Biological Aspects of Late-life Depression

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Chapter 1

General introduction

INTRODUCTION

Depression is the most prevalent and life-threatening mental disorder worldwide. Over the course of a lifetime, major depressive disorder (MDD) afflicts 1 in 6 individuals in the general United States population (1), women twice as often as men (2). Approximately 20% of depressive disorders have a chronic course and most patients experience recurrent episodes (3, 4). Depression is characterized by depressed mood and/or loss of interest or the ability to experience pleasure (anhedonia), plus several additional symptoms that can include change in weight or appetite, insomnia or hypersomnia, psychomotor retardation or agitation, fatigue and loss of energy, excessive feelings of guilt or worthlessness, problems with concentration and indecisiveness, and in more severe cases thoughts of death and suicidality. The Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition,Text Revised) (DSM-IV-TR) (5) further defines MDD as the presence of at least one 2-week depressive episode, and excludes cases in which bipolar disorder, substance use, or a general medical condition is etiologic. Important distinctions in the DSM-IV involve severity, duration, recurrence, melancholic and atypical features. Minor depression is characterized by at least two but fewer than five of the symptoms of MDD. Dysthymic disorder is a syndrome of depression of mild or moderate severity that lasts at least 2 years.

Depression is a major public health problem causing high disease burden for both the community and individuals worldwide, significantly complicating chronic illness (6), affecting social work functioning (7, 8) and resulting in high costs (9). According to the World Health Organization, depression is among the leading disorders causing disability and will be the second most important cause of disability worldwide in 2020, after cardiovascular disease (6). The public health relevance of depression is particularly evident among older persons, who comprise, especially the oldest of the old, the fastest growing segment of our populations in Western Countries (10). The impact of depression on health has been shown to be particularly detrimental in this age group.

LATE-LIFE DEPRESSION

Epidemiology

Depressive symptoms and syndromes are very common in older persons (11, 12). Prevalence rates of depressive problems vary considerably depending on the sample studied and methods used. Studies in clinical settings generally find much higher prevalences than studies in community settings, and studies applying psychiatric diagnostic criteria for depressive disorders find much lower prevalences than studies using symptom checklists. It is possible to score relatively high on a symptom checklist without meeting diagnostic criteria for depression. In fact, symptom checklists identify for the largest part persons who do not fulfill the diagnostic severity threshold of psychiatric depressive or anxiety disorders. This condition is often referred to as 'subthreshold depression' (13). As confirmed in several aging studies, up to 4% of the general older population has major depression, equivalent to an incidence of 0.15% per year (12, 14, 15). Minor depression has a prevalence of 4-13% and dysthymic disorder occurs in about 2% of older persons (14). While psychiatrically

defined depression appear to be less prevalent among older adults than among young and middle-aged adults, the prevalence of subthreshold depression is much more common (up to 21%) (14, 16). In particular, in older adults subthreshold depression is generally at least 2–3 times more prevalent than major depression (13). Approximately 8–10% of older persons with subthreshold depression develop major depression every year and the median remission rate to non-depressed status is only of 27% after more than 1 year (13).As in younger age groups, almost twice as many women as men are affected by depressive disorders and symptoms (14, 16).

Health outcomes

Late-life depression often arises in the context of chronic medical illness, and strongly affects the risk of developing disability and death (12).

About a quarter of individuals who have a myocardial infarction or who are undergoing cardiac catheterisation have major depression, and another 25% have minor depression (17). Meta-analyses and systematic reviews on studies in heart patients and in the general populations suggest that depression is associated with a two-fold increased risk of cardiovascular events and mortality (18-20).

The well-established association between depression and physical disability has also been confirmed by a meta-analysis (21). Not only is there a cross-sectional association (22, 23) but among non-disabled persons an increased risk of disability development over time has been found for both presence of significant depressive symptoms (24) as well as for depression that meets psychiatric criteria for major depression (25). Moreover, depression has been associated with some of the phenotypes that characterize age-related frailty, such as decline in walking speed (26) and muscle strength (27).

Late-life depression is also associated with cognitive impairment and dementia. Approximately 40% of elderly patients with major depression has impairment in some executive functions (28) and depressive symptoms have been found to speed up cognitive decline over time (29). Major depression and depressive symptoms are prevalent in dementia (30), but whether depression is a prodrome or a risk factor for dementia or whether they simply have similar neuropathologic substrates is still a matter of dispute. In older persons, dementia symptoms may arise in the context of a depressive episode and subside after remission of depression (pseudodementia); about 40% of the cases develop irreversible dementia within 3 years (31). Recent cohort studies reported an increased risk of dementia for elevated depressive symptoms (32) and a dose-response relationship between number of previous depressive episodes and the risk of dementia (33).

THE BIOLOGY OF DEPRESSION

From classical hypotheses to new molecular mechanisms

Despite the relevance of depression in terms of public health, its etiology and pathophysiology have not yet been elucidated. Exploring the molecular basis of depression

brings indeed substantial challenges. In contrast to the clear-cut phenotype encountered in other disorders, depressive syndromes are defined only by a list of symptoms that vary across classification systems and encompass a number of different disorders. The heterogeneity is clearly shown by the strongly varying patterns of relationship of depressive symptoms with all kind of validators including personality, demographics, clinical and prognostic characteristics (34). The highly variable compilation of symptoms, course of illness and response to various treatments indicate that depression subsumes numerous disease states of distinct etiology, and perhaps distinct pathophysiology. Moreover, the lack of any clear consensus on neuropathology and of objective diagnostic tests provides no obvious starting point for molecular investigations. Furthermore, in spite of its high heritability (roughly 30-40%), the search for genetic causes has not been successful to date (35).

In this context, the neuropharmacology of depression has been dominated for almost half of a century by the monoamine hypothesis of depression (36), proposed after the twin serendipitous discoveries of the therapeutic benefits of iproniazid (an antimycobacterialwith inhibitory effects on monoamine oxidase) (37) and imipramine (a failed antipsychotic that came to represent a new class of drugs known as tricyclic antidepressants) (38). According to this theory, depressive disorders are caused by a deficit in monoaminergic neurotransmission that can be treated by drugs that enhance the levels of norepinephrine and serotonin in the synaptic cleft. The older monoamine oxidase inhibitor (MAO-I) and tricyclic antidepressants (TCA) served as a template for the development for the newer more specific class of antidepressants such as SSRIs (selective serotonin reuptake inhibitors), NRIs (noradrenaline reuptake inhibitors) and SNRI (serotonin and noradrenaline reuptake inhibitors). Nevertheless, the monoamine-based treatments available nowadays are far from ideal in terms of efficacy. A substantial proportion of depressed patients shows no response to current available antidepressants, and only less than half of drug-responsive patients achieve full remission (39). In the recently completed Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study in which patients with nonpsychotic major depression were followed for up to 6 years through a sequence of alternative treatment regimens, only 37% achieved remission on first-line therapy with a selective serotonin reuptake inhibitor (SSRI), whereas another 16.3% withdrew completely from treatment because of drug intolerance (40). Similar efficacy rates for antidepressants therapies have been reported in older adults and those under the age of 60 (12). Recent meta-analyses including published and unpublished trials reported only modest benefits of antidepressants over placebo (41-43). Interestingly, these meta-analyses showed that significant drug–placebo differences in antidepressant efficacy were detected only for patients with high symptoms severity at baseline, but were relatively small even for severely depressed patients. This may be particularly relevant for older persons, where subthreshold depression is commonly more prevalent than major depressive syndromes.

Despite several decades of research the changes that the drugs induce in the brain that underlie the therapeutic actions of antidepressants remain unclear and the hypothesis that

depression is due to a deficiency in monoaminergic neurotransmission has never been definitively demonstrated. Meanwhile, there has been an impressive accumulation of knowledge about non-monoamine systems that might contribute to the pathophysiology of depression in animal models, and some human evidences are also available (46). An example is represented by the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis during depression, which has been called one of the most reliable findings in all of biological psychiatry (44), although the exact knowledge of this mechanism still remains incomplete. Findings from several studies are generally consistent about HPA hyperactivity in depression, especially among older inpatients who display melancholic or psychotic features (45). However, several findings argue for a reappraisal of this field. Some studies among older frail persons have in fact observed not only hyper-, but also hypoactivity of the HPA-axis in depressed subjects, probably due to the exhaustion of the body's response to stress with physical frailty (46, 47). Therefore, not considering the possibility of reduced cortisol levels among depressed older persons might lead to erroneous conclusions.

More recently, advances in basic science and clinical observations highlighted the potential role of new molecular mechanisms (48) allowing for promising novel insights into depression's neurobiological correlates and its association with adverse health outcome, especially in older persons. Promising new hypotheses about neurobiological correlates of late-life depression include cytokines, nutritional biomarkers, vitamin D and leptin, which will be further discussed in the following sections.

The "cytokine hypothesis" of depression

The "cytokine hypothesis" of depression (49) postulates that inflammatory mediators play a key role in the pathophysiology of depressive disorders. The inflammatory mediators network is represented by a bewildering array of molecules, the most prominent of which are proinflammatory cytokines produced within innate immune cells in the periphery and in the brain (eg, macrophages and dendritic cells). Signals from environmental adversities such as infection, tissue trauma, neoplasm and psychosocial stress activate a wide number of genes in these cells via a limited number of transcription factors, including the nuclear factor kappa B (NFkB) and mitogen-associated protein (MAP) kinases, leading to production and release of inflammatory cytokines including interleukin 1β (IL-1β), tumor necrosis factor α (TNF- α), and IL-6. Other cytokines, known as anti-inflammatory, oppose this response by attenuating the production of proinflammatory cytokine (e.g. IL-10) or by antagonizing their action at the receptor level (e.g. IL-1 receptor antagonist, IL-1Ra). In turn, the actions of proinflammatory cytokines on peripheral cellular targets such as hepatocytes, lead to the synthesis of acute phase proteins (e.g. C-reactive Protein, CRP) responsible for the systemic inflammatory response. Normal aging is a situation characterized by a chronic low-grade inflammatory state, with an overexpression of circulating inflammatory mediators (a condition commonly referred as "inflammaging" (50-52). In the brain, this condition manifests by the chronic activation of perivascular and parenchymal macrophages/microglia expressing proinflammatory cytokines, while the number of astrocytes increases (53-55).

Smith first proposed the "macrophage theory" of depression, based on the observation that cytokines induce symptoms of depression when given to volunteers and have brain effects including HPA axis activation (56). The phenomenon of inflammation-associated depression has received increasing attention during the recent years. This hypothesis was initially based on the quasi-experimental model of immunotherapy in patients receiving injections of recombinant cytokines, mainly IL-2 and/or interferon alfa (IFN-α), for treatment of viral infections (e.g. hepatitis C) or cancer (e.g. kidney cancer, malignant melanoma). In most of these patients, cytokine immunotherapy rapidly induces a core of neurovegetative symptoms such as fatigue, pain, decreased appetite and sleep disorders. This constellation of non-specific symptoms is referred to collectively as 'sickness behaviour' (57). After a few days or weeks depending on treatment modalities (e.g. cytokine, dosage, administration route), 30-50% of the patients develop psychological symptoms including depressed mood, feeling of worthlessness, guilt and suicidal ideation that culminate in a full major depressive episode (58). In contrast to neurovegetative symptoms, these psychological symptoms have been found to be sensitive to preventive antidepressant treatment (58), confirming the dissociation between sickness behavior and depression (57). Patients at risk for developing cytokine-induced depression have been shown to have higher depressive symptoms at baseline (59), poor sleep together with high IL-6 levels (60), exaggerated reactivity of HPA axis to the very first injection of IFNα (61) and genetic vulnerability in the form of functional polymorphisms in the genes coding for the serotonin transporter and for IL-6 (62, 63). These clinical findings were confirmed also in animal models of depression, confirming that inflammation-associated depression develops on a background of sickness behavior (57). Interestingly, in mice the chronic inflammation associated with aging increased the duration of depressive-like behaviors (64). Furthermore, the increasing recognition that psychosocial stress can activate the inflammatory response both peripherally and in the brain, provide the evidence for a source of inflammation, together with nascent inflammatory processes secondary to evolving medical pathologies, that may be related with depressives symptoms also in healthy individuals. A rich literature indicates that chronic stress, including caregiving, bereavement, and perceived stress are associated with increase in inflammatory mediators (65-68). In addition, increased inflammation appears to be a hallmark of early life stress, in that childhood maltreatment and low socio-economic status (SES) have been associated with increased blood levels of inflammatory markers (69-71). More recently, functional genomics studies have indicated that several different types of social adversity, including low SES, social isolation and chronic stress, are all associated with heightened signaling of pro-inflammatory transcription control pathways in both children and adults (70, 72, 73).

The hypothesis that inflammation can cause depressed mood in medically healthy subjects has been confirmed in a recent experimental study on volunteers administered with low doses of endotoxin acutely (74) or vaccinated against typhoid (75), in which increased production of proinflammatory cytokines was associated with occurrence of depressive symptoms. Additional support for the cytokines hypothesis came from some clinical cross-

sectional studies that found an association between major depression and high serum levels of inflammatory markers (76-79). Finally, studies in patients with coronary artery disease and acute coronary syndrome showed that somatic/affective symptoms, associated with less favorable cardiac prognosis (80, 81), predicted inflammation increase (82, 83).

