up to 50% of omega-3 fatty acids out of the total fatty acids, unlike

conventional feed which presents approximately 10% [115]. A recent meta-analysis showed that for cow's milk, there is approximately a 50% higher content of total omega-3 fatty acids (as a percentage of total fatty acids) in organic milk compared to conventional milk [117], confirming in general previous reviews [91][118], while the contents of saturated fatty acids, monounsaturated fatty acids, and omega-6 PUFA were similar in organic and conventional milk [118]. For eggs, it is equally well documented that the FA composition of the feed [115] and consequently access to pasture [119,120] as present in biological systems, is a strong determinant of the fatty acid composition of the egg. However, only a few studies have compared the composition of fatty acids in organic and conventional eggs [121] and a comprehensive view is not available. Furthermore, according to some studies, the content of total PUFA and omega-3 PUFA was significantly higher (23 and 47% respectively) in organic meat compared to conventional meat [122]. As explained previously, the real differences in diets between conventional and non-conventional diets may differ based on the species and country type, therefore the overall reliability of the results is not totally satisfactory. However, the quality of the product and consequently the health of the consumer is not only influenced by the quantity or otherwise of vitamins or fat stability, but also by other compounds such as phenolic compounds. Polyphenols are important secondary metabolites of plants involved in defence against ultraviolet radiation or aggression by as long-term consumption of diets rich in plant polyphenols could offer protection against the development of tumors, cardiovascular diseases, diabetes, and diseases neurodegenerative [123]. According to some studies in the literature, different environmental and agronomic practices could influence the phenolic composition of the crop; among these we find exposure to light, temperature, the availability of some plant nutrients and water management [124]. In conventional agriculture, especially in conditions of high nitrogen and therefore fertilizer availability, many plant tissues show a reduced content of phenolic compounds,

although examples of an opposite relationship still exist [124]. The meta-analyses present to date report how organic farming systems show modest effects on the total phenol content, for example some studies report an increase of 14 – 26% in certain types of crops [91][93] and especially for some groups of phenolic compounds [93]. In a study by Faller et al. the results showed that organic fruits have a higher content of hydrolysable polyphenols than conventional ones, for example with values of around 11.5% in orange peels, to 72.6% in papaya peels. The fruit peels also showed a higher concentration of polyphenols than the fruit pulp, reaching, in the case of bananas and mandarins, double the quantity present in the pulp, therefore with a greater antioxidant capacity [125]. Results also confirmed by the study by Cruz-Carrion et al. which stated that the plant group showed a clear pattern of differentiation between the two types of agricultural systems, where the greater abundance of antioxidant capacity and phenolic compounds, in this case except for orange and tomato, was reflected in the organic samples [126]. Furthermore, significantly higher levels of phenolic compounds have also been found in some studies on organic EVOOs, revealing previously unstudied differences in bioactive components between organic and conventional EVOOs [127]. These contrasting results once again affirm the need to carry out numerous studies in the field. Finally, the excessively widespread prophylactic use of antibiotics in animal production is an important factor contributing to increasing human health problems due to bacterial resistance. Conventional farming is characterized by high production levels with resources such as space, feed etc. limited, causing stress and disease to the animal [128,129]. The use of antibiotics, in contrast, is severely limited in organic farming, aiming instead to provide animal welfare and sufficient space to promote good animal health [128]. In farming practice, however, the health status of organic livestock appears more complex and disease prevention must be tailored to the individual farm [130]. According to EU regulations, routine prophylactic therapy on organic livestock is not permitted, nevertheless, diseases should be treated immediately

to avoid suffering to the animal and therapeutic use of antibiotics may be permitted, but with much longer withdrawal periods. longer than conventional production [131]. The results of a report on the consequences of organic production in Denmark demonstrate how compliance with the regulations required for organic production determines several positive consequences in relation to animal welfare and health [128]. After carrying out a literature review on the nutritional quality and safety of organic foods, in a study by Lairon et al. it was concluded that organic farming has the potential to produce high quality products with some relevant improvements in terms of antioxidant phyto-micronutrient content, nitrate accumulation in vegetables and toxic substances [132]. Naturally, in the absence of nutritional deficiencies, focusing on individual nutrients could be a limited factor for evaluating the impact of a food or diet on human health, which is why studies relating to the effects on health could generally be more information from studies on individual nutrients. There are also some in vivo studies in the literature; the present results and therefore the association between the consumption of organic or conventional foods and the health data are however to be considered partial due to the differences in the quality of the diet and lifestyle. Several observational studies have described the beneficial effects of organic foods on allergic, atopic, eczema but also asthma symptoms, as well as on other hypersensitivity diseases with the use of immunoglobulin E (Ig E) measurements and questionnaires especially in children but also in adults [133-136]. In the Koala cohort study, the exclusive consumption of organic products and especially dairy products during pregnancy and early childhood was associated with a 36% reduction in the risk of eczema at age 2 years, concluding that the content higher fatty acid levels in organically reared ruminants were associated with a higher fatty acid level in breast milk [137]. Furthermore, pesticides have been shown to induce negative health effects, for example neurotoxic effects but also a slowing of intellectual development [138]. Some meta-analyses have identified how dietary exposure to

pesticide residues should be reduced, reducing the risk of developing certain diseases, especially among pregnant women and children. In relation to other pathologies, a study highlighted a lower risk of developing cardiovascular diseases both in men without pathologies and in patients suffering from chronic kidney diseases after an organic diet [139], while in the MOBA cohort study, it was seen that women reported frequent consumption of organic vegetables during pregnancy showed a reduction in the risk of preeclampsia [140]. Similarly, there was no significant association between the overall consumption of organic foods or the other five food groups and preeclampsia. Furthermore, a prospective study, NutriNet-Santé study, was also carried out regarding the change in weight over time based on the level of consumption of organic foods. The increase in BMI over time was lower among habitual consumers of organic foods compared to non-habitual consumers, furthermore, a reduction in the risk of obesity over time was observed [141,142]. A review by Jin et al. also stated that exposure to environmental pollutants can alter the composition of the intestinal microbiome, resulting in disorders of metabolism, nutrient absorption but also the function of the immune system [143]. In a study by Torjusen et al. it has been hypothesized that the incidence of preeclampsia could also be related to an altered microbiota, due to increased hypersensitivity reactions [140]. Furthermore, a prospective cohort study, using a questionnaire, was also carried out on adult individuals to address the effects of organic food consumption on cancer incidence. Overall cancer risks were not associated with the consumption of organic foods, but similarly a significant reduction in the risk of cancer, and particularly Hodgkin's lymphoma, in participants was linked to the habitual consumption of organic foods compared to participants who did not consume never organic foods [144]. Of course, the link with organic food consumption and health remains insufficiently documented in epidemiological studies; similarly, well-designed studies characterized by a prospective design, detailed information, long-term duration and sufficient sampling for good statistical processing are still

necessary. Therefore, given the difficulties for long-term studies regarding dietary intervention on humans, animal studies could currently offer some potential for investigating the long-term effects of organic foods on in vivo health. Studies in this field began almost 100 years ago, precisely for this reason, today it is possible to have a review of many studies [145] regarding the topic. In one of these studies, it was seen that second-generation chickens receiving organically grown feed showed less rapid growth compared to chickens reared using conventional methods [146,147], but at the same time, after an immune test, the chickens that had received organic feed had a better recovery in terms of welfare [147]. This resistance to the immune challenge therefore, in conclusion, was interpreted as a sign of better health. In an agricultural production experiment following a feeding trial on rats, the production system had a clear effect on the immune system and especially on higher plasma IgG concentrations [148]. Another study analysed the effects of an organic or conventional diet on animal physiology and health. The use of organic fertilizers instead of mineral fertilizers in feed production resulted in lower concentrations of cadmium but higher concentrations of polyphenols in feed and higher levels of body protein, white blood cell count, plasma glucose, leptin, insulin like- growth factor 1, corticosterone and lymphocyte proliferation, but with a reduction in plasma testosterone in rats [150]. Most of the identified effects were related to the fertilization regime. None of these studies found that feed production systems were more conducive to animal health. Numerous other studies, especially on rats, have reported some effects of the feed production system on immune system parameters [150,151]. However, the direct relevance of these findings to human health it is uncertain. Collectively, in vitro and animal studies have shown that the agricultural production system impacts some aspects of cellular life, the immune system, and overall growth and development. However, most of the findings observed in animal studies have not yet been examined in humans. On

the other hand, these studies could provide plausibility to the potential effects of conventional and organic foods on human health.

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3. KETOGENIC DIET

3.1 HISTORY AND EVOLUTION

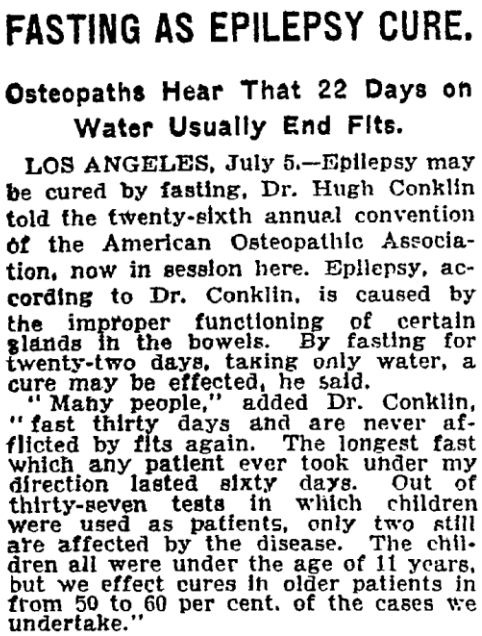
Since the Hippocratic era (460- 370 BC), fasting has always been recognized as a possible non-pharmacological therapeutic treatment for some pathologies, including epilepsy. One of the first scientific reports on the fundamental importance of fasting in epilepsy was published in the 20th century by French doctors Guelpa G. and Marie A., who reported that seizures were less severe with fasting [1]. Subsequently, a second document was published by Bernarr Macfadden, who in 1899 founded his first magazine, Physical Culture, containing the first articles regarding adequate diet and exercise. Macfadden B. claimed that the practice of fasting during a period of three days up to three weeks could improve and cure any disease, including epilepsy [2]. Initially, it was believed that the cause of epilepsy originated in the intestine and was treatable by fasting for 18 to 25 days or up to tolerance by individual, resulting in high cure rates in newborns, but low cure rates in adults over 40 years of age [2]. In 1921, the non-pharmacological treatment of fasting for epilepsy was discussed at the "American Medical Association Convention" where was documented the cognitive improvement that could occur with fasting [3]. Because of the conflicting opinions, delving deeper into the subject in 1922, Dr. W.G. Lennox and Dr. Stanley Cobb began studying why starvation had positive effects for epilepsy and found increased serum uric acid and acidosis, which developed approximately two to three days after the start of fasting; this phenomenon was at the same time accompanied by a decrease in convulsions [3]. These discoveries triggered clinical and research activities, leading to the birth of various theories; dehydration, ketosis, and acidosis were all considered potential mechanisms to explain the effectiveness of fasting [4] (Figure 4). Around the same time, in 1921/1922, Dr. R.T. Woodyatt observed that patients subjected to fasting or diets

(containing extremely low proportions of carbohydrates and extremely high proportions of fat) produced metabolites such as Acetone, AcetoAcetate (AcAc) and Beta-hydroxybutyric acid (BHB) [4]. From this discovery it was hypothesized that the benefits of fasting could be obtained through "ketonemia" and, from this discovery, the term "ketogenic diet" was coined, which referred to diets high in fat and low in carbohydrates. The ketogenic diet (KD), therefore, can be defined as:

"A dietary regime characterized by a high quantity of fats, a good quantity of proteins and a low level of carbohydrates. The macronutrients are divided as follows, approximately an average of 70-80% fat from total daily calories, 5-10% carbohydrate, and 10-20% protein [5]."

Initially, KD was proposed to a series of patients suffering from epilepsy, suggesting that this type of diet had to be maintained for a prolonged period for there to be any effects [6]. KD was widely used especially in the 1920s and 1930s, until "diphenylhydantoin" was discovered in 1938, shifting the attention of doctors and researchers to new antiepileptic drugs [6]. Drugs, of course, were easier to administer while KD was difficult to apply. Subsequently in 1994, KD again received greater attention, thanks to the national media of the United States following a program broadcast by NBC based on the true story of "Charlie Abrahams", a two-year-old boy suffering from pharmacologically intractable epilepsy. Charlie, during his non-pharmacological treatment with KD, presented signs of visible recovery from epileptic seizures and showing progress in development [7]. Following this event, the Charlie Foundation was created to disseminate information and advice on KD together with teams of doctors and dieticians, providing funds for scientific publications and supporting the first prospective multicentre study on the effectiveness of KD [8]. Since then, there has

been a dramatic increase in the number of publications, establishing that KD, therefore, could become an effective treatment for epilepsy. However, only in the last few decades KD has become widespread, not only for epilepsy, but also among the general population gaining clinical interest as a tool for weight loss, thanks to greater research on the effects of diet on the body and the discovery of a wide range of physical, biochemical, and cosmetic benefits.



FASTING AS EPILEPSY CURE.

Osteopaths Hear That 22 Days on Water Usually End Fits.

LOS ANGELES, July 5.—Epilepsy may be cured by fasting, Dr. Hugh Conklin told the twenty-sixth annual convention of the American Osteopathic Association, now in session here. Epilepsy, according to Dr. Conklin, is caused by the improper functioning of certain glands in the bowels. By fasting for twenty-two days, taking only water, a cure may be effected, he said.

"Many people," added Dr. Conklin, "fast thirty days and are never afflicted by fits again. The longest fast which any patient ever took under my direction lasted sixty days. Out of thirty-seven tests in which children were used as patients, only two still are affected by the disease. The children all were under the age of 11 years, but we effect cures in older patients in from 50 to 60 per cent. of the cases we undertake."

Figure 4. Copy of a short newspaper story. The title is "FASTING AS EPILEPSY CURE." and the subtitle "Osteopaths Hear That 22 Days on Water Usually End Fits." July 5, 1922.

3.2 BIOCHEMICAL PRINCIPLES

3.2.1 PROCESS OF KETOGENESIS

The first studies on the mechanism of the KD date back to 1920 in which were reported the use of a high-fat but low-carbohydrate diet to manage diabetes were reported in 1920 [9-11]. In fact, it has been known that, based on previously conducted research [12], diabetic patients often had a higher concentration of ketone in the blood (Ketonemia) and in the urine (Ketonuria). In 1897 it was shown that this increase in blood concentrations of ketone bodies was caused by an increased metabolism of fatty acids, caused by the inability of diabetic patients to metabolize glucose sufficiently and quickly to meet caloric needs [13]. Naturally, this mechanism could become dangerous for diabetics, in fact, uncontrolled ketonemia could lead to a state called "diabetic ketoacidosis", in which an excess of ketone bodies could led to an acidification of the blood, causing a series of side effects such as nausea, weakness and in the most serious cases even death. Precisely for this reason, a diet based on the presence of a low quantity of carbohydrates was suggested to prevent the accumulation of sugar in the blood (hyperglycaemia), which is one of the causes of ketonemia. Of course, the discovery of insulin in 1921 and its possible use for the treatment of diabetes mellitus starting in 1922 reduced the need for other treatment options, however at the same time, the interest in high-fat, low-carbohydrate diets continued to grow [14]. Subsequently there were a series of scientific reports which suggested that the improvement in epileptic parameters following the use of KD may be due to the presence of ketonemia in patients [15,16]. The first protocols published showed the optimized quantities of macronutrients for the diet were formulated, providing approximately 15 g of carbohydrates per day, 1 g of proteins per kg of body weight and the remaining caloric deficit made up of fats [6]. This ratio is almost identical to the modern KD, which is based on a 4:1 ratio of fats,

carbohydrates, and proteins and with a maximum amount of 50 g of carbohydrates per day [17]. Energy for the body comes from digestion and naturally from the metabolic breakdown of macronutrients (fats, carbohydrates, and proteins) taken thanks to the diet [18]. As a priority, carbohydrate metabolism is in the foreground due to their greater availability in the conventional diet, and from the glycogen stored in the liver and in the muscles, which, by hydrolysis into glucose, is broken down oxidatively through the process of glycolysis in S-acetyl coenzyme A (S-acetyl CoA) [19]. S-acetyl CoA is essential for starting the Krebs cycle, through which there is the complete conversion of carbon into carbon dioxide and adenosine triphosphate (ATP), which is the primary source of energy in cells [19]. Similarly to carbohydrates, the fat component can be hydrolytically broken down into fatty acids, resulting in the metabolic production of S-acetyl CoA for the Krebs cycle. This process occurs via a β-oxidation process mainly in the liver mitochondrion [20]. Normally, these two different pathways for S-acetyl CoA production are complementary and metabolically regulated. However, these pathways could become competitive, for example by limiting the availability of dietary carbohydrates, causing the body to switch to using dietary or stored fats to produce S-acetyl CoA needed for the Krebs cycle (Figure 5) [20]. The biochemistry of the ketogenic diet attempts to emulate the body's response to starvation or fasting by attempting to eliminate carbohydrates as a source of S-acetyl CoA.

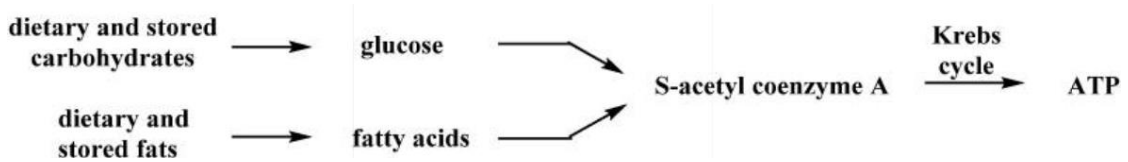


Figure 5. Metabolic Sources of S-Acetyl CoA [19].

When carbohydrates are abundantly available in the diet, the increase in glucose in the blood signals the pancreas to secrete insulin, which is a protein hormone that stimulates cellular absorption of glucose from the blood [21]. This process is "insulin-dependent" and is mainly responsible for the excessive accumulation of glucose into the liver and skeletal muscle as glycogen and for the conversion of glucose into glycerol to be used in the biosynthesis of triacylglycerides to be stored in adipose tissue [22]. However, if following a period of prolonged fasting the glycogen reserves are depleted and at the same time no carbohydrates are consumed through the diet, the body will begin to break down both the triacylglycerides coming from the diet and those contained as reserves in the adipose cells through lipolysis process (Figure 6) [23].

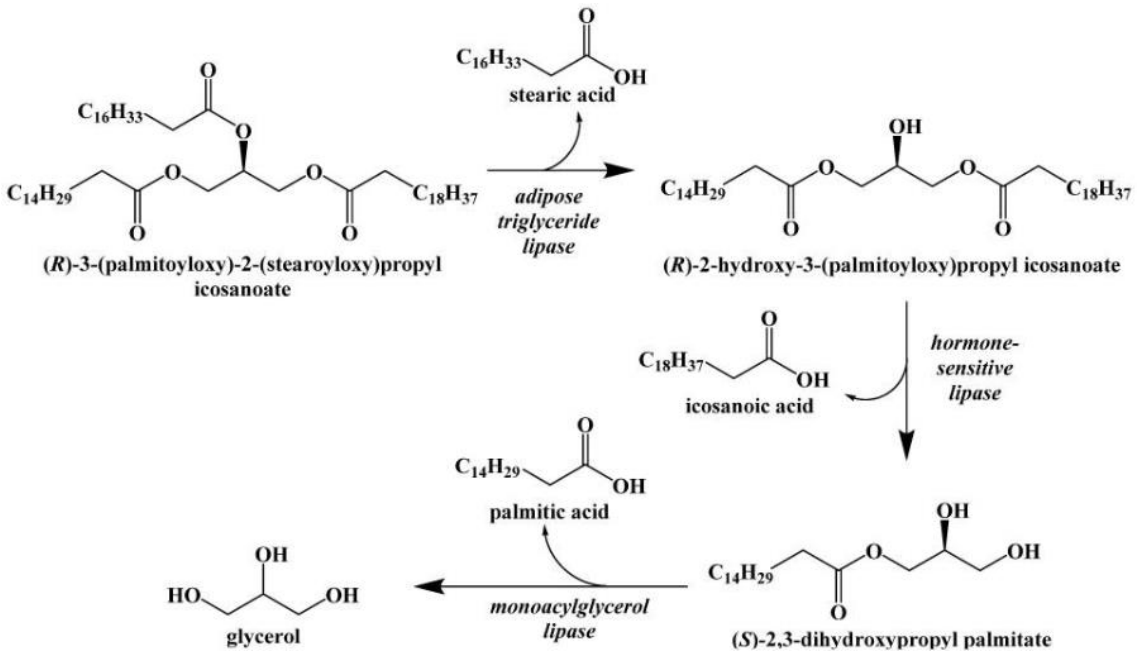


Figure 6. Process of Lipolysis of stored Triacylglycerides [19].

The first step in lipolysis is the removal of stearic acid from the triglyceride by the enzyme "adipose triglyceride lipase" [24]. Although there are three isoforms (stereoisomers) of diacylglycerol, the enzyme shows a preference for hydrolysis of the ester at the sn-2 position [24]. Likewise, the enzyme "hormone-sensitive

lipase" hydrolyzes diacylglycerol instead with a preference for 1,3-diacylglycerols. Monoacylglycerol lipase subsequently hydrolyzes the final fatty acid to generate glycerol and a net total of three fatty acids [24]. Dietary fats undergo a breakdown process using a similar method. In fact, pancreatic lipases and bile salts allow triacylglycerides to be broken down into mono- and diacylglycerides and to emulsify fats to allow their absorption [25]. The free fatty acids will then be absorbed by the cells of the intestinal mucosa and subsequently combined to reform triacylglycerides which can be dissolved into lipoprotein complexes called "chylomicrons" [26]. Chylomicrons will be able to transport insoluble fats through the bloodstream. They will be absorbed by liver, fat and muscle cells where they will be hydrolysed by lipoproteins and hepatic triglyceride lipases to generate new free fatty acids [26]. Glycerol, however, which is released from the degradation of triacylglycerides can be used to create new triacylglycerides or to be converted in the liver to D-glyceraldehyde-3-phosphate and enter the gluconeogenesis pathway to generate new glucose, however, this process will not satisfy completely the body's caloric needs (Figure 7) [27].

