The role of nutritional factors in cognitive aging of older adults

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Index

Abbreviations

•OH: hydroxyl radical

$1/2O_2$: singlet oxygen

13’-OH:13’-hydroxychromanol

24-h-DR: 24-h dietary recall

3’- COOH: terminal metabolite 3’-carboxychromanol (3’- COOH)

AD: Alzheimer disease

ADL: Activities of daily living

APP: amyloid Precursor Protein

ATF4: factors: factor-4

ATP: adenosine triphosphate

Aβ: β amyloid peptide

BBB: blood-brain barrier

BER: base excision repair

BMI: body max index

CAT: Catalase

CEHCs : (2’-carboxyethyl)-6-hydroxychromans

CES-D: Center for Epidemiological Studies Depression Scale

CI: confidence interval

CmR: chylomicron remnants

CNS: central nervous system

COPD: Chronic obstructive pulmonary disease

CoQ: Q coenzyme

DHA: Docosahexaenoic acid

DNA: deoxyribonucleic acid

DSM III-R: diagnostic and statistical manuals of mental disorders, version III-R

ERO1: ER oxidoreductin 1

Fe: iron

Fe^{3+} - $O_2^{-\bullet}$: perferryl radical

FFQ: Food Frequency Questionnaire

FOXO1: Forkhead box protein O1

GI: Gastrointestinal

GSH: Glutathione

GST: Glutathione transferase

GTPx: Glutathione peroxidase

H_2O_2: hydrogen peroxide

HDLs: high-density lipoproteins

HIF-1: hypoxia-inducible factor 1

HPLC: high performance liquid chromatography

HR: Hazard ratio

IADL: Instrumental Activities of daily living

IGF: insulin growth factor 1

IL6: Interleukin 6

InCHIANTI: Invecchiare in CHIANTI

INRCA: Italian National Research Center on Aging

LDLs: low-density lipoproteins

LRP1: low-density lipoprotein receptor-related protein 1

MCI: Mild cognitive impairment

MDA: malondialdehyde

MMSE: Mini Mental State Examination

Mn-SOD: manganese superoxide dismutase

MUFA: monounsaturated fatty acids

NAD: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate (reduced form)

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIH, NIA: National Institute on Aging

NO: nitric oxide radicals

Nrf2: 2-related factor 2

$O_2^{\bullet-}$: superoxide anion

OGG1: enzyme 8- oxoguanine glycosylase

OH: hydroxyl groups

OR: odds ratio

PDI: protein disulphide isomerase

PPARγ: peroxisome proliferator-activated receptor

PRX: Peroxiredoxin

PUFA: polyunsaturated fatty acids

ROS: reactive species of oxygen

SD: standard deviation

Sirt1: sirtuin-1

SOD: Superoxide dismutase

SPPB: short physical performance battery

SRB1: scavenger receptor class B type 1

SU.VI.MAX2 study: Supplementation en Vitamines et Mireraux Antioxydants 2 study

TLR4: Toll-like receptor 4

TNFα: tumor necrosis factor α

TRX: Thioredoxin

UCP2: Uncoupling protein 2

VaD: vascular dementia

VLDLs: very low-density lipoproteins

α-TT: α-tocopherol transfer protein

List of publications performed during the PhD period

Since I have been working in a geriatric research center, and collaborating with other research groups, I contributed to the following publications. Some of them are not strictly related to the thesis topic but still focused on older adults and on various aspects of aging:

1. A. Cherubini, B. Carrieri, P. Marinelli. Advantages and disadvantages of direct oral anticoagulants in older patients. Geriatric Care 2018; 4:7227. DOI: 10.4081/gc.2018.7227

2. N.J. Peladic, P. Orlandoni, G. Dell'Aquila, B. Carrieri, et al. Dysphagia in nursing home residents; management and outcomes. J Am Med Dir Assoc. 2019 Feb;20(2):147-151. doi: 10.1016/j.jamda.2018.07.023

3. C. Del Bo', S. Bernardi, M. Marino, M. Porrini, M. Tucci, S. Guglielmetti, A. Cherubini, B. Carrieri, et al. Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?. Nutrients 2019, 11, 1355; doi:10.3390/nu11061355

4. M. Fedecostante, G. Onder, P. Eusebi, G. Dell'Aquila, E. Zengarini, B. Carrieri, et al. "Predictors of functional decline in nursing home residents: The Shelter Project". J Gerontol A Biol Sci Med Sci. 2020 Jul 13;75(8):1600-1605. doi: 10.1093/gerona/glz296

Abstract

In the last century, due to progresses made by medicine, public health and social-economic conditions, life expectancy at birth increased in high income countries, including Italy, from 45 years to more than 80 years. Aging is a risk factor for chronic diseases which are mistakenly considered an unavoidable stage of aging. The increase in the prevalence of these diseases at a young age means that they are also related to an inadequate lifestyle. In this regard, the reduction of risk factors for certain chronic diseases can lead to a significant reduction in the risk of developing them. The prevalence of cognitive impairment increases with age, and in one third of the cases, it worsens to dementia which is a highly disabling condition associated with high healthcare and society costs. From a public health perspective, it is of primary importance to try to prevent or postpone the onset of cognitive decline and to try to slow its progression to dementia. Given that no pharmacologic treatment can prevent or delay cognitive impairment and dementia, it is mandatory try to intervene on modifiable risk factors, such as nutrition. Oxidative stress appears to play a key role in the pathogenesis of cognitive decline and dementia. The aim of the work carried out during the PhD was to investigate the role of nutritional factors in the cognitive decline associated with aging. Particular interest was addressed to nutrients with antioxidant action such as vitamin E and polyphenolic compounds. Vitamin E, in particular α-tocopherol, is a vitamin introduced with the diet with high antioxidant potential. However, there are conflicting evidence in the literature regarding its association with cognitive decline and dementia. The PhD activity was therefore aimed at verifying the presence of an association between plasma levels of vitamin E and cognitive impairment, in the older adult population, not demented at baseline, participating in the InCHIANTI study (Aging In CHIANTI area), followed for up to 17 years. Our results confirmed the presence of an association between plasma vitamin E level and cognitive status: the increase of 10 μmol / L of vitamin E was associated with a reduced HR of cognitive impairment by 20%. In addition, the research activity carried out was aimed at investigating the role of polyphenol introduced with the diet on chronic diseases that can be risk factors for cognitive impairment. This activity led to the publication of a systematic review entitled “Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?” which focuses on another important antioxidant, polyphenols, and chronic diseases which can be risk factors for cognitive impairment. In this review we reported the inverse association between polyphenols intake, and several outcomes of health and diseases risk. Because to the high heterogeneity of the studies, it was not possible to define a recommended range of daily polyphenol intake. In conclusion, the results

obtained confirmed the role of diet as a risk factor for cognitive impairment. Therefore, it is possible to recommend a diet rich in antioxidants (vitamin E and polyphenols) as a preventive strategy to promote healthy cognitive aging.

1. Introduction

1.1. Aging, chronic diseases and nutrients

The aging of the population is a new phenomenon in human history. In the 1950 there was no country with a percentage of older adults (i.e., age ≥ 65 years) higher than the 11% *(Rudnicka et al. 2020).* In the last century due to progresses made by medicine and public health, life expectancy increased from 45 years to around 80 years *(Cherubini et al. 2005)*. In the 2019 there were 703 million older adults in the world. This number is expected to double in 2050, when projections forecast that one in six people will be an older adult *(United Nations 2019)*. In high income country is estimated that the older adults are 15% of the whole population. This numbers are rapidly increasing (*Livingston et al.2017)*. Aging is a risk factor for chronic diseases, and it is also associated with increased of healthcare costs. With the increase in life expectancy an important impact on the society is expected, such that interest is growing in implementing initiatives aimed at slowing down or even reversing the aging process (*Marsman et al.2018)*. Aging is defined as the result of the accumulation of negative changes in cells and tissues, which occurred over the entire life of an individual, leading to an increased risk of disease, disability and death *(Tosato et al. 2007),* whereas healthy aging is defined as "the process of developing and maintaining the functional ability that enables wellbeing in older age" (*Rudnicka et al.2020, Michel and Sadana 2017, Fallon and Karlawish 2019)*. The abilities of satisfying own needs, to learn, to make decisions, to have relationship, to move, and give own contribute to society are all functional abilities. These abilities are the results of intrinsic capacity and environmental characteristics. Aging is associated with a decline in intrinsic capacity *(Rudnicka, et al. 2020)*. The goal of the society should be the optimization of intrinsic and functional capacities, because healthy aging does not mean being without diseases *(Marsman et al. 2018)*, but increase the years being active and healthy. Chronic diseases are considered an unavoidable stage of aging, however, the increase in the prevalence of these diseases at a young age means that they are also related to an inadequate lifestyle. In this regard, it has been seen that the reduction of risk factors for certain chronic diseases leads to a significant reduction in the risk of developing them *(Shlisky et al.2017).* Nutrition is an important modifiable risk factors for several age-related diseases, among which cardiovascular, metabolic, muscular diseases (*Everitt et al.2006)*. Here is reported a summary of scientific evidence for the main group of nutrients:

-**proteins**: in order to prevent musculoskeletal diseases as sarcopenia and osteoporosis, an adequate intake of proteins is needed. Higher proteins consumption is associated with increased proteins

synthesis. Some studies focused on the leucine supplementation in older adults in order to prevent sarcopenia, but the results obtained were not conclusive *(Gaffney-Stomberg et al.2009, Paddon-Jones and Rasmussen 2009).*

-fats: monounsaturated (MUFA) and polyunsaturated (PUFA) fats are useful to improve cardiovascular risk and insulin sensitivity. On contrary, trans fatty acids negatively affects cardiovascular system. Long chain **Ω**-3 fatty acids reduce oxidative stress and inflammation protecting brain. However, mixed results are available on the effect of fish **Ω**-3 fatty acids on cognitive status. Fish **Ω**-3 fatty acids can positively affect bone, muscles and immune system *(Ning Mak and Caldeira 2014).*

-carbohydrates: some studies suggest that the control of glycemia can be useful to prevent cognitive decline, furthermore observational studies linked diabetes with cognitive decline *(Ning Mak and Caldeira 2014).*

-micronutrients: older adults tend to be at risk of micronutrient deficiency because the reduction of foods rich of them. Micronutrients are important to maintain healthy cognitive status and normal physical functions **(Table 1).**

Table 1. Micronutrients role and deficiency consequences *(Ning Mak and Caldeira 2014)*

Micronutrient	**Scientific evidence**
Vitamin B6, B12 and folic acid	Low vitamin B is associated with high homocysteine and poor cognitive function, high risk of dementia, vascular diseases. Mixed results are available on vitamin B supplementation and the reduction of the risk of the fore mentioned diseases.
Vitamin D and calcium	A deficiency of Vitamin D is associated with calcium malabsorption, metabolic syndrome, muscle weakness, cardiovascular disease, and mortality. Low levels of vitamin D or calcium are associated with bone problems as osteoporosis and fragility fractures. The supplementation of calcium plus vitamin D in older adults seems to be more effective in the reduction of fracture than the supplementation with vitamin D alone. This benefit has been found primarily in institutionalized older adults. However other studies shown an increased risk of kidney stones when the combination of vitamin D and calcium was administered. Inconsistent results are available on the increased risk of cardiovascular events for this combination of supplements.
Vitamin with antioxidant role (i.e., vitamin A, C, E)	A diet rich in these vitamins can reduce the risk of cardiovascular events, dementia, cognitive decline and eye pathologies. Moderate evidence exists on the reduced progression of age-related macular degeneration with antioxidants supplementation. Inconclusive evidence is available on the use of vitamin E for the protection of cognitive status and brain health. Inconsistent results have been found on the effect of antioxidants and prevention of cardiovascular diseases.
Other antioxidants (i.e., Selenium and Zinc)	Low selenium is associated with cognitive impairment, increased risk of mortality and poor immune function. Further studies are needed in order to confirm the association between selenium and chronic pathologies. Low zinc is associated with neuronal injuries typical of Alzheimer disease (AD), whereas there is mixed evidence on the positive effect of zinc in eye health. Zinc seems to reduce infections and the potentially related mortality. When taken within a day from the cold it can reduce the duration of symptoms but not the severity.

-**polyphenols:** catechins from black tea can reduce the low-density lipoproteins (LDLs) and blood pressure. Green tea is associated with reduced cholesterol, LDLs cholesterol and blood pressure. It is not clear if catechins can reduce the risk of cardiovascular disease. Resveratrol (sources are grapes, medical plants and red wine) in animal studies improve performance, reduce fasting glucose, increases insulin sensitivity, increases the duration of life and ameliorates performance, prevents liver injuries and improves performance. Few and recent studies have been carried out in order to investigate the benefits of resveratrol supplementation on human health, however findings obtained are not sufficient to recommend this type of supplementation to increase lifetime and chronic pathologies *(Ning Mak and Caldeira 2014).*

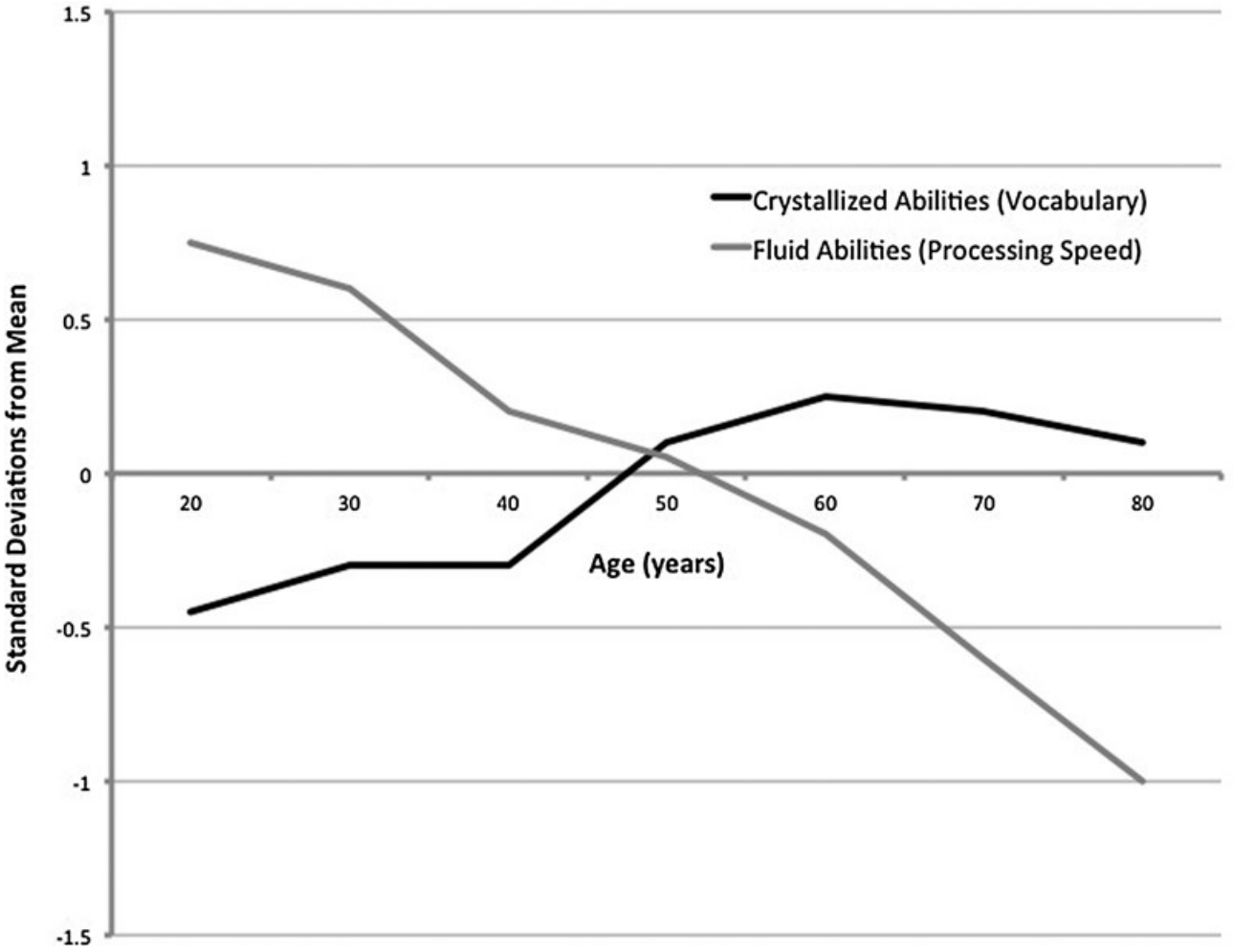
-**probiotics and prebiotics**: modifications in gut microbiota type are associated with metabolic diseases as diabetes and inflammation. The variety of microbiota is reduced with aging, therefore prebiotics (i.e., non-digestible molecules used by probiotics) and probiotics (i.e., *Lactobacilli* and *Bifidobacteria* which can be found in yoghurt and fermented milk-based beverages) can help maintaining intestinal health. Despite few studies investigated probiotics and prebiotics in older adults, some positive effects have been shown (i.e., reduce constipation, prevent the use of antibiotics in diarrheas, increase immunity and therefore reduce allergies and infections) (*Ning Mak and Caldeira 2014*).

In conclusion, the available evidence supports the importance of the intake of adequate levels of nutrients through the diet. It has been shown that Mediterranean Diet (i.e. a diet poor of red meat, and rich of whole grains, fruits, vegetables fish, nuts and olive oil) reduces the risk of various chronic diseases, as type II diabetes and cardiovascular diseases, some types of cancers related to diet and some neurodegenerative diseases, dementia and cognitive decline, depression and stroke *(Ning Mak and Caldeira 2014).*

1.1.1. The aging of brain functions, cognitive impairment and dementia

An intact cognitive status is crucial for maintain autonomy and a good quality of life. Aging is accompanied by a physiologic decline of cognitive ability, but it is important being able to discriminate which changes are part of the normal aging process and which are symptoms of a disease *(Murman 2015)*. The memory reduction rate seems to be about of 0.5% each year *(Salthouse 2010, Morley 2018).* Cognitive abilities can be divided in crystallized and fluid abilities. Crystallized abilities, as math, and reading comprehension, are a sort of acquired knowledge derived from past cognitive processing. Fluid abilities are the results of actual cognitive processing. As can be seen in **Figure 1** crystallized abilities increases until the age of 60 and reach a plateau until the age of 80. Fluid abilities decline from 20 to 80 years *(Murman 2015).*

Figure 1. Trend cognitive abilities with aging *(Murman 2015)*



Memory, attention, language, visuospatial ability and executive cognitive function are cognitive capacities. With age each of these abilities decline as also decline sensory perception and the speed of processing with a subsequent reduction of performance in several cognitive domains. With aging there is also the impairment in the capacity to focus on certain information and ignore others (i.e., selective attention) and also the ability to perform multiple tasks concurrently (i.e., divided attention) is impaired, whereas simple attention as digit span is kept for up to 80 years. Aging is characterized by the impairment of the capacity to learn new things and to retrieve the new learned things, but it does not affect the capacity to maintain the newly learned information. Historical memories are maintained, but the ability to retrieve the source of the information and details declines with aging as working memory and the retention of the new learned information. The ability to remember things to do in the future is called prospective memory, it worsens with aging, whereas the capacity to remember procedures as drive the car or play a musical instrument (i.e., procedural memories) remains stable. Decision making, problem solving, multitasking and planning are parts of executive function. Executive function is another ability affected by aging, this function is associated to the prefrontal cortex and it is important when there is the need of a new type of response, different from a routinely response. Other aspects which decline with aging are the mental flexibility, the ability of abstraction, the concept making, these tasks decline significantly after the age of 70. Speech and language abilities are maintained with aging, however the capacity of comprehension in the presence of noise decreases with age. Verbal retrieval and fluency decline with age, in fact older adults tend to be more repetitive in the spontaneous speech. The visuoperceptual judgment and spatial orientation are affected by aging, but not the ability of recognizing objects, shapes and gestures. The ability to reproduce a simple figure is not affected by aging, but the ability to reproduce of complex picture decreases with age *(Murman 2015).*

Cognitive impairment is a diagnostic entity which describes people who are not cognitively intact but are not sufficiently impaired to be classified as demented *(Tampi et al. 2015)*. Unlike dementia, people with cognitive impairment tend to maintain their autonomy. In one third of the cases, cognitive impairment worsens to dementia *(Albert et al.2011)*. However, the 10-40% of people with mild cognitive impairment (MCI) revert to the normal cognitive status. Because the differences in diagnostic criteria, the prevalence of MCI range from the 3% to the 42% in people aged ≥ 65 years *(US Preventive Services Task Force 2020).*

Dementia is a devastating, chronic and irreversible syndrome leading to negative health outcomes, because of the compromised cognitive function which impairs the ability to perform independently the activities of daily living and social functioning. Dementia is characterized by changes in behavior,

and reduced quality of life for patients and carers. This condition is related to aging, indeed advanced age is an important risk factor for dementia *(Qiu and Fratiglioni 2018*). Dementia is more common in people older than 65 years. In view of the rapid aging of the population, the prevalence of dementia is expected to increase worldwide, representing a public health priority both in high income and low-income countries. The number of people with dementia is expected to exponentially increase to 66 million by 2030, and 131 million by 2050. Currently one third of older subjects who die have dementia (*Livingston et al.2017).* According to a recent meta-analysis, the age standardized prevalence of dementia worldwide has been estimated to be between 5 and 7% in the population aged 60 years or more *(Prince et al. 2011).* The prevalence is low before the age of 60 years, but increases rapidly afterwards, affecting about 30% of subjects over the age of 85 years *(Qiu and Fratiglioni2018).* Dementia has a heavy impact on healthcare costs, greater than other common chronic diseases as cancer and heart diseases. Considering the large burden of unpaid caregiving provided by families, the economic impact of dementia is estimated at $200 billion per year in the US and $600 billion worldwide *(Langa 2015).*

1.1.2. Risk factors of cognitive impairment and dementia

Cognitive impairment and dementia share the same risk factors. Risk factors can be divided in two main groups: non-modifiable risk factors and potentially modifiable (*Livingston et al.2017).*

- Non-modifiable risk factors
  - **Age** *(Livingston et al.2017).*

  - **Biological differences among men and women, and gender differences** related to the exposure to risk and protective factors, are responsible of the differences in the prevalence and incidence of dementia and MCI (*Podcasy and Epperson 2016).*

  - **Genetic** background is clearly a non-modifiable risk factor. APOE gene is on the chromosome 19q13 of the human genome and encodes for a 34kDA astrocytic protein involved in the delivery of cholesterol to neuron. Exist three alleles of APOE, ε2, ε3 and ε4 with three related isoforms. The most common allele is ε3 (with a frequency of 60%), followed by the ε4 and after by the ε2 *(Hickman et al, 2016).* The allele ε4 is associated with increased amyloid plaques deposition and the compromised synaptogenesis *(Medina et al. 2017)***.** The presence of allele ε4 (both in heterozygosity with ε3 or in homozygosity) is related to the increased risk of dementia, low cognitive performance in childhood, and early onset of dementia *(Hickman et al. 2016).*

  - **Family history** is the last non-modifiable risk factor. Having close relatives with dementia is associated with high risk of developing it and with an early onset (*Scarabino et al. 2016).*

- **Potentially modifiable risk factors**

Potentially modifiable risk factors can be modulated by lifestyle interventions. Between modifiable risk factors can be cited cardiovascular risk factors (i.e., hypertension, obesity, diabetes, hypercholesterolemia), lifestyle (i.e., smoking, alcohol consumption, diet, physical activity, social isolation) and other types of risk factors (i.e., level of education, traumatic brain injury, depression, sleep disorders, *(Baumgart et al. 2015),* hearing impairment*) (Livingston et al.2017)*.

1. Cardiovascular risk factors

   - Hypertension is associated with cognitive and attention deficits, impaired memory and slowed mental processing speed *(Iadecola 2014).*

- Midlife **obesity** is associated with an increased risk dementia *(Baumgart et al. 2015).* Furthermore, obesity is associated with the condition of pre-diabetes and metabolic syndrome which negatively affects the amyloid clearance *(Livingston et al.2017)*.

-Diabetes is associated with an increased risk for cognitive decline, mild cognitive impairment and dementia (*Ravona-Springer and Schnaider-Beeri 2011).* Despite that, the association between dementia and diabetes is strong but not conclusive. A recent meta-analysis found that in people with diabetes, the progression from cognitive decline to dementia is greater than in subjects without diabetes *(Baumgart et al. 2015),*

-Systematic reviews found mixed results on the relation between dementia and **hypercholesterolemia.** Also, regarding the effect of statins in the reduction of dementia risk the results from observational studies, a Cochrane review and systematic reviews are mixed *(Baumgart et al. 2015).*

2. Lifestyle risk factors

-Smoking in middle-age doubles the risk dementia in late life, whereas current smoking is associated with increased risk of cognitive decline and possibly dementia *(Baumgart et al. 2015).*

- Data on **alcohol** consumption are not strong and need further investigations. Some recent studies find that alcohol consumption is not associated with the increased progression to dementia, but rather in old age can have a protective role *(Anstey-Am et al. 2009).* However, in a systematic review published in the 2017 alcohol consumption has been related with the early onset of dementia *(Chenget al. 2017).*

-The relation between diet and MCI and dementia is mixed *(Baumgart et al. 2015)*. However **Mediterranean diet** is associated with reduced vascular risk factors, reduced plasma glucose and serum insulin concentrations, insulin resistance and markers of oxidative stress and inflammation *(Livingston et al.2017)* and reduced risk of dementia *(Baumgart et al. 2015)*.

- **Physical activity** is useful to everyone at every age, and allow to improve several health outcomes (i.e., improve balance, mood and function, reduce falls and mortality), and can be a

protective factor for dementia (*Livingston et al.2017)* and cognitive impairment, moreover it is associated with improved cognitive function *(Baumgart et al. 2015)*. Furthermore, it would seem that higher levels of physical activity are associated with greater beneficial effects *(Livingston et al.2017).*

-**Social isolation** increases the risk of dementia, MCI and their risk factors (i.e., hypertension, depression and coronary heart disease), cognitive lethargy and bad mood (*Livingston et al.2017)*. Marriage can be a protective factor to dementia, indeed one meta-analysis of 15 studies showed that people married have lower risk of dementia than widowed and people who have never married *(Sommerlad et al. 2017).*

3. Other factors

- Level of education affects the risk of MCI and dementia. Low education is defined by no secondary school education. It is widely common worldwide with a prevalence of 40%. *(Livingston et al.2017)*. People with better educational level have lower risk *(Baumgart et al. 2015)*.

- **Traumatic brain injury** is a risk factor more common in men than women (*Livingston et al.2017)*, it is associated to the increased risk of dementia and cognitive decline. This risk is increased in the case of repetitive brain injury (i.e. veterans, boxers, etc..) *(Baumgart et al. 2015)*.

- The question if **depression** is an early marker of brain changes or a risk factor for dementia remains open *(Baumgart et al. 2015)*. A study published on JAMA *(Singh-Manoux et al.2017)* carried out for 28 years, investigated if depression is a risk factor for dementia. Data obtained suggested that depressive symptoms in adulthood do not increased the dementia risk, but for symptoms in late life the results are different. Therefore the hypotheses can be two: symptoms of depression may preannounce dementia or dementia and depressive symptoms may have the same causes. However it is known that depression affects stress hormones, neuronal grow factors and hippocampal volume, so it is possible that depression increases the dementia risk. Furthermore researches on antidepressants and dementia suggest that some antidepressants decrease the amyloid production (*Livingston et al.2017)*.

- Several studies showed that **sleep disorders** (i.e., insomnia, sleep apnea, etc…) increase the risk of cognitive decline. The treatment of breathing disorders during sleeping may reduce the risk of cognitive decline *(Baumgart et al. 2015).*

- Considering that **hearing impairment** is widespread after the age of 55, and that also a mild compromised hearing can increases the risk of dementia and cognitive decline, it is easy to understand that hearing loss is an important risk factor for dementia**.** The mechanism underlying cognitive decline and hearing loss is not yet clear, such as whether the use of hearing aids can prevent or delay the dementia onset (*Livingston et al.2017)*.