Previous cross-sectional epidemiological studies on the association between inflammation and depression have produced discrepant results: some found positive associations while other did not (84-89).This discrepancy between these studies is probably attributable to differences in the choice of study populations, assessments of depression, assays of inflammatory markers and in failure to control for important confounding factors. Among these studies, a positive association between depression and inflammation has been found more commonly in samples of older persons (84, 85). Recently, two large meta-analyses (90, 91) based on the cross-sectional data from all the relevant studies, confirmed that inflammatory mediators are positively associated with depression. This association was found in both clinic- and community-based samples, suggesting a dose-response relationship between depression and inflammatory markers. However, to date, only few longitudinal studies examining the relationship between inflammatory mediators and depression in older persons are currently available. This thesis will extend this longitudinal evidence.

Nutrition, nutritional biomarkers and depression

Depression is associated with a poor compliance with healthy lifestyle indications, including dietary recommendations (92), and depression and stress have been shown to promote unhealthy dietary preferences (93, 94). However, depression and diet likely have a bidirectional complex relationship. While there has long been interest in any nutritional contribution to the onset and treatment of mood disorders, there has been increasing scientific evaluation of several candidate nutritional and dietary factors in recent years. Several studies examined the association between depression and omega-3 fatty acids, the vitamin B group (including folate), phospholipids, minerals (including zinc, iron and magnesium), and antioxidants (including vitamin C). However, this line of research has provided so far inconsistent results. An example is represented by the studies that investigated the potential role in mood regulation of the polyunsaturated fatty acid family of omega-3 (*n*-3 PUFAs), for which cold-water oily fish (e.g. salmon, mackerel and trout) is the major source (95). This hypothesis was initially proposed based on findings form epidemiological studies that demonstrated a significant inverse relationship between annual fish consumption and major depression (96). Although meta-analytic reviews confirmed that levels of *n*-3 PUFAs are significantly lower in depressed patients compared with controls (97), several studies opposed the hypothesis that lower *n*-3 PUFA levels are associated with depression (98, 99). Moreover, randomized controlled trials provided only limited support for *n*-3 PUFAs as antidepressants and the proportion of studies showing a significant effect has dropped over the last 5 years (95). Depressive symptomatology in older persons has also been related to plasma PUFAs concentrations and fatty acid composition (100-102).

Recent randomized controlled trials examining the effect of *n*3-PUFA supplementation on depression, showed both no significant effect in 302 Dutch elderly (103) and significant amelioration in 46 older Italian women (104). Further studies are required of sufficient methodological quality, duration, and sample size to confirm these findings.

Among older persons, different factors can lead to a decline in food intake such as eating difficulties, alterations of stomach-fundus compliance, activity of cholecystokinin, depression and inflammatory processes. Decline in overall food consumption increases the risk of inadequate intake of essential nutrients (105, 106). Previous studies examining the relationship of nutrient intake and biomarkers with depression in older persons obtained conflicting results. Lower levels of folate and vitamin B_{12} have been shown to be associated with depression in two cross sectional-studies of Chinese (107) and Greek (108) older adults, and to be a risk for incident depression over a period of 2–3 years in a prospective study of Korean older persons (109). Moreover, in a sample of older disabled women serum levels of vitamin B_{12} were associated with higher risk of severe depression (110). High total intakes of vitamins B_6 and B_{12} have recently been shown to be protective of depressive symptoms over time in community-dwelling older adults (111). However, in a sample of healthy elderly men in the Netherlands, intake of folate and vitamins B_6 and B_{12} were not related to depressive symptoms (112).

An interesting new perspective is represented by the study of nutrient and nutritional biomarkers that play a role in inflammatory processes, which may contribute to the development of depression. In a recent cutting-edge review (113), Kiecolt-Glaser underlined the need of further research aimed at understanding the relationship between depression and nutrition and their interactive influence on inflammation. It has been shown that dietary patterns high in refined starches, sugar, and saturated and trans-fatty acids, poor in fruits, vegetables, and whole grains, and poor in omega-3 fatty acids may cause activation of the innate immune system, while in contrast a diet rich in fruit, vegetables and olive oil is instead associated with lower levels of inflammatory markers (114). Dietary patterns rich in these foods, like the traditional diet of populations living in the Mediterranean area, are indeed widely considered models of healthy eating. Epidemiological studies conducted in different countries have shown that adherence to a Mediterranean type diet is associated with longer survival, and lower risks of cognitive decline, chronic degenerative disease, and mobility decline and reduced cardiovascular and cancer mortality (115, 116). Moreover, trials showed that nutritional interventions based on Mediterranean diet significantly reduced the levels of CRP and IL-6 in participants with cardiovascular risk factors (117, 118). Among the components of this type of diet, major anti-inflammatory effects are supposed to be exerted by the antioxidant components of fruit and vegetables including vitamins and carotenoids, which may be effective in suppressing the proinflammatory pathways through the reduction of reactive oxygen species (119, 120). Dietary carotenoids are powerful antioxidants that are embedded within lipid bi-layers (the two lipid layers that make up cell membranes) and function to quench free radicals generated by intracellular oxidative processes (121). The most prevalent dietary carotenoids are α-carotene, β-

carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene. The first three can be converted into retinol (i.e. vitamin A) and are thus referred to as provitamin A carotenoids, while the others are referred to as non-provitamin A carotenoids. According to the Food and Nutrition Board of the Institute of Medicine, blood concentrations of carotenoids are the best biological markers for consumption of fruits and vegetables (121). In recent studies on older persons, a growing body of evidence has been accumulated showing an association between carotenoids with some of the phenotypes that characterize age-related frailty, such as sarcopenia and mobility disability (122-127), and mortality (128-130). However, whether carotenoid levels are associated with depression in older persons has not been previously studied. This thesis will do so.

Vitamin D and depression

For historical and epidemiological reasons, vitamin D has been classified as a vitamin. However, vitamin D is now being reconsidered as a genuine steroid hormone with a multifaceted function (Figure 1). The term "vitamin D" refers to either vitamin D2 (ergocalciferol) or D3 (cholecalciferol). Ergocalciferol is found in yeast and plants, while cholecalciferol is naturally present in few foods, primarily fatty fish such as salmon, mackerel, and sardines or in fortified foods such as milk and some cereals. However only a small part of the vitamin D requirement comes from nutritional sources. The major source of vitamin D3 for most people is trough the action of UV radiation from the sun on a precursor in the skin related to cholesterol (7-dehydrocholesterol) to form cholecalciferol, which is subsequently hydroxylated in the liver into 25-hydroxyvitamin-D (25(OH)D, or calcidiol). Vitamin D2 is also converted to a dihydroxy active form. As such, the serum level of 25(OH)D is the best indicator of vitamin D body reserve, either by cutaneous synthesis or by ingestion in the diet. The primary active form of vitamin D (1,25(OH)2D, or calcitriol) is then synthesized in the kidney from 25(OH)D by the mitochondrial enzyme 1,α-hydroxylase. Calcitriol is released into the bloodstream as a hormone, circulates bound to a vitamin D-binding protein, enters the cell and binds to the vitamin D receptor (VDR). This is followed by transcription and translation and proteins are formed such as the calcium binding protein or osteocalcin.

Calcitriol has effects on the classic target organs bone, intestine and kidney and stimulates calcium transport through cells of these organs and the blood. The production of 1,25(OH)2D is stimulated by parathyroidhormone (PTH) and there is a direct negative feedback from 1,25(OH)2D to PTH. Severe vitamin D deficiency causes a mineralization problem and osteomalacia and on the other side high PTH levels cause high bone turnover, bone resorption and osteoporosis. Both mechanisms may lead to fractures, especially hip fractures (131).

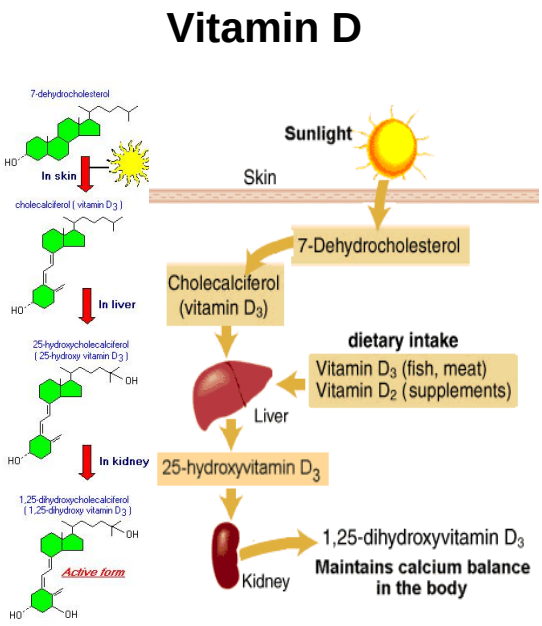

Figure 1. Vitamin D metabolism

Non-classical extra-skeletal actions of vitamin D have recently gained attention since it has been shown that diminished levels of vitamin D induce immune-mediated symptoms in animal models of autoimmune diseases and is a risk factor for various brain diseases. In addition, vitamin D has been increasingly implicated in the pathophysiology of cognition and mental illness (132-136). Difficulties in studying vitamin D are the wide variability in the results obtained by different assays and the lack of consensus on the optimal levels of 25(OH)D. It is now generally agreed that the values of 25(OH)D between 30 ng/ml (or 75 nmol/l) and 20 ng/ml (or 50 nmol/l) are considered to represent vitamin D insufficiency, whereas those less than 20 ng/ml or (50 nmol/l) fall into the frankly vitamin D-deficient. It is usually only in the latter situation where one is likely to observe clinically apparent skeletal effects of the deficient state (137).

The National Health and Nutrition Examination Survey (NHANES) reported a near doubling in the number of subjects in the American population from 1994 to 2004 with 25(OH)D levels less than 30 ng/ml. Currently, only 20–25% of the assayed NHANES population has a serum 25(OH)D level of at least 30 ng/ml, whereas 25–35% of the population has frank vitamin D deficiency (138). Risk factors for vitamin D deficiency are premature and dysmature birth, pigmented skin, obesity, malabsorption and advanced age as a result of reduction of 7-dehydrocholesterol in the skin, reduced sunlight exposure due

to decreased outdoor activity, and reduced vitamin dietary intake (139). Vitamin D deficiency and insufficiency are indeed even more common in older persons, with prevalence between 40% and 90% of persons suffering from hypovitaminosis D (135). A detailed survey (140) of 25(OH)D levels in various populations in Europe, the USA, Australia and other countries showed that vitamin D insufficiency is a frequent finding among community-dwelling elderly, irrespective of latitude, and an almost universal finding among institutionalized elderly. In a study in 824 elderly from 11 European countries (139), 36% of men and 47% of women had 25(OH)D concentrations below 30 nmol/l. In older adults, vitamin D deficiency has been linked to poor health outcomes, such as fractures (141), poor physical function (142), frailty (143), sarcopenia (144), pain (145), nursing home admission (146), mortality (147) and chronic diseases such as osteoporosis, diabetes, cancer, cardiovascular, neurodegenerative, autoimmune, and infectious diseases (133, 134, 148).

Recently, it has been hypothesized that hypovitaminosis D may contribute to late-life depression (135, 136, 149). However there is currently insufficient evidence to support the role of vitamin D as a risk factor for depression. Cross-sectional epidemiological studies based on different international community groups identified associations between depression and low vitamin D levels, although there were a number studies with non-significant results (150). In the Longitudinal Aging Study Amsterdam (151), depressive symptoms were significantly associated with lower 25(OH)D levels, which were comparable across participants with major and minor depression. A recently published national community study of 2070 older people living in England found that vitamin D deficiency was associated with late-life depression in northern latitudes, although increased depressive symptoms were only seen in those with the most severe deficiency state (152). In contrast, a large cross-sectional study (153) of older adults in China found no associations between 25(OH)D levels and depressive symptoms. Vitamin D supplementation has been proposed as treatment for seasonal affective disorders (SAD), a depressive subtype where depression occurs and recurs at the darkest time of the year (i.e. winter) often characterized by hypersomnia, hyperphagia, anergia and worsening in the evening, abating in the spring and summer, and where light deficiency has been hypothesized as causal (149). However the results from several small randomized trials showed only modest support that Vitamin D is effective in treating the symptoms of SAD (150). In a sample of older women, 6-month winter treatment with daily low dose vitamin D supplements did not show any significant improvement in mental health scores (154). Moreover, until recently no longitudinal study examined the association between 25(OH)D concentrations and development of depressive symptoms. Consistently with this level of evidence, the Institute of Medicine of the National Academies (155) stated in their report that **"**whether there is a functional relationship between measures of serum vitamin D or intake and mood or depression has not been determined**"**. This thesis will report on the first study to investigate the longitudinal association between 25(OH)D levels and the development of depressive symptoms in a large sample of older persons.