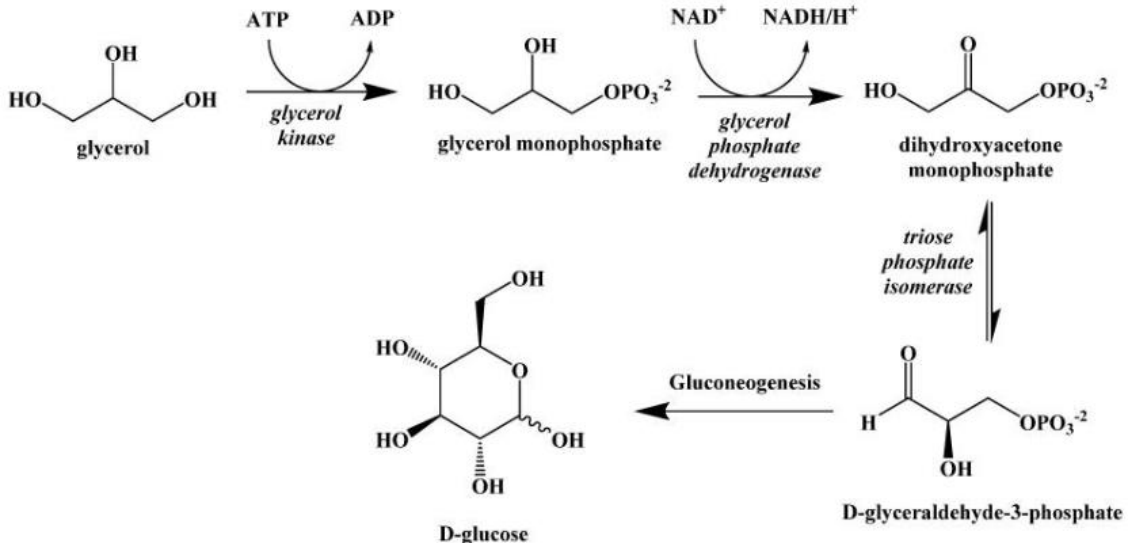


Figure 7. Process of Metabolic Conversion of Glycerol to Glucose [19].

The fatty acids, therefore, released by lipolysis will be absorbed by the cells and subsequently converted into S-acetyl CoA through β-oxidation in the mitochondrial matrix of liver cells. This process is a series of repetitive steps with the aim of converting the β-carbon of the fatty acid into a carbonyl group and then cleaving an S-acetyl CoA, shortening the length of the fatty acid (Figure 8) [20].

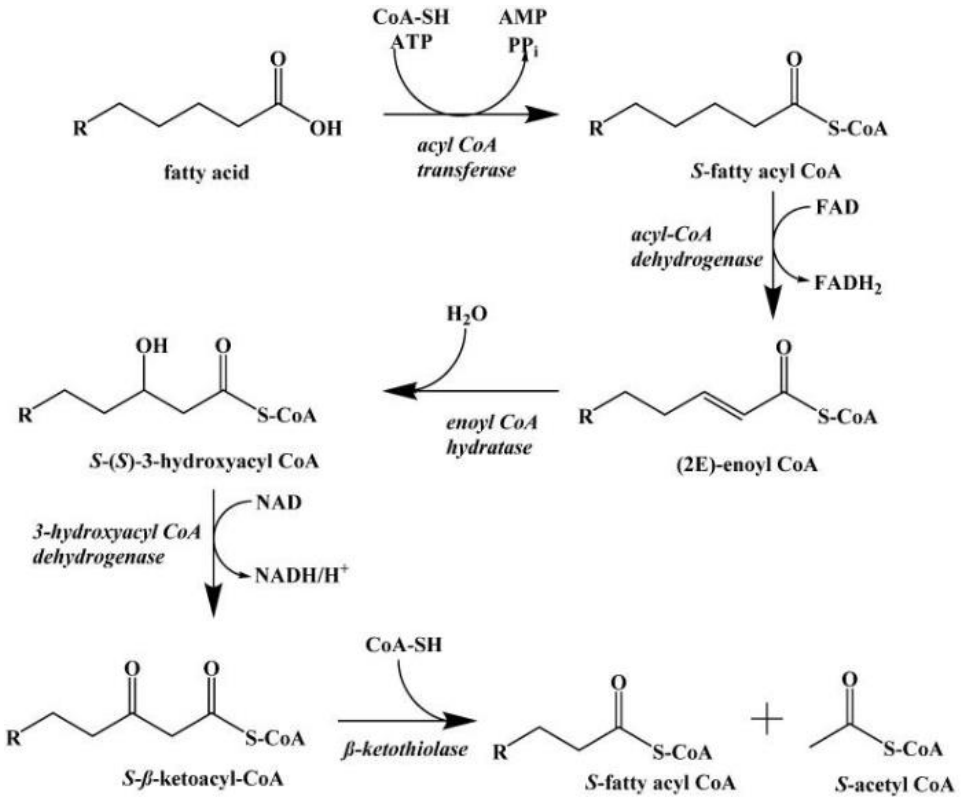


Figure 8. Mechanism of β-Oxidation of a Fatty Acid [19].

S-acetyl CoA generated by cells through β-oxidation can directly enter the Krebs cycle and provide energy in the form of ATP, for most cells in the body, this type of process could meet the individual's energy needs until the reserves of triacylglycerides are exhausted [20]. The brain uses approximately 20% of the energy required by the entire body, and therefore requires an effective method to provide long-term energy if dietary carbohydrate consumption is low [28,29]. The body manages to overcome this obstacle by converting S-acetyl CoA into the

three ketone bodies, i.e. into water-soluble compounds that manage to cross the blood-brain barrier (BBB) and can be absorbed by brain cells to provide energy, following a deprotonation at physiological pH [30]. The metabolic process for generation of ketone bodies starting from S-acetyl CoA starts with the process of ketogenesis and occurs mainly at the liver level (Figure 9) [27].

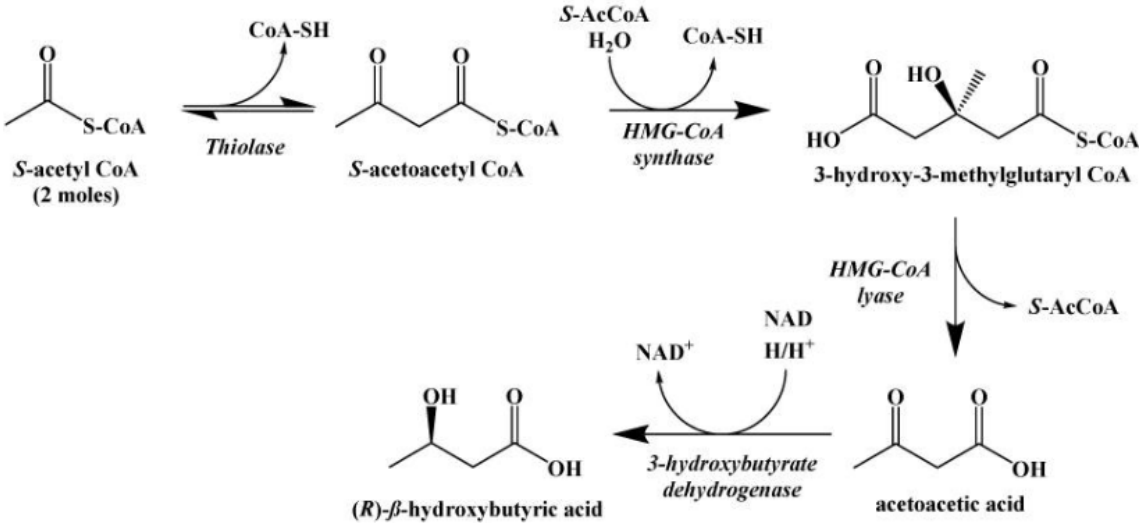


Figure 9. Ketogenesis of Ketone Bodies from S-Acetyl CoA [19].

The first step of ketogenesis is the condensation of two molecules of S-acetyl CoA to form S-acetoacetyl CoA by the enzyme "thiolase". This step is followed by an aldol addition of another S-acetyl CoA to the β-carbonyl and subsequent hydrolysis of one of the coenzyme A thioesters generating a carboxylic acid. S-acetyl CoA is cleaved by hydroxymethylglutaryl-CoA reductase (HMG-CoA) to generate AcAc, which can subsequently be reduced by 3-hydroxybutyrate dehydrogenase to create (R)-β-hydroxybutyric acid [31]. The decarboxylation of AcAc, and subsequent release of acetone and carbon dioxide, can occur spontaneously, although this is the primary method by which ketone bodies that are found to be in excess are removed as waste. Although S-acetoacetyl CoA can be hydrolyzed directly to acetoacetic acid, there is evidence that HMG-CoA synthase plays a key role in limiting the rate at which ketogenesis occurs [32]. The ketone bodies produced in the liver will then be released into the blood

where they can be absorbed by other cells and used to synthesize S-acetyl CoA with the process of ketolysis, occurring in non-hepatic cells, primarily in the heart, brain, and skeletal muscle (Figure 10).

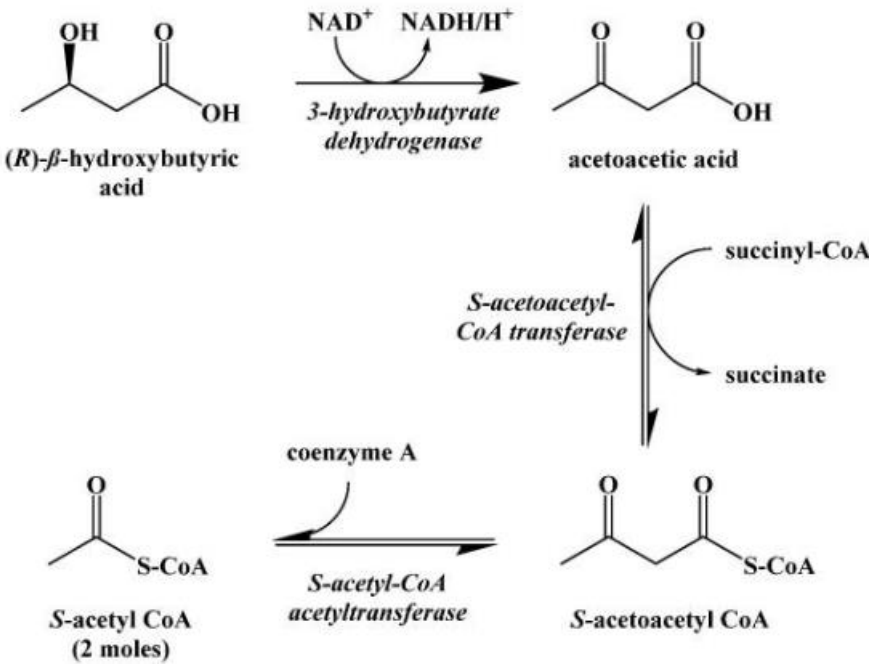


Figure 10. Ketolysis of the Ketone Bodies [19].

The process of ketolysis will begin with NAD+ oxidation, that can convert (R)-β-hydroxybutyric acid to acetoacetic acid, undergoing thioesterification with coenzyme A generating S-acetoacetyl CoA, which will then be cleaved by coenzyme A to provide two molecules of S-acetyl CoA which can enter directly into the Krebs cycle to be used as an energy source [33].

3.2.2 KETONE BODIES: DEFINITION AND PHYSIOLOGICAL ROLE

During the process of ketogenesis, the hormone insulin secretion is low due to low blood glucose levels, resulting in increased fat breakdown and subsequent fatty acid production. The fatty acids are subsequently metabolised into AcAc and converted into BHB and Acetone (Figure 11).

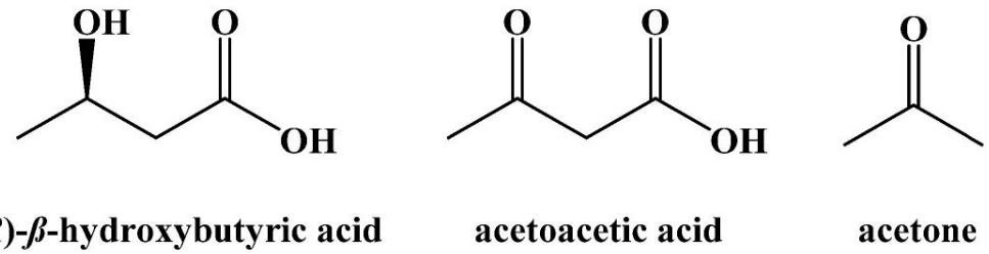


Figure 11. The Keton Bodies [19].

AcAc, BHB and Acetone are defined as primary ketone bodies, they act as an alternative energy source for the body This metabolic state is called "nutritional ketosis," which is generally considered safe as it involves the production of ketone bodies in moderate concentrations without significantly affecting blood pH. In cases of imbalances, however, will appear ketoacidosis, which is a serious and life-threatening condition characterized by excessively high levels of ketone bodies in the blood, that can contribute to the appearance of acidosis [34]. Typically, body ketone concentrations during the process of dietary ketosis are between 0.5 and 3 mM, whereas, following a normal diet with normal amounts of carbohydrates, will result in body ketone concentrations below 0.3 mM [35]. The synthesis of ketone bodies, therefore, occurs mainly in hepatocytes, although it has been proposed that other types of cells, such as astrocytes in the brain or cells in the kidney, could also synthesize ketone bodies to a lesser extent [36]. As previously affirmed, ketone bodies are therefore mainly used as metabolic substrates, occurring mainly in the brain, heart, and skeletal muscle [36]. Both ketogenesis and ketolysis are in turn regulated by the endocrine system, with

both insulin and glucagon preventing and determining ketogenesis and ketolysis, respectively [37]. The use of ketone bodies as an energy substrate is a great advantage from an evolutionary point of view, as it determines the ability to survive even prolonged periods of starvation. In fact, in the absence of ketogenesis, brain cells would depend entirely on the processes of hepatic and renal gluconeogenesis, however the substrates for glucose synthesis are limited [36]. It has been calculated, in fact, that the brain of an adult person could survive 2-3 weeks with only the process of gluconeogenesis but could remain functional for at least 2 months if ketone bodies derived from lipolysis are used as an alternative energy and additional source [38]. Studies state that, after several weeks of fasting, 2/3 of the energy needed by the brain is provided by ketone bodies such as BHB and AcAc [39]. The human brain requires ketone bodies even during the first postnatal phase, in fact the metabolism of newborns, in nature, is ketotic due to the low amount of lactose in colostrum. Approximately half of the energy consumed by the newborn human brain comes from BHB; only after a few days of breastfeeding, the lactose content increases and consequently ketosis disappears [39,40]. Another organ essential for the survival of the individual is Heart. Myocardial cells are strongly dependent on the oxidation of fatty acids which represent 60-85% of the ATP produced, they use glucose/lactate, amino acids but also ketone bodies and use a small amount of glucose for energy production [41,42]. While, as regards skeletal muscle, the contribution of acetoacetate and BHB to the production of ATP could vary substantially. Furthermore, after overnight fasting, ketone bodies could contribute 10–20% of energy needs, increasing up to 50% after several days of fasting [42,43]. The disposal of ketone bodies in skeletal muscle during an aerobic exercise phase could increase up to fivefold, followed by post-exercise ketosis (0.3–2.0 mmol/l) depending on nutritional status and exercise intensity [44]. In addition to their energetic role, ketone bodies have various functions. For example, AcAc and/or BHB can interact with nuclear ribonucleoproteins, inhibiting histone

deacetylases (HDACs), ketone bodies modify histones and other proteins post-translationally, influencing oxidative stress, inhibiting the NLRP3 inflammasome and modulating receptor signalling coupled to G proteins (GPR), interested in signal transductions. BHB, but not AcAc, for example, increases histone acetylation by inhibiting HDACs I, upregulating genes that respond to oxidative stress [45] and interacts with the nuclear ribonucleoprotein hnRNP A1, which causes an increased expression of Lamin B1, a key protective factor of DNA senescence in vascular cells [40]. Both BHB and AcAc modulate signalling via G protein-coupled receptor (GPR). BHB suppresses the activity of the sympathetic nervous system (GPR41) with a consequent reduction in energy expenditure and consequently heart rate [47], while AcAc, but not BHB, activates GPR43, a receptor that is more expressed in adipocytes, whose deficiency inhibits lipid utilization following fasting, compromising plasma lipoprotein lipase activity and decreasing energy expenditure during fasting [48]. Furthermore, ketone bodies are generated also in the intestinal epithelium of mammals through hydroxymethylglutaryl CoA synthase (HMGCS2), a mitochondrial enzyme that catalyzes some reactions of ketogenesis, providing energy in the form of lipid-derived ketone bodies during periods of carbohydrates deprivation, by the addition of a third acetyl group to acetoacetyl-CoA, producing HMG-CoA [49]. In both the small and large intestine, HMGCS2 is regulated by the microbiome, butyrate content, and diet [50,51]. The concentration of butyrate in the colon lumen reaches approximately 10 mM [49]. The intestinal mucosa converts butyrate, which derives from microbial fermentation of complex carbohydrates, into ketone bodies [49], this activity is related to the enzymatic activity of HMGCS2. In both the small intestine and colonocytes, failed HMGCS2 function can be partially rescued by administration of exogenous BHB or a KD, suggesting ketogenesis-dependent HMGCS2 function [52]. Furthermore, HMGCS2 is critical at the physiological level as it is required for postnatal gut development and response to gut damage [52]. It therefore follows that ketone bodies have

different roles in the homeostasis of cells, tissues, organs, and organisms and their physiological roles range from the ability to act as combustible substrates, as modulators of the redox potential and of the substrate supply/demand balance or as signals.

3.2.3 FOCUS ON BRAIN: ROLE OF KETONE BODIES IN NEURODEGENERATIVE DISEASES

In recent years, KD has been the subject of numerous studies, in which increasingly confirm that nutrition and health are closely related, and that the different percentage distributions of macronutrients, especially in the case of KD, could determine positive effects on the individual's health, especially regarding certain pathologies [53]. For example, numerous studies have reported how KD improves obesity [54], diabetes [55], polycystic ovary syndrome (PCOS) [56], and other conditions [57]. Similarly, KD has currently been recognized as a neuroprotective factor, which in addition to epilepsy can benefit in the case of brain lesions and neurodegenerative diseases [58,59]. Neurodegenerative diseases, such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's (AD) and Huntington's disease (HD), appear to be closely related to a progressive loss of material as well as neuronal function [60]. The causes of the appearance of these pathologies can be attributed to various factors including infections and mutations, genetic predispositions, but also to protein aggregates [61], which lead to chronic activation of the central nervous system (CNS) and consequently to high levels of secondary metabolites and mediators of inflammation [62]. As previously mentioned, the brain, with a respective requirement of approximately 20% of daily energy expenditure, in case of glucose deficiency can oxidize ketone bodies and support a large part of its energy needs [39]. This process occurs through monocarboxylate transporters (MCTs), which allow ketone bodies to be transported to provide energy [63]. MCTs are a family of transporters (MCT1, MCT2, MCT3, MCT4) that passively transport metabolic substrates, such as lactate, pyruvate, and ketone bodies. As the only ketone body transporters, MCTs are ubiquitously expressed in the brain. Ketolysis reactions occur in the mitochondrial matrix of brain cells. As a first step, BHB is oxidized into AcAc by a BHD1 enzyme, an enzyme involved in the ketone

body pathway, after its arrival in the mitochondria. Subsequently, AcAc is catalysed and catabolized into two molecules of acetyl-CoA in such a way as to participate in the TCA cycle following the action of succinyl-CoA-3-ketoacid CoA transferase (SCOT) and thiolase enzyme [64]. Metabolism of ketone bodies does not require ATP but can generate more energy than glucose [65,66]. Many neurological pathologies (including naturally neurodegenerative ones) are characterized by disorders of the energetic metabolism and use of glucose in neurons and acute damage that causes inflammatory processes in the brain. It has been found through numerous studies that, in these cases, the number of MCT channels responsible for the transport of ketone bodies in brain cells increases by 85%, and at the same time there is an increase in the number of enzymes that metabolize ketone bodies and in particular BHB [67,68]. Based on these studies, considering that the brain requires another source of energy in case of damage and energy imbalances, current studies aim to research how ketone bodies could determine a protective effect at the neuronal level. Current research, actually, demonstrates that ketone bodies would cover not only a role as an energy source but would promote the function of stem cells, promote vascular function, moderate acute and chronic inflammation, reduce tissue fibrosis, protecting from oxidative and hypoxic stress and providing resilience against hypoglycaemia and energy stress [69]. Among the most recent research it has emerged that the application of the KD, through the mediation of ketone bodies and, in particular, of BHB, can reduce both the demyelination of nerve fibres, the death of oligodendrocytes (myelin producers) but also the axon degeneration caused by glucose deficiency [70]. The broad beneficial effect of ketone bodies and the KD has been documented by a large collection of meta-analyses from 1979 to 2023. In fact, it has been demonstrated that ketone bodies determine a strong neuronal protection against possible "acute damage" to the central nervous system resulting in a reduction in death rate, brain damage, neuronal dysfunction, and neuroprotective potential [71]. KD, in fact, increases the number

of neuroprotective factors and mediators, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), neutrophin-3 (NT-3) and molecular chaperones. Ketone bodies also improve mitochondrial function by increasing the efficiency of energy production and similarly reducing the production of ROS. From various meta-analyses it has been found that the use of ketone bodies at the brain level determines an anti-inflammatory potential deriving from the inhibition of the activities of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), the reduction of the synthesis of proinflammatory interleukins (IL-1β, IL-2, IL-4, IL-6) and tumor necrosis factor alpha (TNFα). Basically, ketone bodies also reduce one of the central components of the inflammatory process, such as the transcriptional nuclear-factor-kappaB (NFκB) [58] [72], demonstrating a multifaceted anti-inflammatory activity [73]. Ketone bodies and in particular BHB can inhibit neuritis by suppressing the cyclooxygenase-2 (COX-2)-dependent pathway by reducing the activation of peroxisome proliferator-activated receptor γ (PPARγ) [74]. Mouse studies have shown that BHB also interacts with the RNA binding protein hnRNPA1, stabilizing the Oct4 factor, which determines reduced senescence in vascular endothelial cells, this was found in mouse studies [46]. BHB has two cell surface receptors, HCAR2 and FFAR3 [75], resulting in neuroprotection; FFAR3 also has a role in reducing and modulating inflammatory activation in other contexts such as lung and intestinal diseases [75]. BHB results in upregulation of stress response genes, in particular Nrf2, Foxo3 and Mt2, resulting in cytoprotection from oxidative stress [76,77]. As regards the blood-brain barrier (BBB), although no unequivocal evidence is currently available, it has emerged that in the case of neurodegenerative pathologies can occur a breakdown of the integrity of the membrane, which in a phase of a higher concentration of ketones in the blood, together with inflammation processes, the permeability of the BBB for BHB would also increase [78]. The condition of ketosis, therefore, has proven to be a key factor in causing

the restoration of the integrity of the BBB following an increase in the content of connexin-43 (Cx43) involved in the construction of the barrier, and an increase in transporters of MCTs [79]. Furthermore, KD and ketone bodies would favour the outflow of "amyloid plaques", extracellular formations made up of a central part in which amyloid protein accumulates and a peripheral part in which neuronal debris typical of neurodegenerative pathologies is deposited, following the increase in concentration of proteins that participate in the clearance of amyloid plaques, such as LDL receptor-related protein 1 (LRP1), P-glycoprotein (P-gp), and phosphatidylinositol-binding clathrin assembly protein (PICALM) [80]. Furthermore, from an energetic point of view, it has been demonstrated that BHB can provide key components that participate in the reconstruction of the respiratory chain [81,82], as well as improving brain functioning (also demonstrated in healthy individuals) through an increase in cerebral blood flow of approximately up to 30%, with unchanged oxygen consumption. Thus suggests a possible neuroprotective effect of ketone bodies [83]. Inherent in the energetic and inflammatory context, another possible mechanism of activity of ketone bodies in the nervous system is the ability to modify the cerebral metabolism of glutamine. Yudkoff et al. state how the condition of ketosis can intensify the metabolism of astrocytes, converting more glutamate into glutamine [84]. This process could contribute to a reduction in the concentration of glutamate, considered as the main neurotransmitter, with a consequent increase, on the contrary, of the main inhibitory neurotransmitter GABA. This neurotransmitter follows a greater intensification of the processes of conversion of glutamine into GABA [85,86], resulting in a decrease in excitotoxicity and a consequent improvement in mood. On the other hand, it could be stated that β-hydroxybutyrate increases mitochondrial respiration and promotes mitochondria biogenesis in the hippocampus [87], leading to a change in the expression of BDNF [88], which could generally lead to greater calm, better concentration, improved mood and increased cognitive abilities [89,90]. Finally,

ketone bodies show the ability to promote mitochondrial biogenesis in the hippocampus [87]. They are not, ketone bodies could promote brain regeneration, the development of new nerve cells and their connection in neuronal networks [91]. Therefore, it can be considered that ketone bodies and KD could lead to an improvement in cognitive functions through a close correlation with the intestinal microbiota, which, according to new studies, has a direct axis with the brain and the modulation of neuronal functions (Figure 12).