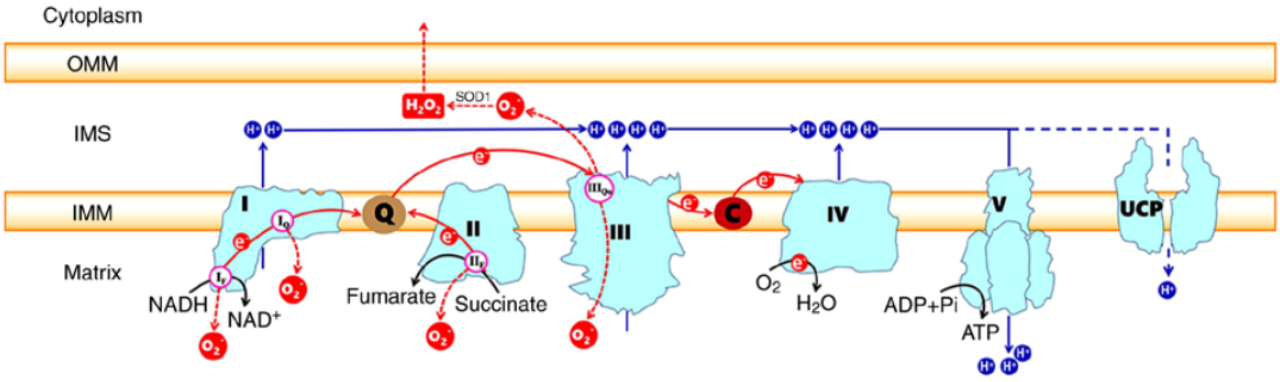
1.2. Redox state, oxidative stress and mechanisms of defense in neurodegenerative disorders and dementia

Brain aging is the critical and common factor among several neurodegenerative disorders and dementia. Cellular, biochemical and molecular studies have shown intimate links between oxidative stress and cognitive dysfunction during aging and age-associated neuronal diseases. Brain aging is accompanied by oxidative damage and impairment of antioxidant defense.

1.2.1. ROS and their role in physiologic conditions

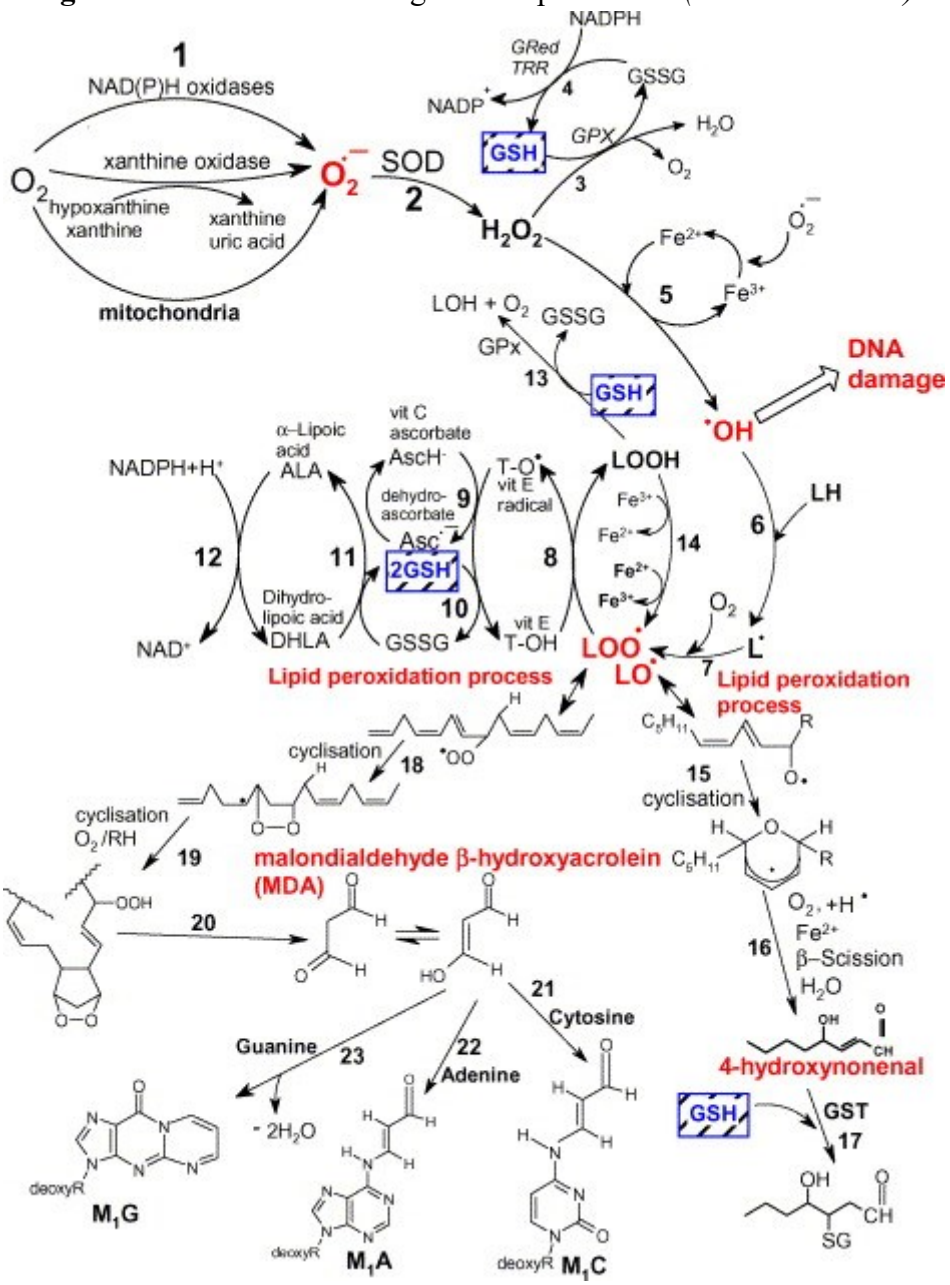
In organisms with aerobic metabolism, the production of a certain amount of the reactive species of oxygen (ROS) e.g., instable toxic molecules with an atom of oxygen with at least an unpaired electron in the external orbit *(Wojsiat et al. 2018)*, is physiological. ROS can be classified as free radicals and non- free radicals. Non- free radicals occur when two free radicals share their unpaired electrons *(Birben et al 2012)*. Example of ROS are the superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($1/2O_2$). ROS, mainly produced by the mitochondrial electron transport chain **(Figure 2)** and the nitric oxide synthase reaction *(Poljsak et al. 2013, Alfadda and Sallam 2012)*, but also from activated phagocytes, ionizing radiations, smoke, and as products of enzymes as NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase, xanthine oxidase and others (*Alfadda and Sallam 2012)*.

Figure 2. Electron transport chain in mitochondria *(Zhao et al. 2019)*



In addition to mitochondria, there are non-mitochondrial ROS formation pathways **(Figure 3):** Fenton's reaction, cytocrome P450 enzymes of microsomes, β- oxidation in perossisomes and the oxidative burst of phagocytes (*Poljsak et al. 2013)*.

Figure 3. Mechanisms leading to ROS production *(Valko et al.2007)*



Under normal conditions ROS participate in physiologic cell processes *(La Fata et al. 2014):*

- Regulation of the vascular diameter→ it is known that ROS affect the vascular physiology, for example during shear-stress. Moderate ROS levels participate at regulating vessel function whereas a high levels of ROS are associated with oxidative damage and inflammation *(Alfadda and Sallam 2012).*

- Role in the oxygen sensing →oxygen sensing help cells initiating adaptative responses in order to increase survival chances before the oxygen shortage. In case of hypoxia, the electrons transport chain can work as sensor releasing ROS which can increase the production of the hypoxia-inducible factor 1 (HIF-1). HIF-1 normalize ROS level and stimulate the formation of new vessels in order to resolve the hypoxia *(Alfadda et al. 2012).*

- ROS participate in the innate and acquired immunity→ Following exposure to pathogens, massive ROS production occurs in activated phagocytes in this way the ROS participate in the first defense against the entry of the pathogen. However, this type of immunity may sometimes not be enough as the pathogen could escape and proliferate. During the acquired immunity, the activated phagocytes produce ROS, the T lymphocytes intracellular signal transduction cascade is stimulated and the threshold to be activated is reduced *(Alfadda and Sallam 2012).*

- Role in the skeletal muscle→ During the muscle contraction, ROS can be involved in the signaling of the glucose uptake. Studies have shown a that physical exercise is associated with the increases in circulating antioxidants involved in the ROS levels control. Whereas after immobilization the production of ROS increases *(Alfadda and Sallam 2012).*

- Genomic stability and cell signaling→ Red-ox status is involved in the stability of genome and in the process of transcription. For example, the post-translational modifications of histone and non-histone proteins depends on the red-ox state of the cellular environment. Oxidative stress and energy consumption are important regulators of transcription and consequently can regulate important cell functions. For example, the sirtuins, (i.e. histone deacetylases) are dependent from red-ox status and NAD+ (nicotinamide adenine dinucleotide). When an excessive level of ROS is produced, the deactivation of the deacetylation function of Sirt1 occurs, therefore pro-apoptotic factors such as p-53 are activated, and the cell undergoes death *(Alfadda and Sallam 2012).*

- Role in the aging process→ ROS would be responsible for the accumulation of cellular damage that would lead over time to the loss of functions. Oxidative damage to deoxyribonucleic acid (DNA), cellular components and mitochondria could lead to cell death. However, in animal models (i.e. rats and *drosophila melanogaster*), moderate level of ROS it is associated with a whole series of adaptive responses that result in the lengthening of life (*Choi and Kim 2019).*

- Differentiation→ ROS are molecules involved in signaling for the cellular differentiation of various types of cells including stem cells. ROS are essential in the differentiation of heart muscle cells. Through the insulin growth factor 1 (IGF1) signaling pathway, ROS promote cell differentiation and cellular hypertrophy *(Choi and Kim. 2019).*

- Autophagy→ this process allows the cell to eliminate damaged components with the help of lysosomes. Mitochondrial ROS are essential for the induction of autophagy in response to certain stressful conditions for the cell, such as nutrients deficiency, the presence of tumor necrosis factor α (TNFα), etc *(Choi and Kim. 2019).*

- Benefits in cancer: ROS are pro-tumor factors as they can cause DNA damage and have pro-inflammatory effects. There is evidence of the efficacy of antioxidants against tumor proliferation. In the same way ROS can induce the transcription of the 2-related factor 2 (Nrf2) which promotes the transcription of genes involved in the redox detoxification mechanism.

1.2.2. Oxidative stress and cell defense

The balance of ROS and antioxidant defense is defined red-ox state *(Kerksick and Zuhl 2015).* Oxidative stress occurs when the production of ROS, is higher than the capacity of the antioxidant defenses to counteract them *(Burton and Jauniaux 2011).*

As previously described, moderate levels of ROS produced under physiological conditions are associated with a whole range of cellular functions. While when produced at high levels, ROS take part in the onset of cellular pathologies or dysfunctions. The oxidative damage of cells is considered one of the mechanisms responsible of aging *(La Fata et al. 2014)*, it is associated with several age-related diseases *(Luca et al. 2015)* among which:

- Role in macromolecular toxicity→ diabetes and obesity are characterized by a condition of "glucolipotoxicity" which increases oxidative stress. Glucose and fatty acids, through the ROS, activate the transcription of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) factor thus activating an inflammatory state. In the mediation of lipotoxicity are involved the Toll-like receptor 4 (TLR4) and the nuclear peroxisome proliferator-activated receptor (PPARγ) *(Alfadda and Sallam 2012)*.

- Role in insulin resistance→ this condition is typical of diabetes but also of obesity, sepsis, metabolic syndrome. On the other hand, oxidative stress it is also associated with these conditions. Animal studies suggest that reducing the ROS level is associated with the reduced risk of insulin resistance. The insulin resistance due to ROS toxicity is associated with the translocation of the pancreatic and duodenal homeobox-1 from the nucleus to the cytosol, which is associated with the blockage of insulin synthesis. Whereas the transcription factor Forkhead box protein Foxo1 (Foxo1) is translocated to the nucleus stimulating the gluconeogenesis *(Alfadda and Sallam 2012)*.

- Role in diabetes → with aging, the ROS accumulation in mitochondria and their dysfunction occurs. With aging the β-cells (i.e., pancreatic cells secreting insulin) are more sensible to ROS because the reduced expression of protective enzymes. The uncoupling protein 2 (UCP2), which is a negative regulator of secretion of insulin, increases in obesity, favoring β-cells dysfunction and the diabetes onset. Also, the complications related to diabetes, as blindness, are associated with oxidative stress *(Alfadda and Sallam 2012).*

- Obesity → obesity is a risk factor for cardiovascular diseases. It is associated with a chronic inflammation status with high activity of monocytes and macrophages. In this intense inflammatory status, an excessive accumulation of ROS occurs, causing diffuse oxidative stress. The main responsible of ROS production is NADPH oxidase which is expressed in phagocytes and endothelial cells. NADPH oxidase stimulates TLR4 (e.g., lipid and endotoxin receptor) to overexpress proinflammatory cytochines as interleukin 6 (IL-6) and TNF-α. The overexpression of these factors is associated with inflammatory status, obesity and insulin resistance *(Alfadda and Sallam 2012).*

- Inflammation→ In response to inflammation, ROS enhance the activation of multiprotein cytoplasmatic complexes called inflammasomes. At the same time the ROS excess inhibits the mitophagy process, e.g., a sort of autophagy used to remove damaged mitochondria. It follows a further production of ROS and activation of inflammasomes in a vicious circle. Alternatively, cells containing damaged mitochondria can undergo to apoptosis, which is regulated by ROS. However, mechanisms leading to the choose of inhibition of mitophagy or apoptosis is not clear. Some pollutants can induce chronic inflammation or cancer by the activation of inflammasomes via ROS *(Alfadda and Sallam 2012).*

- Infections→ Viral and bacterial infections are associated with ROS production and inflammatory status *(Alfadda and Sallam et al. 2012).*

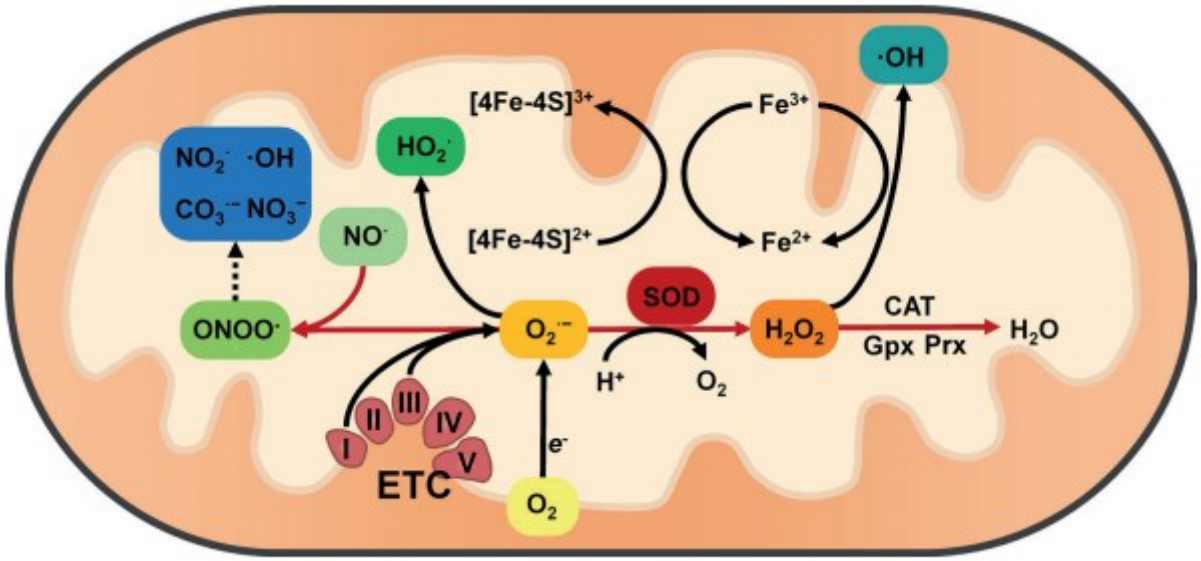
Cells have defense mechanisms to try to contain oxidative stress and its related damage. These defense mechanisms can be enzymatic and non-enzymatic **(Table 2)**.

Table 2. The main enzymatic and non-enzymatic defenses against ROS

Enzymatic defenses	**Non-enzymatic defenses**
Superoxide dismutase (SOD)	Vitamin E
Catalase (CAT)	Vitamin C
Glutathione peroxidase (GTPx)	Glutathione (GSH)
Thioredoxin (TRX)	Carotenoids (β-carotene)
Peroxiredoxin (PRX)	Uric acid
Glutathione transferase (GST)	Polyphenols
	Selenium
	Zinc
	Transferrin
	Metallothionein
	Haptoglobins
	Albumin
	Myglobin
	Ferritin
	Caeroplasmin
	Bilirubin
	Coenzyme-Q
	Polyphenols

The enzymes with antioxidant action have a transition metal in the core, which assumes different valences during the transfers of electron **(Figure 4).**

Figure 4. ROS transformation in the mitochondria by enzymatic defenses *(Choi and Kim 2019)*



Among the various enzymatic defense mechanisms against oxidative stress, below we report some of the most important:

- **SOD** includes enzymes with one or two atoms of transition metals. SOD exists in three forms: the copper/zinc form sited in the cytosol and the manganese form (Mn-SOD) in the mitochondrial matrix, and the SOD sited at the extracellular level. There are two types of SOD which convert $O_2^{\bullet -}$ into H_2O_2, the Mn-SOD in the mitochondria and the copper and zinc isoform in the cytosol. High levels of mitochondrial ROS stimulate the SOD expression into mitochondria, which act against the ROS produced by the electron transport chain *(Lee et al. 2020).* SOD represents the first defense against oxidative stress.

  $M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow M^{n+}\text{-SOD} + O_2$

  $M^{n+}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$ *(Birben et al. 2012)*

- **CAT** is abundant in the subcellular compartments as peroxisomes *(Krishnamurthy and Wadhwani 2012)*. It has an intense activity in red blood cells, kidney and liver

(Pisoschi et al.2020). This enzyme decomposes hydrogen peroxide into oxygen and water using iron (Fe) as cofactor.

$2\ H_2O_2 \rightarrow O_2 + 2H_2O$

$H_2O + Fe\ (III)\text{-}E \rightarrow H_2O + O = Fe\ (IV)\text{-}E\ (.+)$

$H_2O + O = Fe\ (IV)\text{-}E(.+) \rightarrow H_2O + Fe(III)\text{-}E + O_2$ *(Birben et al. 2012)*

CAT is not saturated by H_2O_2, therefore it is involved in the adaptation to oxidative stress *(Lee et al. 2020).*

- **GTPx** are enzymes that split hydrogen peroxide into water, and organic hydro peroxides to the corresponding alcohol, using GSH as hydrogen donors. The GSH is involved in a series of detoxifying reactions forming glutathione disulfides which is converted in GSH by the glutathione reductase and the consumption of one NADPH which is generated back by the pentose phosphate pathway (*Burton and Jauniaux 2011).*

  $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

  $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$ *(Birben et al. 2012)*

  GTPx are sited in mitochondrial matrix and cytoplasm. Mammals have five isoenzymes of GTPx. These enzymes are ubiquitous with different expression in relation to the tissue *(Krishnamurthy and Wadhwani 2012).* GTPx1 is the most abundant form, it can be localized in peroxisomes, cytosol, mitochondria *(Pisoschi et al.2020),* neurons and astrocytes *(Lee et al. 2020).*

- The **Trx** system includes thioredoxin reductase and Trx, and NADPH as donor of electrons. This system repair DNA and proteins and is also involved in the immunity. Trx is a pyridine nucleotide-disulfide oxidoreductase together with the glutathione reductase. Trx and TrxR catalyze the reduction of active site disulfide in oxidized Trx (Trx-S_2) to obtain a Trx-$(SH)_2$ in a NADPH dependent reaction. Trx-$(SH)_2$ is a donor of protons regulating thiol redox. This system is used to maintain red-ox cell level. GSH and Trx system act against the oxidative stress removing several ROS. Trx exists in three isoforms, the Trx1 sited in the cytosol, nucleus, cell membrane, Trx2 in the mithochondria and the Trx3 in the in mammalian testicular cells (*Lee et al. 2020).*

Whereas among the non-enzymatic antioxidants it must be cited:

- **Lipoic acid:** it is a cofactor in the oxidative decarboxylation of α-chetoacids. It acts quenching ROS and chelating transition metals. In addition, it is also able to block the Fenton reaction *(Pisoschi et al.2020).*

- **Uric acid:** it is an important plasma scavenger produced by the metabolism of purines. It acts on the formation of hydrogen peroxide or superoxide anion by means of xanthine oxidase reactions *(Pisoschi et al.2020)*. Uric acid has also a role in the protection of the central nervous system (CNS) *(He et al.2017).*

- **Metallothioneins**: these are proteins characterized by the presence of numerous cysteine residues with thiolate ligands. Lipid peroxidation counteracted by the release of zinc from metal-thiolate complexes. Metallothioneins can counteract the superoxide radical, the hydrogen peroxide and also nitric oxide (NO) radicals and nitrogenated reactive species. Metallothioneins are also able to reduce the toxicity of heavy metals *(Pisoschi et al.2020)*.

- **Bilirubin:** it can act as a ROS scavenger; however, its intracellular concentration is rather low. It inhibits the activity of NADPH oxidase which is associated with the antioxidant activity of heme oxygenase *(Pisoschi et al.2020).*

- **Vitamin C:** is a scavenger of several type of ROS and nitrogenated species. It is able to remove ROS from the aqueous medium before the start of lipid peroxidation *(Pisoschi et al.2020).* Vitamin C not directly scavenges the lipophilic radicals, but with tocopherol can participate to the removal of lipid peroxide radicals. Furthermore, Vitamin C has a role in the regeneration of the tocopherol *(Pisoschi et al.2015).*

- **Vitamin E:** its antioxidant role will be discussed the 3.1.4. section of this thesis

- **Carotenoids:** are fat soluble molecules which can act as physical and chemical quenchers of the singlet oxygen and act as radical scavengers. At the cell membrane level can strengthen the antioxidant action of vitamin E *(Pisoschi et al.2020).*

- **Q- Coenzyme**: is a liposoluble molecule localized in the inner mitochondrial, Golgi and Lysosome membranes. In addition to acting as a scavenger, it is also an enzymatic cofactor. It is synthesized by the membranes of numerous cell types and is a component of the electron transport chain. Q-coenzyme detoxify H_2O_2 converting it into water *(Lee et al. 2020)*. Q-coenzyme can also react with the perferryl radical (Fe^{3+} - $O_2^{-\bullet}$) *(Pisoschi et al.2015)*. This coenzyme is also involved in the regeneration of other antioxidants as vitamin E.

  $CoQH_2 + O_2^{-\bullet} \rightarrow CoQ^{-\bullet} + H_2O_2$

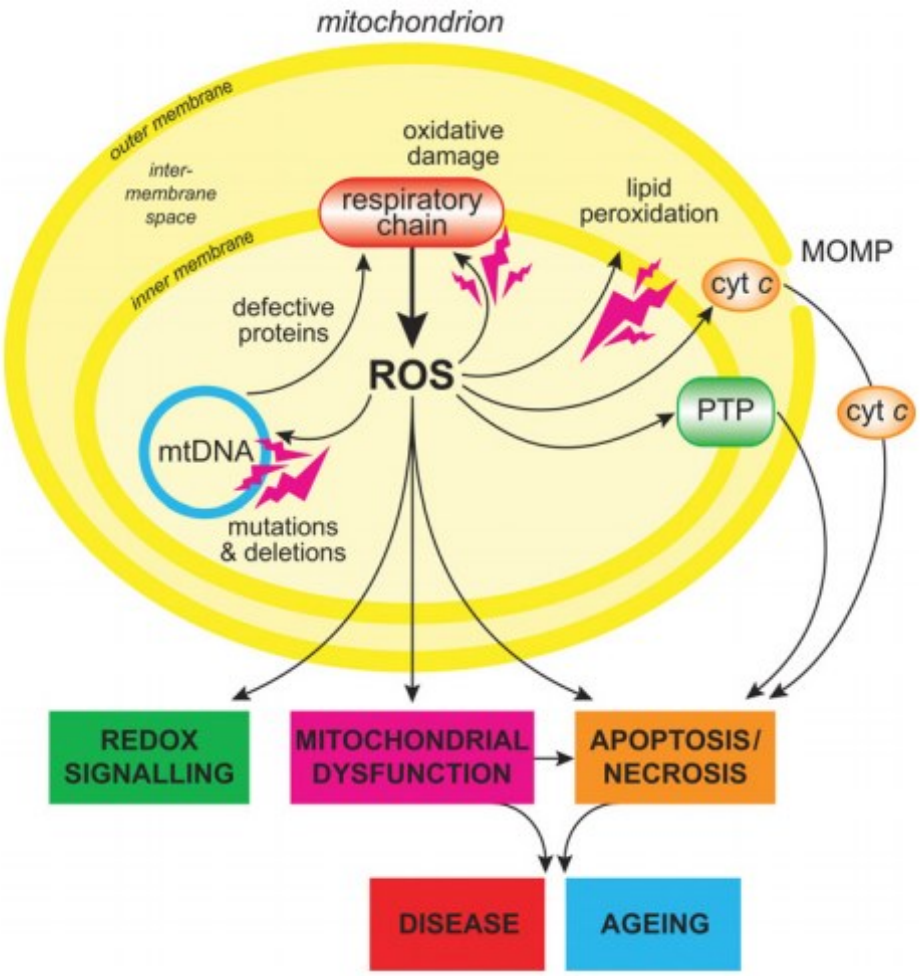
  $CoQH_2 + Fe^{3+} - O_2^{-\bullet} \rightarrow CoQ^{-\bullet} + Fe^{3+} + H^2O^2$*(Pisoschi et al.2015)*.

- **Polyphenols:** their antioxidant property will be treated in the 4.1.3 section of this thesis

- **Dietary minerals** as selenium and zinc are known to have antioxidant properties. Zinc inhibit NADPH oxidases, using NADPH as electron donor to obtain singlet oxygen radical. Zinc is also part of the SOD, it inhibits the NF-κB activation acting as an ant inflammatory and antioxidant factor and stimulates the metallothionein synthesis which are scavenger of the hydroxyl radical *(He et al.2017).*

When the redox balance is lost and oxidative stress takes over, oxidative stress damage can occur. ROS can cause oxidative damage at different cell levels including at the proteins sited in the mitochondria, at the DNA and membranes **(Figure 5).** ROS can also compromise the mitochondria capacity of adenosine triphosphate (ATP) synthesis and of other metabolic activities. ROS can be associated with the activation of the cell apoptosis by increasing the permeability of cell membrane, consequently the release of protein outside of the cell occurs. Also, the permeability of transition pore is increased, this condition has a role in several diseases as for example ischaemia. Furthermore, ROS intervening in the redox signaling, can affect various activities in the cell (*Murphy 2009)*.

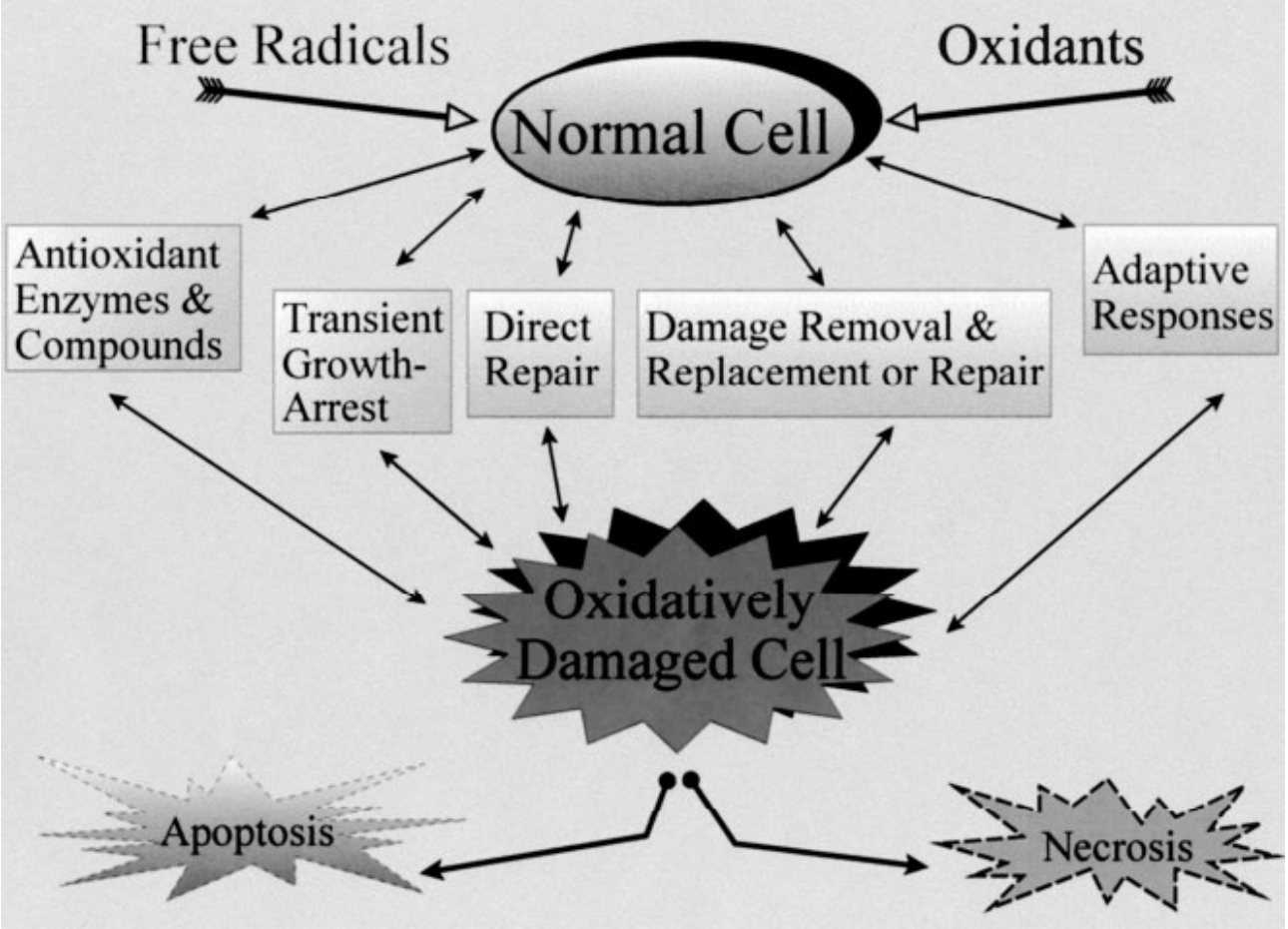
Figure 5. Mitochondrial ROS can cause oxidative damage *(Murphy 2009)*



Cell membranes are highly sensitive to oxidative damage. Final products of lipid peroxidation such as malondialdehyde (MDA), are highly mutagenic in mammalian and carcinogenic in rodents. Also, proteins can be the target of oxidation. Cysteine and methionine residues are highly sensitive to oxidation. If proteins are exposed to ionizing ray, it can witness the formation of ROS *(Valko et al.2007).*

So once the oxidative stress damage occurs, the cell puts in place a whole series of strategies to repair the damage **(Figure 6).** The damaged cell can undergo to apoptosis. Antioxidant enzymes represent the first line of defense and help the cell to transiently arrest its growth in order to protect it. DNA, proteins and lipids damaged can be repaired, removed or replaced. If these defense mechanisms are not enough, the cell remains damaged and can undergo apoptosis. With apoptosis, the damaged cell protects the surrounding cells *(Davies 2000).*

Figure 6. Defences mechanisms in a damaged cell by oxidative stress *(Davies 2000)*



- Oxidized DNA repair → oxidative DNA damage includes single and double strand breaks, base modifications, sister chromatid exchange, cross link DNA-DNA or DNA-proteins. In the strand break, can be produced atypical ends which however cannot be the substrate for DNA polymerase, therefore they must be excised before repair *(Davies 2000)*.