The "leptin hypothesis" of depression, leptin resistance and abdominal obesity

The recently proposed "leptin hypothesis" (156) contends that the adipose-derived hormone leptin may have a potential role in the regulation of mood. Leptin is a peptide hormone and its most-well-known effect is control of energy homeostasis through a negative feedback adiposity signal. Leptin is synthesized in white adipose tissue in proportions to fat stores. Circulating leptin serves to communicate the state of body energy repletion to the central nervous system in order to suppress food intake and permit energy expenditure on the processes of reproduction, tissue remodeling and growth. Leptin was discovered in 1994 (157) studying strains of highly obese mutant mice, which have been found to be homozygous for single gene mutations causing lack of leptin signaling due to mutation of leptin (as in *ob/ob* mice) or the leptin receptor (as in *db/db* mice). The principal homeostatic site of action of leptin is the hypothalamic arcuate nucleus.

More recently, several peripheral and extra-hypothalamic effects have been described, expanding leptin's action far beyond energy balance. In animal models of depression, leptin have been shown to improve cognition and mood suggesting antidepressive properties (158). The protective effect of leptin on mood found in animal model of depression was not clearly replicated by the few available human studies, which instead provided conflicting findings with studies showing increased, decreased or no differences in leptin levels (156, 159). These conflicting findings may be partly explained by the phenomenon of leptin resistance that is observed in persons with common obesity (160, 161). The conundrum of hyperleptinemia observed in obese persons has given rise to the notion of the existence of a mechanism of physiological leptin resistance, similar to the one that links type 2 diabetes and insulin resistance. In synthesis, the failure of high levels of leptin to suppress food intake and decrease body weight/adiposity in obese persons suggests a relative central resistance to the effect of leptin despite its increasing circulating levels. Several mechanisms have been proposed to explain leptin resistance, including impaired transport across the blood–brain barrier, reduced function of the leptin receptor and defects in leptin signal transduction (160, 161). The phenomenon of leptin resistance has been confirmed by the findings of a randomized trial in which treatment with recombinant leptin has been shown to be more effective in terms of weight loss in lean subjects as compared to obese subjects (162). Similarly, in a prospective study from the Framingham cohort (163), a neuroprotective effect of leptin against the development of dementia was observed only among lean individuals. Based on these observations, it has been hypothesized that it is not the absolute serum leptin concentration but rather its impaired central action that is correlated with mood (156, 159), especially in obese persons.

The association between obesity and depression has repeatedly been established. A recent meta-analysis (164) confirmed a bidirectional associations between depression and obesity: obese persons had a 55% increased risk of developing depression over time, whereas depressed persons had a 58% increased risk of becoming obese. In two intriguing studies (165, 166), Vogelzangs et al. showed similar result patterns in a large US cohort of

community-dwelling older persons aged 70 to 79. In the first study (165), depressed persons showed significantly larger increase in abdominal obesity, especially visceral fat, than non-depressed persons. Such an association was not found for an increase in overall obesity and also appeared to be independent of changes in overall obesity. The second study (166) showed that visceral fat, independent of overall obesity, was a risk factor for depression onset in older men. Taken together, these findings suggest that depressive symptoms, at least in older men, are rather specifically associated with abdominal obesity, in particular with fat in the visceral region. Biological mechanisms linking abdominal obesity and depression are not well-delineated. Adipose tissue, especially in the visceral area, is an active endocrine organ that produces cytokines and adipokines such as leptin (167, 168). It has been hypothesized that leptin resistance may serve as a common biological factor for the comorbidity of depression and obesity (156).

While proposing the "leptin hypothesis", Lu (156) underscored the need for future studies to clarify the role of leptin insufficiency versus leptin resistance in depression. To our knowledge the study presented in this thesis is the first to examine the longitudinal relationship between leptin, abdominal obesity and depression in a large cohort.

GENERAL AIM

The main aim of this thesis is to examine the potential role of these newly proposed molecular mechanisms in late-life depression. We examine whether inflammatory markers are associated with, predict or follow depressive symptoms increase. In addition, we investigate whether adherence to a healthy nutritional pattern may buffer the inflammatory process boosted by depression, and whether blood levels of the antioxidant class of carotenoids are associated and predict depressive symptoms via the mediation of inflammatory pathways. Additionally, this thesis examines whether hypovitaminosis D is prospectively associated with depression and, finally, whether the phenomenon of leptin resistance, indicated by hyperleptinemia in obese persons, may be a risk factor for depression onset over time. To study these research questions an epidemiological approach is taken, based on large prospective cohort studies of older persons. By identifying new molecules likely related to late-life depression, this research can suggest future directions for research focused to understand the exact mechanism of these associations and to clinical studies aimed at modulating these new biological risk factors in order to reduce depressive mood in the elderly and prevent its deleterious consequences on morbidity and mortality.

STUDIES USED IN THIS THESIS

In the present thesis, data from two large prospective cohort studies of older persons were used. The InCHIANTI (**In**vecchiare in **Chianti**, aging in the Chianti area) Study is a prospective population-based study among 1155 community-dwelling older persons, aged 65 years and older, living in the Chianti area (Tuscany), Italy, originally designed to investigate factors contributing to decline in mobility in later life. Data from the baseline

measurement and 3 and 6 years follow-up were available. Another study that provided data was the Health, Aging and Body Composition (Health ABC), a prospective population-based cohort study among 3075 well-functioning white and black older persons, aged 70-79 years, from Memphis, Tennessee and Pittsburgh, Pennsylvania (US). The primary goal of this study is to identify determinants and consequences of body composition changes during the aging process. Both baseline and 5-year follow-up data were used in this thesis.

OUTLINE OF THIS THESIS

Bringing together the observations presented in this General Introduction, a schematic presentation of the research model for the present thesis is presented here. Studies in Chapters 2-5 use data from the InCHIANTI Study. In **Chapter 2** we test the hypothesis that in older persons higher plasma levels of inflammatory mediators predict the development of depressed mood over time. In **Chapter 3**, we examined (1) whether older individuals with depressive symptoms are more likely to develop a pro-inflammatory state and (2) whether such an excess risk of developing a pro-inflammatory state is lower in those who have a healthy (Mediterranean-style) diet. In **Chapter 4**, we examined the cross-sectional and longitudinal relationship between plasma carotenoids and depressive symptoms in older persons. We tested the hypothesis that participants with low carotenoid levels would be significantly more likely to have higher depressive symptoms and to develop clinically relevant depressed mood over time. Additionally, we tested whether inflammatory markers mediated this relationship. **Chapter 5** examines the longitudinal relationship between vitamin D and depressive symptoms. Specifically, we hypothesized that participants with lower 25(OH)D levels at baseline would experience a steeper increase in severity of depressive symptoms and would be significantly more likely to develop clinically relevant depressed mood than those with higher 25(OH)D. Using data from the Health ABC Study, **Chapter 6** tests whether serum leptin concentration in older adults are associated with an increased risk of developing clinically relevant depressive symptoms. Since the presence of hyperleptinemia in obese persons may be considered an indicator of leptin resistance, we hypothesized that the risk of depression onset would be especially increased for participants with high levels of leptin and visceral fat. Finally, in **Chapter 7** the results of Chapter 2 through 6 are summarized, discussed and the underlying biological pathways are described in details. Moreover, the different results are related to each other and integrated within a framework based on the current knowledge of the biological underpinning of late-life depression.

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Chapter 2

Il-1ra and incident depressive symptoms over six years in older persons: the InCHIANTI Study

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ABSTRACT

Background: We test the hypothesis that in older persons higher plasma levels of inflammatory markers predict the development of depressive symptoms during a 6-year follow-up.

Methods: This study is part of the InCHIANTI Study, a prospective population-based study of older persons. The sample consisted of 991 participants aged 65 years and older. Serum levels of CRP, IL-1β, IL-1ra, TNF-α, IL-6, IL-6R and IL-18 were measured. Depressive symptoms were assessed at baseline and at the 3 and 6 year follow-ups using the Center for Epidemiological Studies-Depression Scale (CES-D). Depressed mood was defined as CES-D > 20. Potential confounders were baseline variables related to sociodemographics, somatic health and functional status.

Results: At baseline, IL-1ra levels were significantly higher (p = 0.004) in depressed compared to non depressed participants. After adjustment for confounders, among subjects free of depression at baseline, those in the third and fourth IL-1ra quartiles compared to those in the lowest quartile, had respectively a 2.32 (95%CI:1.21-4.42, p = 0.01) and 2.78-fold (95%CI:1.47-5.26, p = 0.002) higher risk of developing depressed mood during a 6 years follow-up.

Conclusions: In old age, persons with high plasma levels of IL1-ra had a higher risk of developing depressive symptoms over time. These findings suggest a potential causal role for inflammation in the development of depressive symptoms in older persons.

INTRODUCTION

The "cytokine hypothesis of depression" (1-6) assumes that inflammatory mediators play a key role in the pathophysiology of depressive disorders. This hypothesis was initially based on clinical findings that depression is accompanied by direct and indirect evidence of upregulated inflammatory response, such as an increased production of pro-inflammatory cytokines (IL-1, IL-6) and an acute phase response indicated by the release of CRP or other acute phase reactive proteins (1-3,7-10). Administration of cytokines as treatment produces symptoms such as dysphoria, fatigue, psychomotor retardation and impaired cognitive function which may be alleviated by withdrawing the cytokines administration or by antidepressants treatment (11,12). In a study (13) of healthy young men, experimental endotoxemia caused both increasing levels of inflammatory markers and depressive symptoms. In spite of this evidence, epidemiological studies have generated contradictory results, and some of them have confirmed and some of them rejected the hypothesis of a connection between inflammatory markers and depressive symptoms (14-17).

Older persons are often affected by a chronic "low-grade proinflammatory state" (18) and have a high prevalence of chronic syndromes of depression (19), and cross-sectional studies have found that high levels of inflammatory markers are associated with depression (17,20,21). However, because most of the available data on the relationship between inflammation and depression are cross-sectional, it is unclear whether cytokine abnormalities precede or follow the onset of depressive symptoms. In a recent study (22) higher production of IL-1β and IL-1ra, determined by ex vivo whole blood stimulation with bacterial lipopolysaccharide (LPS), was identified as an independent risk factor for the development of depressive symptoms in older persons. In the present study we test the hypothesis that in older persons higher plasma levels of inflammatory markers predict the development depressive mood during a 6-year follow-up.

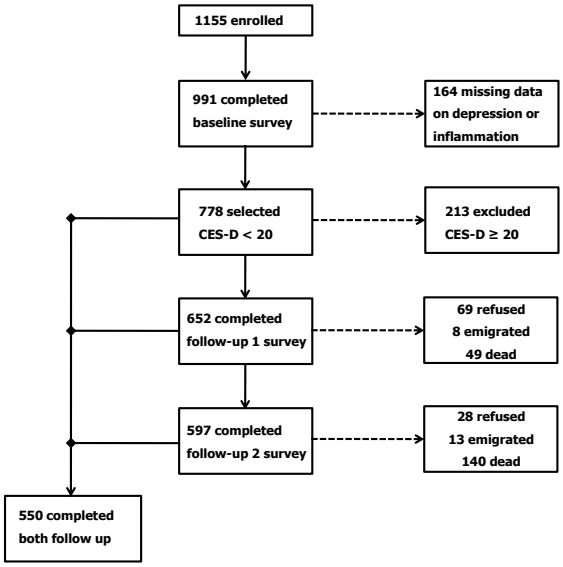
METHODS AND MATERIALS

Study Population

Participants were part of the InCHIANTI (Invecchiare in Chianti, aging in the Chianti area) study, a prospective population-based study of older persons in Tuscany (Italy) designed to investigate factors contributing to decline in mobility function in later life. A description of the study rationale, design and method is given elsewhere (23). Briefly, in 1998-1999 the sample was randomly selected from two sites, Greve in Chianti and Bagno a Ripoli, using a multistage stratified sampling method. Data collection included: 1) a home interview concerning demographics, health-related behaviors, functional status and cognitive function; 2) a medical examination including several performance-based tests of physical function conducted in the study clinic; 3) 24-h urine collection and blood drawing. Participants were seen again for a three-year follow-up visit (2001-2003) and six-year follow-up visit (2004-2006). All respondents signed an informed consent and the Italian National Institute of Research and Care on Aging Ethical Committee approved the study protocol.

Of the 1155 participants aged ≥65 enrolled in the study, we excluded 164 participants because of missing data on CES-D or inflammatory markers. In cross-sectional analyses we included all 991 remaining participants. For the longitudinal analyses we also excluded 213 participants with depressed mood at baseline. Among the remaining 778 subjects, 652 completed the first follow-up (69 refused, 8 emigrated, 49 dead), 597 the second follow-up (28 refused, 13 emigrated, 140 dead) and 550 both follow-up. The study population selection is summarized in Figure 1.

Figure 1. Flow chart of study design.
CES-D, Center for Epidemiological Studies-Depression Scale.