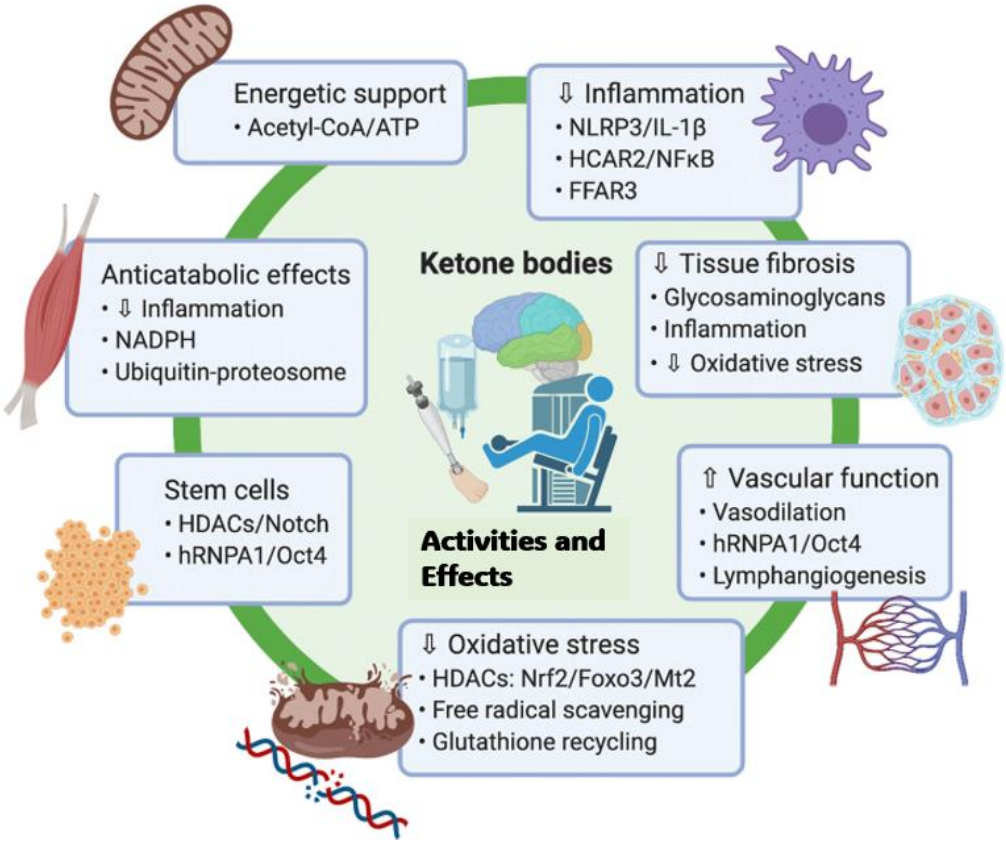


Figure 12. Relevant mechanisms of ketone bodies [92].

3.2.3.1 MICROGLIAL IMMUNOMODULATION

The processes and effects of ketone bodies in the brain have been demonstrated particularly in Microglia. In fact, microglia are the main immune cells of the central nervous system (CNS) [93], they are classified as mononuclear phagocytes resident in the parenchyma of the central nervous system [94,95] and represent one of the first lines of cellular defence aimed at immune surveillance and defence of the individual at the brain level [96]. Microglial cells contribute to neurogenesis and, therefore, to brain development through the phagocytosis processes of apoptotic neurons [97], also supporting the development of neurons and the vascular system through the release of trophic factors. Therefore, these multiple functions make microglia crucial for maintaining CNS homeostasis. Microglia are characterized by their acute sensitivity in perceiving even the slightest variations in the surrounding environment, which could determine their activation [98]. If microglia are not stimulated, they appear in a "resting" state, characterized by a branched morphology and a reduced cytoplasm [99]. The resting state contributes to brain homeostasis by regulating synaptic remodelling but also neurotransmission [100]. Microglial activation in the CNS can be classified into two opposite phenotypic types: the classic phenotype (M1) or the alternative phenotype (M2) [101]. M1-type activation is known as classical-type activation, typically induced by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) [102]. The M1 phenotype produces inflammatory cytokines and chemokines, such as tumor necrosis factor alpha (TNF-α), interleukin IL-6, IL-1β, IL-12, and CC chemokine ligand (CCL) 2 [102], furthermore the M1 type expresses NADPH oxidase which produces superoxide, ROS, inducible nitric oxide synthase (iNOS) which in turn produces nitric oxide (NO), major histocompatibility complex II (MHC-II), integrins (CD11b, CD11c) and costimulatory molecules (CD36, CD45, CD47) [102], which together contribute to neurological damage [103]. The proinflammatory M1 phenotype is characterized

by a larger soma and reduced ramifications, of an "amoeboid shape" [104]. In contrast, M2 phenotype, however, known as alternative activation, is induced by anti-inflammatory cytokines such as IL-4 and IL-13 [102]. The M2 phenotype produces anti-inflammatory cytokines as IL-10, transforming growth factor (TGF-β), growth factors as insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), colony-stimulating factor (CSF)−1, but also neurotrophic growth factors as nerve-derived growth factor (NGF), BDNF, neurotrophins and glial cell-derived neurotrophic factor (GDNF) [102]. Furthermore, they can induce the mannose receptor (CD206), chitinase-3-like protein 1 (CHI3L1), Arginase 1 (Arg1) [102], as neurotrophic factors. M2 microglia promote the phagocytosis of cellular debris and misfolded or damaged proteins, promoting the reconstruction of the extracellular matrix and the repair of brain tissues, supporting neurons' survival through neurotrophic factors [105]. Finally, M2 anti-inflammatory phenotype is characterized by a branched morphology and a small soma [99]. Metabolic and phenotypic reprogramming of microglia refers to characteristic changes in various metabolic processes in the face of alterations in the microenvironment [105], in which cells assume different metabolic profiles to provide energy and biological materials [106]. Thus, microglia generally undergo programmed metabolic changes exhibiting different functions and phenotypes in response to various environmental and cellular stresses [107]. A possible persistent activation of microglia in a proinflammatory M1 state could determine the transition of microglia to an amoeboid morphology which is associated with neuronal damage and overproduction of proinflammatory cytokines [108], which appear to be the main factors responsible for the development of neurodegenerative diseases, such as Parkinson's disease, AD and ALS [109,110]. Furthermore, it has been found that microglia in a homeostatic resting state show dependence on oxidative metabolism, while in a proinflammatory phase the metabolism is shifted towards glycolysis [111], in which succinate functions as

an intermediate in the Krebs cycle promoting the production of IL-1β, which is a proinflammatory cytokine [112].

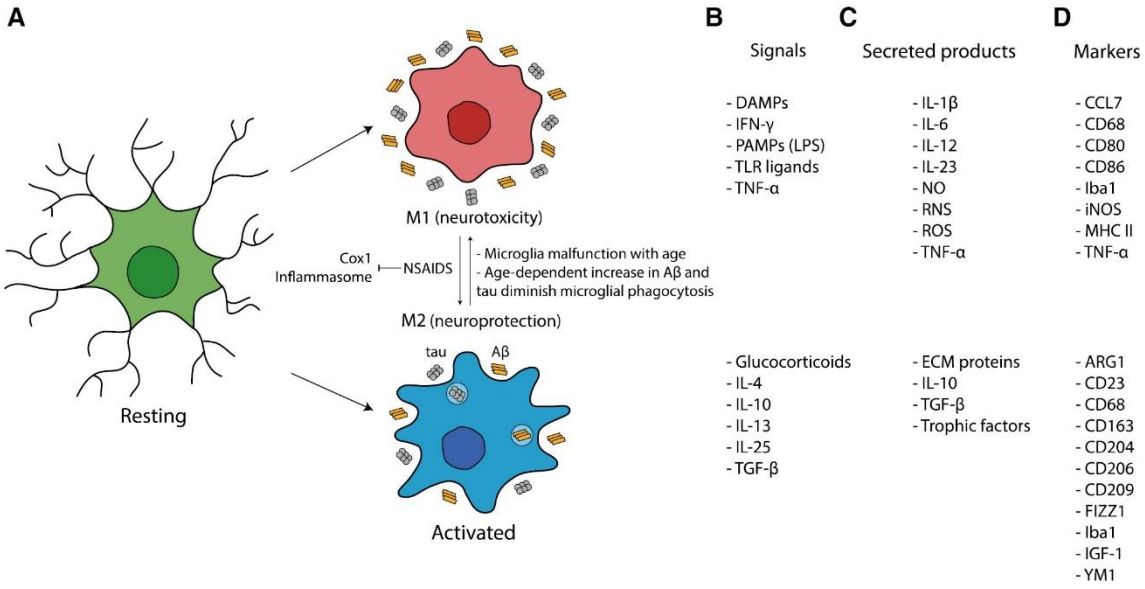


Figure 13. Polarization of Microglia from resting state to M1/M2 phenotype [113].

In this context, KD and BHB appear to have a predominantly neuroprotective role [114]. The mechanisms of action are currently still unclear, however, research in recent years has shown that BHB could inhibit the expression of the NLR family, in particular NLRP3 inflammasome, involved in microglial inflammation processes. [115]. The reduction of NLRP3 activation would lead to a consequent decrease in the production of proinflammatory secondary metabolites, such as the cytokines IL-1β, TNF-α, ROS, iNOS and COX-2 [116]. The NLRP3 inflammasome is assembled and activated when the intracellular sensor NLRP3 recognizes damage-associated pathogenic molecular patterns such as PAMP/DAMP resulting in the release of proinflammatory cytokines such as IL-1β and IL-18. Recent findings have suggested that the inflammasome has a crucial role in neurodegenerative disorders, as the consequences due to its activation act as a key factor for the growth and spread of misfolded β-amyloid protein aggregates as in neurodegenerative diseases such as AD [117,118]. The ability of BHB to control its deactivation and/or identify inhibitory pathways

associated with NLRP3 could open the possibility of new therapeutic breakthroughs to slow the progression of neurodegenerative diseases [119]. In a study on transgenic AD mice, BHB could reduce the formation and activation of the NLRP3-dependent inflammatory cascade by decreasing the amount of secreted Il-1β [119]. Furthermore, it is important to highlight that BHB inhibition of NLRP3 is independent of GPR109A, a butyrate receptor with anti-inflammatory properties for macrophages that allows them to induce the differentiation of regulatory T cells (TREG cells) and IL-10-producing T cells, indicating that BHB has broad effects and could modulate several pathways simultaneously [115]. Furthermore, regulation of the NLRP3 inflammasome is also linked to autophagy. In fact, it has been discovered that autophagy, if modulated by factors such as BHB, exacerbates the activation of the NLRP3 inflammasome with less release of IL-1β [120]. In fact, by reducing the levels of IL-1β and caspase-1, decreasing the production of ROS and reducing the cell death observed in vitro and in vivo [121], the likelihood of damage to the brain decreases. Increased serum BHB levels, and subsequent increase in fibroblast growth factor-21 (FGF-21), could significantly reduce inflammation [122]. Similarly, BHB inhibited the generation of IL-6 and TNF-α and promoted the production of BDNF and TGFβ [123,124]. BHB treatment significantly reduces the marker IBA-1, a microglia/macrophage-specific marker [125] and widely used for microglial detection [125], in mouse microglia with PD and AD [125]. Furthermore, BHB is associated with greater oxidation of NADH [58], which determines an increase in glutathione levels, which, being the main intercellular antioxidant, can prevent possible damage caused by ROS [126]. Indeed, reduced levels of glutathione are associated with greater cognitive impairment, such as in Alzheimer's disease and epilepsy [127]. Therefore, microglial activation, can be defined as a key event in neuroinflammation and it could be defined as a central process in neurological disorders, and ketone bodies, in particular BHB, could be a treatment in this field.

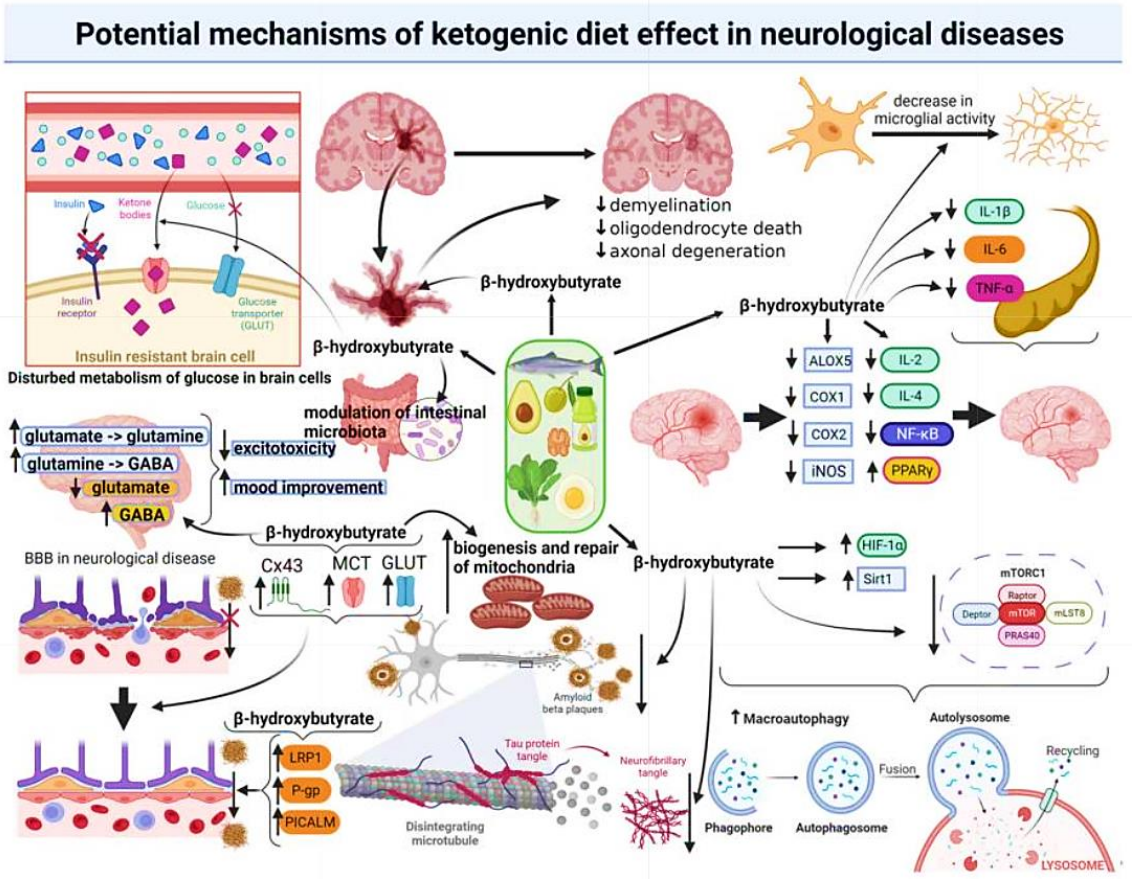


Figure 14. Potential mechanisms of the ketogenic diet effect in neurological diseases [84].

3.2.4 FOCUS ON LUNG: ROLE OF DIFFERENT FORMULATIONS OF LITHIUM AND KETONE BODIES ON LUNG CANCER

Lung cancer (LC) is currently the leading cause of cancer-related death globally. Every year there are approximately 14.1 million new cases and approximately 8.2 million deaths [128]. The incidence appears to be variable but equally distributed, with a tendency to increase among the male gender compared to the female gender [129] as well as the incidence increases with advancing age [130]. Ethnicity is also a fundamental factor for genetic predisposition, it is estimated that black men are 20% more susceptible to develop LC than white men, but at the same time, 30% less susceptible to develop LC characterized by small cells [131]. In fact, lung cancer can be divided into two main subtypes: small cell lung cancer (SCLC); non-small cell lung cancer (NSCLC). Non-small cell lung cancer accounts for approximately 85–90% of all LC cases [132]. Furthermore, beyond the first division in cellular terms, LC can be divided into four major histological subtypes: adenacarcinoma, squamous cell carcinoma, small cell carcinoma, and large cell carcinoma. The first tumor subtype includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma [133]. Thanks to numerous recent studies, it has been discovered that LC with the small cell histological subtype appears to be the deadliest among tumors as it is more likely to cause metastasis [134]. Unfortunately, in most cases, approximately 57% of lung cancers already metastasize at the time of diagnosis, resulting in a reduced five-year survival rate for approximately 4.7% of total cases. Furthermore, only 18.6% of individuals diagnosed survive after 5 years [135]. Physiologically, ROS are in balance with the individual antioxidant defence system, which is essential for the survival of organisms and their health [136]. However, in some cases, ROS could act as anti-carcinogens, for example, promoting apoptosis or cell necrosis or inhibiting angiogenesis [137]. At the same time, however, they could have a carcinogenic effect, as cells would show metabolic disorders under a condition of

oxidative stress, damaging the fundamental constituent elements of their structure such as proteins, carbohydrates, DNA and RNA, or even lipid components of the cell membrane [138-140]. This process results from the coupling of their unpaired electrons to the target molecule, which in turn is made unstable [141]. The process of carcinogenesis can be divided into different phases such as initiation, promotion, and subsequent progression, determining the formation of malignant tumors [142,143], in which various genetic but also epigenetic events occur which lead to the progressive conversion of cells from a physiological to a tumor state. ROS play a key role, especially in the promotion phase, during which gene expression, and in particular the genes that regulate cell differentiation and growth, is modulated. The consequences of ROS in this phase are mutations, chromosomal aberrations, resulting in cellular degeneration, carcinogenesis, and aging [144]. In this first phase of progression, benign tumors are stimulated to grow very quickly and transform into malignant [35,36], furthermore, in this phase, the process of inflammation with consequent production of inflammatory cytokines which determines tumor birth could also be linked to immunosuppression, allowing tumor cells to deflect recognition by the immune system [145]. Resistance to cell death but also imbalance in cell division processes or in the regulation of cell death pathways by apoptosis and necrosis can contribute to uncontrolled tumor proliferation [146]. Current therapies that restore the ability of tumor cells to undergo apoptotic processes could represent a promising new treatment opportunity. Traditional approaches to the treatment of LC currently involve chemotherapy, radiotherapy but also surgical therapy to remove residual tumor tissue, but with acute side effects and episodes of drug resistance [147,148]. Compared to traditional therapy, new therapeutic frontiers have currently emerged regarding treatment with metal ions and BH3 mimetics [149], which could increase the apoptosis of tumor cells with fewer side effects and less related drug resistance [150]. BH3 mimetics are considered a new class of anticancer therapeutics that can mimic the binding of

BH3 proteins to the "hydrophobic groove" belonging to anti-apoptotic proteins [151], but with the ability to trigger cell death [152]. BCL-2 family proteins can interact with each other through the process of dimerization, and apoptosis is subsequently initiated by BAX and BAK homo- or heterodimers. To prevent apoptosis, pro-survival members such as BCL-2 could form heterodimers with BAX and BAK [153] inhibiting this process. ABT-737 falls into this category, binding and inhibiting BCL-2, BCL-xL and BCL-w with high affinity [154]. Through its mechanism of action, ABT-737 can kill cells thanks to the presence of BAK or BAX in the cell [155]. ABT-737 and its orally available analogue ABT-236 [156] have already shown preclinical efficacy in some tumor cell models and are currently tested in clinical trials [156-157]. While, metal ions are involved in the regulation of the tumor immune microenvironment [158,159], these mechanisms, therefore, would suggest that in the future metal ions could play a role of fundamental importance in tumor therapy. Lithium is currently a type of drug used for the treatment of some pathologies, stable in most conditions and suitable for transport and storage [160]. For some years now, lithium treatment has been considered the gold standard for bipolar disorder (BD) and psychosis [161,162]. Since then, lithium has gained increased attention, and in recent years research has evaluated potential tumor modulating properties [163-165]. The role of lithium (Li) and lithium formulations, according to current studies in the literature, in cancer proliferation/inhibition presents a controversial issue in the literature. There is growing evidence identifying the effects of Li on cancer proliferation via inhibition of glycogen synthase kinase-3β (GSK-3β) [166], but also modulations of redox state [167], inflammatory changes [168], pro/anti-apoptotic mechanisms[169], and changes in mitochondrial function[170]. Despite this, there is the presence of contradictory data. Furthermore, a growing number of studies report that ketone bodies, through the metabolic change and reprogramming of cancer cells, could also be an effective therapy against some types of tumors and improve the effectiveness of current standard cancer

therapies [171]. However, these mechanisms underlying the anti-tumor benefits of ketone bodies are not yet fully understood [172]. One of the possible mechanisms in tumor cells could be metabolic alterations such as aerobic glycolysis and reduced oxidative phosphorylation [173], known as the Warburg effect [174], which could be affected using ketone bodies depriving the cells of glucose and inducing ketosis as a primary source of energy [175]. Acetoacetate (AcAc), one of the two main ketone bodies, is generally used in research in the form of LiAcAc salt in both in vitro and in vivo experiments, but also in the form of other formulations such as sodium acetoacetate (NaAcAc) [176]. Some studies have provided some plausible molecular mechanisms by which AcAc could modulate the growth of tumor cells and among these would be the inhibition of ATP production but also the promotion of tumor cell apoptosis [177]. Despite this, the different formulations of AcAc and lithium, such as lithium AcAc (LiAcAc), sodium AcAc (NaAcAc), lithium chloride (LiCl) and lithium carbonate (Li_2CO_3), despite having been studied as a treatment or adjuvant of various cancer types currently present unclear and unconvincing results, especially due to the multipotent and diverse effects of Li on various cellular processes and given the different response between different cell lines [178].