  The 8-oxoguanine, i.e. the oxidized form of guanine is one of the most common DNA base modification induced by the oxidative stress. It is associated with mutagenesis, aging and tumors *(Valko et al.2007)*. The reparation of this mutation occurs with the base excision repair (BER) mechanism with the enzyme 8- oxoguanine glycosylase (OGG1) both in the nucleus and mitochondria.

  In vitro studies suggest that GTPx can repair DNA hydroperoxide. DNA methylase can restore the methylation pattern following oxidative demethylation, maintaining the epigenetic methylation state. Both in eukaryotic and prokaryotic cells there are enzymes capable of directly repairing the DNA damage, as the endonuclease III in *Escherichia coli (Davies 2000)*.

- Protein modifications→ For the protein folding process, in the endoplasmic reticulum, energy and an oxidizing environment are required to form disulfide bridges. This bond formation is carried out by two enzymes; the protein disulphide isomerase (PDI) and ER oxidoreductin 1 (ERO1), which determine the oxidation of the cysteine residues in the forming protein and transfer electrons to the final oxygen acceptor which gives rise to H_2O_2. An intense mechanism of protein folding is accompanied by a strong production of ROS and a pro-inflammatory state. Under stress conditions, numerous unfolded and misfolded proteins are produced. To correct these errors, a lot of GSH is consumed, a mechanism that leads to further stress. However, there are antioxidant defense mechanisms: by activating the transcription of the following two factors: factor-4 (ATF4) and Nrf2 *(Alfadda and Sallam 2012).*
  Another defense mechanism includes the methionine sulfoxide reductase enzyme reduces oxidized methionine and restores protein function.

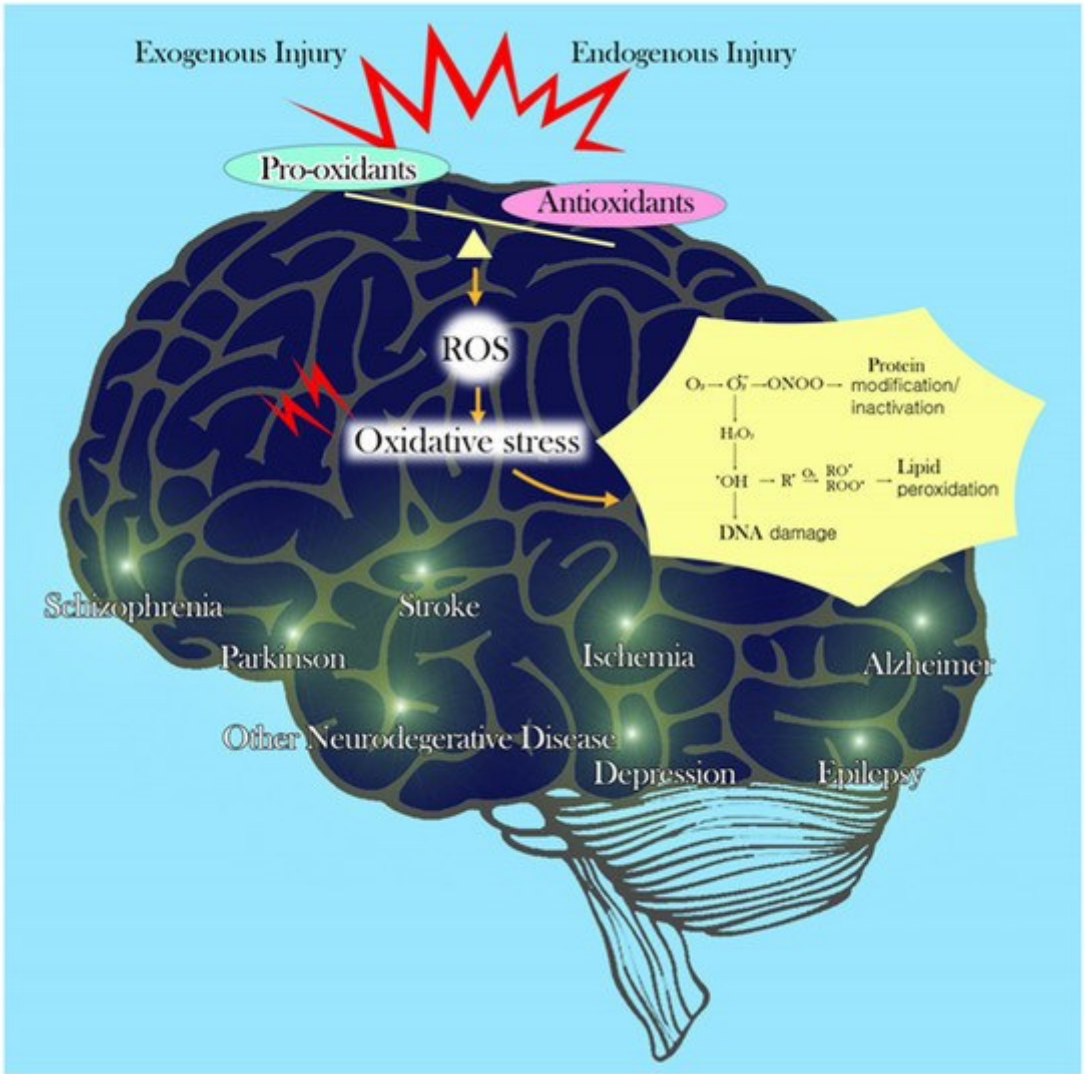
  Indirect repair mechanisms include the recognition of the oxidized molecule, its cutting and removal or degradation. Proteases are enzymes involved in indirect repair *(Davies 2000).*

- Defense mechanism at membrane lipids level→ membrane lipids are very sensitive to oxidative damage, they undergo a mechanism called lipid peroxidation which leads to membrane stiffness, the loss of its selective permeability and sometimes even its integrity. Moreover, the accumulation of damage due to the peroxidation of membrane lipids is associated with various pathological mechanisms including atherosclerosis. The changes in the membrane characteristics due to lipid peroxidation make the lipids more sensitive to the action of the phospholipase A2 which allows to block the propagation of the peroxidation reaction removing the fatty acid hydroperoxides. GTPx is able to detoxify fatty acid hydroperoxides by reducing them. While the lysophospholipids remaining at the membrane level are used for the regeneration of new phospholipids (reacylation reactions) *(Davies 2000)*.

1.2.3. Role of the oxidative stress in the brain

In the brain, oxidative stress can cause of neurodegeneration and therefore, AD and dementia *(La Fata et al. 2014)* **(Figure 7)**. Oxidative stress and its associated damage are involved in the pathogenesis of age-dependent cognitive loss. Aged animal brains showed higher levels of ROS and oxidative stress markers if compared with younger brains *(Sohal R.S. and Brunk 1992, Serrano and Klann 2004)*. The appearance of behavioral deficits or cognitive impairments in temporal and spatial memory, learning and retention of memory displayed strong associations with increasing oxidative species and stress in aged animal models *(Fukui and Onodera et al. 2001)*.

Figure 7. Oxidative stress in the brain is associated with neurodegenerative diseases *(He et al. 2017)*

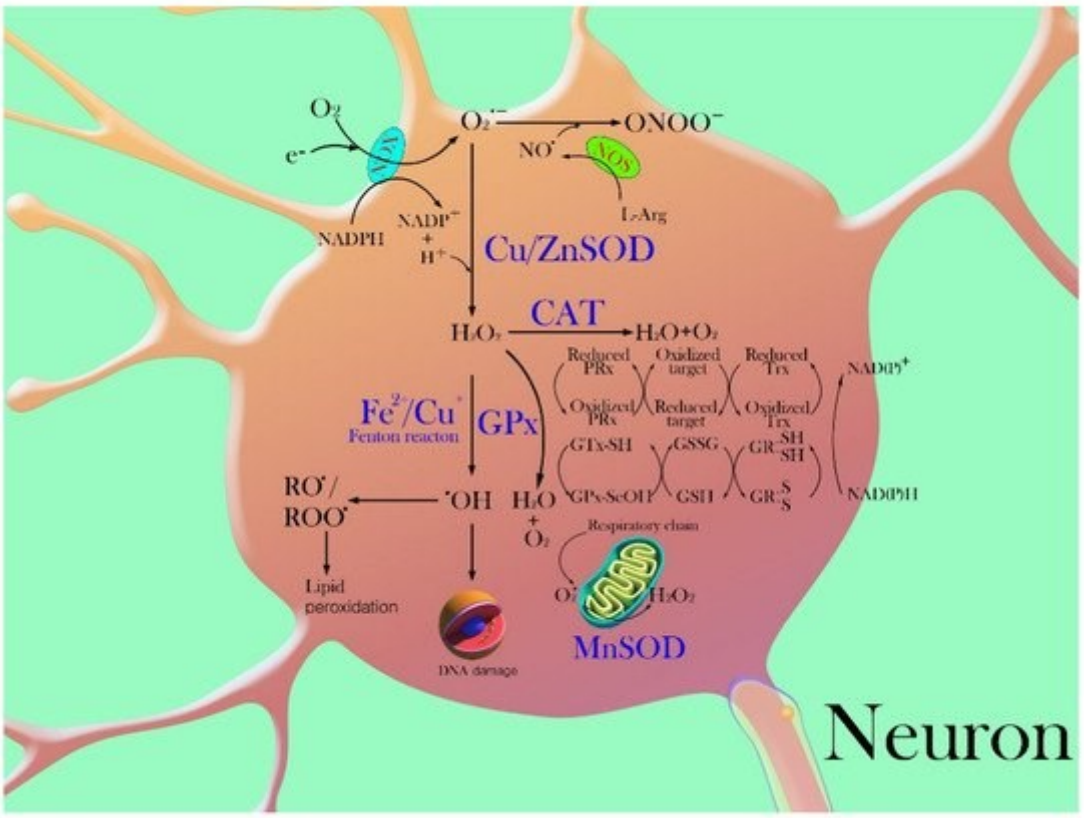


The brain is an organ which is highly sensitive to oxidative stress because:

- It consumes about the 20% of the basal oxygen;
- The membranes of neurons contain high levels of PUFA which are extremely vulnerable to oxidation. With the increasing of oxidative stress, docosahexaenoic acid (DHA) may be oxidized into neuroprostanes which are molecules like prostaglandins that causes a further production of ROS;
- The activation of the microglia is associated with a production of ROS and inflammatory cytokines which increases the oxidative stress of the brain by activating additional microglia and other central nervous system (CNS) cells;
- High extracellular levels of the neurotransmitter glutamate can cause neuronal damage through an exocitotoxic process during which the sustained activation of glutamate receptors causes the entry of calcium ions which triggers a series of processes, including mitochondrial dysfunction, that are associated with the increase in the production of ROS;
- The self-oxidation process of some neurotransmitters can cause the reduction of GSH in the surrounding tissues, increasing the vulnerability to oxidation;
- In the human brain there are about 60mg of non-heme iron, which under normal conditions is bound to ferritin and hemosiderin. In conditions of neurodegenerative diseases such as AD there is an increase in free iron levels which is associated with oxidative damage.

In the brain there are antioxidant defense mechanisms with non-enzymatic nature represented by reduced thiols, metabolic compounds, or from fat-soluble or water-soluble molecules. Moreover, during the antioxidant action, the molecules damaged by oxidative stress are also removed before that their aggregation produces cell damage. Antioxidants are ROS scavenger which acts to regenerate oxidized molecules *(He et al. 2017).* Other antioxidant brain defenses are enzymes with an antioxidant role (i.e., endogenous antioxidants) with the task of preventing the damage caused by ROS, e.g. as seen in the section 1.2.2. of this thesis, can be cited the CAT, SOD, GTPx and the peroxiredoxins *(Guest and Grant 2012)* **(Figure 8)**.

Figure 8. Enzymatic defenses against oxidative stress at neuron level *(Lee et al. 2020)*

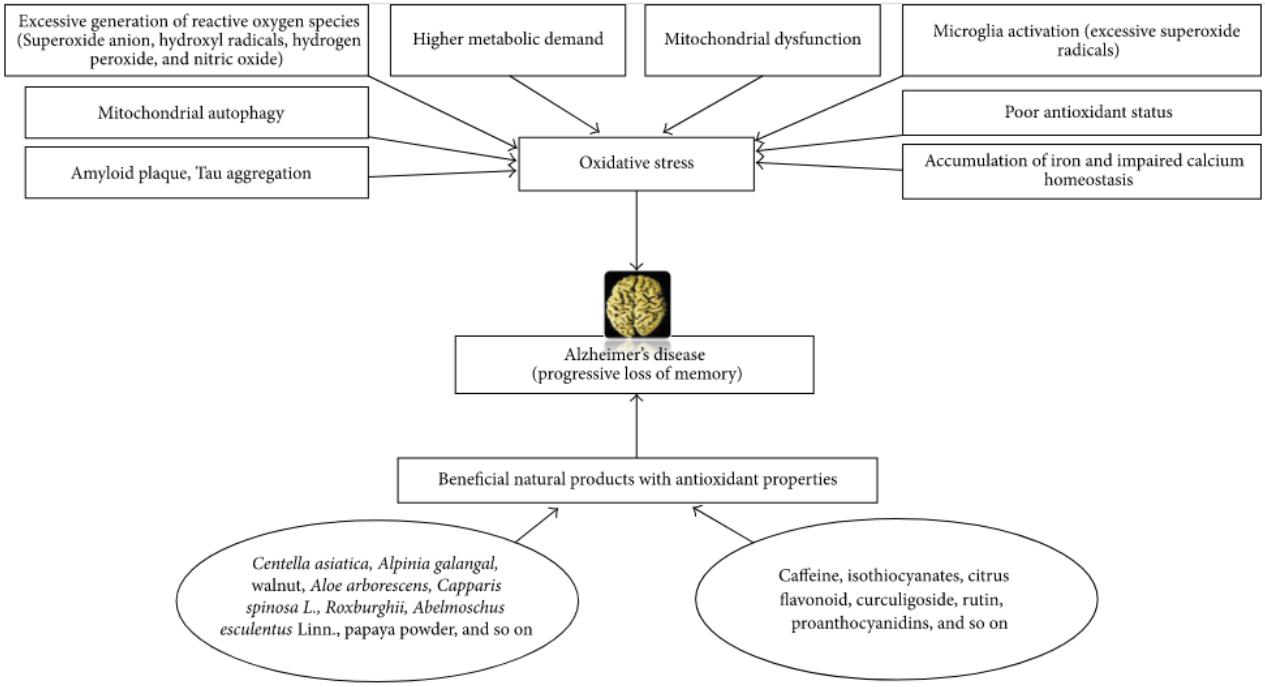


However, there are areas of the brain where these enzymes are present at low levels, such as in the hypothalamus which is characterized by low levels of CAT. Moreover, the CAT is in the microperoxisomes, therefore they cannot act on the hydrogen peroxide present at the sub cellular level. In the case of the rapid increase of the ROS, the enzymatic defense system would be in difficulty, therefore the contribution of antioxidant agents coming from the outside, or from the diet as vitamins with antioxidant properties *(Guest and Grant 2012)* (i.e. vitamins A, E, and C, flavonoids, phenolic acids, and carotenoids, and synthetic antioxidants, such as butylated hydroxytoluene, octyl gallate, butylated hydroxyanisole, propyl gallate, tert-butylhydroquinone, and ethylenediaminetetraacetic acid *(Teleanu et al. 2019)* would be useful *(Guest and Grant 2012).*

Several studies in humans revealed a strong link between oxidative stress and cognitive decline with aging (*Berr et al.2000*). It is not clear if oxidative stress is the cause of consequence of neurodegeneration, however it is associated with neural death and in its propagation. Oxidative stress is involved in the neuronal degeneration as well as decline in cognition in two most common type of dementia: AD **(Figure 9)** and vascular dementia (VaD) *(Luca et al. 2015).* Mitochondrial dysfunction

and apoptosis together with poor antioxidants occur during the AD pathogenesis *(Manoharan et al.2016).*

Figure 9. Mechanisms leading to oxidative stress in AD ***(****Manoharan et al.2016)*



In the brain of people with AD, have been found typical lesions of ROS *(Engelhart et al. 2002*) characterized by lipid peroxidation, DNA damage and protein oxidation, the presence of metals (i.e. zinc and iron) producing ROS *(Luca et al.2015)*. Brain of demented people had iron accumulations which are linked to oxidative stress. These metal deposits can produce H_2O_2 following the Fenton's reaction. Furthermore, the level of oxidative stress is increased by the binding between β-amyloid and iron. Scientific evidence exists on the relation between amyloid plaques and makers of lipid peroxidation as MDA *(Manoharan et al.2016).* In case of implicated neurons in AD, oxidative damage is brought about by the free radicals generated by the entry of mutant amyloid precursor protein (APP) and soluble β amyloid peptide (Aβ) into mitochondria and causes further impair mitochondrial metabolism. AD patients show a higher level of oxidized lipids and lipoproteins and oxidative damaged proteins in peripheral fluids such as plasma and serum (*Keller et al.2005, Bacchetti et al.2015, García-Blanco et al. 2017).*

AD is characterized by high lipid peroxidation and low enzymatic and non-enzymatic defenses. The production of ROS in AD is due to mitochondrial disfunctions, inflammation status, microglia

activation performed by Aβ, the linkage of activate red-ox metals to the deposits *(Luca et al. 2015).* The activated microglia generates massive amounts of superoxide radicals. The high metabolic needs of microglia and the post-mitotic cells are associated with high sensibility to oxidative stress which is favorited by a low rate of brain regeneration a poor antioxidant potential. The autophagy of mitochondria is one of the main sources of ROS *(Manoharan et al.2016).*

Postmortem studies have highlighted the deficiency of cytochrome C oxidase in the brains of subjects with AD. This deficiency is associated with damage to the mitochondria which could produce ROS which further damage them. Another neurodegenerative mechanism involved in the neurodegeneration process, is the uncontrolled inflammation of the cells of the microglia, due to the phosphorylation of the peroxisome proliferator-enabled receptor gamma (PPAR-γ) by oxidative stress. Furthermore, a mild oxidative state would be involved in the early stages of AD favoring the Aβ forming process. The formed Aβ produce ROS, and so on. The formation of ROS also activates a certain antioxidant response; however, the enzymatic and non-enzymatic defense is weak and compromised in subjects with AD. ROS cause neuronal mortality because modifications in the membrane characteristics, as fluidity and transport.

The second more prevalent dementia is VaD. Oxidative stress is associated with VaD, to its risk factors, to blood-brain barrier (BBB) damage, to VaD typical lesions in white matter and to alterations in the cerebral perfusion and permeability. Also, in VaD the enzymatic defense is compromised *(Luca et al. 2015)*.

Several evidence are available on the benefits of dietary antioxidants molecules for dementia and cognitive status, although not always the results are univocal. It is suggested that vitamins with antioxidant role may protect BBB integrity. Vitamin A, C and E are vitamins with antioxidant properties. Vitamin A acts on the Aβ fibrils reducing their aggregation and thus preventing the formation of amyloid plaques. It is known that vitamin C prevents oligomerization of Aβ, and its administration reduce oxidative stress *(Luca et al.2015).* The antioxidant role of vitamin E and polyphenols will be discussed in dedicated sections (3.1.4. and 4.1.3. respectively) of this thesis. Other antioxidants showed positive effects on brain, among which, but not limited to vitamin B, D, and K *(Mehta et al 2018)* polyphenols *(Vauzour 2012),* and carotenoids *(Feart et al.2016).*

2. Hypothesis and Aims

Oxidative stress and decrease of antioxidant defenses are implicated in age-related cognitive impairment and dementia.
Understanding the role of dietary antioxidants may provide strategies to prevent neurodegeration and to promote healthy cognitive aging.

The main aims of the thesis are:

- To investigate the longitudinal relationship between plasma levels of **vitamin E** at baseline and cognitive impairment in the non-demented older adult population enrolled in the "Invecchiare in Chianti" (InCHIANTI) study and followed for 17 years **(Study 1)**.

- To investigate the role of **dietary polyphenols** on chronic diseases and cognitive impairment **(Study 2)**.

3. Study 1: Relationship between plasma levels of vitamin E and cognitive impairment (InCHIANTI) study

This study aims to investigate the longitudinal relationship between plasma levels of vitamin E at baseline and cognitive impairment in the non-demented older adult population enrolled in the "Invecchiare in Chianti" (InCHIANTI) study and followed-up for 17 years.

3.1. Vitamin E

3.1.1. Vitamin E: structural characteristics and food sources

Vitamin E includes eight fat-soluble vitamins *(Lloret et al.2019)* (i.e., α, β, γ and δ classes of tocopherol and tocotrienol) *(Rizvi et al. 2014)* with antioxidant, anti-inflammatory and neuroprotective properties *(Lloret et al.2019)*. Their properties and function are different in relation to the level of structure saturation *(Boccardi et al. 2016).*

It was discovered in 1922 by Evans and Bishop *(Evans and Bishop 1922).* Vitamin E is not produced by the body; therefore, it must be introduced with the diet. It is present in fat-containing foods and is stored in human and animal fatty tissues. Important dietary sources are nuts, seeds, edible vegetable oils (i.e., coconut, maize, palm, olive, peanut, soybean, wheatgerm and sunflower), green leafy vegetables and fortified cereals *(Rizvi et al. 2014)* **(Table 3)**.

Table 3. Content of vitamin E in selected food *(modified from Martin et al. 2002)*

Food	Vitamin E content (mg/100g)
Wheat germ oil	119-128
Sunflower oil	49
Safflower oil	40
Sunflower seeds, raw	50
Corn oil	26
Olive oil	13-22
Almonds	13-27
Peanut oil	19
Mayonnaise	13
Soybean oil	8.1
Butter	2.2
Brown rice, boiled	1.3
Bread, whole wheat	0.5
Bread, white	0.1
Corn flakes, cereal	0.1
Peanuts, dry roasted	7.4
Peanut butter	6.2
Liver, grilled	0.6
Shrimp, frozen	0.6
Eggs	0.5
Bacon	0.5
Apples	0.2
Bananas	0.2
Strawberries	0.1
Asparagus	1.8
Spinach	1.8
Peas	0.6
Broccoli	0.5
White rice, boiled	Trace
Whole milk	Trace

Giving its lipophilic structure *(Farina et al. 2017)*, it can be found at the cell membrane level *(Böhm 2018).* α- and γ-tocopherols can be found in serum and erythrocytes, whereas β and δ tocopherols can be found in little quantities in plasma *(Rizvi et al. 2014)*. Plasma levels of vitamin E isoforms are

related to their absorption, distribution and excretion rate. The vitamin E isoform with longer half-life and most diffused in tissues is α-tochopherol (i.e., 20hours half-life) *(Browne et al. 2019).* Furthermore, the α form is one of the most important, because the high level of its transporter in the brain, α-tocopherol transfer protein (α-TT) *(Lloret et al.2019).* α-TT is a cytosolic protein of 30500 Da, mainly expressed in the liver which is able to recognize the α-tocopherol *(Kono and Arai 2015)* and regulates and deliver vitamin E in tissues *(Lloret et al. 2019).* In the brain α-TT is abundant in the Purkinje cell *(Kono and Arai 2015).*

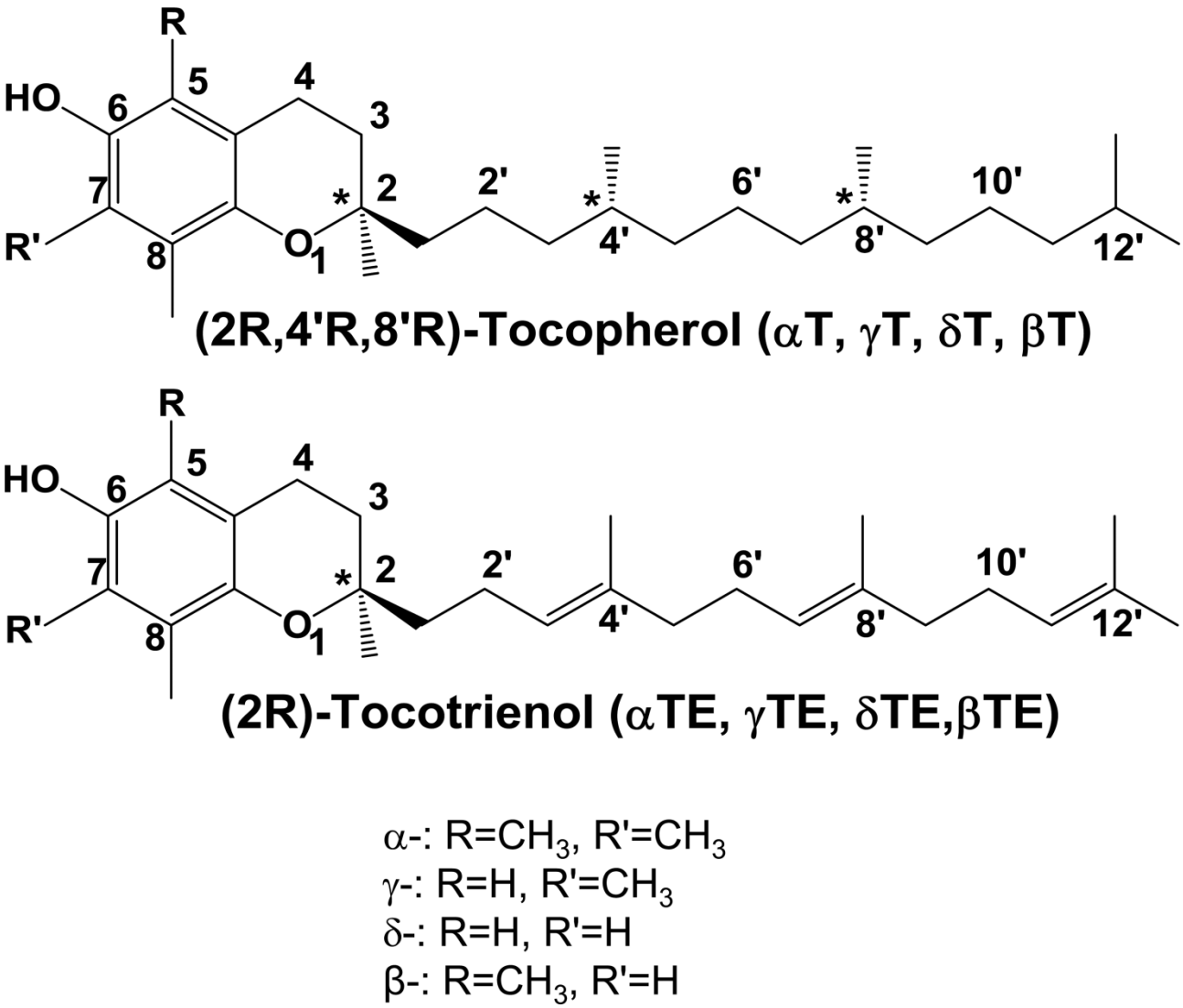
Mutation in the α-TT gene is related to progressive spinocerebellar ataxia, areflexia, loss of proprioception, and extremely low vitamin E levels. This evidence highlights the neuroprotective role of vitamin E *(Lloret et al.2019).* Other causes of vitamin E poor status with neurological problems can be fat malabsorption and diseases associated with to oxidative stress *(Lee and Ulatowski 2019).*

Dietary supplements of vitamin E are composed mainly by α-tochopherol, whose recommended daily dose in adults is 15 mg. Whereas the upper tolerable intake level to avoid side effects as hemorrhage is of 1000 mg/day (*Boccardi et al. 2016, Yap et al. 2001)*. However, there is evidence to discourage the intake of vitamin E supplements even at low doses because it can be related to several negative health outcomes, i.e., the increase the risk of bleeding (for example interacting with aspirin) or the risk of hemorrhagic stroke, prostate cancer, heart failure, hospitalization for heart failure in people with chronic diseases as diabetes *(Farina et al. 2017).* Indeed, a cochrane reported that taking vitamin E supplements, alone or together with other antioxidants, would significantly increase mortality *(Bjelakovic et al.2010).* The normal plasmatic levels of vitamin E range from 11 and 37μM *(Lee and Ulatowski 2019).*

The term "tocopherol" means methyl-substitutes derivates of tocol. Tocochromanols include tocopherols and tocotrienols, with saturated side chain and unsaturated side chain, respectively. Tocotrienols and tocopherols share the same basic chemical structure (i.e., long isoprenoid side chain attached at the 2 position of a 6-chromanol ring **(Figure 10**). The difference among tocopherols and tocotrienols is that tocotrienols have a farnesyl whereas tocopherols have a saturated isoprenoid C_{16} side chain. Tocotrienols have only one stereocenter at C-2. Natural tocotrienols possess 2R, 3'R, 7'E configurations. The body receptor and enzymes are highly stereoselective, so only one enantiomer has the desired effect whereas the others may have no effect or have an adverse effect. Vitamin E isoforms are not interconvertible inside the body (*Rizvi et al. 2014).*

A diet rich in vitamin E cannot has an optimal effect if are not provided vitamin C, vitamin B3, selenium and GSH. This because vitamin E is highly dependent from these molecules *(Rizvi et al. 2014)*.