Depressive symptoms

Depressive symptoms were assessed at baseline and at the 3 and 6 year follow-up using the Center for Epidemiological Studies-Depression Scale (CES-D) (24). The CES-D is a 20-item self report scale, ranging from 0 to 60. The CES-D has been shown to have good psychometrics properties in assessing depressive symptoms in older population-based studies (25), also in an Italian sample (26). A score ≥20 was operationally defined as clinically relevant "depressed mood". While a cut-off of 16 is generally considered to

represent relevant depression, we selected a cut-off of 20 that has been shown to avoid overestimation in older subjects (27).

Inflammatory markers

Measures for the cytokines were obtained from frozen plasma samples originally collected at baseline. Morning, fasting blood samples were collected after a 15-minute rest. Aliquots of serum were stored at -80°C and never thawed before analysis. Serum levels of IL-6, soluble IL-6 Receptor (sIL-6r, 80kDa), IL-1β, IL-1 receptor antagonist (IL-1ra), TNF-α (kits from BIOSOURCE International, Camarillo, California) and IL-18 (kits from Quantikine HS, R&D Systems, Minneapolis, MN) were measured by enzyme linked immuno-absorbent assays (ELISA). Serum C Reactive Protein (CRP-high sensitivity) was measured in duplicate using an ELISA and colorimetric competitive immunoassay. The lowest detectable concentration was 0.1 pg/ml for IL-6, 8 pg/ml for sIL-6r, 0.09 pg/ml for TNF-alpha, 0.01 pg/ml for IL-1β, 4 pg/ml for IL1ra, 0.7 pg/mL for IL-18 and 0.03 mg/L for CRP. The inter-assay coefficient of variation was 4.5% for IL-1ra, 5% for CRP and 7% for other inflammatory markers.

Other variables.

The following variables were also selected. Age, gender, site, education (years), smoking habit (current/former versus non smoker), alcohol use (< 30vs≥ 30 g per day), MMSE score, BMI, number of drugs, use of NSAIDs and use of antidepressants coded according to ATC codes. Major chronic diseases ascertained according to previously validated algorithms (28) using information on self-reported history, pharmacological treatments, medical exam data and hospital discharge records included: congestive heart failure, coronary heart disease including angina and myocardial infarction, stroke, chronic obstructive lung disease, hypertension, diabetes, cancer and hip arthritis. Number of ADL and IADL disabilities was defined as self-report of inability or needing personal help in performing any basics or instrumental activities of daily living (29). Level of physical activity in the previous 12 months, based on response to multiple questions, was classified as sedentary/light/moderate-high. The Short Physical Performance Battery (SPPB) was used to assess lower extremity function using a standard protocol as described elsewhere (30).

Statistical Analyses

Variables were reported as percentage, means ± SD, or median and interquartile range as appropriate. Because plasma levels of inflammatory markers were non-normally distributed, log-transformed values were used in the analyses. Differences in baseline characteristics according to depressed mood were analyzed using chi-square and t-test statistics as appropriate. Pearson's correlation tests were used to evaluate correlations between inflammatory markers. To explore the functional form of the association between inflammatory markers and depressive symptoms, we divided the CES-D scores into four levels: the highest level corresponded to the depressed mood category and the remainder

of the CES-D scores were divided into three groups of equal size. Levels of inflammatory markers across CES-D subgroups were compared using sex/age adjusted analysis of covariance. Linear regression analyses were used to estimate regression coefficients per standard deviation increase in (log) plasma inflammatory markers associated to baseline CES-D score after adjusting for multiple confounders. The RRs (Relative Risks) of developing depression at 1) 3-years follow-up, 2) 6-years follow-up and 3) at 3 or 6-years follow-up according to baseline quartiles of cytokines were calculated. Recent articles (31) pointed out that when the outcome event occurs in more than 10% of the participants it is desirable to estimate RRs directly instead of the ORs (Odds Ratios) approximation. Therefore, we estimated RRs and CIs (Confidence Interval) using the "modified Poisson" approach proposed by Zou (32). These analyses were restricted to subjects free of depression at baseline and were adjusted for confounders in parsimonious models that only included variables with a p value <0.1.

All analyses were performed using the SAS statistical package, version 8.2 (SAS Institute Inc., Cary, North Carolina) with a significance level set at $p<0.05$.

RESULTS

The mean age of the study sample was 75 ± 7 years and 55.9% were women; 21.5% had depressed mood at baseline. As shown in Table 1, depressed persons were older, more often women, less likely to be smokers or heavy alcohol drinkers, took more drugs, antidepressant and NSAID, had a lower MMSE score and were more likely to have hypertension, CHF and hip arthritis. Furthermore persons with depressed mood were more likely to be disabled and sedentary, and had lower SPPB scores. Among inflammatory markers only levels of IL-1ra were significantly higher in depressed participants. Pearson's correlations between inflammatory markers are shown in Table 2. Figure 2 shows age and sex adjusted means ± SE of (log) IL-1ra levels across different CES-D scores subgroups, calculated using analysis of covariance. Adjusted levels of IL-1ra significantly increased from 4.88 ± 0.04 in the CES-D < 6 group to 4.99 ± 0.04 in the CES-D ≥ 20 group. Adjusted regression coefficients for the association of inflammatory markers with baseline CES-D scores are shown in Table 3. Higher levels of IL-1ra were associated with higher CES-D scores.

Table 1. Characteristics of the study population at baseline

	Not Depressed CES-D < 20 (n=778)	**Depressed CES-D ≥ 20** (n=213)	*p**
Age *(years)*	74.3 ± 6.8	77.5 ± 7.1	<.0001
Sex female	50.6	75.1	<.0001
Site *(BR)*	51.4	55.4	0.3
Education *(years)*	6.9 ± 35.8	5.1 ± 3.5	0.17

Continued on next page

	Not Depressed CES-D < 20 (n=778)	**Depressed CES-D ≥ 20** (n=213)	*p**
Smoking status	15	11.3	
non smoker	54.4	73.7	
former smoker	30.6	15	
current smoker	15	11.3	<.0001
Alcohol use	17	8.45	0.0021
BMI	27.5 ± 4	27.4± 4.4	0.71
MMSE scores	25.5 ± 3.2	24.4 ± 3.4	<.0001
N of drugs	2 ± 1.9	3.2 ± 2.2	<.0001
AD use	2.7	11.3	<.0001
NSAID use	6.8	12.1	0.01
Hypertension	33.3	41.8	0.0214
Angina/MI	7.5	8.9	0.48
Stroke	6	7	0.59
CHF	3.7	8	0.0089
Cancer	5.9	7.5	0.39
COPD	8.1	6.6	0.46
Diabetes	11.1	9.4	0.49
Hip arthritis	4.1	9.86	0.001
N of ADL	0.1 ± 0.4	0.3 ± 0.9	0.0002
N of IADL	0.4 ± 1.3	1.4 ± 2.1	<.0001
SPPB Scores	10.5 ± 2.4	8.0 ± 3.5	<.0001
Physical activity			
Low	13.8	40.9	
Medium	80.5	55.4	
High	5.8	3.8	<.0001
IL-1RA *(pg/mL)*	130.9 (94.7-180.8)	136.8 (99.8-194.8)	0.0041
TNF-α *(pg/mL)*	2 (1.5-3.4)	2 (1.5-2.9)	0.41
IL-6 *(pg/mL)*	1.4 (0.9-2.2)	1.4 (0.8-2.2)	0.82
IL-6R *(ng/mL)*	92.7 (67.7-129.1)	97.8 (71.7-122.2)	0.71
IL-18 *(μg/mL)*	381.8 (299.6-484.9)	381.8 (308.8-461.5)	0.91

Based on chi-square for dichotomous variables and independent t test for continuous variables. Values are shown as means ± SD for continuous variable or percentage for categorical variable; Variables with a skeweddistribution are presented as value and interquartile range (IQR) and were log-transformed for the analysis.CES-D, Center for Epidemiological Studies-Depression Scale; BMI, body mass index; MMSE, Mini Mental State Examination; NSAID, nonsteroidal anti-inflammatory drugs; MI, myocardial infarction; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; ADL, activity of daily living; IADL, instrumental activity of daily living; SPPB, Short Physical Performance Battery; CRP, C-reactive protein; TNF, tumor necrosis factor; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; IL-6r, interleukin-6 receptor.

Table 2. Pearson's correlations between (log) inflammatory markers

Inflammatory markers	Il-1β	IL-1RA	IL-6	IL-6R	CRP	TNF-α	IL-18
Il-1β	1	0.094**	0.115**	0.059	0.088**	-0.036	0.106**
IL-1RA		1	0.317**	0.068*	0.359**	0.063*	0.247**
IL-6			1	0.062	0.500**	0.093**	0.211**
IL-6R				1	0.046	0.027	0.025
CRP					1	0.058	0.194**
TNF-α						1	0.098**
IL-18							1

* *p* value <.05** p value <.01
Abbreviations as in Table 1.

Figure 2. Age- and gender-adjusted levels of interleukin-1 receptor antagonist (IL-1ra) (mean ± SE) across Center for Epidemiological Studies-Depression Scale (CES-D) score subgroups.

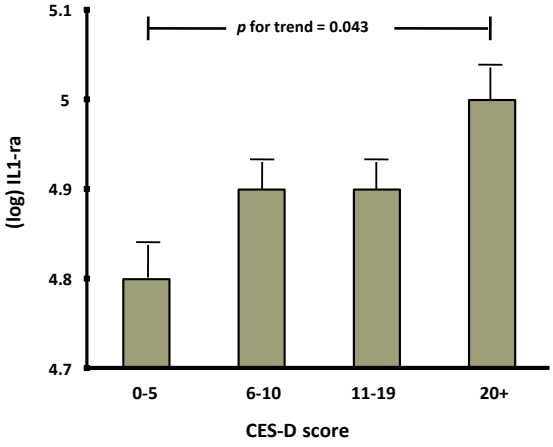


Figure 2. Age and sex adjusted levels of IL-1ra (mean SE) across CES-D score subgroups

Table 3. Adjusted regression coefficients per SD increase in (log) plasma inflammatory markers in relation to CES-D Scores.

	CES-D Scores			
	Adjusted for Sociodemographic* Standardized Reg Coefficients	*p* value	**Fully Adjusted**** Standardized Reg Coefficients	*p* value
CRP	0.041	0.17	0.014	0.65
Il-1β	0.0002	0.99	0.012	0.69
IL-1RA	0.089	0.0027	0.064	0.0352
TNF-α	-0.02	0.51	-0.025	0.40
IL-6	0.023	0.46	-0.007	0.83
IL-6R	-0.015	0.62	-0.029	0.35
IL-18	0.024	0.45	0.001	0.97

Abbreviations as in Table 1.

*Adjusted for age, gender, site, education, alcohol use, smoking status,MMSE.

**Adjusted for age, gender, site, education, alcohol use, smoking status, MMSE, BMI, number of drugs, use of antidepressant, use of NSAID, hypertension,angina/MI, CHF, stroke, cancer, diabetes, COPD, hip arthritis, ADL,IADL, SPPB, and physical activity

Subjects not-depressed at baseline (778) were selected for longitudinal analysis. Subjects who did not complete one or both the follow up had at baseline significantly higher mean levels of IL-6, CRP, TNF-α , IL-18 and IL-1ra compared to those who completed both follow-up. We calculated the RRs for the development of depressed mood during the follow-up according to baseline quartiles of inflammatory markers. The parsimonious multivariate models shown in table 4 were adjusted for potential confounders that were independently associated with the outcome with a p level of <0.1. Among the tested inflammatory markers, only levels of IL-1ra predicted the development of depressed mood over 6-years follow-up. However, this effect of IL-1ra was not detectable after the first 3 years. Among the 550 subjects who completed both follow-up interviews, 22% developed depressed mood at 3 years or 6 years follow-up. Estimated RR for persons in the third and fourth IL-1ra quartiles, compared to those in the lowest quartile, was respectively 1.7 (95%CI:1.07-2.68, $p = 0.03$) and 1.7 (95%CI:1.05-2.64, $p = 0.02$) after adjustment for age, sex, hypertension, COPD, diabetes, ADL disabilities, use of antidepressants and NSAID. Among the 597 subjects that completed the interview after six years, 14.2% developed depressed mood. Estimated RR for subjects in the third and fourth IL-1ra quartile, compared to those in the lowest quartile, were respectively 2.32 (95%CI:1.21-4.42, $p = 0.01$) and 2.78 (95%CI:1.47-5.26, $p = 0.002$), after adjustment for age, sex, COPD, diabetes, ADL disabilities, hip arthritis and use of antidepressants. We repeated the analysis also using Logistic Regression and the results were substantially the same.

Table 4. Adjusted relative risks for depressed mood Associated with quartiles of IL-1ra.