3.3 ANTIOXIDANT COMPOUND OF KETOGENIC DIET

The classic KD, as previously stated, was designed in 1923 for the treatment of epilepsy by Dr. Russell Wilder at the Mayo Clinic [179]. Currently, KDs are made with a high amounts of fat, a good amount of protein and few carbohydrates. Typically, the macronutrient ratio is 3–4:1 (4 g of fat per 1 g of protein and carbohydrate combined) which is approximately 90% of the total calorie intake, which comes primarily from fat, 6% from proteins and 4% from carbohydrates [180]. KD is based on the use of foods such as fatty foods, seasoning oils and seeds, meat, fish products and eggs, milk, and dairy products and finally a small percentage of vegetables [181]. Although KD has been recognized, in the literature, as a non-medical treatment for neurodegenerative diseases thanks to the neuroprotective action of ketone bodies, however, its composition presents a variety of foods characterized by many antioxidant compounds which further determine its protective and beneficial capacity [182]. The foods present in the KD can be divided into food groups, and each food group has its own characteristics and antioxidant compounds:

- Fats and oil: Olive oil, and in particular EVOOs, is known for its high content of antioxidants compounds, that help protect the cells of the body from oxidative stress and the harmful effects of ROS [183], as well as reducing the risk of chronic conditions including heart disease [184]. The main antioxidants present in olive oil include phenolic compounds such as "hydroxytyrosol" [185] and "oleuropein" [186], respectively, phytochemical compounds expressing very strong antioxidant properties, and the main polyphenol present in the leaves and fruits of the olive tree. Oil is also an excellent source of Vitamin E (alpha-tocopherol), a fat-soluble antioxidant that helps protect cell membranes from oxidative damage [187]. Among the antioxidants we also find carotenoids, such as beta-carotene and lutein, known for their potential role in protecting

against various diseases [188]. Finally, squalene, precursor of cholesterol and steroid hormones, has antioxidant properties, helping skin cells from damage caused by UV radiation [189]. Furthermore, olive oil is in fact mainly composed of monounsaturated fats, in particular oleic acid, a monounsaturated fatty acid which belongs to the family of Omega-9 fatty acids, known for its positive impact on health [190]. Furthermore, the presence of linoleic acid (LA 18:2) is fundamental in oil, an essential lipid with 18 carbon atoms which, together with gamma-linolenic acid (GLA 18:3), dihomo-gamma-acid linolenic acid (DGLA 20:3), and arachidonic acid (AA 20:4), constitutes the group of omega 6 essential fatty acids [191]. Linoleic acid is a fundamental precursor of some endogenous bioregulators including prostaglandins, which play a very important function in inflammatory processes and thromboxanes, involved in blood coagulation [192]. The resistance of the oil to high heat makes it a suitable choice for cooking especially at high temperatures and its nutritional content increases its overall value [193]. Among the condiments used in KD there is also butter, a dairy product obtained from milk fat. Unlike olive oil, which contains a good number of natural antioxidants, butter is mainly made up of saturated fats. However, butter contains some nutrients such as Vitamin A, Vitamin D (fat-soluble vitamin that supports bone health and the immune system) and Vitamin E, even if present in minimal quantities [194]. Important to note is that the specific nutritional content of butter can vary depending on the source and how it is processed. In recent years, coconut oil and avocado have become popular among cooking oils and as a dietary supplement. Coconut oil consists mainly of saturated fat, contains small amounts of vitamins and minerals, such as Vitamin E. Although Vitamin E has antioxidant properties, the amount present in coconut oil is relatively low [195]. The main types of fatty acids found in coconut oil are lauric acid, caprylic acid and caprinic

acid, which exhibits antimicrobial and antifungal properties [196]. As coconut oil, avocado contain a variety of essential nutrients and important phytochemicals. A serving of 30g of product avocado provides Vitamin C, E and K, folate, Vitamin B6, lutein/zeaxanthin, phytosterols, and also high-monounsaturated fatty acids and polyunsaturated fatty acids (PUFA) [197].

- Seafoods: As stated previously, the KD contains the right amount of protein. Fish and shellfish can be good sources of antioxidants, vitamins, and other beneficial compounds. Naturally, the specific content of these molecules can vary depending on the type of fish, but in general the category can be characterized by common antioxidants [198]. Among these we find selenium; in fact many varieties of seafood are excellent sources of selenium, which helps protect cells from oxidative damage by supporting the body's antioxidant defence system [199]. Mainly sardines, tuna and cod are among the fish options high in selenium. Fish is known to be a rich source of Omega-3 fatty acids and in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [200]. Fatty fish such as salmon, mackerel and trout are particularly rich in it [200]. Furthermore, among the compounds we find Vitamin E, in fact some types of seafood, such as shrimp, provide modest quantities of Vitamin E, important for protecting cell membranes from oxidative damage [201]. Furthermore, these types of fish are also rich in Astaxanthin, a red pigmented carotenoid antioxidant with functions like Vitamin E, which is found in some fish such as krill and in some species of shrimp and salmon [202]. Finally, zinc and copper, essential cofactors for antioxidant enzymes in the body [203]. Other antioxidants and vitamins present in fish include taurine [204], with antioxidant and anti-inflammatory effects, Vitamin D

[205,206], and Vitamin B3 (plays a crucial role in energy metabolism and in maintaining healthy skin) [207].

- Meat: Meat also plays a key role in KD, where especially lean cuts are characterized by the content of some antioxidants and other beneficial nutrients, albeit minimally [208]. Among the minerals present, zinc stands out, especially in red meat, which is an essential mineral and an antioxidant cofactor involved in various enzymatic reactions aimed at protecting against oxidative stress [209]. Also selenium is in a good amount, especially in some meats such as poultry, Coenzyme Q10 (CoQ10), that is an enzyme produced naturally in the body, used by cells to produce and manage energy) [210], present in organs such as heart and liver, and Carnosine, an antioxidant dipeptide presents mainly in red meat, in particular in beef, with strong antioxidant properties [211]. Furthermore, meat is a good source of numerous essential vitamins, in particular B vitamins including Vitamin B12, Vitamin B6 (important for various metabolic processes, including the production of neurotransmitters and the breakdown of proteins) [212], riboflavin (Vitamin B2, plays a role in the production of energy and in the protection of cells from oxidative damage) [213], Pantothenic acid (Vitamin B5, involved in various biochemical reactions, including the synthesis of fatty acids and cholesterol) [214,215]. Finally, Vitamin A [216], Vitamin D [217], Vitamin K2 (has a role in bone health and blood clotting) [218] and Vitamin E, are present [219]. Vitamin E content varies depending on the breeding method and the feed used, many meta-analyses state that the fat component of meat from organic and non-conventional farms increases the Vitamin E content (alpha-tocopherol) [220]. It's important to note that the method by which meat is prepared and cooked, can affect its antioxidant content [221]. For example, grilling or frying meat at high

temperatures can produce compounds such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), which are associated with oxidative stress and can increase the risk of certain diseases. [222] Therefore, choosing lean cuts of meat, using healthier cooking methods and pairing meat with antioxidant-rich fruits and vegetables in meals can help balance your antioxidant intake.

- Milk and dairy products: Among the fat and protein sources present in KD, milk, and derivatives could play an important role in this field. Milk and dairy products are not generally considered significant sources of antioxidants when compared to foods such as fruits, vegetables, nuts and some types of oils [223]. However, they also contain some antioxidant compounds including, for example, some vitamins such as Vitamin A (beneficial for eyesight, skin, and the immune system) and E [224]. Milk and dairy products contain small quantities of Vitamin E which derives mainly from the fatty part of the milk [225], which is why the content will vary based on the type and skimming of the milk, the animal origin of the dairy products and above all the feed used for breeding [226]. Among the antioxidants present in these products are selenium, Vitamin B2 and conjugated linoleic acid (CLA), isomer of linoleic acid (LA) and polyunsaturated fatty acid [227]. CLA is defined as an essential fatty acid as the human organism does not have the enzymes responsible for its synthesis [191]. Some studies suggest that some types of dairy products, in particular those produced with milk from organically raised cows, may contain greater quantities of CLA [228] and Vitamin E, which is present in reduced and minimal quantities in milk and dairy products. Finally, glutathione, a small antioxidant molecule involved in the body's defence against oxidative stress, is also present [223].

- Eggs: Although they are not rich in antioxidants like other foods, eggs can also offer a good support of antioxidants and nutritional compounds useful to the individual for health [229] such as Lutein and zeaxanthin [230], antioxidant carotenoids important for eye health which contribute to reducing the risk of age-related macular degeneration and cataracts [230], selenium and choline [231], an organic substance classified as an essential nutrient which is sometimes combined with group B vitamins. Choline is a constituent of the phospholipids that make up the cell membrane and the neurotransmitter acetylcholine [232]. Among the vitamins we find Vitamin E, contained mainly in the yolk and therefore in the fatty dietary part of the egg, together with carotenoids including beta-carotene which contribute to the colour and nutritional value of the yolk [233].

- Nuts and seeds: Nuts and seeds are excellent sources of antioxidants, as well as essential nutrients. The antioxidants present in this food category are fundamental for the reduction of oxidative stress and the prevention of possible chronic pathologies, as they are rich in antioxidant compounds [234]. Among the antioxidants present we find Vitamin E (in dried fruit, and in particular almonds and hazelnuts) [235] and selenium (a mineral with antioxidant properties that helps positively modulate the immune system). Among the antioxidant compounds in nuts and seeds there are polyphenols and omega-3 fatty acids, polyunsaturated fatty acids defined as essential since the body is not able to produce them, and for this reason, they must necessarily be ingested through the diet [236]. There are three main omega-3 fatty acids: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA); ALA is referred to as 18:3, which means it has a molecule made up of 18 carbon atoms in which there are 3 double bonds. EPA is a 20:5 fatty acid, while DHA is a 20:6 [237].

Omega-3 are commonly defined as good fats, due to their supposed beneficial properties, many of which have been confirmed by scientific studies which highlight their anti-inflammatory activity [238], their ability to maintain normal levels of cholesterol in the blood [239] and their protective action of normal brain and heart function [240]. Lignans are also present in nuts and seeds (especially in flax seeds), a type of phytoestrogen with antioxidant properties [241]. Among the minerals we find copper and manganese and they play roles as essential cofactors for the body's antioxidant enzymes [242].

- Vegetables: Vegetables are excellent sources of antioxidant compounds, essential vitamins, and minerals, they can be considered together with fruit as the primary source from which to draw these compounds [243]. Among the main vitamins present in vegetables, Vitamin C is found par excellence (antioxidant par excellence which supports the immune system) [244], contained mainly in peppers, broccoli, and Brussels sprouts, but also Vitamin E, present in mainly green leafy vegetables such as for example in spinach, chard etc [245]. Carotenoids (Vitamin A) can be found in carrots, sweet potatoes, squash, spinach, kale and other dark leafy vegetables [245]. Among the vitamins we find Vitamin K in green leafy vegetables such as cabbage, spinach and kale are rich in Vitamin K, important for blood clotting and bone health [246]; Vitamin B9, Vitamin B6 in vegetables such as potatoes, sweet potatoes, and avocados [247] and finally Vitamins B1, B2, B3 and B5 are found in various vegetables and are involved in numerous biochemical reactions [247]. Among the antioxidants there are also flavonoids, including quercetin, in vegetables such as onions, broccoli and spinach [248]; Phytochemicals but also resveratrol, for example, cruciferous vegetables such as broccoli and cauliflower contain sulforaphane, a phytochemical known for its

antioxidant and potential anticancer properties [249]. Minerals with antioxidant power include selenium, zinc, copper, manganese, iron, potassium, magnesium, and calcium.

- Beverages: Some types of drinks are also present in KD and can be a source of antioxidants, depending on their components. In KD the recommended drinks include tea and coffee [250]. Green tea and black tea are rich in antioxidants, especially catechins in green tea and theaflavins and thearubigins in black tea [251]. Coffee, on the other hand, is a drink that contains chlorogenic acid, a natural organic substance that plays the role of antioxidant [252]. Moderate coffee and tea consumption has been linked to potential health benefits, such as reducing the risk of type 2 diabetes and some neurodegenerative diseases [253].

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4. VITAMIN E

Ruggiero, M. Panaro, M,A. la Torre, M,E. Messina, M. Porro, C. Villano, I. Monda, V. Polito, R. Benameur, T. Monda, M. Messina, A. Chapter 78: Effects of tocopherols and tocotrienols on microglia-mediated neuroprotection. Natural Molecules in Neuroprotection and Neurotoxicity. 2023.

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4.1 DEFINITION AND CHEMICAL COMPOSITION

Vitamin E is defined as an antioxidant that acts as a ROS scavenger, protecting against the peroxidation of PUFAs (polyunsaturated fatty acids) in cell membranes [1]. In 1922 there was the first publication by Evans and Bishop, subsequently in 1936 it was isolated for the first time from wheat germ oil. In this first publication, Vitamin E was regarded as "factor X", the function of which was linked to its anti-sterile effects on laboratory rats [2]. Researchers later renamed it with the suffix "ol" that indicated the chemical nature of the compound, which is an alcohol [3]. Subsequently, other studies have shown that in addition to the effects on fertility, Vitamin E could play various fundamental roles in the

development of tissues and organs, such as in the brain but also for nerve endings [4], muscles [5], skin [6], bone marrow [7] and blood [8]. Researchers have confirmed some of these functions in humans, while others are still being studied [9]. Vitamin E has eight isoforms, divided into four tocopherols and four tocotrienols; the isoforms vary according to the functions and activities performed, due to their bioavailability in the human body [10]. Of course, the main function of Vitamin E isoforms is their antioxidant action, as they are excellent scavengers of ROS and reactive nitrogen species (RNS) [11] (Figure 15). The formula and chemical structure of the four tocopherols is C28H48O2 (α-, β-, γ-, and σ-tocopherols) while for the four tocotrienols the molecular formula is C26H38O2 (α-, β-, γ-, and σ-tocotrienols) [12]. These molecules are synthesized starting from photosynthetic organisms including, for example, plants, algae, cyanobacteria [13]. All homologs are derivatives of "6-chromanol" and differ from each other in the number and position of the methyl groups on the chemical ring structure [14]. In fact, the four tocopherol homologs have a saturated 16-carbonphyl side chain, while the tocotrienol homologs have three double bonds on the side chain. It follows that tocopherols and tocotrienols have the same chemical structure, the only main difference lies in the saturation of the aliphatic side chain attached to the chromanol ring [14,15]. The “6-hydroxy” group of the chromanol ring is the active site and primarily responsible for ROS scavenging [16]. In vivo, however, not all Vitamin E compounds are biologically available as their availability depends on the methylation state of the chromanol ring and consequently on the degree of saturation of the side chain [13]. The various isoforms are not interchangeable with each other and only α-tocopherol (RRR-α-tocopherol) satisfies the individual need for Vitamin E, therefore it is the most bioavailable [13].

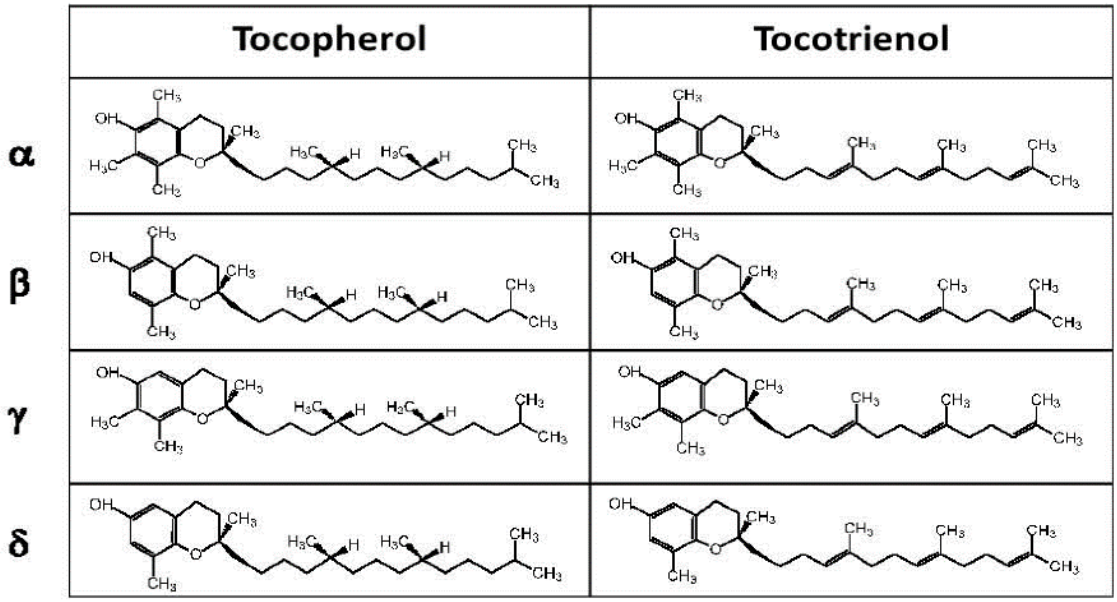

Figure 15. Isoforms of Vitamin E. Structure differences between tocopherols (TOCs) and tocotrienols (TCTs). [17].

Natural and synthetic Vitamin E are not equivalent as the composition, structure, or bioavailability changes. Natural Vitamin E (RRR-α-tocopherol) is a single entity, while synthetic Vitamin E (all-rac-α-tocopherol or dl-α-tocopherol) is a mixture of eight stereoisomers in equal quantities with activity and bioavailability different (Figure 16).

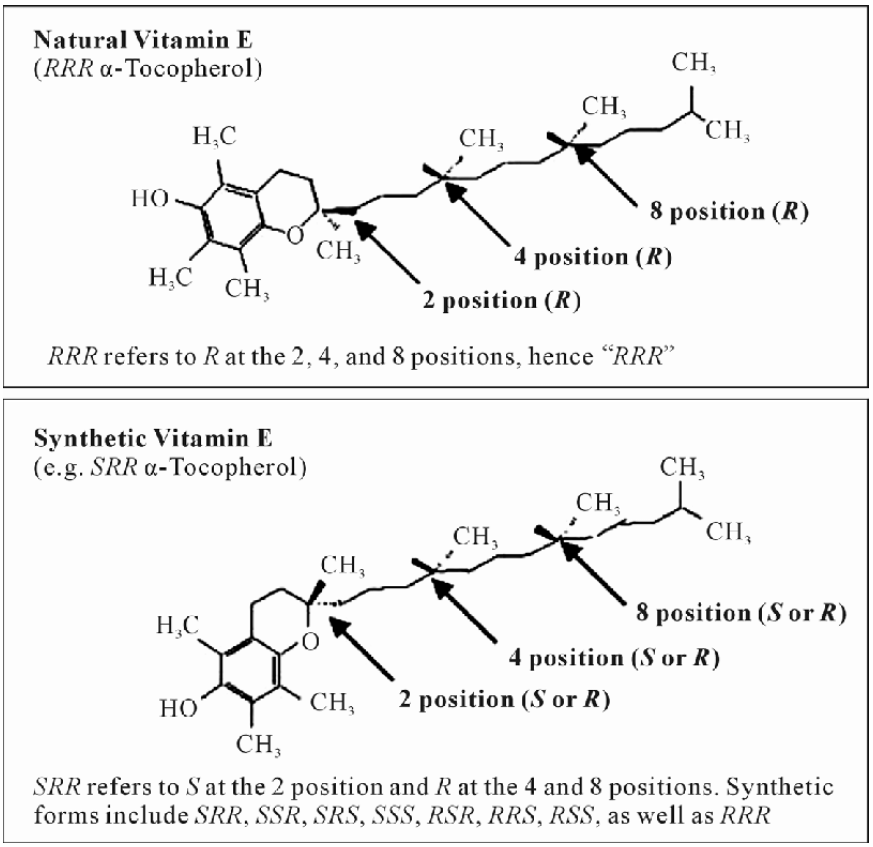

Figure 16. Different structure of Natural and Synthetic Vitamin E [18].

Given the importance of Vitamin E in the human diet for its biological functions, over the years the need to identify the daily intake levels of this vitamin has become important. To facilitate research in this field, several separation techniques have been developed to quantify Vitamin E in different food matrices. The techniques used are gas chromatography (GC), liquid chromatography (NP-HPLC), and reversed-phase HPLC (RP-HPLC) [16].

4.1.1 VITAMIN E AVAILABILITY

Vitamin E and its various isoforms can be found in many food sources that we consume daily or weekly in our diet. Many food groups in which Vitamin E has been shown to be present are considered essential intakes in KD. Vitamin E therefore appears to be one of the fat-soluble vitamins most present in this type of dietary protocol. The content of Vitamin E in food sources has been reported in the following Table (Table 2):

FOOD SOURCES KD OF VITAMIN E	Content of vitamin E (mg/100g)	References
OIL:		
Olive oil	23.50 mg	[19]
Flaxseed oil	0.0-9.11 mg	[20]
Hazelnut oil	20.53 mg-22.79 mg	[21]
BUTTER	2.1-3.3 mg	[22]
COCONUT OIL	0,20 mg	[23]
NUTS:		
Almond	21.90-29.90 mg	
Hazelnut Raw	25.77-31.90 mg	[24]
Hazelnut roasted	25.41-31.99 mg	[25]
Peanut	7.45-12.97 mg	[26]
Ripe Pistachio	0.6 mg	[27]
Dried Ripe	4.0 mg	[28]
Walnuts	2.83-3.87 mg	
AVOCADO	3.22-5.06 mg	[29]
OLIVES	26.7 mg	[30]
VAGETABLES:		
Tomato	0.10-0.26 mg	
Broccoli	3.33-3.77 mg	[31]
Carrot	0.53-1.03 mg	[32]
Cauliflower	0.09 mg	[33]
Cauliflower (dry weight)	7.46-17.97 mg	[34]
Parsley	0.75 mg	[35]
Parsley (dry weight)	14.72-30.32 mg	[36]
Red sweet pepper	2.72-3.78 mg	[37]
Green sweet pepper	0.63 mg	
Spinach	3.97 mg	
SEAFOOD and AQUATIC PRODUCTS	0. 10 to 4. 01 mg	[38]
MEAT		
Pork	0.35 mg	[39]
Lean pork	0.15 mg	
Beef	0.22 mg	
Veal	0.30 mg	
Chicken	0.89 mg	
EGGS	1,05 mg	[40]
Egg, Whole, Raw	2,58 mg	

Egg Yolk, Raw		
MILK Whole milk Low fat milk Skim milk	 0,100 ATE 0,070 ATE 0,040 ATE	[41]
DAIRY PRODUCT Yoghurt whole milk Feta Mozzarella (whole milk) Ricotta (whole milk) Edam Cheddar Brie Camembert Cottage Gruyere Brick Parmesan Munster Port Salut Limburger Gouda Tilsit	0,088 ATE 0,030 ATE 0,350 ATE 0,350 ATE 0,751 ATE 0,360 ATE 0,655 ATE 0,655 ATE 0,122 ATE 0,350 ATE 0,500 ATE 0,800 ATE 0,465 ATE 0,500 ATE 0,640 ATE 0,350 ATE 0,700 ATE *ATE: Alpha tocopherol equivalent	[41]
SEEDS Flaxseed Poppy seed Sesame seed, decorticated Sesame seed, decorticated Sunflower seeds, dry roasted Sunflower seeds, oil-roasted	 0,31 ± 0.01 2,24 1.21 34,50 26.10 ± 4.83 36.33 ± 2.24	 [42] [43] [44] [45]

Table 2. Content of Vitamin E in food sources.