Figure 10. Chemical structure of tocopherols and tocotrienols *(Jiang 2014*)

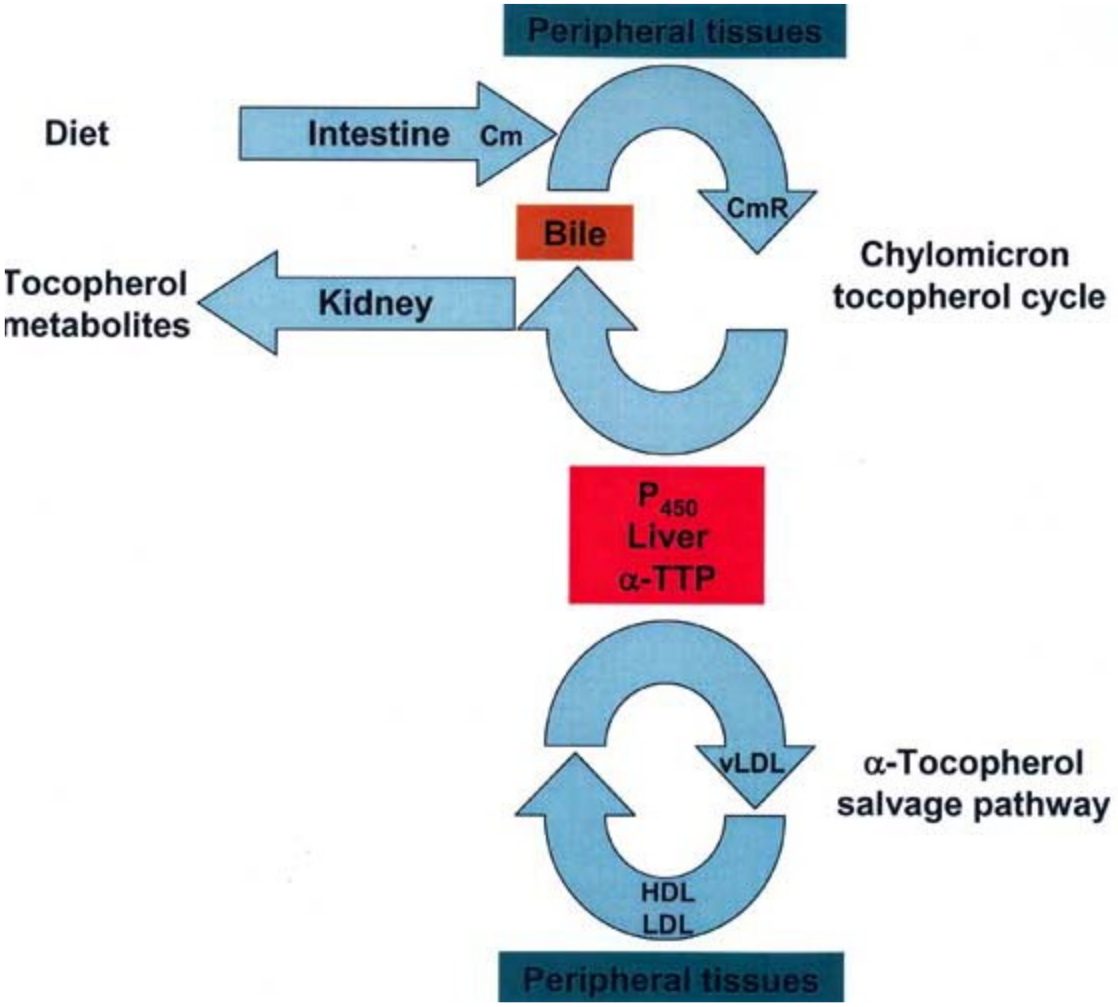


3.1.2. Absorption, metabolism and excretion of Vitamin E

Vitamin E has a clearance rate of 24-48 hours *(Azzi and Stocker 2000)*. Tocopherols introduced with the diet are absorbed in the intestine with fats, then they are secreted in chylomicrons with triacylglycerol, phospholipids and cholesterol *(Jiang 2014).* Later, chylomicrons are secreted in the lymph *(Dror and Allen 2011).* When chylomicrons lipolysis by lipoprotein lipase occurs, part of vitamin E reaches the peripheral tissues (i.e., muscular and adipose tissues, bone marrow, skin and brain), and the liver where the chylomicron remnants (CmR) capture the other part *(Zingg and Azzi 2004).* With the formation of CmR part of the surface components are transferred to high density lipoproteins (HDLs), then other circulating lipoproteins (*Dror and Allen 2011)*. CmR are secreted with very low-density lipoproteins (VLDLs). The other tocopherols and the excess of α isoform are metabolized (e.g., ω-hydroxylation, oxidation and β-oxidation) by the cytochrome P450 (CYP3A) enzyme and cleared by kidneys *(Zingg and Azzi 2004).* Therefore, from the catabolism performed by the cytochrome P450, 13'-hydroxychromanol (13'-OH), various carboxychromanols and terminal metabolite 3'-carboxychromanol (3'- COOH) or (2'-carboxyethyl)-6-hydroxychromans (CEHCs) are generated. When there is high concentration of circulating vitamin E, contemporary to the β-oxidation may take place conjugation processes (i.e., sulfation and glucuronidation) of the phenolic on the chromanol. The main routes of excretion are urine for short chain and conjugated carboxychromanols, feces for unconjugated carboxychromanols. Unmetabolized forms of vitamin E are also eliminated through the biliary excretion *(Jiang 2014)*. Up to 14% of the liver tocopherol is secreted with the bile and the 60% of α isoform in the bile is reabsorbed and go to a second chylomicron circle (*Zingg and Azzi 2004)*.

The α-tocopherol salvage pathway begin in the liver where the α-TT with the ATP binding cassette transporter A1 assemble the α-tocopherol with the lipoproteins VLDLs, to be transported via the circulation system *(Zingg and Azzi 2004, Jiang 2014).* Among the other vitamin E isoforms, α-TT has the highest affinity for α-tocopherol (i.e., affinity of 50% with β-tocopherol, 10-30% for the γ form, and 1% for the δ) (*Jiang 2014)*. In the blood, VLDLs are converted in LDLs and HDLs by lipoprotein lipase and deliver α-tocopherol to the tissues. The excess of tocopherols is brought back to the liver in LDLs and HDLs, and another cycle start. The α-tocopherol salvage pathway allows a continued increased level of the plasma α isoform due to the temporary permanence of α-tocopherol in chylomicrons after diet intake. The α-TT transporter is very important to bring α-tocopherol to the brain and to the periperal nervous system. Instead, muscles and other tissues receive sufficent tocopherols with the chylomicron tocopherol circle **(Figure 11)** *(Zingg and Azzi 2004).*

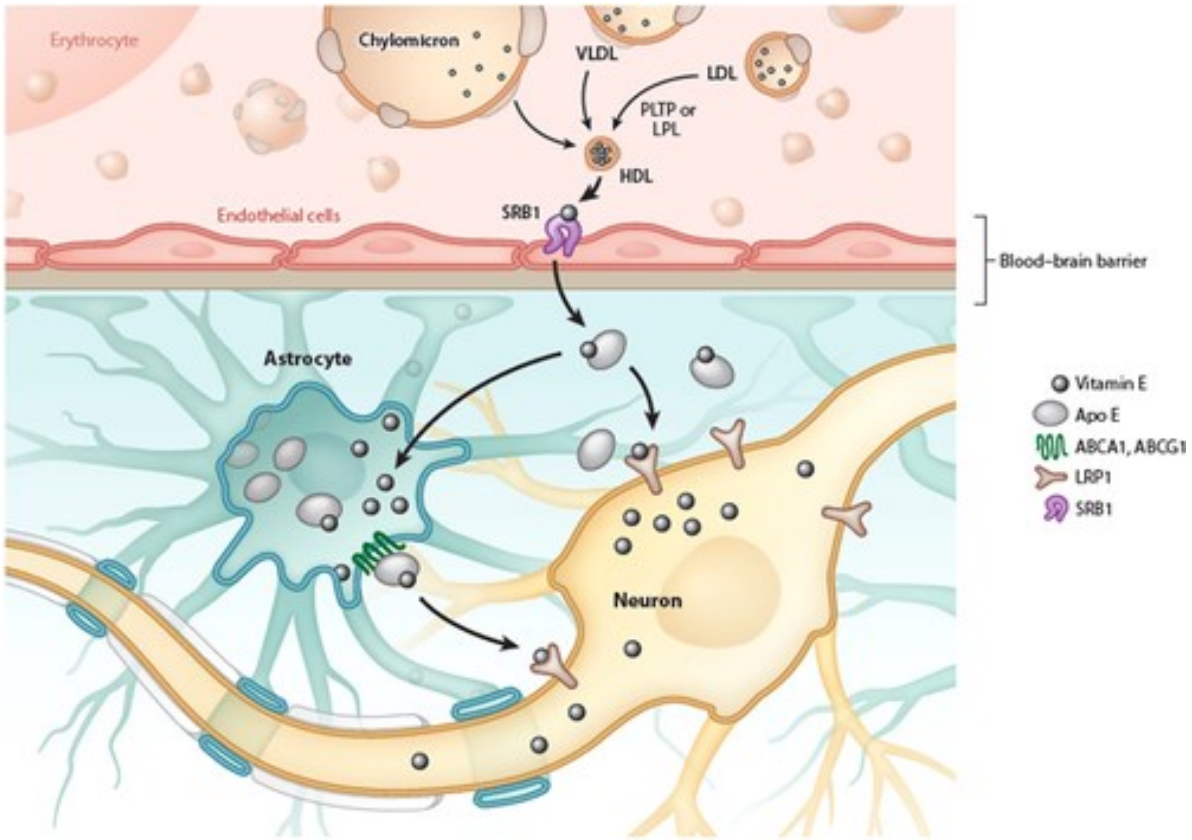
Figure 11. Metabolism of vitamin E *(Zingg and Azzi 2004)*



3.1.3. Mechanism of transport of Vitamin E in the central nervous system

In order to reach the brain, vitamin E needs to pass the BBB **(Figure12).** This important step is possible thanks to the fat solubility of vitamin E *(Lee and Ulatowski 2019).*

Figure 12. passage of vitamin E through the BBB and transport into the CNS *(Lee and Ulatowski 2019)*



Vitamin E may cross the BBB with HDLs through the scavenger receptor class B type 1 (SRB1) expressed on endothelial cells. Studies on animal models suggest that the α-tocopherol regulates the expression of the (SRB1) which is an HDLs receptor. If SRB1 is blocked, the α-tocopherol cannot be delivered in the brain. After passing through the BBB, vitamin E arrives at astrocytes located near the BBB, called Bergmann glia cells, enters in the cell with an unknown mechanism. Given the role of α-TTP, scientists supposed that they help Bergmann glia cells to secrete the vitamin E to the Purkinje cells and to other cells in the brain.

ApoE is synthetized by astrocytes and secreted through membrane proteins (i.e., ABCA and ABCG). ApoE binds the vitamin E transporting it via the cerebral spinal fluid to other cells of the CNS and to the neurons via low-density lipoprotein receptor-related protein 1 (LRP1) both for maintenance or during oxidative stress. It is not clear the mechanism of the movement of vitamin E from astrocytes to neurons.

Oxidative stress may up-regulate the expression of α-TTP *(Lee and Ulatowski 2019)*, i.e., the increasing levels of ROS stimulate the α-TTP expression *(Lee et al.2020).* This results in the regulation of the Vitamin E transport in the CNS to brain site where this molecule is needed.

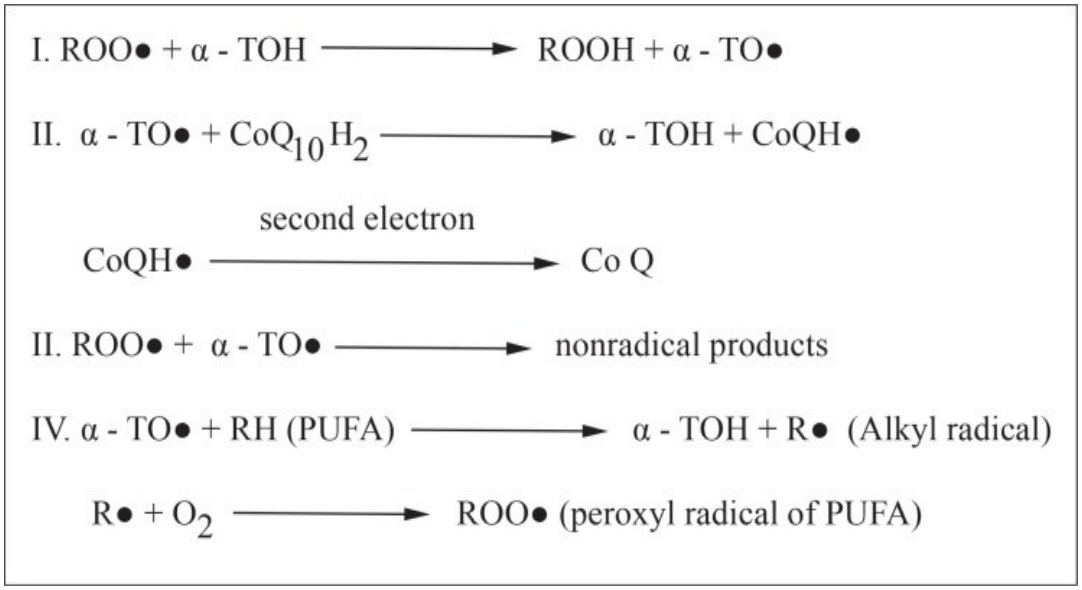
3.1.4. Properties of vitamin E

- Antioxidant and neuroproptective:

  One of the most important property of the vitamin E is the antioxidant property. It is necessary to protect the cell membrane from oxidative damage preventing lipids peroxidation *(Meydani et al. 2018)* **(Figure 13)** and to maintain a healthy nervous system. In fact, the neuroprotective effects of this vitamin are known in conditions of considerable oxidative stress and in conditions of disease (*Mustapha et al. 2020)*. Animal studies have shown the importance of this vitamin in maintaining the integrity of Purkjinie cells *(Ulatowski et al.2014)* and in embryonic brain development *(Head et al.2020)*. Further human studies appear to support the neuroprotective role of this vitamin *(Pace et al.2003, Mangialasche et al.2010)*. Vitamin E activates the differentiation of the neuroglia, preventing the ataxia with deficiency of vitamin E. This vitamin is also a protector of neurons of the hippocampus and regulates inflammatory mechanisms of the neuroglia *(Teleanu et al.2019)*. The distribution of vitamin E in the brain is not homogeneous, which suggests its role of protection from oxidative stress. Its concentration is very high at the level of nuclear membranes, suggesting a role of vitamin E in the nuclear functions of cerebellum and striatum *(Lee et al. 2020)*.

  Vitamin E antioxidant role is given by the presence of a hydroxyl group on its phenolic group on the chromanol ring which can donate hydrogen atom neutralizing a great variety of ROS and obtaining a vitamin radical and a non-radicalproducts *(Lloret et al.2019)*. The antioxidant role of Vitamin E can reduce the negative effects of ROS converting them into less reactive compounds *(La Fata et al. 2014)* inhibiting the lipidic peroxidation *(Farina et al. 2017)*. The vitamin E radical can react with another free radical lipid or be regenerated back from their native form by vitamin C (*Lloret et al.2019)*, ubiquinols and thiols *(Lee et al. 2020)*. In this way vitamin E neutralizes peroxyl radicals and blocks lipid peroxidation, protecting cellular membranes. Vitamin E antioxidant capacity is higher than GSH or β-carotene (*Lloret et al.2019)*. The α-tocopherol is the isoform with highest antioxidant property *(Brigelius-Flohé 2009)*.

Figure 13. Mechanism by which vitamin E protects membranes from lipid peroxidation *(Rizvi et al.,2014)*



- Other properties

Vitamin E is involved in the regulation of signal transduction and the expression of numerous genes *(Böhm 2018).* At post translational level inhibits protein Kinase C, 5-lipoxygenase and phospholipase A2 and activates protein phosphatase 2A and diacylglycerol kinase. Modulates at the transcriptional levels several genes (e.g., scavenger receptors, alpha-TTP, alpha-tropomyosin, matrix metalloproteinase-19 and collagenase). It also has an immune-modulator role enhancing the lymphocytes proliferation *(Kemnic and Coleman 2020).* Among other non-antioxidants properties of vitamin E there is the inhibition of cell proliferation, platelet aggregation, monocyte adhesion. antioxidant signaling and cellular functions *(Zingg and Azzi 2004).*

3.1.5. The relationship between vitamin E and cognitive status: evidence from scientific literature

The relationship between vitamin E levels and the presence of certain pathologies has been demonstrated in the literature. In the same way seems that the supplementation with this vitamin can give some improvements to some pathological conditions (i.e., ataxia, diseases affecting motor activity). Instead, the association between vitamin E and cognitive decline still not conclusive, due to the difficult to perform high quality long-term studies in humans *(La Fata et al. 2014).* Here are reported some evidence from the scientific literature.

Low levels of vitamin E have been found in people with the allele apolipoprotein E- ε4 (ApoE-ε4) *(Huebbe et al.2010),* and in people with AD *(La Fata et al. 2014, de Wilde et al. 2017),* and mild MCI *(La Fata et al. 2014).* People with the allele ApoE-ε4 have higher risk of AD and cardiovascular pathologies. In subjects with this allele, it is observed that vitamin E is less able to be retained in the tissues, furthermore a greater inflammatory state is observed *(Huebbe et al.2010).*

The relationship between and adequate nutrition and healthy brain has been established in the 80s in 260 healthy older people aged > 60 years, where good cognitive status was associated with higher plasma concentrations of folate, vitamin B12, vitamin C, etc. Some years later a study reported improvements in cognitive performance in people with good nutritional status, after the supplementation with vitamin E, C, and thiamine. These associations just described, however, were modest, probably due to the involvement of healthy subjects rather than carrying out the same study in subjects with nutritional deficiencies *(La Fata et al. 2014).* Later, a study in the U.S. using data of multiethnic older adults participating in the cross-national Third National Health and Nutrition Examination Survey (1988-1994) showed the association between the reduction of the serum vitamin E levels and impaired memory (delayed recall score < 4). Whereas the association with memory and vitamin A, C, β-carotene and selenium was not showed *(Perkins et al. 1999).*

Moreover, another study reported significant high level of vitamin E, and high level of the activity on antioxidants enzymes as catalase and erythrocytes glutathione reductase in the plasma of Polish centenarians with healthy cognitive status *(La Fata et al. 2014).* In 14968 older women aged 70-79years participating at the Nurses' Health Study, and followed from 1980 to 2000, cognitive performance was significantly better in women which received supplementation (vitamin E and C) at long term (>10 years) and in any case better than those who did not take supplements. People which received high level of vitamin E had better cognitive performance than those who received low levels. Whereas the improvement obtained was little if only vitamin C was taken, the association with

vitamin E was crucial to obtain better results *(La Fata et al. 2014, Grodstein et al.2003).* The Supplementation en Vitamines et Mireraux Antioxydants 2 (SU.VI.MAX2) study was a French observational study with duration of 13 years, that investigated the effects of foods rich of antioxidants, mainly fruit and vegetables, on cognitive performance. 2533 adults aged 45-60 years at baseline were selected. They had to record their nutrition every two months over 24 hours. Antioxidants introduced with supplements were not considered. In the SU.VI.MAX2, vitamin E consumption was associated with better cognitive performance at 13 years *(La Fata et al. 2014, Péneau et al.2014).*

An interesting study was performed on 2889 older adults aged 65-102 years in order to verify if antioxidants (i.e., vitamin E, C, carotene) were associated with changes in cognitive status. This research was conducted from 1993 to 2000 (average follow-up 3.2 years). Results showed that vitamin E was associated with lower cognitive impairment, whereas a not significant effect was observed when vitamin C and carotene were analyzed *(Morris et al. 2002).* Again, a publication using data of the large epidemiological Italian study titled InCHIANTI (Invecchiare in Chianti, aging in the Chianti area), investigated the presence of dementia and cognitive impairment in relation to plasma vitamin E levels on 1033 older adults aged ≥ 65 years. People with levels of vitamin E in the bottom tertile had the high probability to have dementia and cognitive impairment (*Cherubini et al., 2005)*.

Negative results were obtained in the cognitive sub-study of the Women's Health Study, which was a randomized, double-blind, placebo-controlled trial carried out on 6377 women aged ≥ 65 years which were randomly assigned to 600 IU vitamin E supplementation to be taken on alternate days, with four years of follow-up. There were not significant differences in cognitive change in placebo vs intervention group during the period of the study. Authors concluded that long-term intake of vitamin E was not associated with cognitive benefits in healthy older women *(Kang et al.2006).* A recent Cochrane by *Farina et al. 2017* found no evidence supporting the use of α-tocopherol in cognitively impaired people in order to prevent the progression to dementia, or in order to improve the cognitive function in people with MCI or dementia caused to AD. This result was supported by a subsequent Cochrane *(McCleery et al. 2018*) aimed at evaluating the effect of vitamin and mineral supplements on cognition and incidence of dementia in people with MCI.

3.2. Rationale and aim

From a public health perspective, it is of primary importance to try to prevent or postpone the onset of cognitive decline and to try to slow its progression into dementia *(Cherubini et al. 2005).* Given that a pharmacologic treatment to prevent or delay cognitive impairment and dementia does not exists *(Gorelick 2018)*, it is mandatory try to intervene on non-modifiable risk factors, such as nutrition.

The aim of the **study 1** is to verify the presence of an association between plasma vitamin E levels and cognitive impairment, in the older adults' population non-demented at baseline of the InCHIANTI (Invecchiare InCHIANTI, aging in the Chianti area) study, and followed for a maximum period of 17 years.

Although the InCHIANTI study focuses mainly on mobility disability, it is a large Italian epidemiological study, with a long follow-up that allows us to have a large amount of clinical and biological data available useful to analyze other aspects related to aging.

3.3. Materials and methods

3.3.1. The InCHIANTI study

The InCHIANTI Study (Invecchiare in Chianti, aging in the Chianti area) is an Italian study performed in two small towns near Florence, e.g., Greve in Chianti and Bagno a ripoli. The project is now financed by the National Institute on Aging (NIH, NIA, Bethesda, USA), but it was initially coordinated by the: Italian National Research Center on Aging (INRCA, Florence, Italy) with the financial support of the Italian Health Ministry and NIH. The project is actually coordinated by a collaboration among the the Florence Health Care Agency, the Tuscany Regional Health Agency, the general pratictioners and administrators of Greve in Chianti and Bagno a Ripoli. The study focus on the changes associated with aging leading to mobility disability in order to translate research evidence into clinical tools to improve the diagnosis and treatment of older adults with impaired mobility.

More in depth, the project plans to:

- study the interaction among the different physiological subsystems associated with mobility and age in determining the onset of disability;
- develop reference values to be used in clinical practice in order to assess the state of the physiological subsystems involved in mobility;
- study the risk factors for the development of mild neurological impairment;
- estabilish dysregulation biomarkers of signals that maintain a homeostasis after a distressful event;
- produce relevant scientific evidence *(www.inchiantistudy.net).*

The study began its data collection in 1998 which lasted two years. Five successive follow-ups were then carried out. The first in 2001-2003, the second in 2004-2006, the third in 2007-2009, the fourth in 2013-2015, the fifth 2016-2017. The data of the last follow-up are not yet available.

3.3.2. Study sample

The InCHIANTI study was conducted in Greve in Chianti and Bagno a Ripoli, two small towns near Florence, with a significant percentage of people aged ≥ 65 years on the entire population, 19.3% on 11709 inhabitants and 20.3% on 4704 inhabitants respectively *(Ferrucci et al.2000).*

At baseline 1270 people with ≥ 65 years were randomly selected from the population registry. People aged ≥ 90 were randomly selected until reach 30 men and 30 women of this age. For the following age groups, 20-29, 30-39, 40-49, 50-59, and 60-64 years, 30 men and 30 women for each decade from 20-59 were randomly invited, whereas 10 men and 10 women for the age group 60-64 *(Ferrucci et al.2000)* **(Table 4)**.

Table 4. Older adult participants at baseline from Greve in Chianti and Bagno a Ripoli *(modified from Ferrucci et al.2000)*

	Greve in Chianti *(11.709 residents)*		**Bagno a Ripoli** *(4.074 residents)*	
	Sample (n)	**N. of people of the specific age group living in the town**	**n**	**N. of people of the specific age group living in the town**
Men				
65-69 years	76	327	83	133
70-74 years	86	329	72	119
75-79 years	55	249	48	75
80-84 years	26	102	28	48
85-89 years	21	100	23	35
90+ years	15	25	20	20
Women				
65-69 years	104	395	86	153
70-74 years	83	368	85	130
75-79 years	73	301	86	112
80-84 years	42	181	48	82
85-89 years	41	179	45	64
90+ years	24	66	29	33

3.3.3. Data collection

Potential participants were invited to participate by a letter, followed by a phone call which fixed the interview at home. In case of refusal a maximum of four additional attempts were made before retaining the potential participant as excluded. The home interview was performed by three experienced interviewers. Before starting the questionnaire, the participant was invited to sign the informed consent. The informed consent was also used to allow researchers to access information related to hospitalization, drugs use, etc., and for the conservation and use of biological samples for research. In people with Mini Mental State Examination (MMSE) score ≤ 18 the interview was made to a proxy. Information collected at the home interview were on: architectural limitations, family and social network, depressive symptoms, ability to perform the activities of daily living (ADL), foot problems, anamnesis of fears of falling and falls, medications taken regularly and in the past, present and past health-related behaviors, urinary incontinence, sleep quality, eating habits. After home interview two more appointments have been set, and the 24h urine sampling has been explained with the recommendation not to eat meat and fish during sampling and in the previous 48 hours. Within three weeks of the home interview, the participant had to go to the study office to bring the container with urine collection of the previous 24 hours and being fasting for 8 hours to do some blood test.

- Part of the blood (i.e., 60mL) was used to perform several hematological and chemistry tests, and the other part was used for the biobank (Presidio Ospedaliero Piero Palagi, in Florence). The biobank was used for subsequent analysis.
- 0.3 mL of plasma and serum aliquots and 10 mL of the 24h urine aliquots were stored at -80°C (*Ferrucci et al.2000).*
- Leukocytes DNA was extracted by saline method and stored at 4°C *(Lahiri et al. 1992, Ferrucci et al.2000).*

On the same occasion, clinical tests were carried out, among which we cite as an example, the electrocardiogram and peripheral quantitative computed tomography (*Ferrucci et al.2000)*.