	Risk of Depressed Mood (CES-D ≥ 20) at 3 years Follow-up (n = 652)					
	Model 1[a]			**Model 2[b]**		
	R.R.	**95% C.I.**	***P***	**R.R.**	**95% C.I.**	***P***
IL-1RA						
quartile 1	1					
quartile 2	0.88	(0.45 - 1.72)	0.71	1.00	(0.51–1.96)	1.00
quartile 3	1.18	(0.63 - 2.21)	0.61	1.08	(0.58 - 2.03)	0.85
quartile 4	0.96	(0.49–1.90)	0.92	0.77	(0.38 - 1.59)	0.49

	Risk of Depressed Mood (CES-D ≥ 20) at 3 or 6 years Follow-up (n = 550)					
	Model 1[a]			**Model 2[c]**		
	R.R.	**95% C.I.**	***P***	**R.R.**	**95% C.I.**	***P***
IL-1RA						
quartile 1	1					
quartile 2	1.24	(0.76 - 2.03)	0.40	1.18	(0.73–1.91)	0.49
quartile 3	1.72	(1.08–2.74)	0.02	1.70	(1.07–2.68)	0.03
quartile 4	1.77	(1.10–2.83)	0.02	1.66	(1.05–2.64)	0.03

	Risk of Depressed Mood (CES-D ≥ 20) at 6 years Follow-up (n = 597)					
	Model 1[a]			**Model 2[d]**		
	R.R.	**95% C.I.**	***P***	**R.R.**	**95% C.I.**	***P***
IL-1RA						
quartile 1	1					
quartile 2	1.72	(0.84–3.49)	0.13	1.59	(0.78 - 3.22)	0.20
quartile 3	2.34	(1.20–4.57)	0.01	2.32	(1.21–4.42)	0.01
quartile 4	3.12	(1.63–5.95)	0.001	2.78	(1.47–5.26)	0.02

RRs were estimated using a modified Poisson regression approach.

CI, confidence interval; other abbreviations as in Table 1.

[a] Adjusted for age and sex.

[b] Adjusted for age, sex, years of education, BMI and use of NSAID.

[c] Adjusted for age, sex, hypertension, COPD, diabetes, ADL disabilities, use of antidepressants and useof NSAID

[d] Adjusted for age, sex, COPD, diabetes, ADL disabilities, hip arthritis and use of antidepressants.

We performed the same analyses using the cut-off of 16 for the CES-D and we obtained the same results: only IL-ra predicted the development of depressed mood at 3 or 6 years follow-up and at 6 years follow up.

DISCUSSION

Using data from a population-based study in older persons, we examined the relationship between plasma inflammatory markers and symptoms of depression. We found evidence of a cross-sectional and prospective independent association between IL-1ra and depressive symptoms assessed by CES-D. Several cross-sectional studies (1,2,10) found a significant association between depression and high serum IL-1ra. In an experimental study in healthy young men (13) positive correlations were found between IL-1ra levels and depressed mood in people with endotoxemia.In a previous prospective study of older individuals (22) elevated levels of IL-1ra preceded the onset of depressed mood. In our study, subjects in the two highest quartiles of IL-1ra at baseline, compared to those in the lowest quartile, had a 2.32 and 2.78-fold higher risk of developing depressed mood after 6 years.

IL-1ra, the pure antagonist of IL-1α and IL-1β, is a reliable marker of immune system activation. There is evidence that IL-1ra is an acute phase protein (33). As a member of the IL-1 gene family, IL-1ra production increases under the same inflammatory conditions that stimulate IL-1α and IL-1β. However, while these molecules are produced locally, rapidly metabolized and their serum concentrations is often below the detectable limits with standard methods, IL-1ra is produced by the liver in larger quantities and remains in the circulation for long time (34-36). Therefore, IL-1ra is considered a marker of inflammation even more reliable than IL-1. Regardless of the mechanism, our findings suggest that serum IL-1ra may capture aspects on inflammation that are most relevant to the development of depressive mood. If these findings are confirmed, IL1ra and may become some day a valuable clinical tool for risk assessment.

Interestingly, in our study baseline plasma levels of IL-1ra were not predictive of depressed mood at 3-years follow-up. This could be partly explained by the small mean increase in CES-D scores after 3 years (2.6 points). We could hypothesize that the influence of inflammation on the development of depressive symptoms is a slow process that takes several years to cross the threshold of clinical manifestation.

Previous epidemiological studies on the association between inflammation and depression have produced discrepant results (14-17). This discrepancy between studies is probably attributable to differences in the study populations, assessments of depression and measures of cytokines (clinical versus population-based samples, questionnaire versus DSM diagnosis, and choice of the inflammatory markers/technical limitations of assay). Our findings are consistent with the "cytokine hypothesis of depression" (1-6). IL-1 network molecules could communicate with the brain directly crossing the blood brain barrier (37,38) or indirectly via the afferent projections of the vagus nerve (39). This central action may account for neurochemical and neuroendocrine features of depressive disorders (40-44).

Cytokines have been found to induce serotonin depletion by lowering the availability of tryptophan through activation of TRP-metabolising enzyme (IDO) (42-44).

To date the exact mechanisms through which inflammation plays a role in the pathophisiology of depression is still unclear. Further research in this area is needed.

One limitation of this study is the loss of participants to follow-up. Those lost had high levels of inflammatory markers at baseline. Therefore, censoring of these participants probably led to and underestimation of the relationship between inflammation and depression. Another limitation is that depressive symptoms were evaluated by the CES-D questionnaire and the diagnosis of depression was not confirmed by a clinical psychiatric diagnosis. However, the CES-D is a commonly used scale to measure depressive symptoms and has been widely used in older population-based studies (25,27). Moreover DSM affective disorders are not highly prevalent among elderly persons in the community, while subsyndromal chronic depression is more common (19). Another limitation is that the study design has not allowed us to detect depressive episodes that started and remitted between subsequent follow-up visits. Furthermore, the results could have been affected by the use of antidepressants; some studies (45,46) found that antidepressant agents have negative immunoregolatory effect through stimulation of IL-10 release. However, when we adjusted the analysis for antidepressant medication use, the results did not change substantially. Finally, in our database there was no measure of cognition more selective than MMSE in order to test the confounding effect of cognitive decline on the association between inflammation and depression.

Despite this limitation, we believe that our findings suggest a potential causal role for inflammatory process in the onset of depressive symptoms in the elderly. Accumulation of diseases and cardiovascular risk factor with age or dysregulation due to immunosenescence could slowly increase the "low-grade proinflammatory state"(18), this could lead over time to the development of depression that worsen the prognosis of the patient. Modulation of the inflammatory process may become in the future a strategy to reduce depressive mood in the elderly and prevent its deleterious consequences on morbidity and mortality (47).

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Chapter 3

Depressive symptoms and inflammation increase in prospective study of older adults: a protective effect of a healthy (Mediterranean-style) diet

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ABSTRACT

Background: Both depression and poor dietary habits may contribute to a pro-inflammatory state but their joint contribution to inflammation has not been studied. We determined whether depressive symptoms were associated with a pro-inflammatory state and whether this association was modified by adherence to a healthy (Mediterranean-style) diet.

Methods: This study is part of the InCHIANTI Study, a prospective population-based study of older persons. The sample consisted of 793 participants aged 65 years and older. Depressive symptoms were assessed at baseline with the Center for Epidemiologic Studies-Depression scale. Adherence to the Mediterranean diet was assessed at baseline by a well-validated dietary questionnaire. Levels of Interleukin-6 and C-reactive protein were assessed at baseline, 3-year and 6-year follow-up.

Results: Higher depressive symptoms were associated with prospective increase in Interleukin-6 in participants non-adherent ($\beta=0.13$, $SE=0.03$, $p<.0001$), but not in those adherent ($\beta=0.04$, $SE=0.03$, $p=0.17$) to a healthy diet.

Conclusion: A healthy (Mediterranean-style) diet can buffer the detrimental effect of depression on inflammation among older adults. These findings may have important implications for health in older persons.

INTRODUCTION

Depression is very common in older persons, and both major depression and subthreshold depressive symptoms are major risk factors for cardiovascular disease, cancer and diabetes (1-4). It has been hypothesized that inflammation may be considered the shared link between these conditions (5-7). Previous studies have shown that depression and stress are associated with upregulated inflammatory response, characterized by increased levels of pro-inflammatory cytokines and other acute phase proteins (8-10). Diet may also influence inflammation: dietary patterns high in refined starches, sugar, and saturated and trans-fatty acids, poor in fruits, vegetables, and whole grains, and poor in omega-3 fatty acids may cause an activation of the innate immune system (11). A diet rich in fruit, vegetables and olive oil is instead associated with lower levels of inflammatory markers, perhaps because of the anti-inflammatory properties of antioxidants (11-13). Indeed, trials showed that nutritional interventions based on Mediterranean diet (characterized by high intake of vegetables, fruits and olive oil) significantly reduced the levels of inflammatory markers (14,15).

A previous study on a sample of older persons (16) suggested that depressive symptoms and a high blood Omega-6/Omega-3 ratio of polyunsaturated fatty acidswere synergistically associated with upregulated inflammation. The author recently (17) underlined the need of further research aimed at understanding the relationship between depression and nutrition and their interactive influence on inflammation. In fact, if the synergistic effect is confirmed it could be hypothesized that nutritional interventions may reduce the detrimental influence of depression on health.

In the present study, we examined firstly whether depressive symptoms were prospectively associated with increase in levels of inflammatory markers in a large population-based cohort of older people with 6 years of follow-up. Secondly, we examined whether this association was different in participants who were adherent, compared to those who were not adherent, to a Mediterranean-style diet. We hypothesized that the prospective association between higher depressive symptoms and inflammation would be stronger in participants non-adherent as compared to those adherent to a healthy diet.

METHODS AND MATERIALS

Study Population

Participants were part of the InCHIANTI (**In**vecchiare in **Chianti**, aging in the Chianti area) Study, a prospective population-based study of older persons in Tuscany (Italy) designed to investigate factors contributing to decline in mobility in later life. A description of the study rationale, design and method is given elsewhere (18). Briefly, in 1998-1999 the sample was randomly selected from two sites, Greve in Chianti and Bagno a Ripoli, using a multistage stratified sampling method. Data collection included: 1) a home interview concerning demographics, functional status, dietary habits, cognition and mood; 2) a medical examination including several performance-based tests of physical function conducted in the study clinic; 3) 24-h urine collection and blood drawing. Participants were

evaluated again at three-year (2001-2003) and six-year follow-up visits (2004-2006). All respondents received an extensive description of the study and signed an informed consent. The study protocol complies with the declaration of Helsinki and was approved by the Italian National Institute of Research and Care on Aging Ethical Committee.

Of the 1155 participants aged≥65 enrolled in the study, we excluded 188 because of missing data on depressive symptoms or inflammatory markers at baseline. Among the remaining 967 participants, at 3-year follow-up 716 subjects had available data for CRP and 709 had data for IL-6 (10 had missing data on both markers and another 7 had additional missing data on IL-6, 77 refused to participate in the survey, 3 were not found, 11 were emigrated and 66 were deceased). At 6-year follow-up, 642 participants had available data for CRP and 625 had data for IL-6 (4 had missing data on both markers and 17 others had additional missing data in IL-6,33 refused, 17 emigrated and 185 deceased). Overall, 172 participants had no data on both inflammatory markers at both follow-up, and another 2 had no data on IL-6 at both follow-up (total n=174, 18%). Those not assessed at both follow-ups, as compared to participants assessed at least at one follow-up, were significantly older, more often sedentary, had poorer cognitive function and higher levels of inflammatory markers, but did not differ in terms of depressed mood. After the exclusion of the 174 subjects lost to follow-up, the remaining 793 participants who had at least one follow-up measure available for each inflammatory marker were included in subsequent analyses.

Depressive symptoms

Depressive symptoms were assessed at baseline using the Center for Epidemiological Studies-Depression scale (CES-D) (19). The CES-D is a 20-item self report scale, ranging from 0 to 60. The CES-D has been shown to have good psychometrics properties in assessing depressive symptoms in older population-based studies, also in an Italian sample (20). A score ≥20 was operationally defined as clinically relevant "depressed mood". While a cut-off of 16 is generally considered to represent relevant depression, we selected a cut-off of 20 that has been shown to avoid overestimation in older subjects (21).

Inflammatory markers

Measures for the cytokines were obtained from frozen plasma samples originally collected at baseline, at 3-year and at 6-year follow-up. Morning fasting blood samples were collected after a 15-min rest. Aliquots of serum were stored at −80°C and never thawed before analysis. High sensitivity C-reactive protein (CRP) was measured in duplicate with the Dade Behring BNII nephelometer (Dade Behring Inc., Deerfield, IL, USA), utilizing a particle-enhanced immunonepholometric assay and monoclonal antibodies to CRP. The lowest detectable concentration was 0.15 μg/mL The inter-assay coefficient of variation (CVs) was between 2.1 to 5.7% for baseline, follow-up 1 and follow-up 2 assessments. At baseline, Interleukin-6 (IL-6) was measured with an ultra-sensitive ELISA (CytoScreen Human IL-6, Biosource International Inc., Camarillo, CA, USA). Minimum detectable

threshold was 0.10 pg/mL and the inter-assay CV was 7%. At 3-year and 6-year follow-up, IL-6 was measured using a solid phase high-sensitivity quantitative sandwich ELISA (Quantikine HS Human IL-6 Immunoassay, R&D Systems Inc, Minneapolis, MN, USA). Minimum detectable threshold was 0.10 pg/mL and the inter-assay CV was 7%. In order to make IL-6 measures obtained at baseline comparable with measures from both follow-ups, a pilot study based on 100randomly selected serum baseline specimens was run usinga solid-phase high-sensitivity quantitative sandwich ELISA as described for follow-ups. A regression equation (r-square= 0.79) was developed at the National Institute on Aging to predict the IL-6 sandwich ELISA results from the original ELISA results for the pilot subjects. This equation was then used to predict sandwich ELISA results for all subjects at baseline. The correlation between the estimated high-sensitivity measure of IL-6 and the original measure was high (Pearson R=0.89). The estimated high-sensitivity baseline measure of IL-6 was used in the present study.