The absorption of Vitamin E follows the same path described for dietary fats, naturally the absorption rate varies from 20% to 80% depending on the food matrix [46]. One of the first enzymes involved in the metabolism of Vitamin E, which carries out the hydrolysis of esters, is gastric lipase, although there are currently no studies describing its exact role in the metabolism of the vitamin. In fact, recent evidence states that the hydrolysis of esters into free α-tocopherol occurs in the duodenum through the action of carboxyl ester hydrolase [47] and in the intestine through pancreatic lipase, trypsin, and α-amylase. These enzymes support the release of Vitamin E from the food matrix, determining its

subsequent absorption by intestinal cells [48]. Subsequently to the gastric lumen, Vitamin E and other fats interact with bile salts, forming micelles to facilitate their transport into the intestinal lumen. As cited, the esterified forms of Vitamin E can interact with the enzyme pancreatic lipase especially in the initial phase of metabolism [49] to be more degraded and have the possibility of migrating towards the intestinal epithelial cells [50]. Initially, early research stated that Vitamin E absorption occurred by passive diffusion across enterocyte membranes; however, new research by Reboul et al. found that α- and γ-tocopherol isoforms can be transported into intestinal cells by certain types of scavenger receptor class B type I (SR-BI) [51]. This SR-BI receptor correlates with high-density lipoprotein (HDL) metabolism and is expressed on the apex of enterocytes [52]. Studies state that SR-BI presents Vitamin E absorption activity in vivo and in vitro leading to a notable increase in the bioavailability of α-tocopherol [51], although, this hypothesis requires further research [53,54]. Vitamin E could share the same diffusion routes as cholesterol and other fatty components as it could follow the same absorption path. For example, the Niemann-Pick C1 intracellular cholesterol transporter (NPC1-like 1 - NPC1L1), might participate in the migration of Vitamin E to intestinal epithelial cells [55,56]. Cluster of differentiation 36 of integral membrane proteins (known as CD36), with scavenger receptor function, is another molecule that could determine Vitamin E efflux [57,58]. In a study by Lecompte et al. found that CD36 modulates α-tocopherol concentrations in the blood. Therefore, CD36 might be involved in the absorption of Vitamin E in the intestine or even in tissues [58]. Vitamin E molecules from foods are enclosed within the chylomicron (lipoprotein), synthesized in the endoplasmic reticulum (ER) and by the Golgi apparatus [59,60]. The secreted chylomicrons subsequently enter the lymphatic vessels, the vena cava, through the thoracic duct, transporting Vitamin E into the blood, heart, adipose tissue, muscles, and liver [55]. Chylomicrons (capsules filled with triacylglycerols (TAG), cholesterol, cholesteryl esters, Vitamin E,

surrounded by a layer of phospholipids) are embedded with the apolipoprotein ApoB48 [61]. The chylomicron that is formed is absorbed through the intestinal wall and then reaches the bloodstream, where it acquires further Apo-proteins (Apo E and Apo C II), which subsequently activate the enzyme lipoprotein lipase (LPL) and hydrolyse the TAG [62]. Finally, as showed in Figure 17, via the LDL receptor and via apolipoprotein E (ApoE), Vitamin E molecules in chylomicron are endocytosed in the liver, where discrimination between Vitamin E isoforms begins [63]. Vitamin E metabolism occurs in the liver and α-tocopherol is then transported into lipoproteins to be distributed to other organs and tissues. The hepatic cytosolic transport protein by which α-tocopherol is transported is α-tocopherol transfer protein (α-TTP). This protein shows a high affinity for the α-tocopherol isoform, compared to other isoforms [64,65]. The affinity of α-TTP for tocopherols is due to the presence of the hydrophobic pocket and the correspondence with a tail derived from phytyl-pyrophosphate, its R configuration in C-2, and finally, its fully methylated chromanol ring. Therefore, if there is a change in this protein configuration this could lead to an incompatibility with α-tocopherol. In humans, suppression of the α-TTP gene causes severe Vitamin E deficiency which, according to recent studies, could lead to neurodegenerative diseases [66].

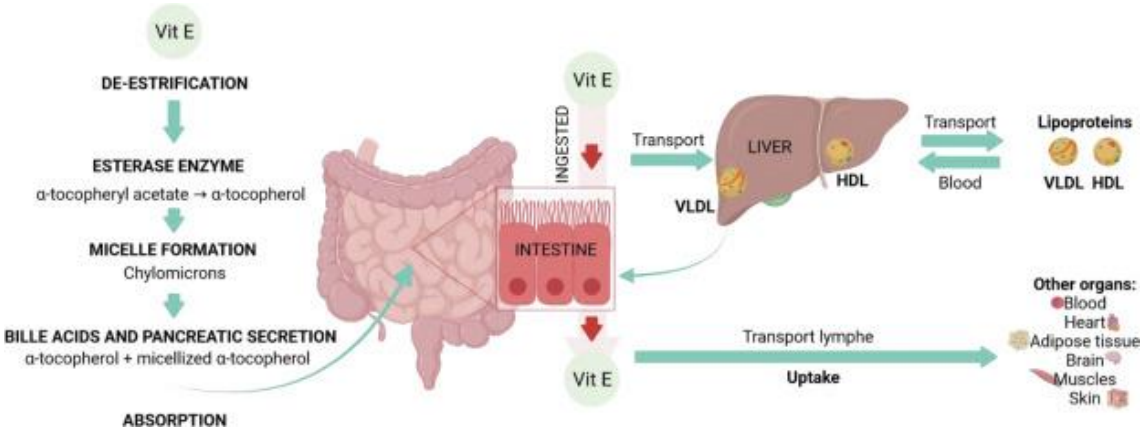

Figure 17. Schematic transport of vitamin E from the intestine to other tissues [67].

Given their widespread diffusion in nature, deficiency syndromes are secondary to absorption disorders, which can occur in the lack of bile salts or in the presence of excessive quantities of unsaturated fatty acids, to fibrocystic disease of the pancreas and to abetalipoproteinemia [68]. The manifestations of deficiency consist of modest haemolytic anaemia which can be found especially in premature newborns. Manifestations of excess, however, occur at high doses, usually showing antagonisms with Vitamin K with consequent haemorrhagic phenomena [69]. It should be remembered that Vitamin E is resistant to acids and alkalis and is sensitive to heat and light. The recommended daily requirement is approximately 0.50 mg/kg for children and 10-30 mg/kg for adults [70].

4.2 BIOLOGICAL ACTIVITIES OF VITAMIN E

4.2.1 ANTI-INFLAMMATORY ACTIVITIES AND MECHANISMS OF VITAMIN- E

Vitamin E compounds have a number of important roles in human metabolism and include, for example, acting as an antioxidant to protect cells and other body components from ROS attack [71]; stimulus of the immune response [72]; reducing the severity of prostaglandin-mediated disorders such as inflammation [15], premenstrual syndrome but also circulatory irregularities [73]; inhibitor for the conversion of nitrites (contained in predominantly smoked, pickled or cured foods) into nitrosamines in the stomach [74]; and regulator of gene expression (in particular of the proliferation of smooth muscle cells) [75]. A possible Vitamin E deficiency does not lead to diseases with rapidly developing symptoms, however, there could be notable effects of inadequate Vitamin E intake, which could develop over a long time, often linked to the development of degenerative diseases. Vitamin E is generally recognized as the main fat-soluble antioxidant in lipid tissues and as a primary defence against lipid peroxidation and fat oxidation, especially at cell membrane level, however, Vitamin E has various roles, result of years of research conducted starting in 1950 with Angelo Azzi et al., who also highlighted non-antioxidant capacities and for α -tocopherol (α-TOH) [76]. The focus of research over the years has been to evaluate the effects of Vitamin E towards the modulation of genetic regulation, enzymatic activities, transport, degradation, metabolism and excretion, the absorption of lipoproteins, the effect in inflammation, and so on [77]. For example, different forms of Vitamin E could modulate the inflammatory process in various phases, such as at the level of transcription factors (NF-κB), peroxisome proliferator-activated receptor gamma (PPAR-κ), in the modulation of gene expression and related signalling cascades through signal transducer and activator of transcription 3 (STAT3), cyclooxygenases (COX), and finally the production of secondary signalling

molecules such as prostaglandins (PG) [78]. It must be considered that the different forms of Vitamin E regulate the activation of NF-κB in a different way based on the saturation of the side chain (tocopherols and trienols) and on the methylation of the chromanol ring (α, β, γ and δ), with δ-tocotrienol and γ-tocotrienol being the most potent tocotrienol forms, followed by β-tocotrienol, while α-tocotrienol has no effects on NF-κB activity [79]. Regarding tocopherols, α-TOH and β-TOH failed to determine any effect on NF-κB activation, while it was discovered that a combination of Vitamin C with high doses of α-tocopherol decreased the activity and the expression of NF-κB without affecting the phosphorylation of IκBα [79]. If the activation of the NLRP3 inflammasome depends in part on the pattern of NF-κB, it can therefore be considered that different forms of Vitamin E could interfere both with NF-κB and with the effective and subsequent activation of the NLRP3 complex [78]. Generally, ROS activate NLRP3, with subsequent cleavage of caspase-1 and subsequent secretion of IL-1β and IL-18 [80]. Since Vitamin E is an antioxidant capable of reducing the action of ROS, in this case it blocks the activation of NLRP3 induced by ROS, blocking the respective formation of pro-inflammatory interleukins [81]. Since Tocotrienols and TOH have a different capacity to regulate NF-κB, with δ and γ-T3 being the most active forms, it has been reported that predominantly δ-T3 attenuates the process induced by NF-κB with consequent reduction of the activation of the NLRP3 inflammasome and its subsequent cascade [82]. In addition to inhibiting NF-κB and NLRP3 inflammasome activation, Vitamin E also acts on signaling pathways. It has been described that the CD36 cluster of differentiation, known as a macrophage surface marker which is responsible for the uptake of oxidized LDL (oxLDL), can interact with the NLRP3 inflammasome and the α-TOH isoform [83]. Furthermore, COX-2/microsomal prostaglandin E2 synthase-1 (mPGES-1) and 5-lipoxygenase (LOX) may contribute to the activation of NLRP3 [84,85]. The interaction of α-TOH with COX-2 and mPGES-1 could influence by modulating the signalling molecules formed by this

pathway [86]. Among the effects inducible by Vitamin E there is also the stimulation of the PI3Kγ/Akt pathway, which could lead to the induction of the expression of VEGF, an actor of growth of the vascular endothelium, involved in the process of angiogenesis and lymph angiogenesis, or in modulation of Sec14L2/TAP1, Sec14L2/TAP2 and Sec14L2/TAP3 proteins, which could bind and inhibit the PI3Kγ complex in vitro [87], and be associated with protection against some tumors (human prostate and breast) [88,89]. Furthermore, γ-tocopherol could significantly mitigate the inflammation modulating the infiltration of neutrophils into lung tissue and the accumulation of neutrophils in the bronchoalveolar lavage fluid [90,91]. γ-tocopherol also inhibited the secretion of mucin and some cytokines, including neutrophil chemotactic cytokines (MIP-2 and GRO-KC) and mucus-producing cytokines (Muc5AC) [90]. Some forms of Vitamin E may be involved in cancer modulation processes. In some recent studies it has been seen how tocopherols, and in particular γ-tocopherol, suppressed tumorigenesis, and in particular inflammation and colon eicosanoids (PGE2 and LTB4) in mice. In addition to colon cancer, Barve et al. [92] demonstrated how tocopherols could suppress the incidence of prostate cancer as, in mouse studies, tocopherols modulated the Nrf2 transcription factor and Nrf2-regulated antioxidant genes in the TRAMP model of murine prostate cancer. In addition to tocopherols, γ-tocotrienol and δ-tocotrienol also document anti-inflammatory and immunomodulatory effects in various pathologies. A study by Shibata et al [93] demonstrated how tocotrienols, but not α-tocotrienol, suppress UVB-induced PGE2 and cytokines by blocking the proinflammatory signalling pathway in keratinocytes, which increases UVB-induced skin thickness resulting in induction of COX-2. Furthermore, dietary supplementation of mixtures of tocotrienols, in murine models, increased the proliferation and production of lymphocytes [94], promoting the regeneration of bone marrow and progenitor cells [95]. Finally, Tsuduki et al [96] studied how tocotrienols can

attenuate allergic dermatitis in mice by suppressing degranulation and histamine release in mast cells.

4.2.1.1 THE EFFECT OF VITAMIN E ON NEURODEGENERATIVE DISEASES: MICROGLIA NEUROPROTECTION

La Torre ME, Villano I, Monda M, et al. Role of Vitamin E and the Orexin System in Neuroprotection. Brain Sci. 2021;11(8):1098.

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Vitamin E is known for its antioxidant properties and for its role in neuroplasticity, which could explain its neuroprotective effects. An "antioxidant" is any substance that can protect against oxidative stress damage caused by free radicals. Older cells decrease their ability to prevent and reduce oxidative damage. Thus, cellular senescence is associated with increased levels of oxidants, decreased body defences against ROS and decreased effectiveness of repair mechanisms; factors that result in increased end products of oxidative damage [97]. The neuroprotective role of Vitamin E in the brain, therefore, has been linked to neurogenesis, neuronal differentiation, hippocampal synaptic function and cell signalling pathways [98]. Antioxidants act in the following two ways: they prevent neuronal death due to oxidative stress (scavenging free radicals and, thus, preventing lipid peroxidation) and reduce the activation of transcription factors [99]. Transcription factors (themselves activated by oxidative stress) [100] are involved in the control of nerve cell survival and antioxidant-induced neuroprotection. Tocopherol is the most effective fat-

soluble antioxidant, breaking chain reactions initiated by free radicals between Polyunsaturated fatty acids (PUFAs) in biological membranes, i.e., counteracting free radical reactivity by donating a hydrogen atom from an intact hydroxyl group to the free radical, thus stabilizing it [101]. Each tocopherol molecule can donate two electrons before being "consumed"; the tocopherol molecule is then reduced to its previous state and can then be reused. Importantly, this reduction process is most likely carried out by ascorbic acid [102], which is why there are many studies reporting the antioxidant capacity of Vitamin E linked to Vitamin C [103]. A combination of different antioxidants, therefore, may offer additional benefit [104] because antioxidants may together have different protective effects. The long-term treatment with Vitamin E tends to increase the concentration of tocopherol in the brain over time, thus, increasing its effectiveness as an antioxidant [105]. Clinical studies suggest that Vitamin E would play an important role in the prevention of neurodegenerative diseases [106] thanks to its ability to act at the level of the microglia, causing a reduction in its activation, reducing inflammation [107-109]. The neuroprotective roles of Vitamin E have been well documented in both in vivo and in vitro studies [105] [108-110]. As antioxidants, tocopherols and tocotrienols protect tissue lipids from free radicals by reducing chemical species such as peroxyl, hydroxyl and superoxide radicals and singlet oxygen. Vitamin E has often been referred to as nature's best chain antioxidant. Typically, one molecule of the vitamin protects about 100 membrane phospholipids [111]. In vivo, Vitamin E and other endogenous antioxidants work in concert or synergistically by maintaining a reduced environment [112]. The effects of Vitamin E on microglial cells have been studied in the short term, most studies, in line with the idea that microglial activation is a harmful process, have shown that Vitamin E suppresses inflammatory activation of microglia, thus providing some neuroprotection (Table 1) [107][113]. Recent studies highlight how Vitamin E can improve the various vital functions of N9 microglial cells; this includes the enhancement of protein turnover, the regulation of oxidative

activity, the amount of proinflammatory agents and the absorption and degradation of extracellular protein material [114-116]. These effects could be explained not only by pure antioxidant effects [117], but also by the role of Vitamin E as a hormone-like substrate, as proposed by Azzi et al. [76]. As regards the various isoforms at the level of the central nervous system, alpha tocopherol is the most biologically active form; in fact, a-tocopherol reduces the radicals of intracellular peroxide induced by stimulation with LPS at the level of the microglia [75]. As far as other isoforms are concerned, for example, tocotrienols may offer a greater bioavailability than tocopherols because their unsaturated hydrocarbon tails allow for better penetration into fatty tissue such as the brain [118]. A drastic decrease in ROS production by α-tocopherol has already been demonstrated in macrophages and is related to the inhibition of protein kinase C (PKC) [119]. The inhibition of PKC leads to the inhibition of NADPH-oxidase assembly [120] and, thus, reduces the production of superoxide. α-tocotrienol protects neurons from a glutamate-induced death better than γ-tocotrienol [121]. Interestingly, this effect is not related to the differential uptake of δ-tocotrienols in cells: γ-tocotrienols are absorbed more efficiently by neurons than α-tocotrienol [122]. To directly show the involvement of Vitamin E in neuroprotection through the modulation of microglial responses, several papers have treated microglia cells with Vitamin E alone, or with LPS alone, or pre-treated with Vitamin E and then stimulated with LPS [107], the most used pro-inflammatory stimulus for microglia, both in vitro and in vivo. Various studies show that Vitamin E significantly suppressed LPS-induced microglia activation by decreasing the associated NO production and induction of IL-1α, TNF-α and iNOS expression. Indeed, Li et al. showed that incubating cells with 50 μM of Vitamin E for 24 h significantly inhibited LPS-induced NO production (68%) and reduced the expression of IL-1α (89%), TNF -α (32%) and iNOS (55%) [110]. More specifically, it was seen that the δ-tocotrienol taken up by BV2 microglia was 71% retained in BV2 cells even 24 h after its removal. The inhibitory effects of δ-

tocotrienol on NO production by BV2 microglia could be partly attributable to δ-tocotrienol retention, as inhibition continued 48 h after stimulation with LPS [123]. Indeed, it showed that although various isoforms of tocotrienol at various concentrations were able to reduce the NO produced by BV2, δ -tocotrienol has the most potent effect, reducing NO levels by approximately 50%, even after 48 h [118]. Some studies have reported that Vitamin E reduces the expression of iNOS, on human monocytic cells [124]. Indeed, Vitamin E inhibits the phosphorylation of p38 MAPK and the activity of NFκB [107]. The Vitamin E-induced inhibition of microglial responses after stimulation with LPS is, therefore, linked to the suppression of activation of p38 MAPK and NFκB, both of which regulate cytokine and iNOS expression [107]. In addition, Vitamin E has also been reported to interact with the cyclooxygenase-2 (COX-2) signaling pathway, which is linked to pro-inflammatory signals [125] in BV2 cells. In this regard, several studies, including seminal observations from the Ames laboratory [126,127], suggest that c-tocopherol possesses significant anti-inflammatory activities that are distinct from its classical free radical defense action. Both c-tocopherol and δ-tocotrienol are more potent than a-tocopherol in inhibiting the catalytic function of Cyclooxygenase (COX) in BV2 cells [126]. COX, particularly inducible COX-2, are key inflammatory enzymes that mediate the conversion of arachidonic acid to prostaglandin E2 (PGE2) [128]. Finally, among the protective effects, a recent publication demonstrated that δ-tocotrienol can inhibit inflammation activation and subsequent IL-1β production in iJ774 macrophages [109]. The production of IL-1b, a key cytokine that mediates the inflammatory response, was found to be significantly reduced in Vitamin E-treated microglia after 7 days in vitro, confirming the results of previous studies [107][113]. These findings indicate that antioxidants can be used to mitigate cytokine expression in the brain and protect against damage due to microglia activation. Regarding the ability of tocopherol to modulate the cell signalling pathways, in vitro and in vivo models have shown that Vitamin E lowers the

inflammatory responses that induce activation of microglia [107][129]. The chronic activation of microglia probably plays an important role in neurodegenerative disorders related to oxidative stress such as PD, AD [130,131] and ALS [107][132]. Vitamin E would, thus, suppress the harmful activation of microglia, thus offering possible neuroprotection [113].

Type of Vitamin E	Biological Activity	Study Model	References
α-tocopherol	Reduces astrocytosis and microglia activation	Cell rat hippocampus	Ambrogini et al. [133]
α-tocopherol	Inhibits Microglia Activation	Pheochromocytoma cell line: PC12 cells	Li et al. [110]
α-tocopheryl acetate	Increases microglial activation and RAGE expression	Astroglial cell of mice	Bialowas-McGoey et al. 2008 [134]
α-tocopherol	Blocks glutamate release	Sprague Dawley rats	Barger et al. 2007 [135]
α-tocopherol	Attenuates expression of COX-2 and the production of proinflammatory cytokines	Cell rat hippocampus	Annàhazi et al. 2007 [136]
α-tocopherol	Reduces proinflammatory cytokines and production of ROS	Primary glial cultures	Stolzing et al. [137]
α-tocopherol	Decreases lipid peroxidation and IL-6 secretion	BALB/c mice	Godbout et al., 2004 [138]
Tocotrienols	Prevents death of HT4 cells treated with glutamate	HT4 hippocampal neuronal cells	Sen et al., 2000 [139]
δ-tocotrienol	Reduces NO production and IL-1β expression, inhibits PGE2 expression	BV2 microglia cells	Tan et al., 2020 [140]
α-, γ- and δ-tocotrienol	Reduce NO release	BV2 microglia cells	Tan et al., 2011 [141]
α-tocopherol	Attenuates COX-2 protein synthesis	BV2 microglia cells	Egger et al. [142]
γ-Tocopherol	Inhibits cyclooxygenase activity and nitrite accumulation	Murine RAW264.7 macrophages	Jiang Q et al., 2000 [143]

Table 1. Biological activities of each type of Vitamin E.