After two weeks from sampling, the participant met a geriatrician or a rehabilitation therapist to be subjected to a clinical and functional evaluation **(Table 5)**. Also, the six subsystems (i.e., bones, joints, muscles, CNS, peripheral nervous system, energy production and delivery) affecting walking performance were analyzed.

Table 5. Functional evaluation (modified from *Ferrucci et al.2000)*

Functional evaluation assessment
Height
Weight
Manual dexterity (Purdue Pegboard)
Evaluation of the ability of use stairs
Time needed to perform 5 chair-stands
Dynamic baropodometric analysis
Balance evaluation
Walking 4 meters and 7 meters at usual pace/fast as possible
Walkjng 4 meters within the limits of 25 and 15 cm wide path fast as possible
Walking 7 meters with steps long as possible
Walking 7 meters stepping over 2 obstacles with and without sunglasses
Walking 7 meters carrying a large package which non allows to see the feet
Talking and walking
Collect specific items during 7 meters walk
Walking 400 meters fast as possible
Walking 60 meters fast as possible wearing heavy jacket
Lower extremity muscle power
Range of motions of all the main movements of hips, knees, ankles and shoulders
Muscle strength of low extremity 8 muscle groups, and upper extremity 2 muscle groups
Grip strength (Jamar dynamometer)

Main diseases were identified using standard algorithms based on medical history, medical documents, hospital discharge records, drug therapy, sign and symptoms (*Ferrucci et al.2000)*. A further evaluation has been planned for those with MMSE score < 26 after the administration of recovery tests (e.g., possible cognitive issues), subjects who reported at least one episode of incontinence in the previous three months, and who referred recurrent foot pain while walking.

Participants were followed longitudinally for 19 years **(Table 6)**.

Table 6. Baseline and follow-ups of the InCHIANTI study. The last follow-up was carried-out in the 2016-2017, but the data still not available *(modified from the InCHIANTI project's website http://inchiantistudy.net/wp/)*

	Baseline	**Follow-up 1**	**Follow-up 2**	**Follow-up 3**	**Follow-up 4**
Years	1998-2000	2001-2003	2004-2006	2007-2009	2013-2015
N. of participants	1453	1167	1067	900	687

3.3.4. Evaluation of cognition

Compromised cognition and dementia were assessed during the home interview using the MMSE *(Cherubini, et al. 2005, Folstein et al.1975)*. The MMSE *(Folstein et al.1975)* is an eleven questions tool, which can be administered in 5-10 minutes, it is no timed. It is composed by two sections (maximum total score 30). The first section (maximum score 21) covers orientation, memory and attention, it requires voice answers. The second section (maximum score 9) assess the ability to name, to follow voice and written commands, to write a sentence chosen by the participant, copy a polygon which reminds the Bender-Gestalt Figure.

A MMSE score > 26 identify non-demented people whereas a result ≤ 21 identify probable dementia. More specifically a MMSE score of 20-24 suggests mild dementia, a score 13-20 suggests moderate dementia whereas a score < 12 suggest severe dementia. The final score can be adjusted based on age and years of education **(Table 7)**.

Table 7. Factor to consider adjusting the MMSE score *(modified from Magni et al.1996)*

Years of education	**Score adjustment**				
0-4	+0.4	+0.7	+1.0	+1.5	+2.2
5-7	-1.1	-0.7	-0.3	+0.4	+1.4
8-12	-2.0	-1.6	-1.0	-0.3	+0.8
13-17	-2.8	-2.3	-1.7	-0.9	+0.3
Age groups	**65-69**	**70-74**	**75-79**	**80-84**	**85-89**

People with MMSE ≤ 21 underwent to a second assessment. Subject with a MMSE of 22-26 underwent to the memory assessment by the paired word test *(Wechsler 1987)* and to the attention assessment through the digit test from the Weschler adult intelligent test (*Wechsler 1958)*, and to the visuo-spatial capacity assessment with the Caltagirone drawings (*Carlesimo 1996).* If based on the memory test the participant was considered as normal, a full score of MMSE was given. Five points were given back at the voice "subtract seven five times from 100" and one point was given back to the item "pentagon drawing" if in other test evaluating the same neuropsychological functions were considered intact. At this point people with MMSE score > 26 were considered cognitively intact, whereas people with a score 22-26 continued the evaluation. In participants with MMSE 22-26 a diagnosis of dementia using the criteria of the diagnostic and statistical manuals of mental disorders,

version III-R (DSM III-R) *(American Psychiatric Association. 1987)* criteria was conducted by a geriatrician and a psychologist (*Cherubini, et al. 2005).*

In the DSM- III-R the diagnostic criteria of dementia are *(American Psychiatric Association. 1987)*:

A. evidence of impaired long-term and short-term memory;

B. compromised abstract thinking (1), and/or compromised judgement (2), and/or aphasia, apraxia, agnosia, problems in "constructional" abilities (3), and/or changes of the personality (4);

C. A and B criteria affect work and/or social activities and/or relationships;

D. these disturbances not occour only during delirium;

E. Both (1) or (2):
(1) anamnesis, physical assessment, laboratory test evidence etiologically related with the problem
(2) if these evidence do not exist, an etiologic organic element can be considered if the problem cannot be related to any nonorganic mental disorder

3.3.5. Vitamin E measurement

The method used to dose Vitamin E (α-tocopherol) from plasma was reverse-phase high performance liquid chromatography (HPLC). Vitamin E (e.g., α-tocopherol) cocentration was expressed in μmol/l. The American Association for Laboratory Accreditation, Washington, DC, USA provided a sample with known concentration of α-tocopherol, to be used to examine the reproducibility and accuracy of the methodology. This sample was tested in triplicate. The intra and inter-lot coefficients of variation were 3% and 4.2% respectively.

100 μL of plasma were mixed with 100 μL ethanol and vortexed. Tocopherol was extracted into 500 μL hexane containing 0.002% butylated hydroxyl toluene (Sigma, St. Louis, MO). Tocol (a gift from Hoffman La Roche, Nutley, NJ), was added as internal standard. After the centrifugation (800 rpm for 5 min at 4 ◦C) the supernatant was collected. The surnatant was dried by a stream of nitrogen gas and reconstituted in 100 μL of methanol. Tocopherols were separated by HPLC with a 3 μm C18 reverse phase column (Perkin- Elmer, Norwalk, CT). The mobile phase (e.g., 1% water in methanol, containing 10 mmol/l lithium perclorate) was delivered with a flow rate of 1.0 ml/min. An autosampler, 1100 series, Hewlett-Packard, was used to inject samples. An applied potential of +0.6V by a LC 4B amperometric electrochemical detector (Bioanalytical System, West Lafayette, IN) was used to the detection of the eluted peaks. Peaks were integrated with a ChemStation software (Hewlett-Packard) *(Cherubini, et al. 2005).*

3.3.6. Statistical analysis

Continuous variables were described with means and standard deviations, while categorical ones were reported as count and percentages. Student T test or one way ANOVA was used for continuous variables and chi-square test for categorical ones. Bonferroni correction was used to adjust multiple comparisons Nonparametric alternatives were used whenever appropriate. Predictors of cognitive decline were assessed using multivariate Cox proportional hazards regression models. In the absence of a conversion event, data were censored at the last available follow-up. After having assessed the role of each risk factor, a p-value lower than 0.20 was used as screening criterion to consider the risk factor as candidate for the multivariate analysis. In case p-value > 0.20 for clinically relevant variables, these will in any case be considered in the multivariate analysis. This decreased the probability of incorrect rejection of potentially important variables due to uncontrolled confounding. Backward elimination was used to select a final model. Significance level of 5% was assumed for all the analyses. Statistical analyses were performed using SPSS v. 25 (International Business Machines Corporation).

3.4. Results

The subjects who accepted to participate to the survey at baseline were 1453 (age 20-102 years). People aged ≥ 65years at baseline were 1155.

3.4.1. Description of the participants of the InCHIANTI study at baseline

In the tables presented in this section, the characteristics of the 1453 participants of the InCHIANTI study at baseline are reported.

The main demographic and clinical characteristics of the baseline total sample are shown in **Tables 8-10**.

At baseline, participants were manly women, with mean age 68.9 (±15.7) years **(Table 8)**. The schooling of most of the participants stopped at the elementary school. The group of participants over 85 was the one who experienced widowhood with a higher percentage. With regards to lifestyle, participants aged < 65 years is the group with higher percentage of smokers (35.9%), whereas the age group more sedentary was made with participants >85 years (58.2%). The group aged > 85 years has also the lowest BMI (26.0 ±4.0 Kg/m^2). The group for which a BMI > 30 was more prevalent (26.1%), was the group aged 65-75 years.

Table 8. Socio-demographic characteristics and baseline lifestyle of the participants in the InCHIANTI project, in relation to age.

	Total (n=1453, 100%)	**<65years** (n=298, 20.5%)	**65 – 75 years** (n=610, 42 %)	**75 – 85 years** (n=366, 25.2%)	**≥ 85 years** (n=179, 12.3%)	**P**
Sex (F)	811 (55.8%)	156 (52.3%)	322 (52.8%)	216 (59.0%)	117 (65.4%)	0.008
Age (years)	68.9 (±15.7)	43.7 (±13.0)	69.5 (±2.8)	78.8 (±3.0)	88.8 (±3.1)	<0.001
Education						
None	360 (24.8%)	4 (1.3%)	115 (18.9%)	134 (36.6%)	107 (59.8%)	<0.001
Elementary school	660 (45.4%)	63 (21.1%)	353 (57.9%)	185 (50.5%)	59 (33.0%)	
Secondary school	158 (10.9%)	82 (27.5%)	58 (9.5%)	15 (4.1%)	3 (1.7%)	
Other	259 (17.9%)	147 (49.3%)	77 (12.7%)	27 (7.4%)	8 (4.5%)	
Marital status						
Unmarried	169 (11.6%)	84 (28.2%)	39 (6.4%)	29 (7.9%)	17 (9.5%)	<0.001
Married	883 (60.8%)	200 (67.1%)	458 (75.1%)	183 (50.0%)	42 (23.5%)	
Widow/er	381 (26.2%)	4 (1.3%)	103 (16.9%)	154 (42.1%)	120 (67.0%)	
Lives alone	229 (15.8%)	15 (5.0%)	77 (12.6%)	103 (28.1%)	34 (19.0%)	<0.001
Smokers	268 (18.4%)	107 (35.9%)	112 (18.4%)	43 (11.7%)	6 (3.4%)	<0.001
Sedentary	290 (20.1%)	12 (4.1%)	64 (10.5%)	111 (30.4%)	103 (58.2%)	<0.001
BMI (Kg/m^2)	27.2 (±4.1)	26.2 (±4.1)	27.8 (±4.1)	27.3 (±4.1)	26.0 (±4.0)	<0.001
BMI ≥30	295 (23.4%)	52 (18.4 %)	147 (26.1%)	80 (26.1%)	16 (14.8%)	<0.001

All data are n (%) except age and BMI (mean± sd)

BMI: body max index

At baseline, the group of participants aged > 85 years had higher number of concomitant diseases (1.6 ±1.3) and severe sensorial deprivation (i.e., severe hearing loss 1.9%, severe low vision 1.5%). In participants aged >85 years the most common disease was hypertension (45.7%) followed by knee arthrosis (41.2%), depressive symptoms (37.2%), heart failure (35.8%), chronic ischemic heart disease and ApoE4 (16.2% each), COPD (15.1%), diabetes (12.8%), stroke (10.6%), severe low vision (10.1%), osteoarthritis of the hip and severe hearing loss (9.5% each), femur fracture (6.4%), Parkinson (4.5%) and cancer (3.4%). Whereas the most common diseases among all ages participants was hypertension (53.5%), followed by knee arthrosis (29.9%), heart failure (19.8%), diabetes (10.4%), osteoarthritis of the hip (10.3%), COPD (9.6%), chronic ischemic heart disease (9.4%), stroke (6.1%), cancer (4.5%) and femur fracture (3.0%). The 19.8% of older participants had depressive symptoms. The ApoE4 (one allele at least) was present in the 16.7% of the older population participating at the InCHIANTI study. The age group with higher percentage of this allele was the group of people younger than 65 years (19.6%) **(Table 9)**.

Table 9. Clinical characteristics of the participants in the InCHIANTI project, in relation to age

	Total (n=1453, 100%)	**<65 years** (n=298, 20.5%)	**65 – 75 years** (n=610, 42%)	**75 – 85 years** (n=366, 25.2%)	**≥ 85 years** (n=179, 12.3%)	**P**
Diseases						
COPD	140 (9.6%)	6 (2.0%)	71 (11.6%)	36 (9.8%)	27 (15.1%)	<0.001
Chronic ischemic heart disease	137 (9.4%)	5 (1.7%)	44 (7.2%)	59 (16.1%)	29 (16.2%)	<0.001
Heart failure	287 (19.8%)	9 (3.0%)	99 (16.2%)	115 (31.4%)	64 (35.8%)	<0.001
Osteoarthritis of the hip	149 (10.3%)	11 (3.7%)	75 (12.3%)	46 (12.6%)	17 (9.5%)	<0.001
Diabetes	151 (10.4%)	11 (3.7%)	79 (13.0%)	38 (10.4%)	23 (12.8%)	<0.001
Hypertension	242 (53.5%)	777 (29.9%)	89 (57.0%)	348 (66.1%)	98 (45.7%)	<0.001
Cancer	66 (4.5%)	2 (0.7%)	36 (5.9%)	22 (6.0%)	6 (3.4%)	0.002
Parkinson	28 (1.9%)	0 (0%)	12 (2.0%)	8 (2.2%)	8 (4.5%)	0.007
Stroke	89 (6.1%)	4 (1.3%)	33 (5.4%)	33 (9.0%)	19 (10.6%)	<0.001
Knee arthrosis	386 (29.9%)	51 (18.1%)	169 (29.8%)	117 (36.4%)	49 (41.2%)	<0.001
Femur fracture	40 (3.0%)	2 (0.7%)	13 (2.3%)	16 (4.9%)	9 (6.4%)	0.001
N. of diseases	1.3 (±1.2)	0.5 (±0.7)	1.4 (±1.2)	1.6(±1.1)	1.6 (±1.3)	<0.001
ApoE4 (one allele at least)	222 (16.7%)	56 (19.6%)	87 (15.3%)	56 (16.8%)	23 (16.2%)	0.460
Depressive symptoms (CES-D≥20)	272 (19.8%)	31 (10.4%)	95 (15.6%)	98 (28.7%)	48 (37.2%)	<0.001
Severe hearing loss	28 (1.9%)	0 (0%)	3 (0.5%)	8 (2.2%)	17 (9.5%)	<0.001
Severe low vision	22 (1.5%)	0 (0%)	1 (0.2%)	3 (0.8%)	18 (10.1%)	<0.001

All data are n (%) except the n. of diseases (mean ±sd)

COPD: Chronic obstructive pulmonary disease, CES-D: Center for Epidemiological Studies Depression Scale

At baseline the group aged > 85 years had more disabilities, i.e., lost more ADL and IADL (1.1 ±1.9 and 3.8 ±3.1 respectively) than the other age groups **(Table 10)**. Also, the MMSE score was worse in this group of older adults (18.8 ±7.9), also if adjusted for the years of education (20.7±7.7). The Trail Making Test score worsen with the increase of age, in fact the age group > 85 years shown the worst results (part A score 166.8±79.0, and part B score 262.6±98.5). Concerning physical performance, the group of people aged >85 years shown the worst results: slower speed on 4m (0.7±0.3 m/s) and slower speed 400 meters at a fast pace (1.0±0.2m/s), the lower SPPB score among age groups (5.6±4.1), the higher prevalence of SPPB score <10 (76.5%), the higher prevalence of experiencing a lot of difficulty or be not able (referred) to walk for 400m (50.9%) or 1 Km (621%) or around home (22.7%) or to need help or be not able to climb stairs (62.6%).

Table 10. Cognitive and functional characteristics in the subjects participating in the InCHIANTI project assessed in this study, in relation to age

	Total (n=1453, 100%)	**<65 years** (n=298, 20.5%)	**65 – 75 years** (n=610, 42 %)	**75 – 85 years** (n=366, 25.2%)	**≥ 85 years** (n=179, 123%)	**P**
N. lost ADL	0.3 (±1.0)	0.0 (±0.2)	0.1 (±0.5)	0.3 (±1.2)	1.1 (±1.9)	<0.001
N. lost IADL	0.9 (±2.1)	0.0 (±0.2)	0.2 (±1.0)	1.2 (±2.2)	3.8 (±3.1)	<0,001
MMSE						
MMSE score	25.6 (±5.4)	29.0 (±1.5)	26.9 (±2.6)	24.0 (±5.7)	18.8 (±7.9)	<0.001
MMSE aduste score	25.2 (±4.6)	26.9 (±1.3)	26.1 (±2.4)	24.3 (±5.5)	20.7 (±7.7)	
Trail Making Test						
part A (sec)	84.8 (±59.8)	40.5 (±21.2)	79.5 (±43.9)	117.9 (±66.0)	166.8 (±79.0)	<0.001
part B (sec)	154.9 (±89.5)	92.8 (±58.4)	163.1 (±79.1)	211.7 (±90.3)	262.6 (±98.5)	<0.001
Speed on 4 m (m/s)	1.1 (±0.3)	1.3 (±0.2)	1.1 (±0.2)	1.0 (±0.3)	0.7 (±0.3)	<0.001
Usual speed at 4m (m/s) <0.8 m/s	179 (14.3%)	1 (0.4%)	37 (6.7%)	72 (23.8%)	69 (61.1%)	<0.001
Speed 4 meters at a fast pace (m/s)	1.5 (±0.4)	1.8 (±0.3)	1.5 (±0.3)	1.3 (±0.3)	1.0 (±0.4)	<0.001
Speed 400 meters at a fast pace (m/s)	1.3 (±0.3)	1.6 (±0.2)	1.3 (±0.2)	1.1 (±0.2)	1.0 (±0.2)	<0.001
SPPB						
SPPB score	10.3 (±3.2)	11.9 (±0.8)	11.3 (±2.0)	9.3 (±3.5)	5.6 (±4.1)	<0.001
SPPB < 10	275 (21.1%)	6 (2.1%)	50 (8.8%)	115 (35.7%)	104 (76.5%)	<0.001
SPPB≥10	1031 (78.9%)	276 (97.9%)	516 (91.2%)	207 (64.3%)	32 (23.5%)	<0.001
Difficulty walking for 00 meters (referred)						
None/some	1212 (87.2%)	297 (99.6%)	573 (97.1%)	262 (77.3%)	80 (49.1%)	<0.001
A lot /not able	178 (12.8%)	1 (0.3%)	17 (2.9%)	77 (22.8%)	83 (50.9%)	
Difficulty walking for 1 Km (referred)						
None/some	1137 (81.2%)	294 (99.3%)	550 (92.3%)	229 (67.3%)	64 (37.8%)	<0.001
A lot /not able	264 (18.8%)	2 (0.7%)	46 (7.7%)	111 (32.7%)	105 (62.1%)	
Difficulty walking around home (referred)						
None/some	1338 (95.6%)	298 (100%)	596 (99.3%)	325 (93.4%)	119 (77.3%)	<0.001
A lot /not able	62 (4.4%)	0 (0.0%)	4 (0.7%)	23 (6.6%)	35 (22.7%)	
Ability to climb stairs (referred)						
Able	1127 (77.6%)	292 (98.0%)	530 (86.9%)	238 (65.0%)	67 (37.4%)	<0.001
Helped /not able	326 (22.4%)	6 (2.0%)	80 (13.1%)	128 (35.0%)	112 (62.6%)	

Data are espressed as mean±SD or N(%)

ADL: activities of daily living IADL: instrumental activities of daily living MMSE: mini mental state examination, SPPB: short physical performance battery

The characteristics of the 1453 participants of the InCHIANTI study based on the cognitive outcome are shown in **Tables 11-13**.

Of the 1453 participants in the InCHIANTI study, 703 were cognitively intact, 357 had cognitive impairment, and 393 had dementia **(Table 11)**.

The 48.6% of the cognitively intact participants were women, with mean age of 60.7±17.4 years. The 39.1% of participants aged < 65 years, the 43.5% of those aged 65-< 75 years, the 12.7% of those aged 75-85 years and 4.7% of participants aged >85 years were cognitively intact. Referring to the level of education, cognitive status was intact in the 9.2% of people without any level of schooling, 41.1% in people with primary school education, 18.1% in people with secondary education and 30.9% in people with other levels of education. The 16.4% of cognitive intact were unmarried, the 67.4% were married and the 14.5% were widow/er. The 11.4% lived alone, the 25.3% were smokers, and the 8% were sedentary. The average BMI for cognitive intact participants was 27.0±4.1 Kg/m^2. The 21.1% had a BMI > 30 Kg/m^2.

At baseline, the 56.0% of participants with cognitive impairment were women with mean age of 75.1±8.4 years. The prevalence of cognitive impairment was 4,8% in the age group of < 65 years, 46.5% in the age group 65- 75 years, 33.3% in the age group of 75-85, 15.4% in the group of people aged >85 years. Concerning the level of education, the prevalence of cognitive impairment was 35.3% in people without any level of schooling, 52.4% in people with primary school education, 5.0% in people with secondary education and 6.4% in people with other levels of education. The 5.9% of cognitive impaired were unmarried, the 59.1% were married and the 33.3% were widow/er. The 18.5% lived alone, the 15.1% were smokers, and the 24.2% were sedentary. The average BMI for cognitive impaired participants was 27.4±4.1 Kg/m^2. The 26.4% had a BMI > 30 Kg/m^2.

The 68.4% of participants with dementia were women with mean age 78.0±8.0 years. The prevalence of dementia was 1.5 % in the age group of < 65 years, 35.1% in the age group 65-75 years, 40.2% in the age group of 75-85, 23.2% in the group of people aged >85 years. Concerning the level of education, the prevalence of cognitive impairment was 43.0% in people without any level of schooling, 46.8% in people with primary school education, 3.3% in people with secondary education and 4.8% in people with other levels of education. The 8.4% of cognitive impaired were unmarried, the 50.4% were married and the 40.7% were widow/er. The 21.1% lived alone, the 9.2% were smokers, and the 37.9% were sedentary. The average BMI for cognitive impaired participants was 27.4±4.3 Kg/m^2. The 25.4% had a BMI > 30 Kg/m^2.

Table 11. Socio-demographic characteristics and lifestyle of the 1453 subjects participating in the InCHIANTI project, in relation to the cognitive outcome

	Total (n=1453)	**Normal** (n=703)	**Cognitive impairment** (n=357)	**Dementia** (n=393)	**P**
Sex (F)	811 (55.8%)	342 (48.6%)	200 (56.0%)	269 (68.4%)	<0.001
Age	68.9 (±15.7)	60.7 (±17.4)	75.1 (±8.4)	78.0 (±8.0)	<0.001
Age group (years)					
<65	298 (20.5%)	275 (39.1%)	17 (4.8%)	6 (1.5%)	<0.001
65-75	610 (42.0%)	306 (43.5%)	166 (46.5%)	138 (35.1%)	
75-85	366 (25.2%)	89 (12.7%)	119 (33.3%)	158 (40.2%)	
≥85	179 (12.3%)	33 (4.7%)	55 (15.4%)	91 (23.2%)	
Education					
None	360 (24.8%)	65 (9.2%)	126 (35.3%)	169 (43.0%)	<0.001
Primary school	660 (45.4%)	289 (41.1%)	187 (52.4%)	184 (46.8%)	
Secondary school	158 (10.9%)	127 (18.1%)	18 (5.0%)	13 (3.3%)	
Other	259 (17.8%)	217 (30.9%)	23 (6.4%)	19 (4.8%)	
Marital status					
Unmarried	169 (11.6%)	115 (16.4%)	21 (5.9%)	33 (8.4%)	<0.001
Married	883 (60.8%)	474 (67.4%)	211 (59.1%)	198 (50.4%)	
Widow/er	381 (26.2%)	102 (14.5%)	119 (33.3%)	160 (40.7%)	
Lives alone	229 (15.8%)	80 (11.4%)	66 (18.5%)	83 (21.1%)	<0.001
Smoker	268 (18.4%)	178 (25.3%)	54 (15.1%)	36 (9.2%)	<0.001
Sedentary	290 (20.1%)	56 (8.0%)	86 (24.2%)	148 (37.9%)	<0.001
BMI (Kg/m^2)	27.2 (±4.1)	27.0 (±4.1)	27.4 (±4.1)	27.4 (±4.3)	0.185
BMI ≥30 Kg/m2	295 (23.4%)	137 (21.1%)	80 (26.4%)	78 (25.4%)	0.301

All data are n (%) except age and BMI (mean±sd)

BMI: body mass index

At baseline, people with dementia had the higher number of concomitant diseases (1.6±1.2) if compared with normal (1.0±1.0) and cognitive impaired participants (1.4±1.2). Participants with cognitive impairment showed a higher prevalence for COPD (12.0%), chronic ischemic heart disease (12.3%), heart failure (28.6%), cancer (5.0%).

Demented participants showed higher prevalence for osteoarthritis of the hip (15.3%), diabetes (13.0%), hypertension (62.1%), Parkinson (5.3%), stroke (11.5%), knee arthrosis (41.9%), femur fracture (4.9%), depressive symptoms (29.4%), severe hearing loss (3.8%) and severe vision impairment (3.3%). The 20.7% of demented participants showed at least one allele apoE4 (**Table 12)**.

Table 12. Clinical characteristics of the 1453 subjects participating in the InCHIANTI project, in relation to the cognitive outcome

	Total (n=1453)	**Normal** (n=703)	**Cognitive impairment** (n=357)	**Dementia** (n=393)	**P**
Dieseases					
COPD	140 (9.6%)	54 (7.7%)	43 (12.0%)	43 (10.9%)	0.044
Chronic ischemic heart disease	137 (9.4%)	53 (7.5%)	44 (12.3%)	40 (10.2%)	0.035
Heart failure	287 (19.8%)	85 (12.1%)	102 (28.6%)	100 (25.4%)	<0.001
Osteoarthritis of the hip	149 (10.3%)	55 (7.8%)	34 (9.5%)	60 (15.3%)	<0.001
Diabetes	151 (10.4%)	55 (7.8%)	45 (12.6%)	51 (13.0%)	0.008
Hypertension	777 (53.5%)	332 (47.2%)	201 (56.3%)	244 (62.1%)	<0.001
Cancer	66 (4.5%)	30 (4.3%)	18 (5.0%)	18 (4.6%)	0.848
Parkinson	28 (1.9%)	4 (0.6%)	3 (0.8%)	21 (5.3%)	<0.001
Stroke	89 (6.1%)	16 (2.3%)	28 (7.8%)	45 (11.5%)	<0.001
Knee arthrosis	386 (29.9%)	159 (24.3%)	89 (28.9%)	138 (41.9%)	<0.001
Femur fracture	40 (3.0%)	12 (1.8%)	11 (3.5%)	17 (4.9%)	0.025
N. of diseases	1.3 (±1.2)	1.0 (±1.0)	1.4 (±1.2)	1.6 (±1.2)	<0.001
ApoE4 (one allele at least)	222 (16.7%)	97 (14.6%)	52 (16.6%)	73 (20.7%)	0.046
Depressive symptoms (CES D≥20)	272 (19.8%)	95 (13.5%)	79 (23.2%)	98 (29.4%)	<0.001
Severe hearing loss	28 (1.9%)	2 (0.3%)	11 (3.1%)	15 (3.8%)	<0.001
Severe low vision ApoE4 (one allele at least)	22 (1.5%)	1 (0.1%)	8 (2.2%)	13 (3.3%)	<0.001

All data are n (%) except the n. of diseases (mean±sd)

At baseline demented people lost more ADL and IADL (0.8±1.7 and 2.1±3.0 respectively) than the other age groups. Also, the MMSE score was worse in this group of older adults, also if adjusted for the years of education (22.1 ±7.6 vs 22.6±7.6 respectively). The Trail Making Test has the worst results with dementia (part A score 125.5±71.1, and part B score 218.4±94.7). Concerning the physical performance, the group of demented participants shown the worst results: slower speed on 4m (0.9±0.3s) and slower speed 400 meters at a fast pace (1.2±0.4m/s), the lower SPPB score among the other groups (8.4±4.3), the higher prevalence of SPPB score < 10 (42.9%), the higher prevalence of experiencing a lot of difficulty or be not able (referred) to walk for 400m (30.7%) or 1 Km (40.3%) or around home (14.2%) or to need help or be not able to climb stairs (43.5%) **(Table 13)**.