Healthy (Mediterranean-style) diet

Daily dietary intake was assessed at baseline by the food-frequency questionnaire created for the European Prospective Investigation on Cancer and Nutrition (EPIC) study, previously validated in the InCHIANTI population (22). The Mediterranean Diet Score was computed according to the method developed by Trichopoulou et. al (23). Intake of each of 9 food groups was dichotomized using sex-specific median values as cut-offs. A score of 1 was assigned for above the median level of presumed beneficial foods (vegetables, legumes, fruits, cereal, fish and ratio of monounsaturated fats to saturated fats) and consumptions below the median level of presumed detrimental foods (meat and dairy products). For ethanol, 1 point was assigned to men who consumed between 10 and 50 g per day and to women who consumed between 5 and 25 g per day. Thus, the total Mediterranean Diet Score ranged from 0 (minimal adherence to the traditional Mediterranean diet) to 9 (maximal adherence).

Covariates

The following covariates assessed at baseline were used in the analysis: age, gender, education (years), Mini Mental State Examination (MMSE) score, and number of prescribed and non-prescribed drugs. Use of antidepressants, non-steroidal anti-inflammatory drugs (NSAID) and serum lipid-reducing agents was coded according to Anatomical Therapeutic Chemical (ATC) classification system. Total number of chronic diseases (heart failure, coronary heart disease including angina and myocardial infarction, stroke, chronic obstructive lung disease, hypertension, diabetes, cancer, dementia and hip arthritis) was calculated as a global marker of poor physical health; diseases were ascertained according to standardized, pre-established criteria and algorithms based upon those used in the Women's Health and Aging Study (24) using information on self-reported history, pharmacological treatments, medical exam data and hospital discharge records. The Short Physical Performance Battery (SPPB) (0-12, higher scores indicate better performance) was

used to assess lower extremity function using a standard protocol as described elsewhere (27).The SPPB is a strong predictor of nursing home admission, disability in self-care tasks and mobility, and death among older adults (27). Body mass index (BMI) was calculated as kg/m^2 andcategorized according to the World Health Organization definition (25): normal (BMI < 25), overweight (25-29.99) and obesity (BMI ≥ 30). Level of physical activity in the previous 12 months was classified as sedentary (completely inactive or light physical activity: ie, walking), light (light physical activity for 2 to 4 h/wk), and moderate to intense (light physical activity for more than 4 h/wk or moderate physical activity (ie, swimming etc) (26). Smoking habit was classified as current, former and non-smoker.

Statistical Analyses

Variables were reported as percentage, or means ± standard deviation (SD). Because plasma levels of inflammatory markers were non-normally distributed, values were reported as medians and interquartile range (IQR) and were log-transformed for analyses. First, age- and sex-adjusted differences in baseline characteristics according to depressed mood were analyzed using logistic regression and general linear model as appropriate. Second, the longitudinal association between depressive symptoms and inflammatory markers increase was estimated using random coefficient analyses, in which intercept and slopes were fitted as random effects. This method takes into account multiple observations per subject that are likely to be correlated. The different inflammatory marker measures were entered as dependent variables in separate analyses. The CES-D scores were entered as independent variables and in different analyses were used as a continuous score (per standard deviation increase) and categorized as depressed mood (CES-D ≥ 20) versus non-depressed. Interaction term between CES-D scores and time were entered in the same models to estimate the rate of change in inflammatory markers over time as a function of depressive symptoms. Analyses were adjusted for covariates significantly associated with depressed mood with a $p < 0.1$ in previous age- and sex-adjusted analyses. Third, to examine whether adherence to a healthy diet modified the association between depressive symptoms and inflammatory markers we entered interaction terms between CES-D score, diet and time to the fully adjusted models, including the interactions term nested within this interaction. Finally, we repeated the analyses stratified by adherence to a healthy diet.All analyses were performed using SAS (v. 9.1, SAS Institute, Inc., Cary, NC) with a statistical significance level set at $P<0.05$.

RESULTS

Participants mean (±SD) age at baseline was 73.5 (±6.4) years and 56.6% were women. At baseline, the mean CES-D score was 12.6 (±8.9) and 167 participants (21.1%) had depressed mood (CES-D≥20). Table 1 describes the baseline characteristics of the 793 participants included in the analyses for the total sample and according to depressive status. Depressed participants were older, more often women, took more medications and were more likely to use antidepressants and NSAIDs. Moreover participants with depressed

mood were more likely to be sedentary, had poorer lower body mobility and were less adherent to a Mediterranean-style diet.

Table 1. Characteristics of the study population at baseline in the total sample and according to depression status.

Characteristics	**Total sample** (n=793)	**Not Depressed** (n=626)	**Depressed mood** (n=167)	*p**
Age *(yrs)*	73.5±6.4	72.9±6.2	75.8±6.7	<.0001
Sex *(F)*	56.6	50.7	77.8	<.0001
Education *(yrs)*	5.6±3.3	5.7±3.3	5.2±3.4	0.73
Smoking				0.23
non smoker	58.4	54.3	73.7	
former smoker	27.5	30.7	15.6	
current smoker	14.1	15.0	10.8	
MMSE scores	25.6±2.9	25.8±2.9	24.9±3.1	0.36
BMI				0.29
normal	29.6	27.8	36.5	
overweight	45.4	47.4	37.7	
obesity	25.0	24.8	25.8	
Med. Diet Sscore	4.6±1.6	4.7±1.6	4.1±1.6	0.003
Physical activity				0.0001
low	15.9	11.3	32.9	
medium	78.6	82.8	62.9	
high	5.6	5.9	4.2	
SPPB scores	10.4±2.5	10.7±2.2	9.2±3.2	<.0001
N medications	2.2±2.0	1.9±1.8	3.0±2.1	<.0001
Antidepressants	4.4	2.6	11.4	0.002
NSAID	7.6	6.4	12.0	0.09
Lipid-red. agents	4.8	5.3	3.0	0.25
N chronic diseas.	1.2±0.9	1.1±0.9	1.2±1.0	0.31
IL-6 *(pg/mL)*	2.85 (1.96)	2.86 (1.93)	2.71 (2.02)	0.08
CRP*(µg/mL)*	2.50 (4.06)	2.55 (3.83)	2.32 (4.53)	0.43

Variables were reported as percentage or means±standard deviation as appropriate.
Variables with a skewed distribution (IL-6 and CRP) are presented as median and interquartile range, and were log-transformed for the analysis.
*Based on age- and- sex adjusted logistic regression or general linear model as appropriate.

Table 2 presents analyses looking at longitudinal associations between CES-D scores (per SD increase) and inflammatory markers. After adjustment for age, sex, physical activity, SPPB score, number of medications, use of antidepressants and NSAIDs, depressive symptoms were associated with higher IL-6 increase over time, but not with CRP change. A significant depression*time effect (β=0.09, SE=0.02, p<.0001) was observed, indicating

that higher depressive symptoms were associated with a steeper IL-6 increase over time. The same findings were obtained using the cut-off of 20 points on the CES-D: participants with depressed mood at baseline, as compared to those nondepressed, had a higher increase in IL-6 levels over the follow-up period (β=0.19, SE=0.05, p=0.0001).

When we tested interactions between CES-D, Mediterranean diet score and time significant interactions were found for depression*diet*time for IL-6 (p= 0.01) but no significant interaction was detected in the analysis focusing on CRP (p=0.52) levels.

Table 2. Adjusted associations between change in inflammatory markers over time and depressive symptoms.

	Model 1			**Model 2**[a]		
	β	*S.E.*	*p*	β	*S.E.*	*p*
			Interleukin-6			
Intercept	3.31	0.23	<.0001	2.90	0.32	<.0001
Depression	-0.24	0.09	0.01	-0.52	0.23	0.03
Time	0.16	0.02	<.0001	0.15	0.06	0.01
Depression*Time	0.09	0.02	<.0001	0.23	0.07	<.0001
Diet				0.27	0.16	0.09
Depression*Diet				0.07	0.05	0.18
Diet*Time				-0.0003	0.01	0.98
Depression*Diet*Time				-0.03	0.01	0.01
			C-reactive protein			
Intercept	5.33	0.71	<.0001	4.13	1.00	<.0001
Depression	-0.29	0.27	0.29	-0.61	0.73	0.4
Time	0.10	0.07	0.11	0.41	0.06	0.04
Depression*Time	0.03	0.07	0.64	0.13	0.19	0.50
Diet				0.10	0.05	0.06
Depression*Diet				0.07	0.05	0.18
Diet*Time				-0.0003	0.01	0.98
Depression*Diet*Time				-0.03	0.04	0.52

All models adjusted for age, sex, physical activity, SPPB score, number of medications, use of antidepressants and NSAIDs.

[a] Includes a depression*diet*time term plus the interactions terms nested within this interaction

Depression: CES-D scores per SD increase; SD = 8.9.

Diet: Mediterranean diet score.

Then, we dichotomized the Mediterranean diet score around the median in order to obtain the following two groups: participants non-adherent (score<5) and participants adherent (score≥5) to a healthy diet. Figure 1 shows that the unadjusted mean change in IL-6 levels after 6 years of follow-up differed significantly (p for trend=0.001) across

depression and diet groups. Mean increase in IL-6 levels was higher among the depressed non-adherent to a healthy diet than in all other groups.

Figure 1. Unadjusted mean change in IL-6 levels after 6 years of follow-up across depression status and adherence to a healthy (Mediterranean-style) diet.Depressed mood: CES-D ≥ 20. Healthy diet: Mediterranean diet score ≥ 5.

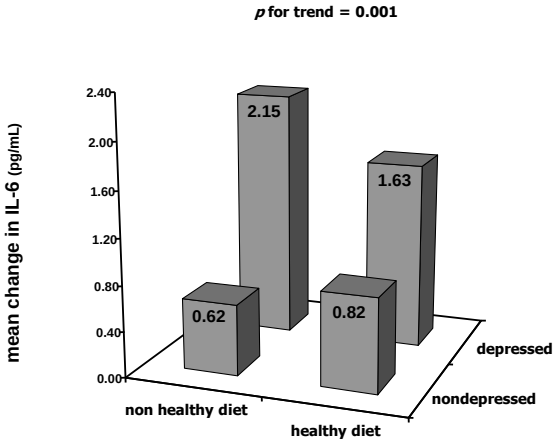

Table 3 presents the adjusted associations of CES-D scores (entered in separate analyses as continuous score per SD increase and categorized as depressed mood versus non-depressed) with IL-6 plasma level stratified by adherence to a healthy diet. Higher depressive symptoms were associated with a major increase in IL-6 levels over time in participants non-adherent to a healthy diet (β=0.13, SE=0.03,p<.0001), but not in those adherent (β=0.04, SE=0.03, p=0.17), after adjustment for age, sex, physical activity, SPPB score, number of medications, use of antidepressants and NSAIDs. Again, similar results were obtained using the cut-off of 20 points on the CES-D.

Table 3. Adjusted associations between change in IL-6 over time and depressive symptoms, stratified by adherence to a healhty (mediterranean-style) diet.

	Interleukin-6					
	NO Healthy diet			Healthy diet		
	β	*S.E.*	*p*	β	*S.E.*	*p*
Intercept	3.57	0.33	<.0001	3.12	0.34	<.0001
Dep symptoms	-0.27	0.12	0.02	-0.16	0.13	0.22
Time	0.14	0.03	<.0001	0.16	0.03	<.0001
Dep symptoms*Time	0.13	0.03	<.0001	0.04	0.03	0.17
Intercept	3.69	0.33	<.0001	3.23	0.34	<.0001
[b]Dep mood	-0.67	0.28	0.02	-0.51	0.30	0.09
Time	0.09	0.04	0.01	0.13	0.03	<.0001
[b]Dep mood *Time	0.27	0.07	0.0002	0.11	0.07	0.13

Adjusted for age, sex, physical activity, SPPB score, number medications, use of antidepressants and NSAIDs

Healthy diet: Mediterranean diet score ≥ 5

[a] CES-D scores per SD increase; SD = 8.9

[b] CES-D ≥ 20

DISCUSSION

In this large community-based population of older persons we found evidence of an association between high depressive symptoms and increase of IL-6 levels over six years of follow-up. This association was not found for CRP levels. Previous studies have shown that depression and stress are associated with higher inflammation. The largest meta-analysis (8) to date of the relationship between depression and prominent inflammatory markers, based on the cross-sectional data from all the relevant studies, confirmed that CRP, IL-6 and Interleukin-1 are positively associated with depression. This association was found in both clinic- and community-based samples, suggesting a dose-response relationship between depression and this inflammatory marker. Few prospective studies are currently available. A 6-year longitudinal study (9) examined the association between chronic stress and IL-6 in 119 participants who were caregivers for a spouse with dementia and 106 noncaregivers. The average annual rate of increase in serum IL-6 wasabout four times as large in caregivers compared to non-caregivers. Another study (10) examined the longitudinal associations between depression and inflammatory markers in a sample of perimenopausal women. In multivariate analyses adjusted for relevant confounders, depressive symptoms assessed by CES-D were associated over time with fibrinogen, but not with CRP. Previous cross-sectional studies on the association between IL-6 and/or CRP and depression have generated mixed results: some found positive associations while other did not (28-33). This discrepancy between these studies is probably attributable to differences in the choice of study populations, assessments of depression, assays of inflammatory markers and in failure to control for important confounding factors. The lack of association

in the present study between depressive symptoms and CRP deserves further comments. CRP is an acute phase protein whose production is upregulated by IL-6 (34,35). Although mostly upregulated by IL-6, the synthesis of CRP is strongly influenced by mechanisms of post-transcriptional regulation that are independent of IL-6 (36). Therefore, CRP may be less specific for the low-grade inflammatory process associated with depression. However, the reasons for the lack of association in the present study remain unknown, and further research is needed.