4.2.2 OVERVIEW OF THE PRO-INFLAMMATORY EFFECTS OF VITAMIN-E

In recent years, the supplementation of vitamins and foods enriched in vitamins or natural compounds has increased, in fact it is estimated that in the United States, the supplementation of antioxidant vitamins including Vitamin E (α-tocopherol) is taken daily by 11% of the adult population [144], while approximately 37% report having taken it at least in the previous month [145]. Recent meta-analyses have evaluated the dose-response relationship that exists between Vitamin E supplementation and the total mortality rate, and it has been demonstrated that there is an increase in mortality with Vitamin E supplementation at high doses and concentrations [146]. Currently, however, the underlying mechanisms to explain these observations are not clear. Thanks to the ability to donate the phenolic hydrogen of the chromanol ring in position 6 to the free radicals of lipids, tocopherols and tocotrienols show a strong antioxidant activity. However, antioxidants and consequently their radicals could often undergo side reactions that can be classified as pro-oxidants [147,148]. One of the mechanisms hypothesized by Miller et al. [149] is that Vitamin E supplementation, particularly the tocopheroxyl radical, could have a pro-oxidant effect [150]. Naturally, these results could be a cause for concern, as the increase in oxidative activity is considered a contribution to the appearance of degenerative diseases of aging such as cancer, but also heart disease, cataracts and finally brain dysfunction [147]. α-tocopherol in high doses could act as a pro-oxidant, increasing DNA damage. Some results in in vitro experiments state how Vitamin E could cause an insult in cultured cells capable of producing reactive oxygen species (ROS) [151,152], which together with α-tocopherol inside the lipids could oxidize by forming its own radical, which if not eliminated by co-factors, increases lipid peroxidation, a process known as tocopherol-mediated peroxidation (TMP) [153]. The now peroxidized lipids produce a series of ROS

capable of damaging DNA. The H_2O_2, generated by the TMP process, even if it does not directly damage the DNA, is able to cross cell membranes by reacting with the transition metals (Fe, Cu) associated with the DNA generating hydroxyl radicals (•OH) and causing damage to the DNA [154,155]. Therefore, when the concentration of the tocopheroxyl radical is sufficiently high, several unwanted secondary and collateral reactions could occur which could trigger a chain reaction increasing lipid peroxidation. In studies of LDL isolated from blood, Vitamin E could accelerate the peroxidation of polyunsaturated fatty acids [156]. Infact, in a study of Reilly et al. the results showed how Vitamin E increased F2 isoprostanes, stable lipid peroxidation products of arachidonic acid, the quantification of which provides an index of oxidative stress, and total plasma PGF2α in smoking subjects who at the same time consumed a diet rich in PUFA [157], therefore it can be deduced that the increase in plasma levels of isoprostanes could be a consequence in response to the increase in the availability of Vitamin E [158]. The results of some studies state that the antioxidant activity of Vitamin E could prevail even in highly oxidative conditions but could evolve and have a pro-oxidant role when the presence of co-antioxidants would decrease in conditions of mild oxidation [159]. The results therefore indicate how the level of oxidative stress and the concentration of co-antioxidants, such as ascorbate, would determine the main factors of the activity of Vitamin E towards oxidation both in human plasma and in LDL [160]. The pro-oxidant radicals of vitamin E analogues could cause intracellular oxidation of LDL-lipid peroxide LPO, GSH and cytotoxicity [161]. A pro-oxidant effect of Vitamin E associated with high doses could explain the increased mortality in adult individuals who take high-dose Vitamin E supplements for a period of time greater than 1 year [149]. Furthermore, the use of high doses of Vitamin E could have an impact on cardiovascular outcomes [162] with a possible increase in the rate of heart failure especially in subjects already predisposed to high risk of cardiovascular events [163]. Furthermore, several other studies have reported that Vitamin E could

behave as a carcinogenic agent that would initiate and promote the appearance of tumors [164,165], in fact in some studies it has been reported as the Chronic subcutaneous administration of Vitamin E on mice could lead to the induction of tumors [166], significantly increasing the incidence of liver cancer [167], the incidence of lung cancer, and an increase in mortality resulting from hemorrhagic stroke, thus illustrating the controversial results observed on Vitamin E supplementation. This suggests that the current recommendations for the maximum Vitamin E dosage of 1000 mg/day of any form of α-tocopherol supplement (corresponding to synthetic Vitamin E 1100 IU/day or natural Vitamin E 1500 IU/day) should be revised considering the new data [168].

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5. OBJECTIVE OF THE DOCTORAL THESIS PROPOSAL

The objective of the doctoral project was to evaluate the antioxidant and anti-inflammatory effects of ketogenic diet enriched of organic food product on BV2, A549 and PC9 cell lines. Was evaluated the combined effect of formulations of ketone bodies, producing during ketogenic diet, such as BHB and AcAc, together with Vitamin E, a fat-soluble micronutrient contained in the main food groups that characterize the ketogenic diet and organically derived foods.

6. MATERIALS AND METHODS

6.1 MATERIALS AND METHODS: MICROGLIA BV2 CELLS

Polito R, La Torre ME, Moscatelli F, et al. The Ketogenic Diet and Neuroinflammation: The Action of Beta-Hydroxybutyrate in a Microglial Cell Line. *Int J Mol Sci*. 2023;24(4):3102.

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6.1.1 PRIMARY MICROGLIAL CELL LINE

A BV2 murine microglial cell line was used in this study. The cells were purchased from the American Type Culture Collection (Manassas, VA, USA). BV2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Euroclone; Milan, Italy), 100 units/mL penicillin, 100 μg/mL streptomycin, (Penicillin-Streptomycin; Euroclone; Milan, Italy) and 2 mM glutamine (Glutamine; Euroclone; Milan, Italy) at 37 °C in a humidified incubator with a CO2 level equal to 5%. For subsequent experiments, BV2 cells were plated at an appropriate number and density after detaching them from the substrate using Trypsin-EDTA (Trypsin-EDTA 1X in PBS, Euroclone, Milano, Italy).

6.1.2 PREPARATION OF B-HYDROXYBUTYRATE SOLUTION

β-hydroxybutyrate (BHB) (DL-β-Hydroxybutyric acid sodium salt, ~98%; Sigma-Aldrich) was initially diluted in sterile PBS (Dulbecco's Phosphate-Buffered Saline w/o Calcium, Euroclone, Milan, Italy) in a 1 M concentration solution. Subsequently, the final concentrations were created from the stock solution and diluted in the DMEM in which the cells were plated. Cells were incubated with BHB for 24 h before each treatment.

6.1.3 PREPARATION OF VITAMIN E SOLUTION

Vitamin E (DL-*α*-tocopherol acetate ≥96% (HPLC); Sigma-Aldrich; CAS: 7695-91-2) was initially obtained by creating a solution with 1 M concentration diluted in ethanol (absolute ethanol; Scharlau). For the following experimental tests, the final concentrations were created, starting from the stock solution, by diluting the

Vitamin E in DMEM. Prior to the experiments, cells were incubated in DMEM containing Vitamin E for up to 24 h [1].

6.1.4 CELL VIABILITY ASSAY

The cytotoxicity of Vitamin E and β-hydroxybutyrate for BV2 cells, was assessed using an MTT assay with Thiazolyl Blue Tetrazolium Bromide purchased from Sigma-Aldrich (CAS: 298-93-1). For the MTT assay, BV2 cells were plated in 24-well plates with a density of 2×10^5 cells and were incubated at 37 °C with 5% CO2 for 24 h with initial Vitamin E concentrations of 50 μM, 100 μM, 200 μM, and 400 μM, and β-hydroxybutyrate concentration 5 mM, 10 mM, 20 mM, and 100 mM to study the cytotoxicity of these substances. For the next MTT assay, the test was performed with 100 μM Vitamin E and 5 mM of β-hydroxybutyrate respectively in the presence or absence of LPS at a concentration of 1 μg/mL for 24 h. 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 are shown as the cell viability (%) based on the control condition.

6.1.5 CELL MORPHOLOGY ASSAY

An analysis of microglial morphology was carried out by means of a morphological image test to evaluate the effect of Vitamin E with a concentration of 100 μM and β-hydroxybutyrate with a concentration of 5 mM respectively in the absence or presence of the pro-inflammatory stimulus (LPS with a concentration of 1 μg/mL). About 5×10^5 cells were plated on a 6-well plate. All morphological tests were performed in triplicate, and the results are expressed

as the average of the areas of three cells from five independent experiments. The plates were evaluated using Leica Microscopy photography (DM IRB Leica Microsystems GmbH, Wetzlar, Germany) at 10× and 20× magnifications. The cell areas (μm^2) were quantified using ImageJ software.

6.1.6 CELL WOUND CLOSURE ASSAY

To assess cell migration, we used a cell wound closure test. A total of 1×10^6 BV2 cells were added to the wells on a 6-well plate. The cells were cultured until confluence. Confluent monolayers were injured with a sterile scratch using a scraper; subsequently, after washing with PBS and changing the DMEM, the remaining cells were incubated for 24 h overnight under different conditions with 100 μM Vitamin E and 5 mM of β-hydroxybutyrate respectively in the absence or presence of LPS with a concentration of 1 μg/mL. All migration tests were performed in triplicate. Closure of the open scar was documented after 24 h with photomicrographs of the various conditions that were analysed. Wound closures were analysed using ImageJ software and are expressed as a percentage of the cell-covered area from the zero-time condition.

6.1.7 ELISA TEST

Vitamin E at a concentration of 100 μM and β-hydroxybutyrate at a concentration of 5 mM respectively in the absence or presence of LPS (1 μg/mL), was added to BV2 cells and incubated at 37 °C with 5% CO_2 for 24 h. After 24 h, the culture medium was collected and used for the evaluation of cytokines, as TNF-α and IL-10 for the supernatants of cells stimulated with 100 μM of Vitamin E, and IL-18 and IL-10 for the supernatants of cells stimulated with 5 mM of β-

hydroxybutyrate using commercially available ELISA kits (R&D Systems a bio-techne brand). The protocols used for the procedures were in accordance with the manufacturers' instructions. The cytokine concentrations (pg/mL) in the medium were determined by referring to standard curves obtained with known amounts of TNF-α, IL-10 and IL-18.

6.1.8 WESTERN BLOT ANALYSIS

After previously described cell treatments, BV2 cells were detached from the plate by scraping and were collected after centrifugation at 2000 rpm for 10 min at 4 °C. The cells were lysed with an ice-cold lysis buffer composed of Tris-HCl (50 mM) at PH 8, 1% (*v*/*v*) Triton X-100, 1.5 M NaCl, 0.1% SDS, 100 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin hemisulfate salt, and 4 U/mL aprotinin (all from Sigma Aldrich). After eight cycles of freezing and thawing, the lysates were obtained by centrifugation at 12,000 rpm for 30 min at 4 °C. The concentrations of the lysates were determined using the Bradford assay, and for each treatment the same amount of protein (20 μg) was subjected to SDS-PAGE (NuPage Electrophoresis System, Invitrogen) mixed with NuPage LDS Sample Buffer (4×, 1:4 (*v*/*v*)) and NuPage Sample Reducing Agent (10×, 1:10) on 4–12% Novex Bis-Tris Midi gel 1.0 mm precast gels (Life Technologies Van Allen Way, Carlsbad, CA 92008, USA). At the end of the electrophoretic run, resolved proteins were transferred from the gel to a nitrocellulose membrane using iBlot Dry Blotting System A (Life-Technologies). Membranes, after blocking with 5% (*w*/*v*) non-fat dried milk for 1 h, were washed 3 times with 0.1% Tween 20-PBS (T-PBS). Primary antibodies directed against β-actin (1:500), CD40 (1:500), CD206 (1:500), TLR4 (1:500), and p-Akt (1:500), all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Heidelberg, Germany), were incubated for 1 h at room temperature on a shaker and then overnight at 4 °C. Next, the

membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, diluted 1:10,000) for 60 min at room temperature in the dark on a shaker. After three washes with 0.1% Tween 20-PBS (T-PBS), immunoreactive bands were visualized using chemiluminescence (BioRad Laboratories, Hercules, CA, USA). Lastly, β-actin was used as a housekeeping protein to normalize protein expression levels [2]. The bands obtained after immunoblotting were submitted to a densitometric analysis using the ImageJ platform, and the results are expressed in arbitrary units.

6.1.9 STATISTICAL ANALYSIS

All data referred to the experimental phase on BV2 cells with Vitamin E and β-hydroxybutyrate respectively were plotted as the means of three independent experiments ± SDs. Statistical analyses were conducted by one-way ANOVA testing, using Graph Prism 9 software (GraphPAD Software, San Diego, CA, USA). Statistical significance was assessed with a p-value < 0.05.

6.2 MATERIALS AND METHODS: A549 AND PC9 LUNG CANCER CELLS

6.2.1 HUMAN LUNG CANCER CELL LINES

A549 (Human cells, epithelial cell, Lung Carcinoma) and PC9 (Human cells, Lung adenocarcinoma) cells were used in this study. The cells were purchased from ATCC. A549 and PC9 cells were cultured in RPMI 1640 1X medium (Corning, Manassas, USA), without L-Glutammine, with low glucose level 1g/L, supplemented with 10% Fetal Bovine Serum (FBS, Biosera, Uruguay), 5% penicillin- streptomycin (Penicillin-Streptomycin Solution, 100x, Corning, Manassas, USA), at 37 ◦C in a humidified incubator with a 5% CO2 level. For the next experimental steps, A549 and PC9 cells were plated with appropriate number and density after detaching them from the substrate using Trypsin-EDTA (Trypsin EDTA 1X, 0.05%, Corning, Manassas, USA).

6.2.2 PREPARATION OF FORMULATIONS OF LITHIUM AND ACETOACETATE

Lithium acetoacetate (Sigma-Aldrich ≥90% HPLC, CAS: 3483-11-2, St. Louis, MO, USA), Sodium Acetoacetate, Lithium Chloride (Calbiochem, CAS: 7447-41-8, Darmstadt, Germany) and Lithium carbonate (Sigma-Aldrich, CAS: 554-13-2, St. Louis, MO, USA) were initially obtained by creating a solution with 500mM concentration diluted in sterile water (VERSOL® Sterile Water, Bristol, UK) and subsequently, for the following experimental tests, the final concentrations of 5mM for Lithium Acetoacetate, Sodium Acetoacetate and Lithium Chloride, while 2.5 mM for Lithium Carbonate, were created, starting from the stock solution, by diluting the lithium acetoacetate into the medium.

6.2.3 PREPARATION OF ABT737 SOLUTION

An initial concentration of 25 mM was created for ABT737 (Biotechne, Tocris, CAS: 852808-04-9, Bristol, UK), diluted in DMSO (Dimethylsulphoxide, Labscan), and stored at a temperature of -20°C. The final concentration of 5uM, for both, was used for the subsequent experimental steps, diluted directly in the medium.

6.2.4 CYTOFLUORIMETRIC ASSAY ON CELL APOPTOSIS

To evaluate the effect of formulations of lithium and acetoacetate on A549 and PC9, we used a Cytotoxicity Assay. A total of 1×10^5 A549 and PC9 cells respectively were added to the wells on a 24-well plate. The cells were left for 24h in a condition of 37°C at 5% CO2 level. Subsequently, after changing the medium (RPMI 1640), cells were incubated for 24 hours with 5mM Lithium Acetoacetate, Sodium Acetoacetate and Lithium Chloride, while 2.5 mM for Lithium Carbonate. After the 24h of incubation, medium was changed with different conditions as 5μM/mL of ABT737 in the absence or presence of 5mM Lithium Acetoacetate, Sodium Acetoacetate, Lithium Chloride, and 2.5 mM Lithium Carbonate respectively. After 24h of incubation, the medium contained in the wells was recovered, the cells were detached with Trypsin-EDTA and with PBS (DPBS, 1X, Corning, Manassas, USA) were performed washings for each well. The cells were centrifuged, resuspended in Annexin V Buffer, and labelled with Annexin V-FITC (Annexin V FITC, Biolegend, San Diego, CA) and 7AAD (7-AAD Viability Staining Solution, Biolengend, San Diego, CA). Subsequently read by Flow Cytometer (CytoFLEX, 2.6).

6.2.5 STATISTICAL ANALYSIS

All data referred to the experiments related to the Apoptosys assay on A549 and PC9 cells were evaluated by FlowJo software (FlowJo 10.09.0). Statistical analyses were performed using analysis of variance (One way- ANOVA) and Tukey's post hoc test, using GraphPad Prism (GraphPad Prism 8.0.2). P-values <0.05 were evaluated as statistically significant. All assays were performed in triplicate and data are expressed as means ± SEM.

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7. RESULTS

7.1 RESULTS: MICROGLIA BV2 CELLS

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7.1.1 RESULTS OF CELL VIABILITY ASSAY

7.1.1.1 INFLUENCE OF B-HYDROXYBUTYRATE ON BV2 CELL VIABILITY

The results of the cell viability analysis (MTT) regarding the concentrations of BHB and their effects on BV2 cells in the presence or absence of LPS (1 μg/mL) are shown in Figure 18 below.

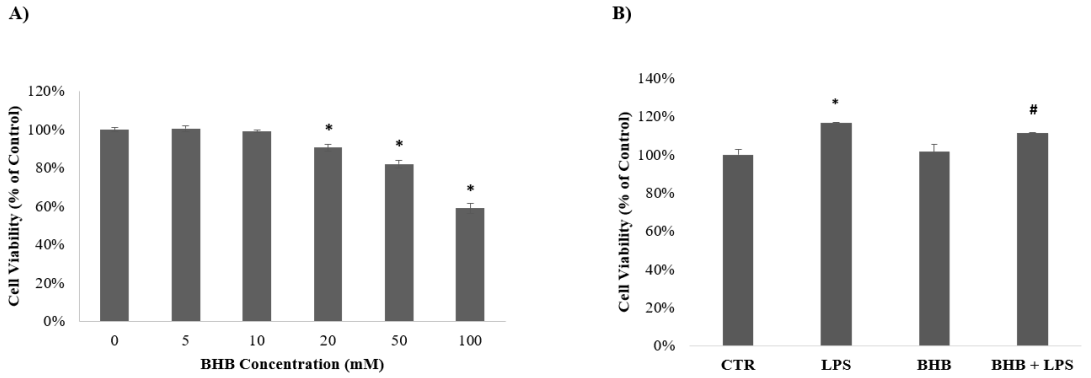

*Figure 18. Cell viability analysis of BHB. Cell viability evaluated by MTT test, initially with BV2 cells, with dose–response curves for concentrations from 5 mM to 100 mM (A). The 5 mM concentration of BHB was used in the next experimental phase to treat cells in the absence or in the presence of LPS (1 μg/mL) (B). Results are presented as means ± SDs of three independent experiments performed in triplicate of percentages compared to control values. * $p < 0.05$ compared to the same time points for the CTR. # $p < 0.05$ compared to the LPS time points.*

The data in Figure 18, Panel A show the dose–response curves in relation to BHB concentrations. As reported in previous studies in the literature, BHB did not significantly interfere with cell viability at a concentration of 5 mM [1]. Meanwhile, as shown in Figure 18, Panel B, pre-treatment of BV2 cells with BHB co-administered with LPS showed a significant ability to reverse the increase in LPS-induced cell proliferation.

7.1.1.2 INFLUENCE OF VITAMIN E ON BV2 CELL VIABILITY

We tested different concentrations of Vitamin E in BV2 cells in a dose–response curve using an MTT assay with concentrations ranging from 50 μM to 400 μM (Figure 19). No cytotoxic effect was detected for any of the concentrations that were used. The lowest concentration that induced greater cellular viability was 100 μM, as also reported in some scientific studies [2]. This final concentration was chosen for the subsequent experimental tests. The MTT test was subsequently reproduced at 24 h with a Vitamin E concentration of 100 μM in the presence or absence of LPS at a concentration of 1 μg/mL, as shown in Figure 19.

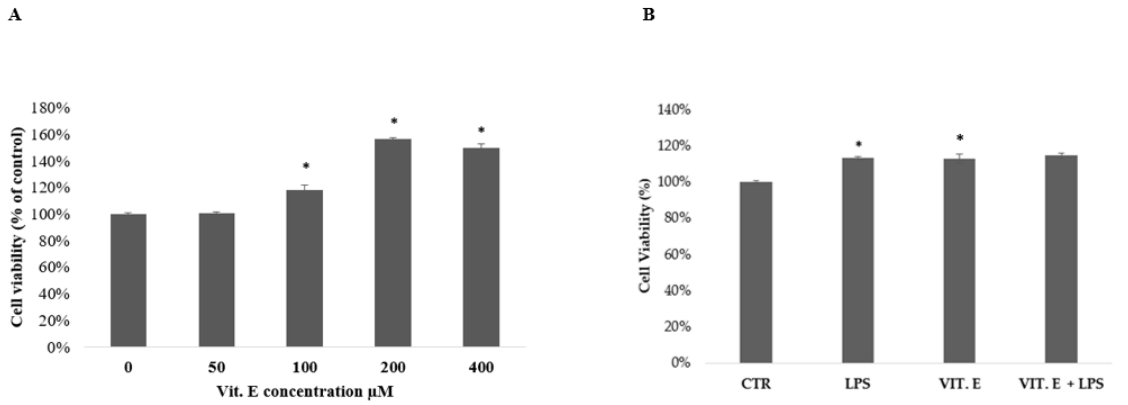

*Figure 19. Cell viability analysis of Vitamin E using an MTT assay. BV2 cells were treated with a Vitamin E dose–response curve from 50 μM to 400 μM (A). Vitamin E at a concentration of 100 μM was used to treat cells in the absence or presence of 1 μg/mL LPS (B). Data are reported as percentages compared to control values and are expressed as means ± SDs. * $p < 0.05$ compared to the control of the same time point.*

As shown in Figure 19, the treatment of BV2 microglial cells with Vitamin E alone did not substantially change cell viability, unlike the LPS treatment, which induced an increase in cell viability after 24 h of treatment [3]. Furthermore, Vitamin E co-administered with LPS did not show the ability to reverse the LPS-mediated increase in cell proliferation.

7.1.2 RESULTS OF CELL MORPHOLOGY

7.1.2.1 ANALYSIS OF THE EFFECT OF B-HYDROXYBUTYRATE ON BV2 CELL MORPHOLOGY

The results concerning the morphological analysis of the BV2 cells following the administration of BHB in the presence or absence of LPS (1 μg/mL) are shown in Figure 20, below.