Table 13. Cognitive and functional characteristics of the 1453 subjects participating in the InCHIANTI project, in relation to the cognitive outcome

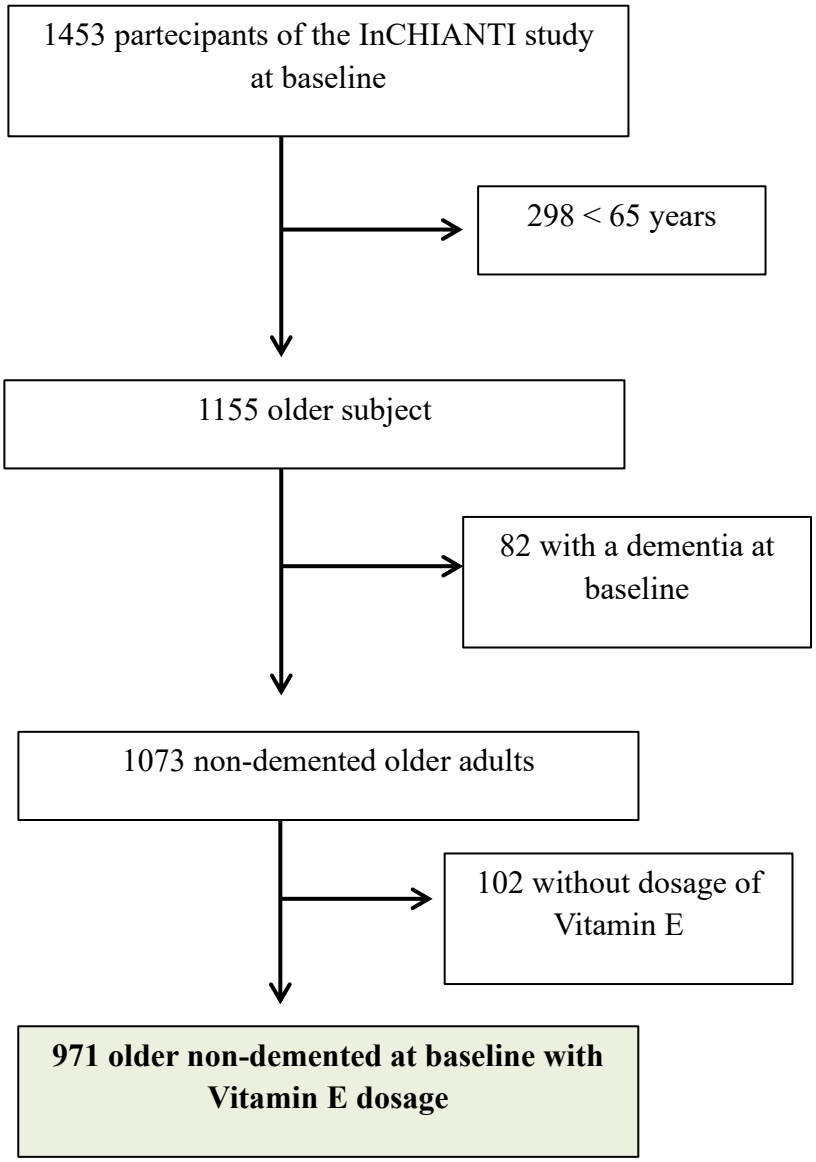
	Total (n=1453)	**Normal** (n=703)	**Cognitive impairment** (n=357)	**Dementia** (n=393)	**P**
N. lost ADL	0.3 (±1.0)	0.0 (±0.3)	0.1 (±0.4)	0.8 (±1.7)	<0.001
N. lost IADL	0.9 (±2.1)	0.2 (±0.8)	0.9 (±2.0)	2.1 (±3.0)	<0.001
MMSE					
MMSE score	25.6 (±5.4)	28.1 (±2.1)	24.7 (±4.4)	22.1 (±7.6)	<0.001
MMSE adjusted score	25.2 (±4.6)	26.8 (±1.7)	24.8 (±3.8)	22.6 (±7.0)	
Trail Making Test					
part A (sec)	84.8 (±59.8)	59.1 (±37.8)	104.7 (± 60.4)	125.5 (±71.1)	<0.001
part B (sec)	154.9 (±89.5)	123.5 (±74.4)	192.8 (± 83.3)	218.4 (±94.7)	<0.001
Speed on 4 m (m/s)	1.1 (±0.3)	1.2 (±0.3)	1.0 (±0.3)	0.9 (±0.3)	<0.001
Usual speed at 4m (m/s) <0.8 m/s	179 (14.3%)	35 (5.5%)	55 (18.2%)	89 (29.0%)	<0.001
Speed 4 meters at a fast pace (m/s)	1.5 (±0.4)	1.6 (±0.4)	1.4 (±0.4)	1.2 (±0.4)	<0.001
Speed 400 meters at a fast pace (m/s)	1.3 (±0.3)	1.4 (±0.3)	1.2 (±0.2)	1.1 (±0.2)	<0.001
SPPB					
SPPB score	10.3 (±3.2)	11.4 (±1.8)	10.1 (±3.0)	8.4 (±4.3)	<0.001
SPPB < 10	275 (21.1%)	56 (8.6%)	72 (23.2%)	147 (42.9%)	<0.001
Difficulty walking for 400 meters (referred)					
None/some	1212 (87.2%)	673 (97.4%)	286 (85.6%)	253 (69.3%)	<0.001
A lot /not able	178 (12.8%)	18 (2.6%)	48 (14.4%)	112 (30.7%)	
Difficulty walking for 1 Km (referred)					
None/some	1137 (81.2%)	649 (94.3%)	267 (77.8%)	221 (59.7%)	<0,001
A lot /not able	264 (18.8%)	39 (5.7%)	76 (22.2%)	149 (40.3%)	
Difficulty walking around home (referred)					
None/some	1338 (95.6%)	692 (99.4%)	338 (98.0%)	308 (85.8%)	<0.001
A lot /not able	62 (4.4%)	4 (0.6%)	7 (2.0%)	51 (14.2%)	
Ability to climb stairs (referred)					
Able	1127 (77.6%)	642 (91.3%)	263 (73.7%)	222 (56.5%)	<0.001
Helped /not able	326 (22.4%)	61 (8.7%)	94 (26.3%)	171 (43.5%)	

Data are espressed as mean±sd or N(%)

3.4.2. Selection of the population for the longitudinal statistical analysis

For our analysis of the relation between cognitive status and plasma vitamin E, it has been selected the InCHIANTI sub-population formed by non-demented older adults (i.e., people aged ≥ 65 years) for which the dosage of vitamin E at baseline was available **(Figure14)**. Therefore 82 older adults with dementia, 102 older adults without vitamin E dosages have been excluded. The final sample was formed by 971 older non-demented participants at baseline with the vitamin E dosage available.

Figure14. Flowchart of the selection of the population for this analysis



3.4.3. Descriptive analysis of the selected sub-population

The mean age of the 971 selected older adults was 74.5±7.0 years (range 65-100 years) **(Table14)**. Participants were mainly women (n= 544, 56.0%). The 58.7% (n=570) never smoked, the 27.3% (n=265) was a former smoker, and the 14.0% (n=136) was a smoker. Only the 26.7% of subjects had 6 or more years of school. MMSE at baseline was 25.3± 3.2 for the adjusted score, and 25.2±3.4 for the raw score. At baseline, the average plasma concentration of vitamin E was 33.8 (±7.5) μmol/L, whereas for lipids the mean values were 218.4 (±39.2) mg/dL for total cholesterol and 128.4 (±71.2) mg/dL for triglycerides. Concerning chronic diseases, 11.4% (n=111) had chronic obstructive pulmonary disease (COPD), 12.2 % (n=118) suffered of diabetes, 2 % (n=19) had Parkinson, 7.1% had had previous stroke (n= 69%) and 3.9% had cardiac disease (n=38). The mean value of BMI was 27.5 (±4.1) Kg/m^2.

Table 14. Descriptive analysis of the selected population, at baseline

VARIABLE	Mean±sd* or N (%)
Age (years)	74.5±7.0
Sex -Women -Men	 544 (56.0%) 427 (44.0%)
Smoke -Never smoke -Former Smoker - Smoker	 570 (58.7%) 265 (27.3%) 136 (14.0%)
Education -Primary school -Secondary school -Other	 711 (73.3%) 223 (23.0%) 37 (3.7%)
MMSE at baseline - MMSE adjusted score - MMSE raw score	 25.3± 3.2 25.2±3.4
Plasma Vitamin E (µmol/L)	33.8 (±7.5)
Total Cholesterol (mg/dL)	218.4 (±39.2)
Triglycerides (mg/dL)	128.4 (±71.2)
COPD- yes	111 (11.4%)
Diabetes -yes	118 (12.2%)
Parkinson-yes	19 (2.0%)
Previous stroke	69 (7.1%)
Cardiopathy	38 (3.9%)
BMI (Kg/m^2)	27.5 (±4.1)

***** *SD: standard deviation*

In the following table **(Table 15)** is reported the mean level of Vitamin E in relation of age groups in the older adult population non-demented at baseline, with at least one follow-up and a dosage of vitamin E. Using Bonferroni correction for multiple comparisons, the difference is statistically significant between the 65-74 years age group and the >85 years group.

Table 15. Mean level of vitamin E in older adults selected for this statistical analysis

Age groups (years)	**Mean (µmol/L)**	**SD**	**p**
65-74	34.1	7.1	0.051
75-84	33.8	7.9	
>85	32.1	7.7	
Total	33.8	7.4	

Men had lower vitamin E level (32.3±7.4 µmol/L). A lower level of education (primary school) was accompanied by lower levels of vitamin E (33.7±7.6 µmol/L) than in those who had continued their studies (34.2 ±6.7 µmol/L). In addition, lower levels of vitamin E were found in: participants with COPD (33.3±8.2 µmol/L), diabetes (33.2±8.5 µmol/L), Parkinson (30.1 ±5.6 µmol/L), underweight (26.9 ±6.2 µmol/L), with MMSE score lower than 24 (33.1 ±8.4 µmol/L) **(Table 16)**.

Table 16. Vitamin E levels in relation to selected variables

Variables	Mean (µmol/L)	SD	p
Sex			
Men	32.3	7.4	< 0.001
Women	34.9	7.3	
Education			
≤ 5 years	33.7	7.6	0.387
>5 years	34.2	6.7	
COPD			
Yes	33.3	8.2	0.501
No	33.8	7.3	
Diabetes			
Yes	33.2	8.5	0.034
No	33.9	7.3	
Parkinson			
Yes	30.1	5.6	0.030
No	33.8	7.5	
Stroke			
Yes	32.2	6.7	0.066
no	33.9	7.5	
Cardiopathy			
Yes	35.5	9.2	0.139
No	33.7	7.4	
Smoke			
Yes	33.8	8.7	0.914
No or former	33.8	7.2	
BMI			
underweight	26.9	6.2	
normal weight	32.9	7.7	0.009
overweight	34.1	6.9	
obese	34.7	7.7	
MMSE			
< 24	33.1	8.4	0.131
≥24	34.0	7.1	

The 4th follow-up (2013-2015) was performed on 751 survivors (all ages participants). Of the 971 older adults selected at baseline the 67.9% (n=659), deceased.

In **Table 17** are reported the MMSE mean score (adjusted and raw) in the various follow-up of the project. Over the years the number of participants has decreased (802 at the follow-up n.1 and 369 at the follow-up n.4). Clearly, analyzing an already older population it is expected that over the years many participants will be lost due to death, or others have no longer wanted to participate. Over time we see the average reduction of the MMSE score, both in its raw and in its correct form.

Table 17. MMSE mean score in the various follow-ups of the project

	N	Mean	sd
MMSE adjusted score at follow-up n1	802	24.9	4.6
MMSE raw score at follow-up n1	802	24.8	4.8
MMSE adjusted score at follow-up n2	696	24.4	5.2
MMSE raw score at follow-up n2	696	24.4	5.2
MMSE adjusted score at follow-up n3	588	23.1	7.4
MMSE raw score at follow-up n3	588	22.9	7.5
MMSE adjusted score at follow-up n4	369	21.6	8.3
MMSE raw score at follow-up n4	369	19.7	9.2

3.4.4. Univariate analysis

From the selected sample of older adults, 108 were eliminated because without at least one follow-up. Participants with at least one follow-up evaluation were 863. The univariate analysis was performed on 863 older participants.

We evaluated the association between participants characteristics at baseline and cognitive decline (defined as the loss of ≥ 2 point at the MMSE in the last available follow-up for each participant) *(Andrews et al.2019)*.

Women had an increased risk of cognitive decline (OR: 1.544, 95%IC 1.176-2.026; p=0.002), in fact the 61.1% of women lost ≥ 2 point at the MMSE vs the 50.4% of men **(Table 18)**.

Table 18. Sex and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
Sex	**Men**	N	186	189	375	1.544	1.176-2.026	0.002
		% in Sex (M, F)	49.6%	50.4%	100.0%			
	Women	N	190	298	488			
		% in Sex (M, F)	38.9%	61.1%	100.0%			

People who lost at least ≥2 point at the MMSE **(Table 19)** were older (74.7±6.7 vs 72.1±6.1 p<0.001), with low educational level (mean years of school 5.1 vs 6.0; p<0.001), had lower plasma concentration of vitamin E (33.5±7.5 µmol/L vs 34.5 ±7.2 p=0.037), low cholesterols level (217.4±37.9 vs 223.1±1.9 p=0.029).

Table 19. Baseline characteristics of people who lost ≥2 point at the MMSE at the last available follow-up

	Lost of ≥2 point at the MMSE	N	Mean	sd	Mean standard error	p
Age at the Baseline (years)	no	376	72.1	6.1	0.3	
	yes	487	74.7	6.7	0.3	<0.001
BMI at baseline	no	371	27.6	3.7	0.2	
	yes	469	27.4	4.3	0.2	0.565
Vit E(µmol/L) at baseline	no	376	34.5	7.2	0.4	
	yes	487	33.5	7.5	0.4	0.037
Total cholesterol (mg/dL) at baseline	no	375	223.1	38.7	1.9	
	yes	486	217.4	37.9	1.7	0.029
Triglycerides (mg/dL) at baseline	no	375	129.2	61.3	3.2	
	yes	486	128.4	78.5	3.6	0.878
Education at baseline (years)	no	376	6.1	3.5	0.2	
	yes	487	5.1	2.9	0.1	<0.001
MMSE adjusted score at baseline	no	376	25.7	2.6	0.1	
	yes	487	25.6	3.1	0.1	0.563
MMSE raw score at baseline	no	376	25.6	2.7	0.1	
	yes	487	25.4	3.3	0.2	0.319

In **table 20** is reported the percentage of participants with COPD who lost at least 2 point at the MMSE. The 60.2% of older adults with COPD lost ≥ 2 point at the MMSE vs the 56.0% without COPD. Considering the last available follow-up: 770 of participants did not have COPD, of these the 56.0% (n=431) lost ≥ 2 point at the MMSE. Older adults with COPD were 93, of these the 60.2% lost ≥ 2 point at the MMSE. The presence of COPD was significatively associated with the risk of develop cognitive impairment, with an OR of 1.190 (95% IC 0.768-1.846 p= 0.044).

Table 20. COPD and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
COPD	**No**	N	339	431	770	1.190	0.768-1.846	0.044
		% in COPD	44.0%	56.0%	100.0%			
	Yes	N	37	56	93			
		% in COPD	39.8%	60.2%	100.0%			

Diabetes was not significatively associated with the risk of cognitive impairment (OR 0.906 95%IC 0.601-1.356 p=0.636) as can be seen in **table 21.** In fact, the 56.7% of older participants without diabetes lost ≥ 2 point at the MMSE vs the 57.0% of diabetic participants.

Table 21. Diabetes and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**IC**	**p**
Diabetes	**No**	N	328	430	758	0.906	0.601-1.356	0.636
		% in Diabetes	43.3%	56.7%	100.0%			
	Yes	N	48	57	105			
		% in Diabetes	457%	543%	100.0%			

Parkinson was significatively associated with the risk of cognitive impairment (OR 5.129, 95%IC 1.150-22.876 p=0.017). As shown in **table 22,** the 86.7% of older participants with Parkinson lost ≥ 2 point at the MMSE vs the 55.9% of older participants without Parkinson.

Table 22. Parkinson and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
Parkinson	**No**	N	374	474	848	5.129	1.150-22.867	0.017
		% in Parkinson	44.1%	55.9%	100.0%			
	Yes	N	2	13	15			
		% in Parkinson	13.3%	86.7%	100.0%			

Previous stroke had a borderline effect (i.e., trend) on the risk of cognitive impairment (OR 1.796, 95%IC 0.981-3.289 p=0.055). The 69.2% of the older participants with stroke lost ≥ 2 point at the MMSE vs the 55.61% of older participants without stroke **(Table 23)**.

Table 23. Stroke and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
Previous Stroke	**No**	N	360	451	811	1.796	0.981-3.289	0.055
		% in Stroke	44.4%	55.6%	100.0%			
	Yes	N	16	36	52			
		% in Stroke	30.8%	69.2%	100.0%			

Cardiopathy was not significatively associated with the risk of cognitive impairment (OR 0.818 %95 IC, 0.399-1.676 p=0.582). The 56.6% of older adults without cardiopathy lost ≥ 2 point at the MMSE vs the 51.6% of cardiopathic older adults **(Table 24)**.

Table 24. Cardiopathy and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
Cardiopathy	**No**	N	361	471	832	0.818	0.399-1.676	0.582
		% in Cardiopathy	43.4%	56.6%	100.0%			
	Yes	N	15	16	31			
		% in Cardiopathy	48.4%	51.6%	100.0%			

Smoke habit was not significatively associated with cognitive impairment (OR 1.058, 95%IC 0.714-1.567 p=0.778 **(Table 25)**.

Table 25. Smoking habits and loss of ≥ 2 point at the MMSE, considering the last available follow-up

			Lost of ≥ 2 point at the MMSE					
			No	**Yes**	**Total**	**OR (no/yes)**	**95% IC**	**p**
Smoke	**No or Former**	N	326	419	745	1.058	0.714-1.567	0.778
		% smoke at baseline	43.8	56.2	100			
	Yes	N	50	68	118			
		% smoke at baseline	42.4	57.6	100			

3.4.5. Multivariate analysis

We performed a Cox regression considering confounders and time. Variable used for the multivariate analysis had a p -value < 0.2 at the univariate tests or were clinically relevant. The stepwise backward method was applied. Of the 971 non-demented older adults with vitamin E dosage and at least one follow-up, 469 lost at least 2 points at the MMSE, 134 were eliminate because had no follow-up, and 368 did not experienced the loss of at ≥2 point at the MMSE. After adjusting for multiple confounders higher Vitamin E plasma levels were associated with lower risk of cognitive decline. The increase of 10 µmol/L of vitamin E was associated with the reduced HR of cognitive impairment by 20% (HR: 0. 801, 95% IC; 0.696-0.921; p=0.002) **(Table 26)**.

Table 26. Multivariate analysis (Cox regression model) of variables associated with cognitive decline, variable used for the multivariate analysis had a p -value < 0.2 at the univariate tests or were clinically relevant.

	HR	**95%IC**	**p**
Age at Baseline	1.123	1.103-1.142	<0.001
Years of school at baseline	0.939	0.906-0.973	0.001
Previous Stroke at baseline	1.817	1.253-2.636	0.002
Vitamin E(µmol/L) at baseline	0.801	0.696-0.921	0.002
Triglycerides (mg/dL) at baseline	1.002	1.000-1.003	0.039
Adjusted MMSE at baseline	1.1060	1.023-1.098	0.001
Smoke at baseline	1.520	1.162-1.990	0.002

3.5. Discussion

Using data of this large epidemiological study, we have shown the presence of a longitudinal association between higher plasma levels of vitamin E and lower risk of cognitive impairment, in 971 non-demented older adults residing in the communities of Greve in Chianti and Bagno a Ripoli, after adjusting the analysis for confounding risk factors. The confounding factors were selected as they could influence the circulating level of vitamin E and/or cognitive decline, while some are clinical confounding factors.

Analogously to a study with 10 years of follow-up published by *Tilvis et al.2004*, at baseline, we observed a relationship between low MMSE score and older age, e.g., older participants had a lower MMSE score than younger participants. To underline the relationship between age and cognitive outcome we have shown the general worsening in the in the MMSE score, both for the adjusted and raw score, observable from follow-up to follow-up.

Oxidative stress seems to play a key role in the pathogenesis of cognitive decline *(Baierle et al.2015, Hajjar et al.2018)* and dementia *(Luca et al.2015).* In the brain of people with AD, have been found typical lesions of ROS *(Engelhart et al.2012).* Brain is an organ at highly risk of oxidative damage because it uses about the 20% of the oxygen present in the body, it is rich in polyunsaturated fatty acids and is relatively poor of antioxidants *(Head 2009)*. *In vitro* studies have shown that exogenous antioxidants can reduce the toxicity of β-amyloids *(Behl 1997, Christen 2000).* Based on this evidence it can be plausible to speculate that antioxidants can reduce the risk of cognitive impairment and slowing or prevent the possibility of the consequent dementia *(Beydoun et al 2014)*. Considering the relationship between vitamin E and cognitive decline found in our longitudinal analysis and having vitamin E a known antioxidant property, it is possible to suggest the use of vitamin E as a non-pharmacological strategy to try to maintain cognitive health for as long as possible, also because at the moment there are no drugs that allow to obtain this type of results. Moreover, since the older adults are at risk of polypharmacy due to the numerous concomitant pathologies, it is good to look for non-pharmacological strategies.

Our results agree with what was found in the longitudinal observational study by *Morris et al 2002* even if in that publication vitamin E was not measured but was estimated by means of a questionnaire that estimates the habitual intake of certain nutrients. However, the relationship between vitamin E and cognitive function in the scientific literature is not conclusive. Epidemiologic studies examined the longitudinal relationship between supplemental or dietary antioxidants and risk of dementia found conflicting results, some studies reporting a reduced incidence of dementia in subjects with high plasma levels or high dietary intake of vitamin E while other studies showing only borderline or little

evidence of a cognitive benefit *(Beydoun et al 2014).* Observational studies which do not report the effect of vitamin E supplementations on cognitive function, may fail because vitamin E supplements may not have an effect in those who do not get enough levels of this vitamin in their diet. These mixed results can be ascribable to methodological issues. In fact, the circulating levels of vitamin E depends to its dietary intake but also to the lifestyle factors as smoking habits, cholesterols and triglycerides, others vitamin as vitamin C which affects its metabolism. Not all studies correct for confounding factors. Therefore, dietary intake may not precisely reflect blood levels that are more directly associated with tissue's levels. In other studies, the analysis was not corrected for lipids. Since vitamin E is carried in the bloodstream by lipoproteins, changes in lipid concentration should always be considered *(Traber and Jialal 2000)*. In our analysis it was not necessary to correct vitamin E for lipids as lipids were used as confounding factors in the multivariate analysis *(Cherubini et al.2005).* Regarding the correction for other molecules that could influence the level of vitamin E in the blood as vitamin C, it was not possible to consider them in our statistical analysis as they were not measured in the InCHIANTI project.

Cherubini et al. 2005 analyzed the InCHIANTI project older adults' population at baseline with the aim of study the cross-sectional relation between vitamin E plasma levels and dementia and cognitive impairment. *Cherubini et al2005* found that older adults in the lower tertile of Vitamin E intake were more likely to be demented and have cognitive decline. Conversely, those who were in the upper tertile of vitamin E intake were more likely to maintain the integrity of cognitive function. However, the design of the analysis of *Cherubini et al.2005* was cross-sectional, therefore the relation cause-effect was suggested but non-demonstrated.

The present new analysis has a longitudinal design. Longitudinal studies are stronger than cross-sectionals, being able to analyze the relative risk of a risk factor in a certain period and to more convincingly suggest the cause-effect relation. In this longitudinal study we consider the subjects until their last available follow-up, up to a maximum of 17 years of project. In this way we avoid losing a significant amount of data at follow-up due to mortality. We used the cox regression model, which is a survival analysis which allows us to evaluate the relationship in question over time, considering confounding factors. Another strong point of this study is certainly that it is a study on the Italian population and has an extended sample size and a long duration of the study, with a lot of variables recorded. In human is not possible measure the vitamin E levels at brain level. Therefore, it has been analyzed the plasma levels of this vitamin (*Cherubini et al.2005, Martin et al. 1999, Vatassery et al.1988)*. Studies on animals showed that following the introduction of vitamin E supplements with the diet, the vitamin level increases proportionally both in the plasma and in the

brain. Accordingly, to that, the analysis of vitamin E in plasma appears to be a correct methodology. On the other hand, it is an observational study. Therefore, although we adjusted for several confounders, we cannot rule out residual confounding. Another limitation is that the vitamin E considered derives from a dosage and is not the intake. However, some studies reported that it is better to consider the dosage, as it provides a more precise estimate that intake, which can be affected by recall bias. Another limitation is that the dosage was performed only at baseline, because to budget limitation, whereas while having the dosage over time would certainly make the association found stronger. Interventional studies on the role of vitamin E in improving the condition of MCI and slowing the potential progression to dementia are available *(Farina et al.2017)*, however these have given negative outcomes. It is also true that the number of studies available is small, and they often had a small sample and short follow-up. Therefore, further interventional studies are recommended to investigate this issue.

3.6. Conclusion

This longitudinal analysis based on the data of the older adult population of the InCHIANTI study showed an association between higher plasma levels of vitamin E and reduced risk of cognitive impairment, after the adjustment of the main confounding factors. These findings support the recommendation of maintaining diet that contains an adequate intake of vitamin E. However, these results derive from an observational study. Interventional studies are needed in order to establish the presence of a preventive effect of vitamin E on cognitive decline.

4. STUDY 2: Role of dietary polyphenols in chronic diseases and cognitive impairment

Another section of my thesis work concerns the collaboration in the publication of a systematic review entitled "Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?" which focuses on dietary polyphenols and chronic diseases which can be risk factors for cognitive impairment. Also, cognitive impairment was included in this review.