We observed that adherence to a healthy diet, such as a Mediterranean-style diet, modified this association between depressive symptoms and IL-6 increase: in participants non-adherent to a Mediterranen-style diet, as compared to those adherent, higher depressive symptoms were associated with a steeper increase of IL-6 levels over time.

A previous study on a sample of 43 older persons (16) examined the interactive contribution of depressive symptoms and the blood Omega-6/Omega-3 ratio of polyunsaturated fatty acids in enhancing inflammation. Although predicted cytokine levels were fairly consistent across Omega-6/Omega-3 ratios with low depressive symptoms, higher Omega-6/Omega-3 ratios were associated with progressively elevated TNF-α and IL-6 levels as depressive symptoms increased.

In the present study, we used data from a well established food frequency questionnaire to measure adherence to a Mediterranean-style diet (22,23), which is widely considered a model of healthy eating. Epidemiological studies conducted in different countries have shown that adherence to a Mediterranean type diet is associated with longer survival, lower risk of cognitive decline, chronic degenerative disease, depression, and reduced cardiovascular and cancer mortality (37). Moreover, recent trials showed that nutritional interventions based on Mediterranean diet significantly reduced the levels of CRP and IL-6 in participants with cardiovascular risk factors (14,15).

The fact that inflammatory response is influenced by both mood and diet suggests the hypothesis of a shared biological pathway. Transcription factor nuclear factor kappa B (NF-kB) activation upregulates proinflammatory cytokines and has been linked to psychosocial stress, sickness behavior and depression (35,38). Psychosocial stress has been shown to promote NF-kB, providing a mechanism for translating psychological distress into mononuclear cell activation (35,38,39). Antioxidants contained in fruit and vegetables, such as carotenoids, by reducing free radical concentration may modulate redox balance and activation of NF-kB (12). Another shared pathway could be represented by central adiposity. It has been shown that depressive symptoms, as well as unhealthy diet, may facilitate visceral fat accumulation, which in turn promotes inflammation directly or through other mechanisms such as hypothalamic-pituitary-adrenal axis dysregulation(40-43). Finally, depression and diet likely have a bidirectional complex relationship. Depression and stress may promote unhealthy dietary preference (44,45). Diet in turn may influence mood: in a recent prospective study (46), adherence to a Mediterranean-style diet was associated with a lower risk of incident depression after a median follow-up of 4.4 years.

A limitation of the present study is that depressive symptoms were evaluated by the CES-D questionnaire and the diagnosis of depression was not confirmed by a clinical psychiatric diagnosis. However, the CES-D is a commonly used scale to measure depressive symptoms and has been widely used in older population-based studies (21). Moreover DSM affective disorders are not highly prevalent among elderly persons in the community, while subsyndromal chronic depression is more common (47,48). Another limitation is the loss of participants to follow-up. Participants lost to follow-up were significantly older, more disabled and had poorer cognitive function as compared to those available for longitudinal analysis; this could limit the generalization of the findings. Furthermore, the results could have been affected by the use of medications that may potentially alter the inflammatory response, such as antidepressants (49,50); however all longitudinal analyses were adjusted for antidepressant and NSAID medication use.

Despite these limitations, we believe that the present study indicate that a healthy (Mediterranean-style) diet can buffer the detrimental effect of depression on inflammation among older adults. More recently it has been hypothesized that the joint contribution of psychological and nutritional factors to inflammatory process is more than simply additive (17). We believe that evidence provided by the current study sustains the latter hypothesis and suggest that intervention aimed at improving the quality of diet may be especially effective in improving health in older persons.

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Chapter 4

The relationship between plasma carotenoids and depressive symptoms in older persons

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ABSTRACT

Objective: We examined the cross-sectional and longitudinal relationship between plasma carotenoids and depressive symptoms over a six-year follow-up in older persons.

Methods and Materials: This research is part of the InCHIANTI Study, a prospective population-based study of older persons in Tuscany, Italy. The sample for this analysis included 958 women and men aged 65 years and older. Plasma total carotenoids were assessed at baseline. Depressive symptoms were assessed at baseline and at the 3- and 6-year follow-up using the Center for Epidemiological Studies-Depression Scale (CES-D). Depressed mood was defined as CES-D≥20.

Results: At baseline, higher total carotenoids level were associated with lower probability of depressed mood (OR=0.82, 95%CI=0.68-0.99, p=0.04) after adjustment for sociodemographic, health and inflammation. After the exclusion of participants with baseline depressed mood and use of antidepressants, higher total carotenoids level were associated with lower risk of incident depressed mood (OR=0.72, 95%CI=0.52-0.99, p=0.04) at 6-year follow-up, after adjustment for confounders plus baseline CES-D. Inflammatory marker Interleukin-1 receptor antagonist partially mediated this association.

Discussion: Low plasma concentrations of carotenoids are associated with depressive symptoms and predict the development of new depressive symptoms in older persons. Understanding the mechanism of this association may reveal potential targets for prevention and treatment.

INTRODUCTION

Depression is a major public health problem causing high disease burden for both communities and individuals worldwide. Chronic depressive syndromes are very common in older persons, especially in those affected by chronic medical illness, and strongly affect the risk of developing disability

and death (1, 2). According to the World Health Organization, depression is among the leading disorders causing disability and will be the second most important cause of disability worldwide in 2020 (3). Previous studies have shown that depression and stress are associated with upregulated inflammatory response, characterized by increased levels of pro-inflammatory cytokines and other acute phase proteins (4, 5). Diet may also influence inflammation: a diet rich in fruit and vegetables is associated with lower levels of inflammatory markers, perhaps because of the anti-inflammatory properties of antioxidants (6-8). Few studies with mixed results investigated the associationsof nutrient intake and biomarkers, such as folate, vitamin B_{12}, vitamin D, and polyunsaturated fatty acids (PUFA) with depressive symptoms and depression diagnoses in older persons (9-20). However, whether carotenoid levels are associated with depression in older persons has not yet been studied. According to the Food and Nutrition Board of the Institute of Medicine, blood concentrations of carotenoids are the best biological markers for consumption of fruits and vegetables (21).

In the present study we examined for the first time the cross-sectional and longitudinal relationship between plasma carotenoids and depressive symptoms over a six-year follow-up in a representative sample of older persons. We tested the hypothesis that participants with lowcarotenoid levelswould be significantly morelikely to have higher depressive symptoms and to develop clinically relevant depressed mood over time. Finally, we tested whether inflammatorymarkers mediated this relationship.

METHODS AND MATERIALS

Study Population

Participants were part of the InCHIANTI (**In**vecchiare in **Chianti**, aging in the Chianti area) study, a prospective population-based study of older persons in Tuscany (Italy) designed to investigate factors contributing to decline of mobility in late life. A description of the study rationale, design and methods is given elsewhere (22). Briefly, in 1998-1999 the sample was randomly selected from two sites, Greve in Chianti and Bagno a Ripoli, using a multistage stratified sampling method: 1270 persons ≥65 years were randomly selected from the population registry of the two sites, another 29 subjects ≥90 years were oversampled. Thirty-nine participants were not eligible because they had already died or emigrated. Among those who were eligible, 1155 (91.6%) were enrolled. Data collection included: 1) a home interview 2) physical performance testing and medical examination at the study clinic; 3) a 24-h urine collection and a blood drawing. Participants were seen again for a three-year follow-up visit (2001-2003) and six-year follow-up visit (2004-2006).

All respondents signed informed consent and the Italian National Institute of Research and Care on Aging Ethical Committee approved the study protocol.

Of the 1155 participants aged≥65 enrolled in the study, we excluded 175 because of missing data on plasma carotenoids or depressive symptoms at baseline. Subjects who did not participate in the blood drawing were generally older and had more comorbidity than those participating, as reported elsewhere (23). Moreover, we additionally excluded 22 participants with dementia at baseline. In cross-sectional analyses we included all 958 remaining participants. In longitudinal analyses, consistent with a previous study based on the InCHIANTI cohort (24), we considered participants with available data on depressive symptoms at 3- and 6-year of follow-up. At 3-year follow-up, 62 participants had already died, 90 were lost (77 refused, 10 emigrated, 3 not found) and 41 had missing depressive symptoms scores. This left 765 participants with available depression measures at the 3-year follow-up. At 6-year follow-up, 180 had died, 52 were lost (35 refused, 17 emigrated) and 65 had missing depressive symptoms scores. This left 661 participants for whom 6-year follow-up data on depression were available. As compared to participants with at least one available follow-up measure of depression, those lost at both follow-up were significantly older, more often sedentary and disabled, had poorer cognitive function, lower concentrations of plasma total carotenoids and higher depressive symptoms at baseline.

Carotenoids

The six major dietary carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene) comprise an important component of the antioxidant defense system in humans, and are considered a good indicator of fruit and vegetable intake (21). Measures for level of carotenoids were obtained from frozen plasma samples originally collected at baseline. Blood samples were collected in the morning after a 12-hour fast. Aliquots of serum and plasma were immediately obtained and stored at -80°C. Aliquots of plasma were shipped on dry ice to Dr. Semba's laboratory for measurements of plasma carotenoids. Carotenoids were measured using high-performance liquid chromatography (HPLC). Total carotenoids were calculated as the sum of *α*-carotene, *β*-carotene, *β*-cryptoxanthin, lutein, zeaxanthin, and lycopene in micromoles per liter (μmol/L). Within-run and between- run coefficients of variation, respectively, were 7.3% and 9.6% for *α*-carotene, 4.5% and 5.4% for *β*-carotene, 2.7% and 3.5% for *β*-cryptoxanthin, 2.6% and 7.1% for lutein, 6.2% and 6.8% for zeaxanthin, and 7.5% and 7.8% for lycopene.

Depressive symptoms

Depressive symptoms were assessed at baseline, at 3- and 6-year follow-up using the Center for Epidemiological Studies-Depression scale (CES-D) (25). The CES-D is a 20-item self report scale, ranging from 0 to 60. The CES-D has been shown to have good psychometric properties in assessing depressive symptoms in older population-based studies, also in an Italian sample (Fava 1983). In addition to a continuous CES-D score, a cut-off score ≥20 was used to additionally define clinically relevant "depressed mood".

While a cut-off of 16 is generally considered to represent relevant depression, we selected a cut-off of 20 that has been shown to avoid overestimation of depressed mood in older subjects (26).

Other variables

The following covariates assessed at baseline were used in the analysis: age, gender, education (years), smoking habit (current/former versus non smoker), alcohol use (< 30 vs≥ 30 g per day) and Mini Mental State Examination (MMSE) score. Use of antidepressants was coded according to Anatomical Therapeutic Chemical (ATC) classification system. Total number of chronic diseases (heart failure, coronary heart disease including angina and myocardial infarction, stroke, chronic obstructive lung disease, hypertension, diabetes, cancer, Parkinson's disease and hip arthritis) was calculated as a global marker of poor physical health; diseases were ascertained according to standardized, pre-established criteria and algorithms based upon those used in the Women's Health and Aging Study (27) using information on self-reported history, pharmacological treatments, medical exam data and hospital discharge records. Presence of activities of daily livings (ADL) disabilities was defined as self-report of inability or needing personal help in performing any basic activities of daily living (28). Body mass index (BMI) was calculated as kg/m^2 and categorized according to the World Health Organization definition (WHO Global Database on Body Mass Index): normal (BMI < 25), overweight (25-29.99) and obesity (BMI ≥ 30). Level of physical activity in the previous 12 months was classified as sedentary (completely inactive or light physical activity: ie, walking), light (light physical activity for 2 to 4 h/wk), and moderate to intense (light physical activity for more than 4 h/wk or moderate physical activity (ie, swimming etc) (29). Daily dietary energy intake was assessed by the food-frequency questionnaire created for the European Prospective Investigation on Cancer and Nutrition (EPIC) study, previously validated in the InCHIANTI population (30).