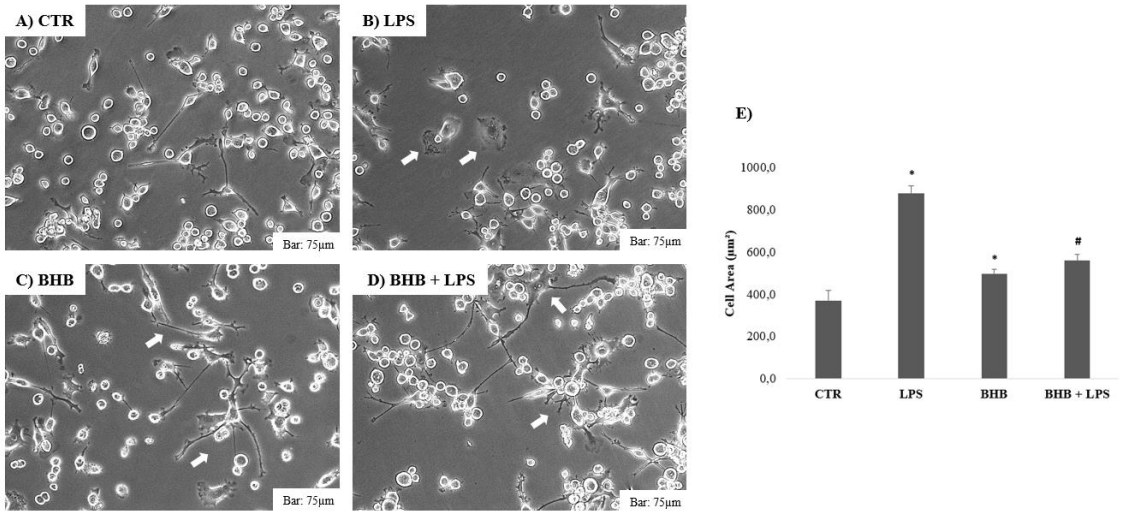

*Figure 20. Morphological assay after administration of BHB with or without LPS. Morphological assay of BV2 cells in different conditions: control (A), 1 μg/mL LPS (B), 5 mM BHB (C) and BHB with 1 μg/mL LPS (D). Bar: 75 μm (20× objective). The arrows indicate the cells that underwent morphological change. Cell areas expressed in μm² were quantified using ImageJ software (Panel E). Data are expressed 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 20, the control BV2 cells (Panel A) show the classic morphology of microglia in a "resting state", characterized by ramifications and small cell bodies [4]. Following proinflammatory or anti-inflammatory stimuli, microglia can assume either the M1 phenotype or the M2 phenotype, mediating functions to maintain the homeostasis of tissues [5]. The M1 phenotype, which is characterized by the absence of branches and an increased cell soma, as confirmed by various studies [6], can be induced by proinflammatory stimuli, such as LPS, as shown in Panel B. The M2 phenotype (alternative or anti-

inflammatory activation) occurred after treatment with BHB and is characterized by significantly elongated branches as compared to the control and a reduced soma (Panel C). The results show that the same condition obtained after pretreatment with BHB with the addition of LPS (Panel D). It was therefore observed that BHB had an anti-inflammatory effect against the microglial BV2 cells, restoring the anti-inflammatory phenotype. The results were also confirmed by the morphological analysis (Panel E): BHB was able to significantly reduce the increase in cellular area induced by the LPS condition, causing the microglial cells to maintain their initial morphology towards an anti-inflammatory state.

7.1.2.2 EFFECT OF VITAMIN E ON BV2 CELL MORPHOLOGY

Since microglia are macrophages capable of modifying their morphology following extracellular signals [7], we performed morphological tests to evaluate how BV2 cell morphology was affected after the administration of Vitamin E with or without LPS. The results are shown in Figure 21.

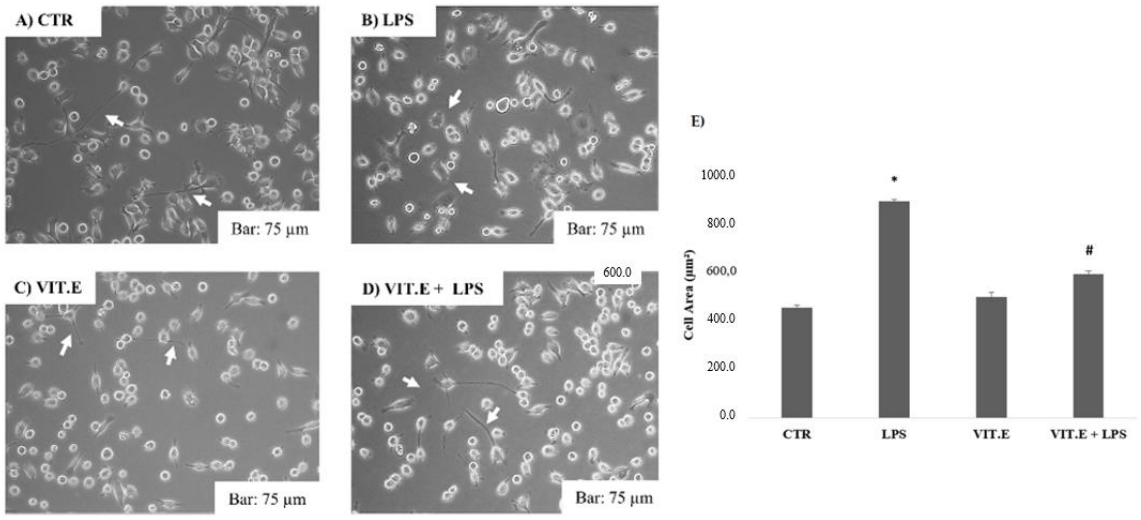

*Figure 21. Morphological analysis after administration of Vitamin E with or without LPS. Morphological analysis of BV2 cells in the control condition (A) and after 1 µg/mL LPS (B), 100 µM Vitamin E (C), or 100 µM Vitamin E in the presence of 1 µg/mL LPS (D). Bar: 75 µm (20 × objective). In the images, the arrows indicate cells that have undergone a morphological change. Cell areas (µm²) were quantified using ImageJ software bounded with Java8 64-bit (E). Data are expressed as the means ± SDs of the cell areas. * $p < 0.05$ compared to control. # $p < 0.05$ compared to LPS.*

Figure 21 shows that untreated cells, corresponding to the control condition (A), had the classic morphology of microglia in a quiescent state, with a small central body and many elongated cell extensions. As expected, based on current morphology studies [8], after treatment with LPS (B), the BV2 cells acquired an amoeboid morphology (linked to the pro-inflammatory condition), with increases in their soma and reductions in their prolongations, thus highlighting an amoeboid form. Treatment with Vitamin E (C) did not affect the cell morphology compared to the control, thus showing a branched morphology with

a small central soma (linked to the anti-inflammatory condition). Similarly, in the cells treated with Vitamin E and LPS (1 μg/mL) (D), it was noted that Vitamin E restored the amoeboid phenotype typical of LPS, causing greater branching of the distal branches. These results were also confirmed by an analysis of the cellular areas (E), which showed how the treatment with LPS increased the size of the BV2 cells, which is typical of the amoeboid form. In fact, they appeared to have significantly increased cellular areas compared to the control condition. Regarding the treatment of Vitamin E in the presence of LPS (1 μg/mL) (E), the results showed that Vitamin E can significantly reduce the increase in the cellular area induced by the LPS condition, inducing microglial cells to maintain their initial morphology, which is typical of the control condition.

7.2 RESULTS OF CELL WOUND CLOSURE ASSAY

7.2.1 B-HYDROXYBUTYRATE AND BV2 CELL WOUND-CLOSURE ASSAY

The results of the cell wound assay for BV2 cells following the administration of BHB in the presence or absence of LPS (1 μg/mL) are shown in Figure 22, below:

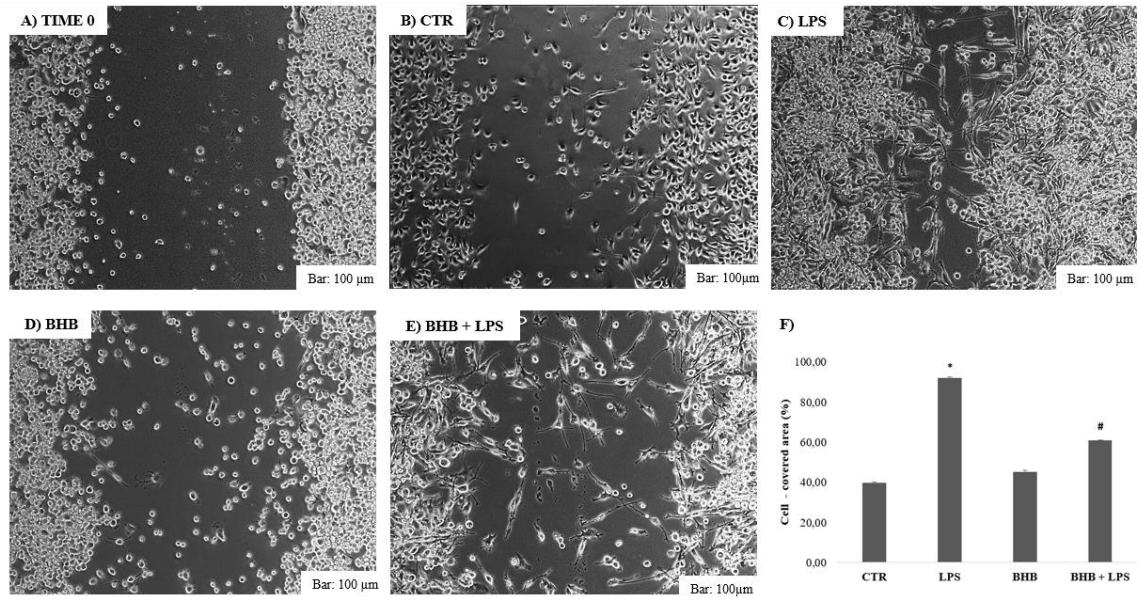

*Figure 22. BV2 cell wound-closure assay results following administration of BHB with or without LPS. The cuts on the BV2 cell monolayer were evaluated 24 h after treatment in the different conditions: BV2 cells at time 0 (A), BV2 control (B), LPS (C), 5 mM BHB (D) and BHB + LPS (1 μg/mL) (E). The images are representative of three independent replicates for each experiment. The results are expressed as means ± SDs of the percentages of wound closures compared to the 0-time condition (F), using ImageJ software. * $p < 0.05$ compared to CTR. # $p < 0.05$ compared to LPS.*

An increased migration capacity of microglial cells, as demonstrated, is mainly associated with inflammatory responses [5][9], as documented by several studies in the literature [10]. The results confirm that stimulation with LPS determines a greater migration of BV2 cells, as can be seen in Figure 22 (Panel C), which significantly reduced the free cell area of the cell monolayer after 24 h of incubation (Panel F). The application of BHB alone (Panel D) did not cause an increase in the migratory capacity of BV2 cells, while the pre-treatment with BHB

with the addition of LPS (Panel E) significantly reduced wound closure by reversing the proinflammatory effect of LPS (Panel E). BHB co-administered with LPS has a protective effect in microglial cells by reducing the migratory capacity induced by a proinflammatory stimulus.

7.2.2 VITAMIN E AND BV2 CELL WOUND-CLOSURE ASSAY

To assess the influence of microglial motility following Vitamin E treatment, we used a wound closure assay as a test, as described above. A cut was made with a scraper. The BV2 cells with the scratch created in the monolayer were allowed to migrate during a 24 h incubation. Consequently, the fixed and free cell areas were measured to understand whether the stimulus of Vitamin E in the presence or absence of LPS induced wound closure. The results are shown in Figure 23.

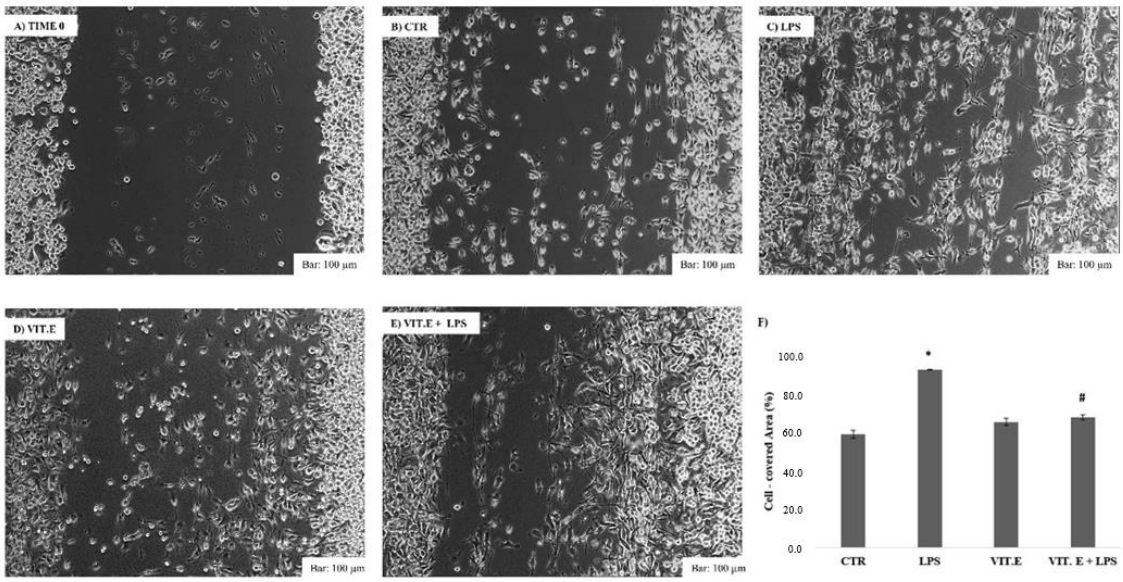

*Figure 23. Analysis of the migratory capacity of microglia following the administration of Vitamin E with or without LPS. A wound was procured from a sub-confluent layer of BV2 cells, and the resulting space was imaged at the time of the wound and 24 h after the treatment: BV2 cells at time 0 (A), 24 h after the cut in the control condition (B), with 1 µg/mL LPS (C), with 100 µM Vitamin E (D), and with 100 µM Vitamin E in the presence of 1 µg/mL LPS (E). The images are representative of an experiment with three independent replicates. The percentage of the wound gap was analysed using ImageJ software and subsequently plotted and statistically analysed as the percentage of wound closure compared to the 0 time condition (F). Values are presented as means ± SDs. Bar: 100 µm (10× objective). * $p < 0.05$ compared to control. # $p < 0.05$ compared to LPS condition.*

The results show that LPS stimulation induced a greater cell migration potential than that of control cells after 24 h of incubation (Panel C), significantly reducing the free wound surface after cutting. The application of Vitamin E (Panel D), as

shown in Figure 23, did not result in major and significant motility of the BV2 cells, showing a free wound surface almost comparable to the control condition after 24 h (Panel F), thus showing a migratory capacity markedly lower than in the LPS condition. Regarding the co-stimulation of Vitamin E with LPS, Figure 23 (Panel F) shows that Vitamin E caused a significant reversal of the LPS effect, leading to a significant reduction in microglial migration compared to BV2 cells treated only with LPS. Therefore, in both migration tests, Vitamin E acted as a modulator of the migratory capacity by reducing BV2 cell motility, which was enhanced by the application of LPS.

7.3 RESULTS OF ELISA TEST

7.3.1 B-HYDROXYBUTYRATE AND MICROGLIAL CYTOKINE EXPRESSION

The results of the ELISA tests of the expression levels of IL-17 and IL-10 by BV2 cells are presented in Figure 24.

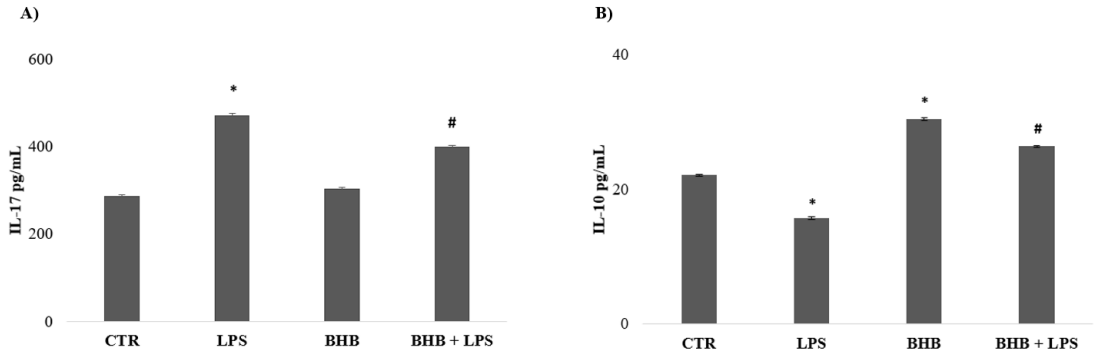

*Figure 24. Effects of BHB on cytokine expression. Analysis of cytokine expression, performed by ELISA assay, of IL-17 (A) and IL-10 (B) with stimulation of 5 mM of BHB in the presence or absence of LPS (1 μg/mL). Experimental data are expressed as the means (pg/mL) ± SDs of three independent experiments.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

Figure 24 (Panel A) shows the expression of IL-17, a proinflammatory cytokine, which was statistically higher in the condition of the cells treated with LPS than in the control condition. The BV2 cells treated with BHB + LPS, on the other hand, showed a significant decrease in the expression of IL-17 compared to the cells treated with LPS. Figure 24 (Panel B) shows the production of IL-10, an anti-inflammatory cytokine, which was significantly higher in BV2 cells treated with only BHB administration. Similarly, the expression of IL-10 in the condition of BV2 cells treated with BHB + LPS was found to be significantly higher than in the LPS condition.

7.3.2 VITAMIN E INFLUENCED MICROGLIAL CYTOKINE CONCENTRATIONS

The anti-inflammatory effect of Vitamin E on the concentrations of pro-inflammatory cytokines of BV2 cells was also evaluated. We analysed the levels of TNF-α and IL-10. The results are presented in Figure 25.

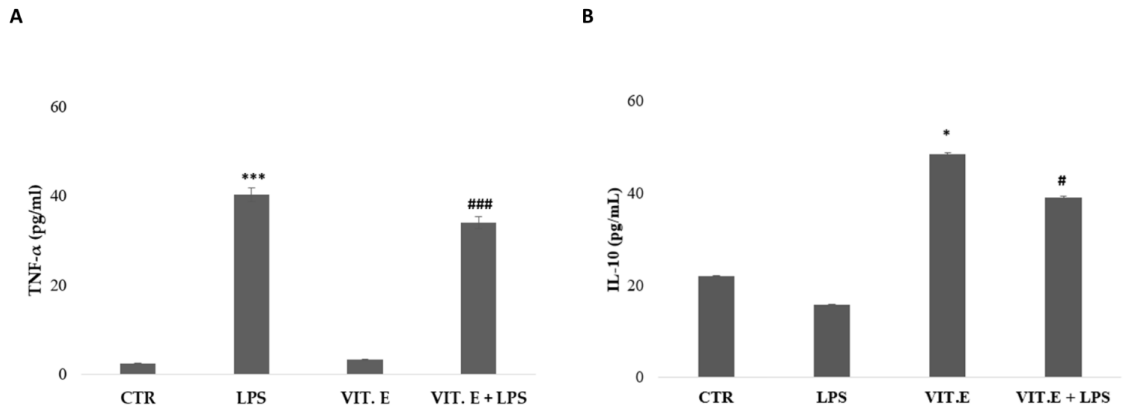

*Figure 25. Evaluation of cytokine concentrations following administration of Vitamin E with or without LPS. Analysis of cytokine concentrations (TNF-α (A) and IL-10 (B)) with stimulation of 100 μM Vitamin E with or without LPS (1 μg/mL). Data are expressed as means (pg/mL) ± SDs. (A) *** $p < 0.001$ compared to the control condition. ### $p < 0.001$ compared to the condition of LPS-stimulated microglia. (B) * $p < 0.05$ compared to the CTR. # $p < 0.05$ compared to the LPS condition.*

As shown in Figure 25, Panel A, the concentration of TNF-α was significantly lower in the control condition and in cells treated with Vitamin E, with almost overlapping results. In the condition of cells treated with LPS, the expression of TNF-α significantly increased compared to the control cells. BV2 cells treated with Vitamin E + LPS showed a lower concentration of TNF-α that was significantly reduced compared to the LPS condition. At the same time, as shown in Figure 4B, the production of IL-10, an anti-inflammatory cytokine responsible for modulating and regulating the immune response, was significantly increased in vitamin-E-treated BV2 cells. Furthermore, the concentration of IL-10 in the Vitamin E + LPS condition was significantly higher than in the LPS condition.

7.4 RESULTS OF WESTERN BLOT ANALYSIS

7.4.1 EFFECTS OF VITAMIN E ON THE EXPRESSION OF PRO-INFLAMMATORY AND ANTI-INFLAMMATORY MARKERS OF ACTIVATED MICROGLIA

The expression levels of markers of the M1 phenotype (CD40) and the M2 phenotype (CD206) were determined using a Western blot analysis. The results, reported in Figure 26, Panel B and C, show that the LPS treatment of BV2 cells induced significant increases ($p < 0.05$) in the pro-inflammatory CD40 and the anti-inflammatory CD206 molecules in comparison to control. We next examined the changes in protein expression after the treatment with Vitamin E.

Interestingly, a densitometric analysis of the immunoblotting bands revealed that Vitamin E caused a significant reduction ($p < 0.05$) in the CD40 protein expression levels, indicating that this compound can negatively modulate this pro-inflammatory marker. Conversely, CD206 showed a significant increase ($p < 0.05$) in microglia in response to the Vitamin E treatment in comparison to the cells stimulated with LPS alone, suggesting that the M2 phenotype may represent the predominant phenotype among BV2 cells (Figure 26). Since the TLR4/Myd88 signalling pathway mediates the inflammatory response, leading to the production of pro-inflammatory cytokines, we evaluated whether the signal mediators TLR4 and p-AKT underwent a modulation in terms of protein expression levels. We found that the expression of TLR4 and p-AKT increased significantly in the presence of LPS ($p < 0.05$) compared to control. As shown in Figure 26, Panel A and B, the pre-treatment with Vitamin E caused a significative downregulation of both TLR4 and p-AKT ($p < 005$) (Figure 26, Panel D and E), suggesting that Vitamin E may act as a modulator of inflammatory responses targeting the TLR4/Myd88 signalling pathway.