This review was published in open access *Nutrients 2019, 11(6), 1355; https://doi.org/10.3390/nu11061355*

4.1. Polyphenols

4.1.1 Generalities and characteristics

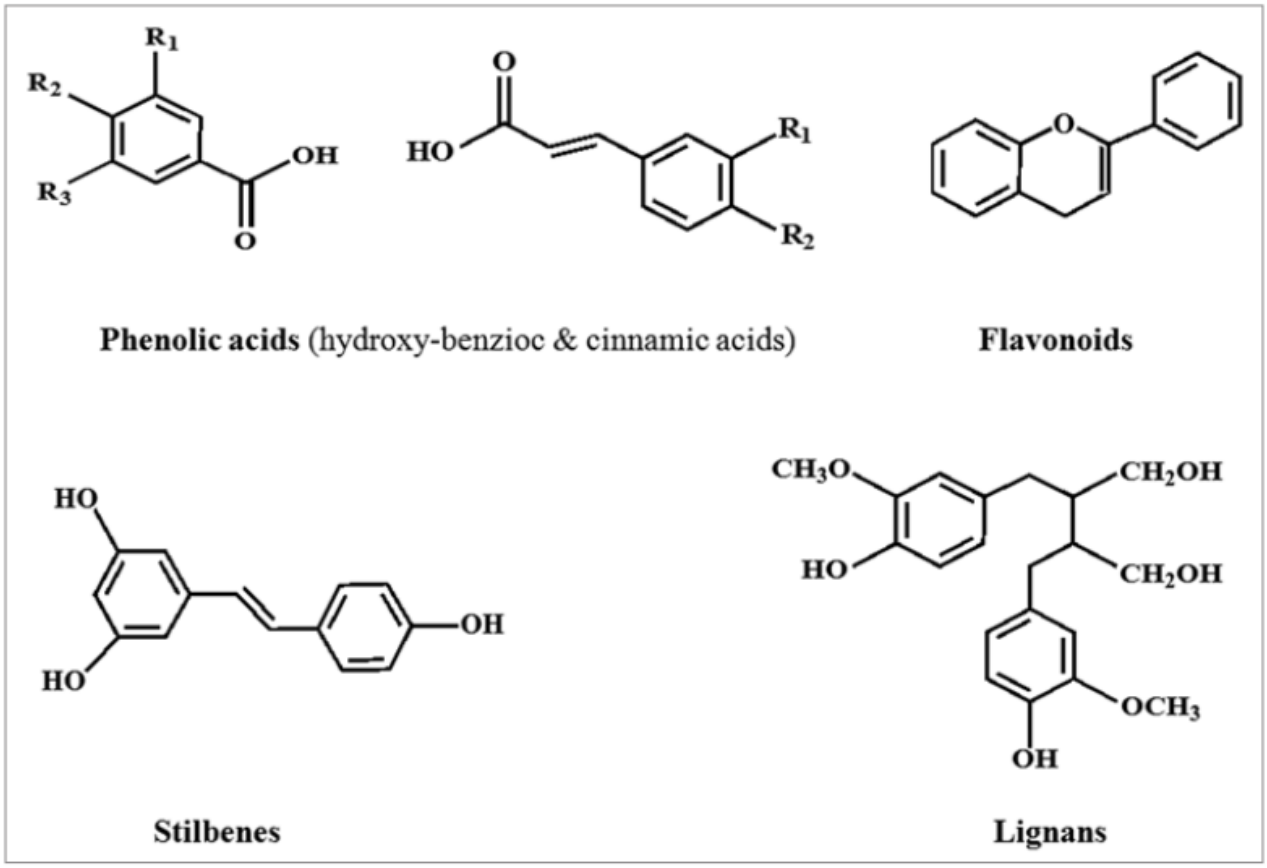
The term "polyphenols" derives from the Greek, "poly" means "many" whereas "phenol" refers to the hydroxyl groups (OH) bounded to aromatic phenyl ring. Generally, have > 12 phenolic OH groups, with 5-7 aromatic rings. These compounds, with molecular weight of 500-4000 Da, are moderately soluble in water. Polyphenols always have a substituent heteroatom different from OH groups. Ester and ether bounds are common (*Abdel-Shafy and Mansour 2017)*. Some can be found as glycosides whereas other in insoluble polymers *(San Miguel-Chávez 2017).*

8000 types of polyphenols are known in nature. Based on the number of phenolic groups and other structural characteristics, polyphenols can be classified into four classes **(Table 27, Figure 15)** (*Ganesan and Xu 2017).*

Table 27. Classes and subclasses of polyphenols (*Modified from Del Bo' Nutrients 2019*)

Classes	**Subclasses**
Flavonoids	• Flavanols (Epicatechin, Catechin, Epigallocatechin-gallate) • Flavanones (Hesperidin, Naringenin, Eriodictyol) • Flavones (Apigenin, Luteolin, Yangeritin, Chrisin) • Isoflavones (Genistein, Daidzenin) • Flavonols (Kaempferol, Myrestin, Quercitin) • Anthocyanins (Cyanidin, Delphinidin, Malvedin, Pelargonidin)
Phenolic acids	• Hydroxybenzoic acid (Protocatechuic acid, Gallic acid, Vanillic acid, Ellagic acid, Salicyclic acid) • Hydroxycinnamic acid (Caffeic acid, Ferulic acid, Sinapic acid, Choloregin acid, P-Coumaric acid, Quinic acid)
Lignans	• Secoisolariciresinol, Pinoresino, Lariciresinol, Syrigaresinol, Matairesinol, Hydroxymataresinol, Sesamin)
Stilbenes	• Resveratrol, Pterostilbene

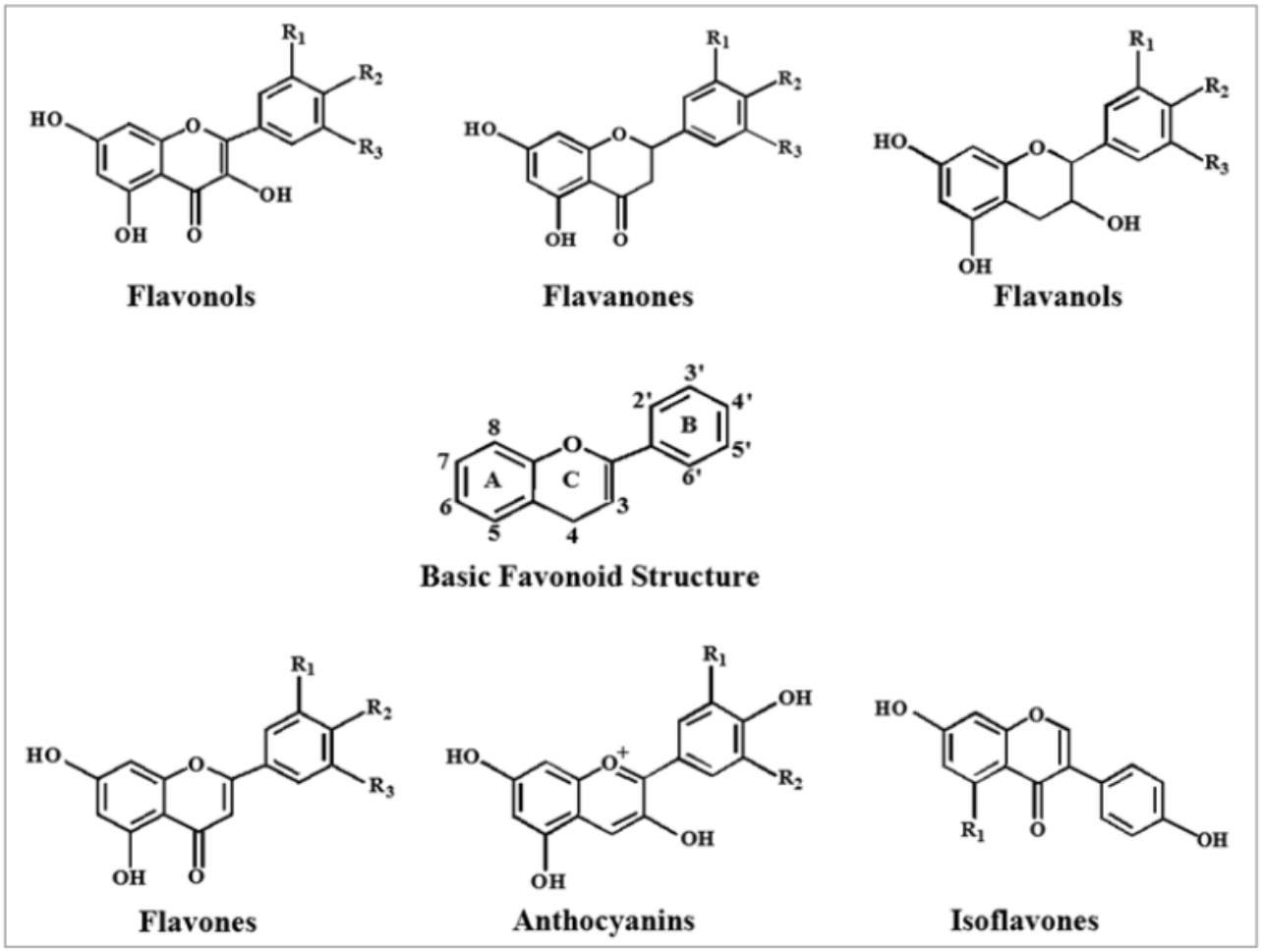
Figure15. Chemical structure of polyphenols classes *(Pandey and Rizvi 2009)*



Flavonoids are the most studied class of polyphenols, their structure is based on a carbonaceous skeleton of 15 elements, with a chromane ring bound to the second aromatic ring *(Fraga et al.2019)*.

The chemical basic structure of polyphenols subclasses is represented in **Figure 16.**

Figure 16. Chemical structure of flavonoid sub-classes *(Pandey and Rizvi 2009)*



Polyphenols are compounds produced by the plants (i.e., phytochemicals) abundant in fruits and vegetables. In plants they are generally concentrated in leaves, bark, flowers, and fruits *(Abdel-Shafy and Mansour 2017).* In fruit they participate in the definition of flavor, color, and nutritional characteristics *(El Gharras 2009)*. They also defend plants from abiotic stress and pathogens *(San Miguel-Chávez 2017).* Among the properties of polyphenols, there is the hormonal regulation, the antimicrobial, antiproliferative, proapoptotic and antioxidant properties *(Chan et al. 2009).*

Other important sources are tea and coffee, cocoa, legumes, cereals *(Ganesan and Xu 2017),* red wine, olives and extra virgin oil. The cooking method influences the nutritional and chemical characteristics of these compounds, for example steam cooking maintains the antioxidant properties of certain polyphenols better than frying, or also, clarifiers added in the production of wine, beer and other non-alcoholic beverages can totally eliminate the polyphenol content *(Abdel-Shafy and Mansour 2017).*

In plants polyphenols are bounded with cell membranes or with the wall, o can be also free. Processing food at high or low temperatures can release them by increasing their bioavailability, but this is not always true. There are some evidence regarding the reduced bioavailability of polyphenols following cooking by boiling. This can be explained by the water solubility of some phenolic compounds which are then lost in the cooking water. At the same time also the type of cooking method in relation to the type of food can determine the increase or decrease of the bioavailability of the chemical compound. In fact, the extractability of the compound from the matrix and the dehydration of the tissues come into play *(Minantel et al.2017).*

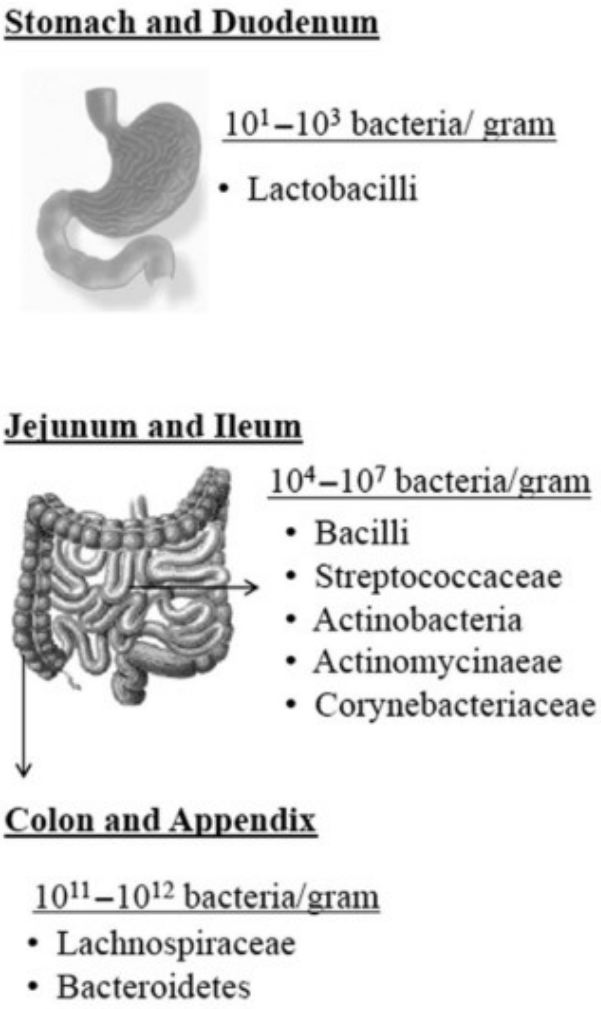
4.1.2 Metabolism of polyphenols

The various classes of polyphenols differ in chemical structure. This translates into a different absorption kinetics. The polyphenols that are most absorbed are isoflavones and phenolic acids whereas the least adsorbed are proanthocyanidins and anthocynidins. In relation to the amount of polyphenols introduced, the site of metabolism differs. In fact, large doses of ingested polyphenols are metabolized in the liver, whereas smaller ingested amounts, are metabolized in the mucosa of the intestine. The liver is involved in the metabolism of the conjugated forms. Polyphenols introduced with the diet are poorly adsorbed in the small intestine (*Pathak et al. 2018)*.

Not all polyphenols contained into food can be adsorbed in their native form (except aglycones) because they often associate in polymers or esters. Therefore, polyphenos, need to be hydrolyzed by intestinal enzymes or by the activity of the GI microbiota *(Pathak et al. 2018, Fraga et al.2019).* Where human enzymes cannot obtain metabolites that can be absorbed from polyphenols, enzymes of the microbial flora succeed.

From the stomach to the colon the microflora has an increasing gradient *(Pathak et al. 2018)*, which reaches the higher concentration in the colon. It is estimated that there are about 1000 microbial species in the GI *(Cardona et al. 2013)*. The microbial composition varies according to the anatomical site *(Pathak et al. 2018),* environmental and genetic factors *(Fraga et al.2019)* **(Figure 17).**

Figure 17. GI microbiota *(modified from Pathak et al. 2018)*

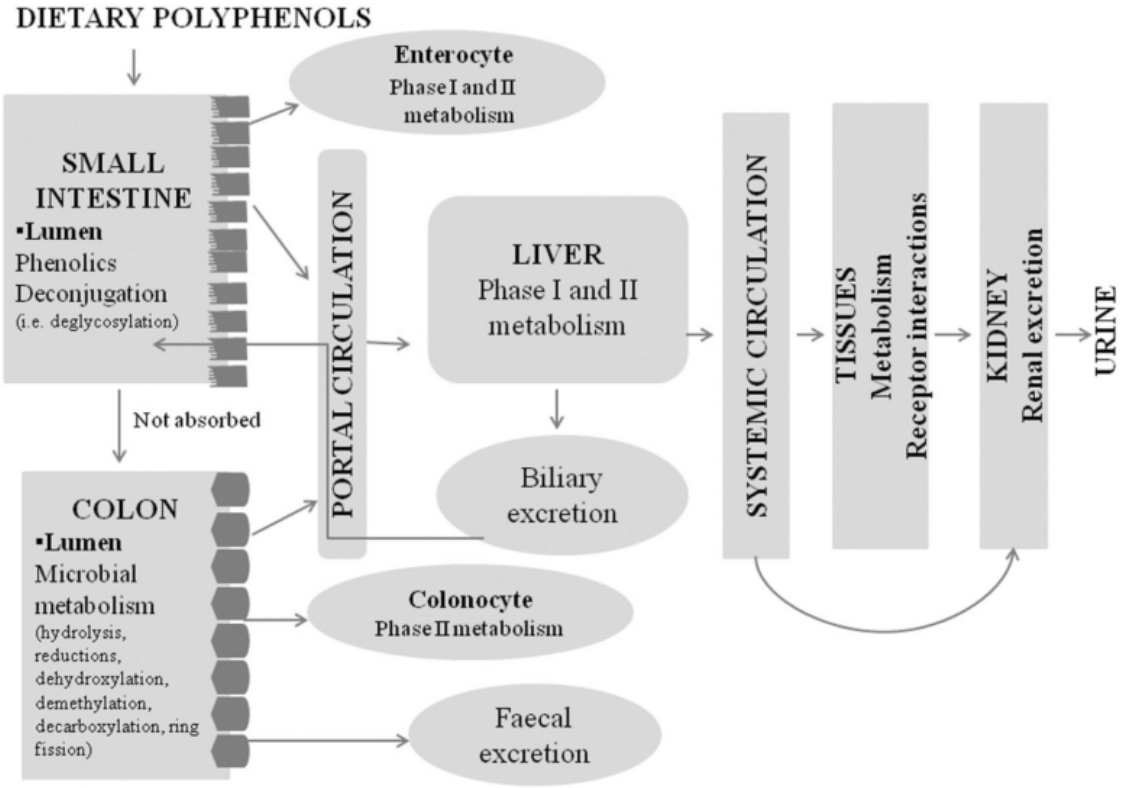


Polyphenols can be metabolized by few bacterial species, e.g., *Bifidobacterium, Lactobacillus, Eubacterium, Bacteroides* and *by Escherichia Coli (Cardona et al. 2013)*.

Polyphenols stay in the colon longer than they do in the intestine; therefore, they affect the health of the colon and of its bacteria *(Fraga et al.2019)*. In the colon polyphenols are deconjugated and metabolized by the resident flora which produce metabolites which enters in the systemic circulation. In general, polyphenols are poorly adsorbed in the small intestine (only the 5-10%) (*Cardona et al. 2013*, *Pathak et al. 2018)*. In the small intestine, less complex polyphenols can undergo to oxidation, reduction, hydrolysis (phase I) and to conjugation (phase II) in enterocytes and hepatocytes. The result are water soluble metabolites, e.g., derivates of methyl, sulfate and glucoronide, which are released into the systemic circulation, and then reach the organs and can be released in the urine

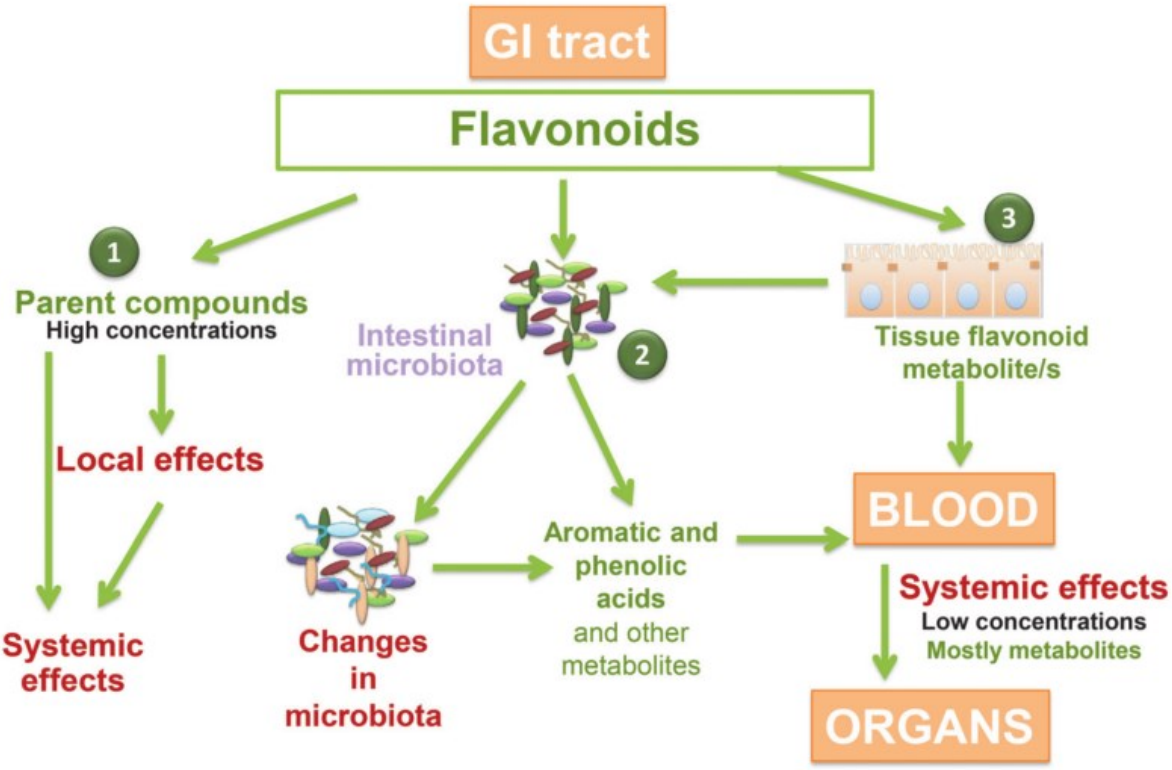
(Cardona et al. 2013). More complex polyphenols which represent 90-95% of the polyphenols are not absorbed in the small intestine. They are transported in the large intestine and metabolized by the resident flora, to be converted in forms more absorbable. Complex polyphenols are mainly metabolized in the gut (e,g. deglycosilation, dehydrolation, demethilation) and transformed into low-molecular-weight more absorbable metabolites. Some classes of polyphenols, as flavones, anthocyanidins and others, are glycosylated. The first step in the degradation of polyphenols includes the release of aglycones and oligomers by microbial enzymes, which increases the absorption. The microflora hydrolyzes the aglycones, and aromatic acids are formed and subsequently cleaved *(Pathak et al. 2018)*. The formed metabolites can be absorbed or excreted with the feces. However, the elimination with feces is limited. These metabolites, if adsorbed, reach the liver by the portal vein. In the liver they undergo to some modifications as glucuronidation, sulfatation, methylation or to a combination of this mechanisms. Then, they reach the systemic circulation, and consequently the organs, or can be excreted with the urine. These phase II metabolites extruded via the bile can be deconjugated by microbial glucuronidase and sulfatase. This helps the reuptake to the enterohepatic circulation and enhance their bioavaiability **(Figure 18)** *(Cardona et al. 2013)*.

Figure 18. Metabolism of dietary polyphenols *(Cardona et al. 2013)*



In general, it is possible to say that polyphenols act on the microbiota of the gastrointestinal system by modifying its composition *(Fraga et al.2019),* activating or inhibiting their growth *(Pathak et al. 2018),* for example altering the permeability of bacterial cell membrane, influencing *quorum sensing*, inhibiting DNA and RNA synthesis, etc *(Cardona et al. 2013)*. Given that intestinal dysmicrobism is associated with the onset of chronic diseases, the influence of polyphenols can determine positive effects on health. From the metabolism of polyphenols by the intestinal microbiota, compounds are formed that carry out biological activities *(Fraga et al.2019),* **(Figure 19).**

Figure19. Polyphenol colon metabolism and adsorption of metabolites *(Fraga et al.2019)*



**GI: Gastrointestinal*

4.1.3. Antioxidant property of polyphenols and chronic diseases

Among the properties of polyphenols, can be cited the hormonal regulation, the antimicrobial, antiproliferative, proapoptotic and antioxidant properties *(Chan et al. 2009).* To the purpose of this thesis, we focus on the antioxidant property.

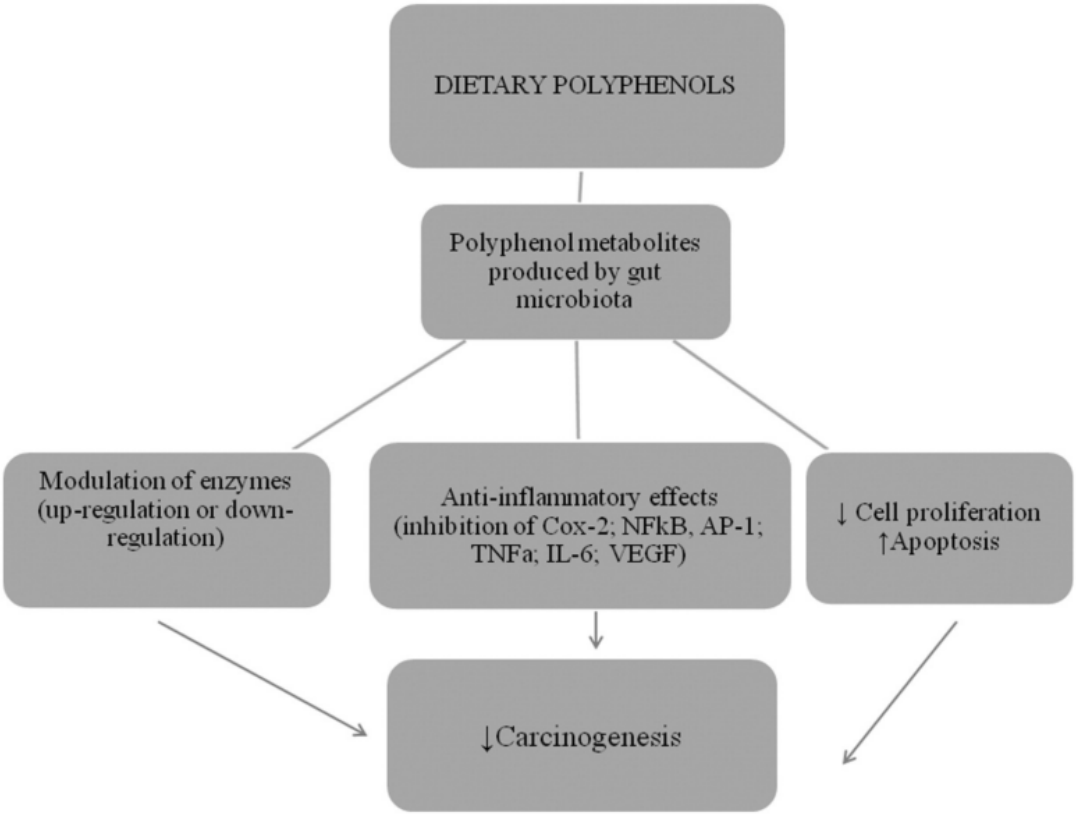
The antioxidant activity of polyphenols helps to avoid platelet aggregation, erythrocytes damage and the oxidation of LDLs (*El Gharras 2009).* This property is due to their ability of scavenging free radicals, chelating metal ions, donating protons and electrons. The position of the OH groups and the type of substituent in the aromatic ring determines the capacity of inactivating free radicals. *(Tsao 2010).*

Based on their structure, polyphenols, can act as scavengers or chain breakers *(El Gharras 2009).* The phenolic groups can accept electron generating a phenoxyl radical which is relatively stable, in this way the oxidation chain reaction is interrupted. Eating foods rich in polyphenols can help increase the antioxidant capacity of the plasma. There is evidence in the literature to support the intake of polyphenols to counteract numerous pathologies associated with the mechanism of oxidative stress *(Pandey and Rivi 2009).* In particular it would seem that the long-term intake of polyphenols would reduce the risk of some chronic diseases *(Arts 2005)*. The mechanism by which the different classes of polyphenols act on the risk of diseases varies between polyphenols. In general, it can be said that polyphenols have a positive effect on the following health conditions:

- Cardiovascular disease→Polyphenols intake is associated with lower risk of coronary heart disease. Inhibiting the oxidation of LDLs, polyphenols counteract the atherogenic process and can stabilize atherogenic plaques which can be associated with acute conditions as myocardial infarction. Other protective mechanisms for cardiovascular health include anti-platelet aggregation preventing the formation of thrombus, anti-inflammatory and the improved endothelial function. The antioxidants through their antioxidant activity can improve endothelial function and can lower blood pressure *(Pandey and Rizvi 2009, Fraga et al.2019).*

- Cancer→ polyphenols reduce the risk of several type of cancer, localized in the mouth, skin, liver, prostate, colon, breast and in other sites, inducing enzymes detoxification the cell cycle blockage or the apoptosis, being involved in epigenetic changes, contributing to changes in the cells signaling, through antioxidant and ant inflammatory properties *(Pandey and Rizvi 2009, Briguglio et al.2020)* **(Figure 20)**.

Figure 20. Proposed mechanisms according to which polyphenols would prevent cancers *(Cardona et al. 2013)*.



- Diabetes →polyphenols can reduce the risk of diabetes inhibiting the gut glucose absorption or glucose uptake in tissues. Furthermore, they can inhibit glucosidase and glucose transporter in the intestine. Concerning polyphenol antioxidant property and diabetes, it has been seen that quercetin can act on the oxidative stress of diabetic patients *(Pandey and Rizvi 2009, Silveira et al.2019).*

- Aging →dietary antioxidant intake can positively affect the oxidative stress typical of aging. The combination of antioxidant and anti-inflammatory polyphenols can be useful of health *(Pandey and Rizvi 2009).* Polyphenols can act inhibiting senescense and increasing lifespan *(Russo et al.2020).*

- Neurodegenerative diseases→scientific evidence exist on the neuroprotective effect of polyphenols for AD and Parkinson. For example, the moderate intake of red wine can reduce the risk of incident dementia by the 80%. Resveratrol is abundant in red wine and has

scavenging property (i.e., lipid hydroperoxyl free radicals, O2- and OH). Resveratrol can protect the microglia from the toxicity of the β-amyloid *(Pandey and Rizvi 2009, Silveira et al.2019).* The dietary supplementation with food rich in polyphenols were associated with the reversing of brain deficits of rats. Furthermore, polyphenols can cross the BBB and carry out a protective action in the aging brain *(Pandey and Rizvi 2009).*

- Other→Polyphenols can protect from obstructive lung diseases as asthma, improving the function of the lungs *(Pandey and Rizvi 2009, Pounis et al.2018)*. Can positively affect bone health avoiding the loss of bone density and volume. Some polyphenols showed antiviral activity, for example polyphenols with anti-HIV activity has been found in black tea *(Pandey and Rizvi 2009).*

4.2. Aim and Rationale

Recently the interest is increasing in studying the effect of polyphenols in diseases related to aging and in healthy aging *(Spencer et al.2009).* Evidence from animal and human studies are stimulating the onset of research on the estimation of polyphenols in specific groups of subjects. The possibility to investigate the impact of the single class or subclass of polyphenols on health is also stimulated by the creation of databases available online, which are focused on the polyphenol composition of certain foods. On the other hand, the low bioavailability of polyphenols and their extensive metabolism makes it difficult to make recommendations on daily intake. Studying the polyphenol intake in target populations with different lifestyle, and consequently with different dietary habits, might help to verify the presence of an intake range associated with the reduced risk of specific health conditions. In order to be able to produce recommendations on the intake of food bioactive it is necessary to know the amount of bioactive necessary to perform the positive function, the quantity of bioactive present in the food and the dietary contribution of bioactive. For this purpose, we carried out a systematic review of the literature considering the tools and methods used to estimate the polyphenol intake, taking into account the difference between total polyphenols and single classes / subclasses. The impact of these bioactive foods on disease risk endpoints was also considered. Finally, we wanted to see if from the evidence found it was possible to provide recommendations on the intake of polyphenols in the diet *(Del Bo' et al. 2019).*

4.3. Methods

4.3.1. Selection of papers

The PUBMED and EMBASE databases were mainly consulted, to find articles in English, published between January 2008 and December 2018. Title and abstract were analyzed in order to verify the studies eligibility. Studies included were prospective, cohort and case-control, analyzing the association between polyphenols intake (individual, classes or total) with end points of diseases and mortality risk. Interventional studies were excluded, also were excluded studies which used urinary excretion to measure polyphenol intake, studies non-performed in human, studies analyzing dietary supplements, studies on cancer risk or mortality, studies not written in English.

Data extracted were mainly: first author and year of publication, study design, number of enrolled participants and their characteristics, the country, method used for the food intake estimation, the database used for polyphenols and study results.

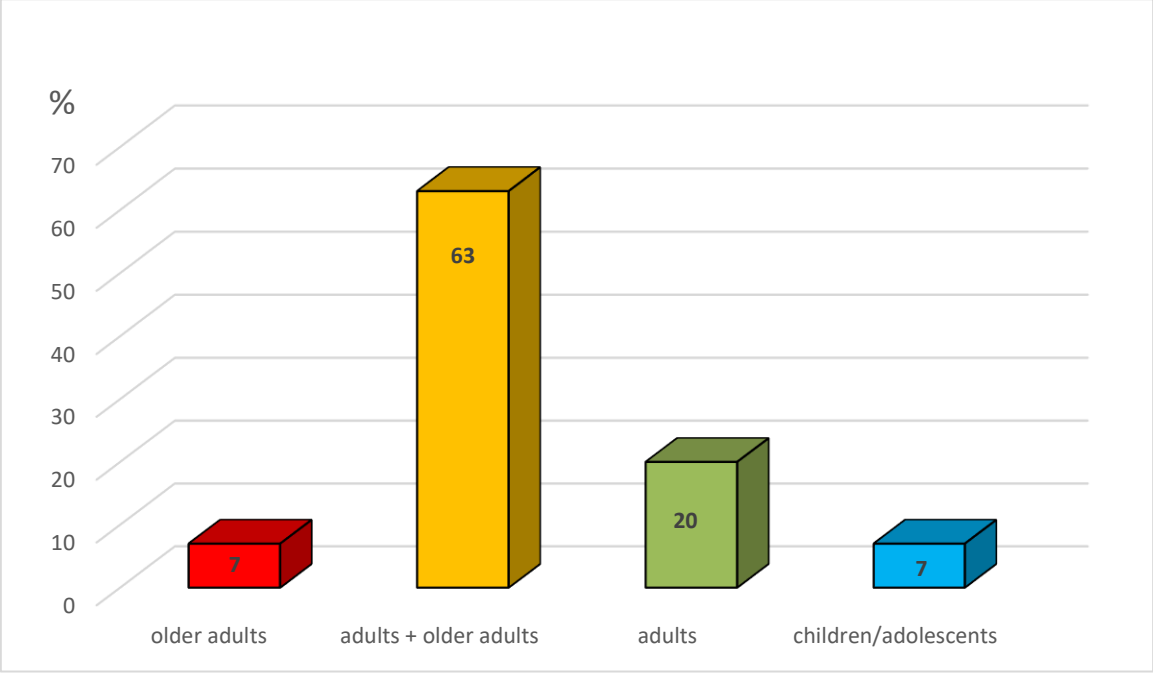
4.4. Results

Of the 3004 articles resulting from consulting the databases: 48 were duplicates 2566 were excluded after controlled the title or the abstract. Of the 390 eligible papers, 299 were excluded because not satisfied inclusion criteria. 91 studies were included:

- 45 on polyphenols intake,
- 24 on the relation with diabetes and/or cardiovascular risk
- 9 on cardiovascular and all-cause mortality
- 17 on other outcomes (included cognitive impairment)

The majority of the 45 studies on polyphenols intake were performed in Europe (53%), to follow, North America (16%) and Asia (16%), Australia (9%), South America (7%). Only the 7% of the studies focused only on older adults **(Graphic 1).**

Graphic 1: Target groups of the selected studies on polyphenols intake



The questionnaire used to verify the polyphenols intake were mainly the 24-h dietary recall (56%, i.e. 24h-DR) and the Food Frequency Questionnaire (FFQ 31%). The databases used to estimate polyphenols dietary intake were mainly the USDA (22%) and Phenol-Explorer (20%). The mean

intake of total polyphenols on the whole population was about 900mg/day. This intake showed differences in the specific group of age as also the alimentary sources varied.

Figure 21. USDA website screenshot taken 12/11/2020 *https://fdc.nal.usda.gov/index.html*

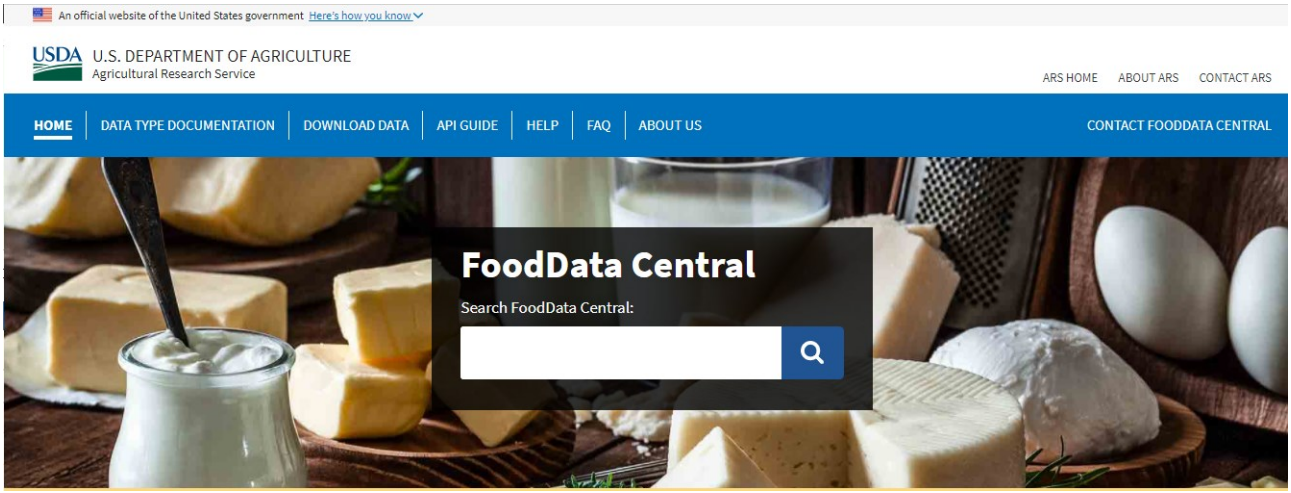
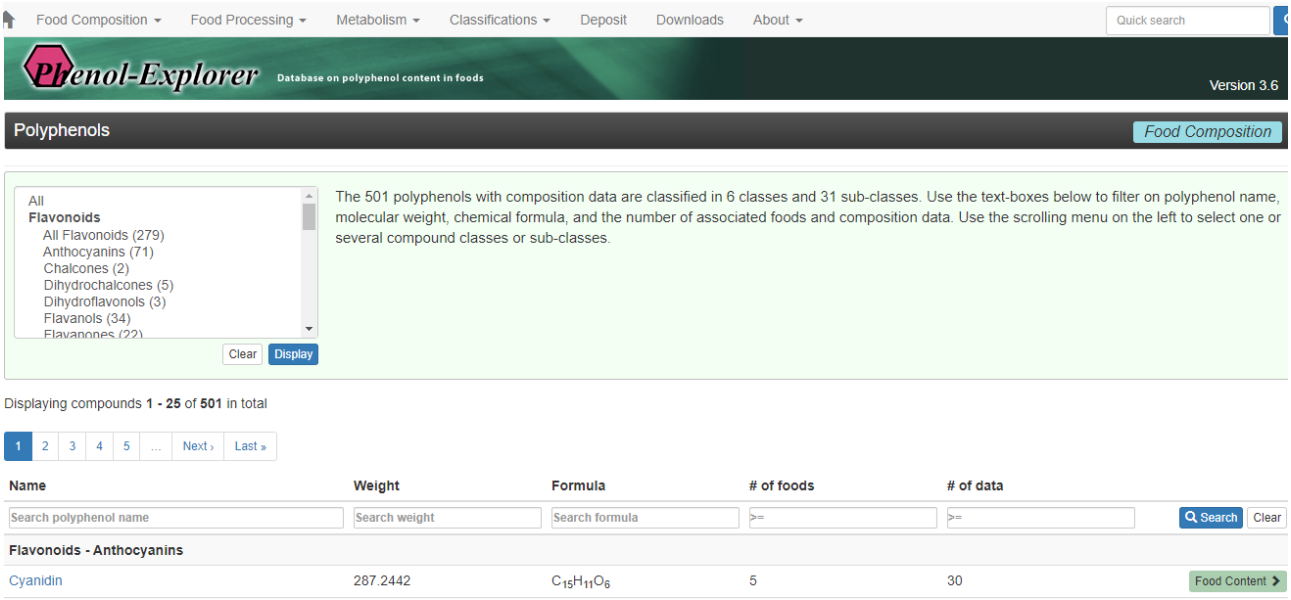
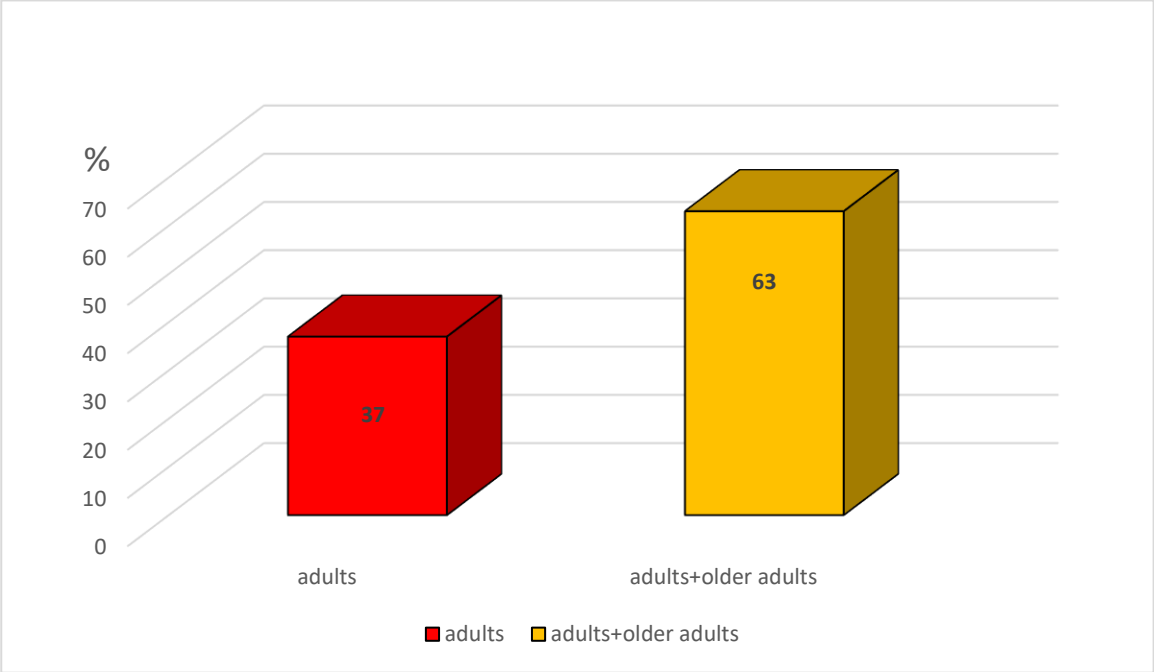


Figure 22. Phenol explorer website screenshot taken 12/11/2020 *http://phenol-explorer.eu/compounds*



Most of the studies on polyphenol intake and cardiovascular and/or diabetes risk were performed in Europe (12 studies). The target populations included mainly both adults and older adults (63%), there was no study focused only on older adults **(Graphic 2).**

Graphic 2. Target groups of the selected studies on polyphenols intake and cardiovascular and/or diabetes risk

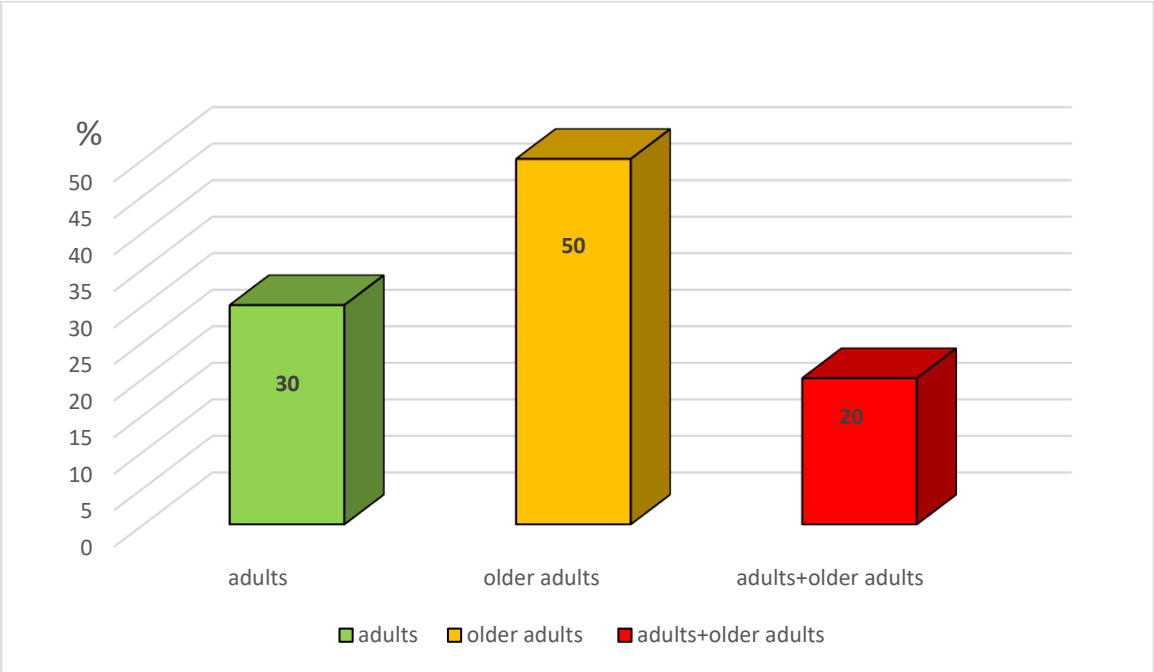


FFQ was used in the 63% whereas the 24h-DR in the 29% of the studies. The 42% of the studies used USDA as database, whereas the Phenol Explorer was used in the 25%. Three studies used both USDA and Phenol explorer, whereas the remaining studies used other databases as for example the Flavonoid Korean database. Diabetes and cardiovascular risks were analyzed referred to the following outcomes: cardiovascular and stroke events, triacylglycerols, HDL-cholesterol ratio, BMI, type 2 diabetes homeostatic model assessment of insulin resistance, hypertension. The inverse association between polyphenol intake and cardiovascular events was showed in all the 12 studies included. In some studies, the effect was reported when higher levels of intake were compared with the lowers (e.g., 1170 mg/day for Spanish and 2632 mg/day for Polish studies). While other studies found no relationship (e.g., 1248 mg/day in Spanish and 2459 mg/day in Iranian studies). 10 studies focused on flavonoids but only 3 showed the association with cardiovascular events with an intake of 115-994 mg/day. One Polish study found a positive effect on type 2 diabetes risk with an intake of total polyphenol high than 2632 mg/day. Other studies focused on total flavonoids or on other subclasses,

but mixed results were obtained. One study found an inverse association for cardiovascular and diabetes risk at an intake of 585mg/day.

10 studies have been found evaluating the relation between polyphenols intake and all causes or cardiovascular mortality. The 50% of them were performed in Europe. Concerning the age of participants, the 50% of the studies were performed in people aged >65 years **(Graphic 3).**

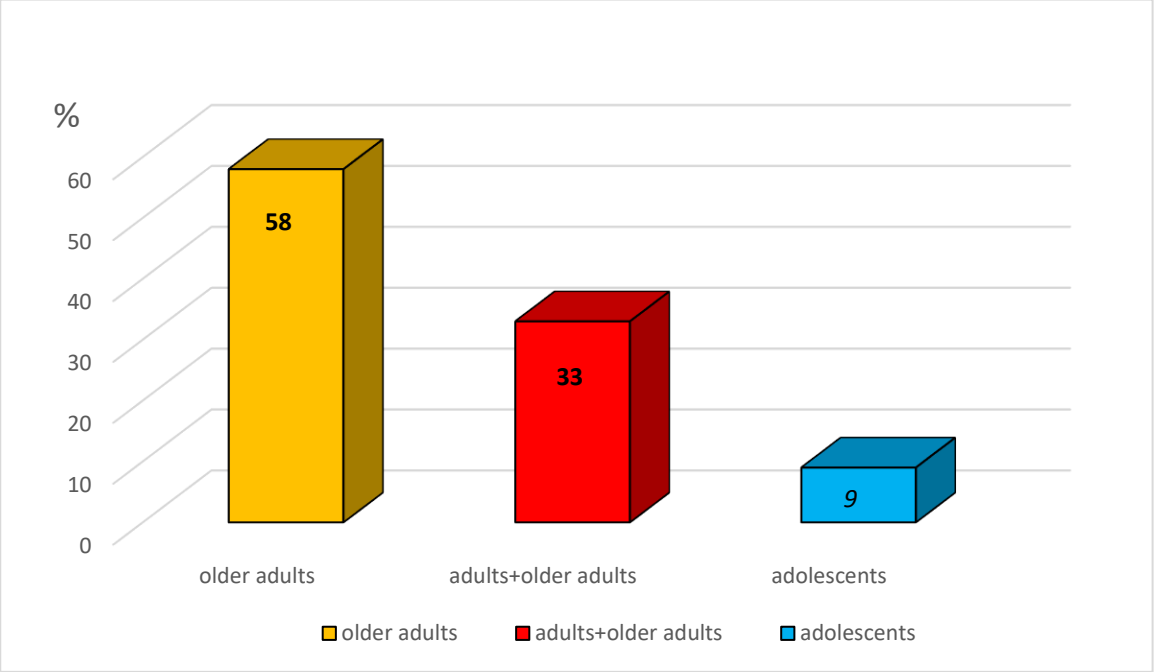
Graphic 3. Target groups of the selected studies on polyphenols intake and all causes or cardiovascular mortality



The 60% of the studies estimated dietary intake of polyphenols with the FFQ, the 30% combined the FFQ with other tools as a questionnaire on dietary history recorded by computer, etc. One study used both the FFQ and the EPIC study questionnaire. USDA database was used in the 30% of the studies, the combination of USDA and other databases in the 40%, USDA and phenol explorer in the 20%. In one study the association between polyphenols intake and all causes of mortality was not demonstrated. Also, the association between flavonoids and cardiovascular mortality was not showed. Whereas total flavonoids were associated with reduced risk of mortality for causes or cardiovascular mortality with the higher levels of intake (e.g.,360-800mg/day). The effect of polyphenol subclasses was different in relation to the participants characteristics and cause of death.

13 studies evaluated the polyphenol intake in relation to other outcomes as renal function, endothelial function, health of the eyes, health of the bones, reduced physical performance, dementia, cognitive impairment and pubertal development. The 46% of the studies were carried out in Europe. The 58% focused on older adults **(Graphic 4).**

Graphic 4. Target groups of the selected studies on polyphenols intake in relation to other outcomes



In the 77% of the cases was used the FFQ, only one study used the 24h-dietary recall whereas 2 studies utilized FFQ together with other tools. The 50% consulted the USDA database, 3 studies used both USDA and phenol explorer whereas 2 studies used USDA together with other database.

Urinary total polyphenols were associated with reduced risk of frailty *(Urpi-Sarda et al. 2015)* and cognitive impairment *(Rabassa et al. 2015).* In the following table **(Table 28)** are reported the other relations founds with flavonoids, proanthocyanidinis and isoflavones.

Table 28. Evidence found regarding flavonoids, proanthocyanidinis and isoflavones.

Flavonoids	**Proanthocyanidinis**	**Isoflavones**
Improved endothelial function (>640mg/day) *(Fisher et al. 2012)*	Renal insufficiency and failure (≥229 mg/day) *(Ivey et al. 2013)*	Improved pubertal development (>3mg/day) *(Segovia-siapco et al.2018)*
Reduced risk of low forced vital capacity and lung spirometric restriction (290 mg/day) (*Pounis et al. 2018)*		
Improved bone density (490 mg/day) *(Zhang et al. 2014)*		
Reduced bone fractures (1500 mg/day) *(Myers et al. 2015)*		
Reduced macular degeneration (875 ng/day) *(Gopinath et al. 2018)*		

4.5. Discussion

The results of the study demonstrated an inverse association between a level >1170 mg/day of total polyphenols and cardiovascular risk and mortality, and with an intake >2632 mg/day for the risk of type 2 diabetes. However, some studies did not report this associations at doses >1200mg/day or higher. Furthermore, results obtained show considerable differences in polyphenols intake. This could be due to the heterogeneity of the study in terms of tools used to evaluate the consumption of polyphenols, the database used for the calculation of the polyphenol intake, type of class / subclass of polyphenols analyzed, the characteristics of the selected population (i.e., age, geographical provenience, gender, etc). Studies including target populations belonging to different age groups, found differences in eating habits that resulted in a different intake of polyphenols.

Nine studies focused only on older adults:

- total polyphenols intake 333mg/day in Spain- 1492 mg/day Japan
- flavonoid intake 170mg/day in Spain- 834mg/day Australia

Age is associated with different foods preferences, for example in the TOSCA.IT study, people aged >65 years had high intake of flavonoids and stilbenes than younger participants, or in a Brazilian study older adult had high intake of tyrosol and fruits. Children and adolescents usually have a diet poor in fruits and vegetables. Clearly also the geographical provenience has an important impact; for example, the main polyphenol food source, in Japan, is green tea, whereas in South Europe alcoholic beverages based on fruits are preferred.

Dietary intake is difficult to measure with a single tool. Furthermore, the two tools mainly used in the selected studies (i.e., FFQ and 24-h dietary recall) have some limitations. The FFQ consists of a predefined list of about 100 foods for which it is possible to record consumption over the period of interest. This method does not consider the cooking process; moreover, being a predefined list, it does not include all foods and brands and, therefore it may not accurately reflect food consumption. The 24h-dietary recall is an open questionnaire administered by expert personnel able to record all the information relating to food consumption in the last 24 hours, also considering the cooking method. This method requires multiple administrations to accurately assess the intake of flavonoids and its subclasses, however the selected studies tended to administer this questionnaire once. Given these premises, it is not possible to exclude any overestimation / underestimation of polyphenols and related classes / subclasses. In any case, the estimated intake of total polyphenols, flavonoids and phenolic acids seems to be similar using FFQ or 24h-dietary recall. Regarding the databases used for the estimation of polyphenols, we have seen that the most used were the USDA and Phenol-Explorer.

While these two databases are fairly accurate in estimating polyphenol intake, they have limitations. First of all, both do not consider all classes of polyphenols, in fact the USDA focuses mainly on flavonoids, while the Phenol Explorer on flavonoids and their precursors, total polyphenols. Moreover, not all food sources are considered and those factors that can influence the availability of polyphenols such as seasonality, cooking and storage processes are not always considered.

4.6. Conclusions

In this review we reported the inverse association between polyphenols intake, and several outcomes of health and diseases risk. The associations were reported with the highest levels of intake compared to the lowest. Because to the high heterogeneity of the studies, it was not possible to define a recommended range of daily polyphenol intake.

In conclusion, the contribution of polyphenols represents the other side of the coin of a food pattern. Therefore, it is advisable to think in terms of food patterns and not focus on the contribution of the single nutrient. Moreover, from the evidence found in this systematic review it is possible to recommend a dietary pattern rich in polyphenols as they seem to prevent numerous chronic diseases. Future studies are needed to standardize the methods of analysis of the contribution of polyphenols, to improve the databases of polyphenols and to validate biomarkers for the contribution of polyphenols. Furthermore, there is the need for intervention studies to clearly define a dose response association.

5. Final conclusion of the thesis

Both studies conducted during the PhD course ("study 1" and "study 2") suggest the presence of a relationship between diet and cognitive impairment. The results obtained strongly suggest the promotion of a diet rich in antioxidants, such as polyphenols and vitamin E, for the maintenance of cognitive health. Given that at the moment there are no pharmacological treatments capable of reducing the risk of developing cognitive decline non-pharmacological strategies, such as nutrition are a mandatory solution especially in light of polypharmacy and the adverse events associated with it. Results obtained strongly encourages interventional studies in order to establish the presence of a preventive effect of vitamin E and polyphenols on cognitive decline.

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7. Abstract of the paper published during the PhD course

- A. Cherubini, B. Carrieri, P. Marinelli. Advantages and disadvantages of direct oral anticoagulants in older patients. Geriatric Care 2018; 4:7227. DOI: 10.4081/gc.2018.7227

  Atrial fibrillation (AF) and venous thromboembolism (VTE), which includes deep vein thrombosis and pulmonary embolism, are conditions that increase with age. Anticoagulant therapy is strongly recommended both in patients with AF for the prevention of cardioembolic stroke, and for treatment of VTE and prevention of recurrent VTE. Until recently, vitamin K antagonists (VKAs) were the only oral drugs for long-term anticoagulation. In the past decade, four direct oral anticoagulants (DOACs) were approved: a direct thrombin inhibitor (dabigatran) and three factor Xa inhibitors (apixaban, rivaroxaban, edoxaban). Despite increasing evidence demonstrating the efficacy and safety of DOACs in older patients, there are still gray areas where the use of VKAs might be valuable.

- N.J. Peladic, P. Orlandoni, G. Dell'Aquila, B. Carrieri, et al. Dysphagia in nursing home residents; management and outcomes. J Am Med Dir Assoc. 2019 Feb;20(2):147-151. doi: 10.1016/j.jamda.2018.07.023

  Objectives: To define the prevalence of dysphagia and its associated factors and to investigate the influence of dysphagia and nutritional therapies performed in dysphagic subjects on clinical outcomes, including nutritional status, pressure ulcers, hospitalization, and mortality.
  Design: A prospective observational study.
  Setting and participants: Thirty-one Italian nursing homes participating in the ULISSE project and 1490 long-stay nursing home residents, older than 65 years, assessed at baseline and reassessed after 6 and 12 months.
  Measures: All participants underwent a standardized comprehensive assessment using the Italian version of the nursing home Minimum Data Set. The activities of daily living Long-Form scale was used to evaluate functional status. Health care professionals assessed dysphagia by means of clinical evaluation. Nutritional status was assessed using the information on weight loss.
  Results: The prevalence of dysphagia was 12.8%, and 16% of the subjects were treated with artificial nutrition. The mortality rate in subjects with dysphagia was significantly higher compared with that of nondysphagic subjects (27.7% vs 16.8%; P = .0001). The prevalence

of weight loss and pressure ulcers was also higher in dysphagic subjects. At variance, dysphagia was not associated with a higher hospitalization risk.

Conclusion/implications: Dysphagia is common in nursing home residents, and it is associated with higher mortality. Therefore, early diagnosis and optimal management of dysphagia should become a priority issue in nursing homes.

- C. Del Bo', S. Bernardi, M. Marino, M. Porrini, M. Tucci, S. Guglielmetti, A. Cherubini, B. Carrieri, et al. Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?. Nutrients 2019, 11, 1355; doi:10.3390/nu11061355

  Growing evidence support association between polyphenol intake and reduced risk for chronic diseases, even if there is a broad debate about the effective amount of polyphenols able to exert such protective effect. The present systematic review provides an overview of the last 10-year literature on the evaluation of polyphenol intake and its association with specific disease markers and/or endpoints. An estimation of the mean total polyphenol intake has been performed despite the large heterogeneity of data reviewed. In addition, the contribution of dietary sources was considered, suggesting tea, coffee, red wine, fruit and vegetables as the main products providing polyphenols. Total flavonoids and specific subclasses, but not total polyphenols, have been apparently associated with a low risk of diabetes, cardiovascular events and all-cause mortality. However, large variability in terms of methods for the evaluation and quantification of polyphenol intake, markers and endpoints considered, makes it still difficult to establish an evidence-based reference intake for the whole class and subclass of compounds. Nevertheless, the critical mass of data available seem to strongly suggest the protective effect of a polyphenol-rich dietary pattern even if further well targeted and methodologically sound research should be encouraged in order to define specific recommendation.

- M. Fedecostante, G. Onder, P. Eusebi, G. Dell'Aquila, E. Zengarini, B. Carrieri, et al. "Predictors of functional decline in nursing home residents: The Shelter Project". J Gerontol A Biol Sci Med Sci. 2020 Jul 13;75(8):1600-1605. doi: 10.1093/gerona/glz296

  Background: The aim of our study was to identify independent predictors of functional decline in older nursing home (NH) residents, taking into account both resident and facility characteristics.

  Methods: Longitudinal observational study involving 1,760 older (≥65 y) residents of NH participating in the SHELTER* study (57 NH in eight countries). All residents underwent a comprehensive geriatric assessment using the interRAI LTCF. Functional decline was defined as an increase of at least one point in the MDS Long Form ADL scale during a 1 year follow-up. Facility and country effects were taken into account.

  Results: During the study period 891 (50.6%), NH residents experienced ADL decline. Residents experiencing ADL decline were older, had lower disability at baseline, were more frequently affected by severe dementia and by urinary incontinence, and used more antipsychotics. In the mixed-effect logistic regression model, factors independently associated with a higher risk of functional decline were dementia and urinary incontinence, whereas the presence of a geriatrician was a protective factor.

  Conclusions: Both resident and facility characteristics are associated with the risk of functional decline in NH residents. Increasing the quality of healthcare by involving a geriatrician in residents' care might be an important strategy to improve the outcome of this vulnerable population.