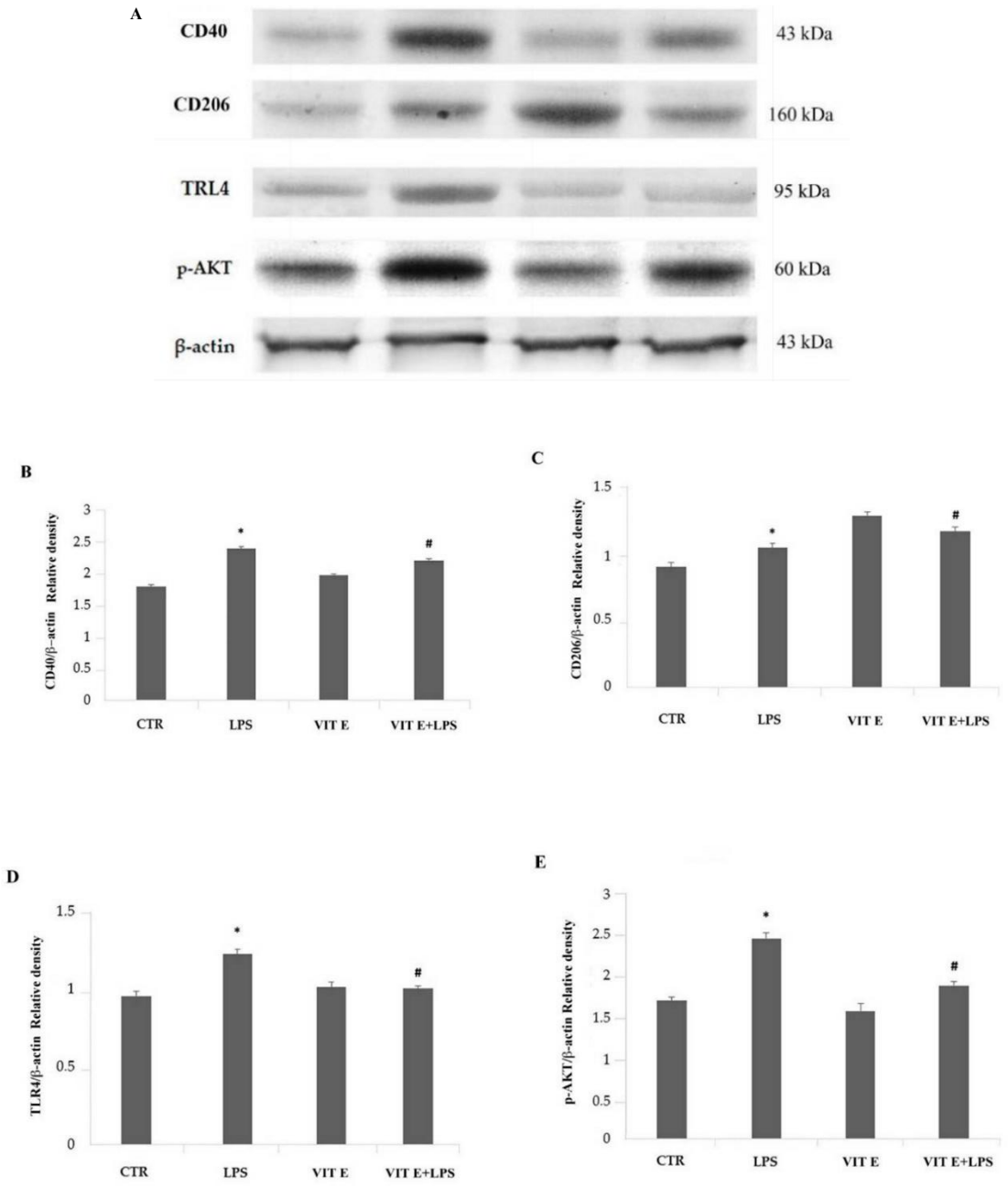

Figure 26. Evaluation of CD40, CD206, TLR4, and p-AKT expression following administration of Vitamin E with or without LPS. Representation of Western blotting detection (A) and densitometric analysis of the expression of the pro-inflammatory CD40 (B), anti-inflammatory CD206 (C), TLR4 (D), and p-AKT (E) in control cells (CTR), BV2 cells treated with Vitamin E (VIT E), BV2 cells treated with LPS (LPS), and BV2 cells treated with Vitamin E + LPS (VIT E + LPS). Protein expression values are expressed in arbitrary units after normalization against β-actin. Data are presented as means ± SDs ($p < 0.05$ vs. CTR; # $p < 0.05$ vs. LPS).*

7.5 RESULTS OF APOPTOSIS ASSAY ON LUNG CANCER CELL LINES

7.5.1 EFFECT OF DIFFERENT FORMULATIONS OF LITHIUM AND ACETOACETATE ON A549 AND PC9 APOPTOSIS ASSAY

In Figure 27, Panel A and Panel B, are shown the results for the cytotoxicity assay evaluated by flow cytometry of A549 and PC9 cells:

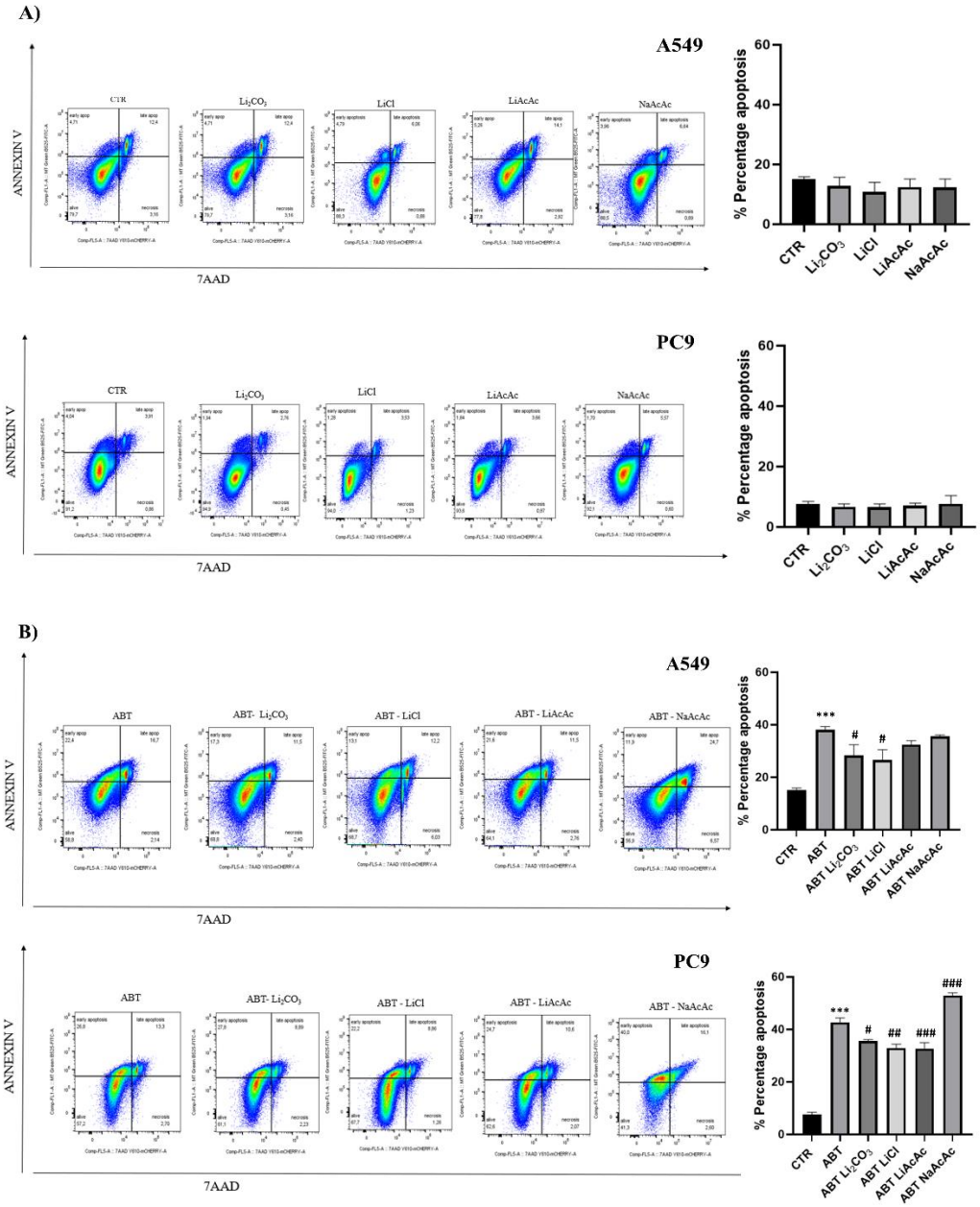

Figure 27. Evaluation of ABT737-induced apoptosis in the absence or presence of LiAcAc, NaAcAc, LiCl, Li_2CO_3. The results show flow cytometric analysis of A549 and PC9 cells treated with 24h pretreatment

*of 5mM Lithium AcetoAcetate, NaAcAc, LiCl and 2.5mM Li_2CO_3 in basal conditions (A) and following stimulation with 5uM ABT737 for 24h (B). Results are expressed as the mean of three independent experiments ± SEM. *p-value <0.05 compared to the control, #p-value <0.05 compared to ABT737.*

As shown in Figure 27, Panel A, compounds such as LiCl, Li_2CO_3, LiAcAc, and NaAcAc showed no significant changes on basal cell apoptosis in A549 and PC9 cells. In Figure 27, Panel B, it is shown how treatment, as expected, with ABT737 induced cell apoptosis significantly in both cell lines; furthermore, for A549 cell line, pretreatment with LiCl and Li_2CO_3 significantly reduced apoptosis, while pretreatment with LiAcAc and NaAcAc did not modulate ABT737-induced apoptosis. At the same time, interestingly, LiCl and Li_2CO_3 significantly reduced PC9 cell apoptosis, and pretreatment with LiAcAc significantly reduced apoptosis, while NaAcAc increased PC9 cell death.

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8. DISCUSSION

The popularity of organic foods is growing systematically, especially in recent decades, thanks to a greater consumer perception of food quality [1]. In recent years, numerous research and meta-analyses have been published regarding organic products and their consumption, together with their effect on the health of the consumer, as organic foods generally appear to be characterized by greater nutritional qualities [2]. Organic foods of plant and animal origin have fewer traces of pesticides and synthetic residues [3], a lower content of nitrates and heavy metals such as cadmium and lead [4] but at the same time a greater content of compounds with an antioxidant action such as polyphenols, phenolic acids, flavanones, stilbenes, anthocyanins [4], vitamins such as Vitamin A, C, E [3], and a greater intake of minerals such as zinc, magnesium, copper, selenium etc [3]. Similarly, as regards organic products of animal origin, they are characterized by a higher content of unsaturated fatty acids, Omega-3s [5], beneficial for the individual's health, a higher protein content [6], Vitamin D and group B [7,8], and other compounds with antioxidant action [6]. Currently, compounds with antioxidant activity have received greater attention from scientific evidence for their possible health benefits associated with the increase in consumption of crops and foods rich in these compounds, especially for preventive purposes of chronic pathologies, such as cardiovascular diseases [9], some types of tumors [10], but especially for neurodegenerative diseases [11]. Similarly, KD, a very low carbohydrate diet through which the body induces the production of ketone bodies, has long established itself as a new dietary and therapeutic approach for the treatment, initially of intractable epilepsy [12], and nowadays rapidly gained the attention of scientific studies in the last decade, thanks to the promising therapeutic potential of KD for various pathologies [12]. The beneficial effects of KD are mainly associated with the production of ketone bodies such as AcAc and BHB following a period of caloric deprivation, being used as a caloric substrate

to provide energy to the cells of the body and mainly to the brain [13], being able to easily cross the BBB via the cell walls of the capillaries [13]. KD shows potential to modulate microglial activation [14]. As reported in numerous studies, microglia, immune cells resident in the CNS, are activated in response to stimuli coming from the surrounding brain environment [15] modifying their phenotype towards the proinflammatory M1 type, characterized by an amoeboid form with increased cytoplasm and reduced branching [54], or the anti-inflammatory M2 phenotype [16], with elongated cell processes and reduced body. Excessive activation of microglia in a proinflammatory state contributes to neuronal damage, the main cause of cognitive impairment [17,18]. When activated in an M1 state, the expression of the proinflammatory enzymes iNOS and COX-2 increases, along with increased production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [19], and a marked migratory capacity induced by Akt/STAT3 signalling pathways [20]. These factors appear to be the main factors contributing to a greater likelihood of neuronal degeneration [21]. In contrast, M2-type activation is mediated by interleukins, such as IL-4 and IL-13 [22], determine the expression of cytokines and receptors involved in the inhibition of microglial inflammation and the restoration of homeostasis in brain environment [19]. This includes the production of anti-inflammatory interleukins, such as IL-10, or the factors TGF-β, VEGF, EGF and Arg1 [23], and a reduced migratory capacity [24]. Therefore, inhibition of microglial activation could be a key therapeutic strategy to improve cellular states and reduce senescence processes in neuronal cells [25] that are defining the hallmarks of neurodegenerative diseases. Research in recent years has demonstrated how the ketone body BHB can modulate the microglial inflammatory response [26], reducing the likelihood of developing neurodegenerative diseases [27], equally improving body composition [28], improving metabolic health [29,30] and presenting anti-aging potential [31,32]. These results were also confirmed by the results of this study, in fact, it was observed that BHB exerted anti-inflammatory power in BV2

microglial cells by modulating the inflammatory response induced by the proinflammatory stimulus, LPS, indicating its possible neuroprotective role against LPS-induced reactive microglia. The results confirm that BHB can modulate the polarization of BV2 from an M1 (proinflammatory) phenotype towards an M2 (proinflammatory) phenotype, reducing the migratory capacity and the production of proinflammatory cytokines, such as IL-17, associated with causes of chronic inflammation and neuronal damage [33]. Likewise, BHB helps increase levels of proinflammatory cytokines, such as IL-10, a key factor in maintaining microglia in an anti-inflammatory state. It can therefore be stated that pre-treatment with BHB before stimulation with LPS prevented the retraction of the microglial cellular processes, with consequent acquisition by the microglia of a branched morphology typical of the M2 inflammatory state [34], reduction of the migratory capacity and modulation of cytokine production in LPS-induced BV2 cells [35,36]. The mechanisms of action remain unclear; however, previous research has shown that BHB can inhibit the expression of the NLR family, particularly the NLRP3 inflammasome, which is involved in microglial inflammation processes [37], resulting in decreased production of proinflammatory secondary metabolites, such as cytokines IL-1β, TNF-α, ROS, iNOS and COX-2 [38]. BHB suppresses LPS-induced inflammation in BV2 cells by inhibiting NF-κB activation and the subsequent increase in glutathione synthesis [14] caused by increased NADH oxidation [39]. Regarding migratory capacity, in some studies in the literature it has been hypothesized that cells stimulated by LPS undergo increased levels of proinflammatory cytokines and/or AKT/STAT3 signalling, while, on the contrary, antioxidant compounds, including BHB, Due to their anti-inflammatory effects, they strongly inhibit LPS-induced BV2 cell migration by inhibiting NF-κB/STAT3, as summarized in Figure 22 [40].

Similar results were also obtained for Vitamin E, which as detailed previously, is a fat-soluble vitamin consisting of four tocopherols and four tocotrienol

derivatives. Each group can be further divided into four different isomers, namely α, β, δ and γ, depending on the presence and position of one or more methyl groups as the side chain [41]. Vitamin E, particularly α-tocopherol, can be easily obtained and consumed by most of the world's population as it is naturally present in various foods. Vitamin E is present in many foods and food groups [42] of the KD and in greater quantities in products of biological origin [43], but with controversial effects in the literature, an anti-inflammatory action, mainly for neurodegenerative diseases, in fact different nutraceutical foods can modulate microglial cells in the CNS [44,45], but at the same time with a pro-oxidant in the case of other pathologies such as cardiovascular diseases and cancer [46]. Despite the studies present in the literature regarding the dual nature of Vitamin E, through the results obtained from this study we can state how Vitamin E does not seem to have a cytotoxic potential towards BV2 microglial cells, as it did not cause alterations in cell viability. Furthermore, it appears that Vitamin E significantly suppressed LPS-induced microglial activation at the morphological and migratory levels and in terms of the pro-inflammatory cytokine production, as observed for TNF-α. Furthermore, Vitamin E modulates the LPS-mediated activation of TLR4, with a consequent reduction in the CD40 molecule acting on the PI3K/AKT pathway. It is well known that LPS is an endotoxin. It is a component of the bacterial membrane [47] and has been described as a classical stimulus for microglial activation [48]. Basically, LPS binds to the TLR4 receptor, a receptor mainly expressed by microglial cells [49] that is responsible for the inflammatory cascade. Members of the TLR family play a key role as regulators of both the innate and adaptive immune responses [50], activating pro-inflammatory signals such as NF-κB and Akt [51] to induce the production of pro-inflammatory metabolites such as TNF-α, IL-6, and NO [52,53], with the subsequent maturation of antigen-presenting cells (APC) [54,55]. Furthermore, LPS induces the expression of the CD40 gene, which is a member of the TNFR family and is mainly expressed by cells such as macrophages,

microglia, and dendritic cells as well as endothelial and tumor cells. The interaction between CD40 and its ligand is fundamental and is responsible for a pro-inflammatory immune response [56,57] with the production of pro-inflammatory cytokines and the enhancement of costimulatory molecule expression [58]. With our results, we can affirm how Vitamin E positively involves the suppression of basic signal transduction events involved in the activation of microglia, such as the reduction in TRL4 activation and the reduction in CD40 expression, resulting in the modulation of the PI3K/AKT pathway, with a consequent reduction in the expression of pro-inflammatory cytokines such as TNF-α, thus enhancing the idea of protection against a pro-inflammatory and potentially neurotoxic state, which is already supported by some studies in the literature [59,60]. Furthermore, although our results did not show a strong ability to upregulate the expression of CD206, a typical anti-inflammatory marker, its reduction was on the verge of significance, suggesting a more favourable action for the alternative response of microglia in the vitamin E treatment. Finally, if it is true that antioxidants reduce inflammatory processes, Vitamin E should also be characterized by the ability to reduce the migration of BV2 microglial cells and the consequent phenotypic change from the activated form to the resting form. Thus, the result that Vitamin E and BHB can play a neuroprotective role by modulating microglial functions opens possible future scenarios for their therapeutic use for neurodegenerative diseases. Furthermore, a growing number of preclinical and clinical studies report anti-tumor effects, through the intervention of metal ions and respective formulations, used in the regulation of the immune microenvironment of the tumor [61,62]. These mechanisms could offer advantages for the future use of metal ions in tumor therapy. Lithium, in particular, is a drug used as a gold standard for bipolar disorder (BD) and psychosis [63,64], as it could directly inhibit inositol mono-phosphatase (IMPase) preventing subsequent inositol production [65] and consequently reducing the signal transmission of aberrant neurotransmitters in

pathological regions [66, 67,68]. Despite this, the results are still controversial and at the same time the use and diffusion of its formulations such as LiAcAc, Li_2CO_3, LiCl in clinical practice is still limited due to possible side effects and potential toxicities. However, at the same time, AcAc, one of the main ketone bodies produced following glucose deprivation, has recently aroused interest for its use in the treatment of tumors thanks to its formulations, commonly used in the form of lithium or of sodium salts, thanks to its ability to modulate tumor metabolism, for example influencing its growth through the inhibition of ATP production and the promotion of apoptosis. In this context, different formulations of AcAc and lithium, such as LiAcAc, NaAcAc, LiCl and Li_2CO_3, have been studied as treatment or adjuvant of different types of cancer, following the induction of apoptosis by ABT737, BH3-mimetic with a pro-apototic action [69]. Our results showed how treatment with ABT737 induced cell apoptosis of both cell lines. A pretreatment with NaAcAc did not modulate ABT737-induced apoptosis in A549 cells but increased PC9 cell death. LiAcAc significantly reduced apoptosis of PC9 cells. Interestingly, LiCl prevented the apoptosis of these two lung cancer cells, while Li_2CO_3 significantly reduced the apoptosis of A549 cells. Taken together, these results suggest that the effects observed using LiAcAc are independent of AcAc and can be attributed to lithium. This preliminary study needs further investigation but highlights the weakness of the potential therapeutic use of lithium and AcAc formulations in a precise context, as it could depend, among other parameters, on the cell type, once again showing the need for further research and insights into this field.

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9. CONCLUSION

Our study highlights interesting aspects regarding the action of some compounds present or produced directly by KD. Considering the results that emerged in this study, we can state that ketone bodies and in particular BHB are generally considered a source of energy for tissues during periods of caloric deprivation and/or adherence to a low carbohydrate diet, such as KD. In fact, in addition to being a caloric source, BHB has many beneficial effects, especially on a brain level. BHB, therefore, could act as an anti-inflammatory agent at the microglial level and could be involved in neuroinflammation and neuroprotective action, although the mechanisms are still partially unknown. We postulate that BHB could be a key molecule in the prevention of neurodegenerative diseases. Furthermore, BHB being a product of KD may indirectly provide evidence for the potential role of the ketogenic diet in neuroinflammation and neuroprotection, although further studies are needed to clarify the molecular mechanisms involved. As regards Vitamin E, a compound present in greater quantities in KD and organic products, the results highlight how, in the presence of a proinflammatory stimulus, Vitamin E can modulate microglial responses from a morphological point of view. functional by reducing microglial migratory properties and influencing the expression of typical proinflammatory markers. Furthermore, Vitamin E has been shown to be able to inhibit the activation of pro-inflammatory molecules and patterns by reducing the production of secondary metabolites typical of the neuroinflammatory response, suggesting its use as a dietary supplement for the treatment of neuroinflammation. Finally, in addition to the treatment of neurodegenerative pathologies, further studies are needed on the use of some compounds and formulations such as LiAcAc, NaAcAc, LiCl and Li_2CO_3 as adjuvants in the treatment of pathologies such as cancer. In this context, greater attention must be applied to the different compositions and formulations of lithium and AcAc,

which, despite being considered new therapeutic frontiers, require further studies due to their potential toxic effects. Our results suggest that the anti-apoptotic or pro-apoptotic effect observed using these different formulations could be dependent on various factors, including cell type and cancer origin, underlines the weakness in the potential therapeutic use of lithium and AcAc formulations in this precise context.

LIST OF PUBLICATIONS

ARTICLES IN SCIENTIFIC INTERNATIONAL OR NATIONAL JOURNALS REFEREE SYSTEM RELATED TO Ph.D PROJECT

- Ruggiero M, Panaro MA, la Torre ME, Messina G, Porro C, Villano I, Monda V, Polito R, Benabeur T, Monda M, Messina A. Effects of tocopherols and tocotrienols on microglia-mediated neuroprotection. Natural Molecules in Neuroprotection and Neurotoxicity. 2024.

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OTHER ACTIVITIES

- Participation in the PRA Unifg project “Vitamin E, Neuroinflammation, Neuroprotection, Microglia, Neurodegenerative Diseases”.

- Participation in the project “Lifestyle & Planet life”: migliorare la propria vita salvando il pianeta” in collaboration with ADISU Puglia.