Finally, measures of serum inflammatory markers were considered as potential mediators. Previously from the InCHIANTI Study, it has been reported (24) that participants with high interleukin-1 receptor antagonist (IL-1ra) had a higher risk of developing depressive symptoms over time. Serum levels of interleukin 6 (IL-6) and IL-1ra were measured by enzyme linked immuno-absorbent assays (ELISA) (kits from BIOSOURCE International, Camarillo, California). Serum C-reactive protein (CRP) was measured in duplicate using the Dade Behring BNII nephelometer (Dade Behring Inc., Deerfield, IL, USA), utilizing a particle-enhanced immuno-nepholometric assay and monoclonal antibodies to CRP. The lowest detectable concentration was 0.1 pg/ml for IL-6, 4 pg/ml for IL1ra, and 0.03 mg/L for CRP. The inter-assay coefficient of variation was 4.5% for IL-1ra, 5% for CRP and 7% for IL-6.

Statistical Analyses

Variables were reported as percentage, or means ± standard deviation (SD) for categorical and continuous variables as appropriate. Continuous variables with a skewed

distribution are shown as median and interquartile range (IQR) and log-transformed values were used in the analyses. Partial correlations between baseline characteristics and total carotenoids were examined using Pearson and Spearman coefficients controlling for age and sex. Multivariate linear regression models were used to analyze the association between total carotenoids (per SD increase) and CES-D score at baseline. Multivariate logistic regressions were used to compare the odds of prevalent depressed mood at baseline per SD increase in total plasma carotenoids and across carotenoid quartiles. Then, we excluded participants with depressed mood and/or use of antidepressants at baseline and logistic regression was used to test whether plasma carotenoids predicted incident depressed mood over the follow-up period. Consistent with our previous study on inflammatory markers and depression (Milaneschi et al. 2009), separate analyses were performed for incident depressed mood at 3- and at 6-year follow-ups, because the mean increase of CES-D score was small after 3 years, while the 6-year interval allowed for characterization a greater mean increase in depressive symptoms. This could influence the findings in terms of risk of developing incident depressed mood at each time point. Finally, to address reverse causation, analyses were repeated to examine the association between plasma carotenoids and 6-year follow-up incident depressed mood after the additional exclusion of participants who became depressed after 3 years. Ancillary analyses were also performed to study the association between single carotenoid compounds and depression. All multivariate analyses were adjusted for age, sex, and for covariates that showed a significant correlation with total carotenoids. All cross-sectional analyses were also adjusted for use of antidepressants. All longitudinal analyses were additionally adjusted for baseline CES-D score in order to correct for "regression to the mean". Finally, we tested whether inflammatorymarkers could be considered mediators in the relationship between carotenoid concentrations and incident depressed mood at 6-year follow-up. We applied meditational analyses that use bootstrapping techniques, a nonparametric resampling procedure (31). Cases were randomly selected, with replacement, from the original sample of N. For each bootstrap sample, the model was estimated and the parameter estimates saved andtheir distribution examined. The indirect effect was deemed significant if the confidence interval around that effect did not include zero. We set the number of bootstrap samples to 1000. We utilized the SPSS macro developed by Preacher and Hayes (31) which allows to estimate models with binary outcome. All other analyses were performed using SAS (v. 9.1, SAS Institute, Inc., Cary, NC) with a statistical significance level set at $P < 0.05$.

RESULTS

Baseline characteristics and their correlation with total plasma carotenoids are shown in Table 1. The mean (±SD) age of the study sample was 74.3 (±6.8) years and 55.7% were women. The mean plasma level of total carotenoids was 1.8 (±0.7) μmol/L. At baseline, higher plasma concentrations of total carotenoids were associated with female gender, being non-disabled, higher level of physical activity, lower number of chronic diseases, lower BMI and lower serum inflammatory markers. The adjusted Pearson's correlation

coefficient between total carotenoids and depressive symptoms assessed with CES-D was -0.09 (p<.01).

Table 1. Characteristics of the study population at baseline and partial correlations with total carotenoids.

Characteristics	**Total sample** (n=958)	**Correlation with Total carotenoids**
Age (yrs)	74.3±6.8	- 0.05
Sex *(F)*	55.7	0.15**
Education *(yrs)*	5.5±3.3	0.01
Alcohol *(≥3 drink/d)*	15.3	- 0.04
Smoking habit		- 0.06
non smoker	58.5	
former smoker	27.4	
current smoker	14.0	
MMSE scores	25.4±3.1	0.03
BMI		- 0.08*
normal	28.1	
overweight	46.4	
obesity	25.5	
Physical activity		0.14**
low	18.6	
medium	76.0	
high	5.4	
CES-D score	12.8±8.8	- 0.09**
Antidepressants use	4.4	0.02
Vit supplementation	3.3	0.05
Total carotenoids *(µmol/L)*	1.8±0.7	1
ADL disabilities	4.7	- 0.09**
No. of chronic diseases	1.2±1.0	- 0.09**
Energy intake *(Kcal/day)*	1931.5±564.8	0.06
CRP *(µg/mL)*	2.7 (4.3)	- 0.11**
IL-6 *(pg/mL)*	1.4 (1.3)	- 0.13**
IL-1ra *(pg/mL)*	132.0 (87.4)	- 0.10**

Values are shown as means ± SD for continuous variable or percentage for categorical variable; continuous variables with a skewed distribution are shown as median (IQR) and were log-transformed for the analysis.

Partial Correlations based on Pearson or Spearman coefficient as appropriate and adjusted for age and sex.

MMSE, Mini Mental State Examination; BMI, Body Mass Index; CES-D, Center for Epidemiological Studies-Depression Scale; ADL, Activities of Daily Living; CRP, C-Reactive Protein; IL-6, Interleukin 6; IL-1ra, Interleukin 1 receptor antagonist.

* p< .05

** p< .01

Table 2 shows the relations between total carotenoids and other covariates with depressive symptoms at baseline. Higher plasma level of total carotenoids (per SD increase) was significantly associated with lower CES-D score after adjustment for age, sex and antidepressants use (β = -0.76, SE= 0.26, p = 0.004). Additional adjustment for BMI, physical activity, number of chronic diseases and disabilityreduced by 24% the strength of the association, which nevertheless remained significant (β = -0.57, SE= 0.27, p = 0.03). Analyses were additionally adjusted for inflammatory markers. Collinearity between inflammatory markers was examinedusing correlation and variance inflation factors in regression models. Correlation coefficient were low (from 0.32 to 0.50) and all varianceinflation factors were below 10, indicating non-significant multicollinearity. Additional simultaneous adjustment for all inflammatory markers marginally reduced the association between carotenoids and depressive symptoms (β = -0.55, SE= 0.27, p = 0.047). When we considered the single compounds separately, only higher plasma levels (per SD increase) of lycopene (β = -0.84, SE= 0.27, p = 0.002) and β-cryptoxanthin(β = -0.52, SE= 0.27, p = 0.05) were significantly associated with lower baseline CES-D score after full adjustment for confounders. Non-significant negative associations were also found for lutein and zeaxanthin.

Figure 1 shows the percentages of participants with prevalent depressed mood at baseline and incident depressed mood over follow-up across quartiles of carotenoids.At baseline, 21% of participants had depressed mood. Table 3 reports the odds ratios (ORs) for prevalent depressed mood at baseline and incident depressed mood at 3- and 6-year follow-up per SD increase in total plasma carotenoids and across carotenoids quartiles. Higher total plasma carotenoids (per SD increase) concentrations were associated with a lower probability (OR=0.82, 95%CI=0.68-0.99, p=0.04) of depressed mood at baseline after adjustment for age, sex, antidepressants use, BMI, physical activity, number of chronic diseases, disability and inflammatory markers. Estimated ORs for participants in the lowestcarotenoid quartile, compared to those in the highest quartile, were 1.72 (95%CI:1.05-2.83, p=0.03) after full adjustment for confounders.

For the incidence analyses, we excluded 220 participantswith depressed mood and use of antidepressants at baseline. Incident depressed mood was developed by 17.1% of the participants available at 3-year follow-up and by 14.8% of those available at 6-year follow-up. In multivariate analyses, plasma levels of total carotenoids significantly predicted incident depressed mood status after 6 years. However, this relationship between carotenoids and depressive symptoms was not detectable after the first 3 years. At 6-year follow-up, higher total carotenoid concentrations (per SD increase) were associated with a lower risk (OR=0.72, 95%CI=0.52-0.99, p=0.04) of depressed mood after adjustment for age, sex, baseline CES-D, BMI, physical activity, number of chronic diseases, disability and inflammatory markers.Participants in the lowest quartile of plasma carotenoids, as compared to those in the highest quartile, had a higher risk of developing depressed mood (OR=2.63, 95%CI=1.16-6.00, p=0.02) after 6 years. To test whether this association was consistent across gender we included a "carotenoid-by-sex" interaction term in the previous

logistic regression models. The interaction term was not statistically significant (all p>0.15) suggesting that the nature of association between serum carotenoids and depression is substantially similar in the two sexes. For all individual carotenoids, higher plasma concentrations (per SD increase) of all compounds were associated with lower probability of incident depressed mood, although these associations were non-significant after full adjustment. To verify that the longitudinal findings were not biased by reverse causation (that is, participants who were subclinically depressed at baseline and could have had lower levels of carotenoids as a consequence of depression), the 6-year follow-up analyses were repeated after the additional exclusion of 79 participants, among those available after 6 years, who became depressed after 3 years. Again,risk of incident depressed mood decreased by 37% for each SD increase in plasma total carotenoids concentrations (full adjusted OR =0.63, 95%CI=0.43-0.94, p=0.02).

Finally, we tested the role of inflammatory markers as potential mediators of the relationship between blood total carotenoid concentrations and incident depressed mood at 6-year follow-up. Among the inflammatory markers, bootstrapping analyses identified IL-1ra as a significant mediators between carotenoids levels and subsequent depressed mood (point estimate = -0.04, bias corrected 95%CI=-0.09 - -0.01). The findings were essentially unchanged when age, sex and baseline depressive symptoms were added to the model (IL-1ra point estimate = -0.04, bias corrected 95%CI=-0.1 - -0.004).

DISCUSSION

Using data from a population-based study in older persons, we found evidence of a strong cross-sectional and prospective independent association between plasma carotenoid concentrations and depressive symptoms. Participants with lower total carotenoids level had higher depressive symptoms at baseline and were more likely to develop incident depressed mood after six years of follow up. To our knowledge, this is the first study examining this relationship. Previous studies examining the relationship of nutrient intake and biomarkers with depression in older persons obtained mixed results. Lower levels of folate and vitamin B_{12} have been shown to be associated with depression in two cross sectional-studies of Chinese (9) and Greek (10) older adults, and to be a risk for incident depression over a period of 2–3 years in a prospective studies of Korean older persons (11). Moreover, in a sample of older disabled women serum levels of vitamin B_{12}were associated with higher risk of severe depression (12). High total intakes of vitamins B_6 and B_{12} have been recently shown to be protective of depressive symptoms over time in community-dwelling older adults (13). However, in a sample of healthy elderly men in the Netherlands, intake of folate and vitamins B_6 and B_{12} were not related to depressive symptoms(14).Decreased serum vitamin D levels have been shown to be associated with depression status in the Longitudinal Aging Study Amsterdam (15) and with higher risk of developing depressed mood over time in the InCHIANTI Study (16). Depressive symptomatology in older persons has also been related to plasma PUFAs concentrations and fatty acid composition (17-19).

Table 2. Relationship between total carotenoids and other covariates with baseline depressive symptoms.

	Baseline CES-D scores								
	Model 1			**Model 2**			**Model 3**		
	β	*S.E.*	*p*	β	*S.E.*	*p*	β	*S.E.*	*p*
Tot Carot *	- 0.75	0.26	0.005	- 0.57	0.27	0.03	- 0.55	0.27	0.047
Age	0.21	0.04	<.0001	0.15	0.04	0.0003	0.16	0.04	0.0002
Sex	5.46	0.53	<.0001	5.14	0.55	<.0001	5.13	0.60	<.0001
Antidep use	0.21	0.06	<.0001	0.17	0.07	0.002	3.90	0.07	0.004
N chronic dis				0.39	0.29	0.18	0.29	0.30	0.32
Physical activ				- 2.26	0.63	0.0004	- 2.27	0.62	0.0004
ADL				3.17	1.52	0.04	3.05	1.52	0.05
BMI				- 0.26	0.37	0.48	- 0.43	0.38	0.26
(log) CRP							-0.41	0.38	0.27
(log) IL-6							0.04	0.30	0.89
(log) IL-1ra							1.41	0.50	0.005

* Per SD increase; Total Carotenoids SD = 0.68 µmol/L.

CES-D, Center for Epidemiological Studies-Depression Scale; SE, Standard Error; MMSE, Mini Mental State Examination; BMI, Body Mass Index; ADL, Activities of Daily Living; CRP, C-Reactive Protein; IL-6, Interleukin 6; IL-1ra, Interleukin 1 receptor antagonist.

Table 3. Relationship between total carotenoids with prevalent depressed mood at baseline and with incident depressed mood at 3- and 6-year follow-up.

	Baseline Prevalent Depressed Mood (201/958)								
	Model 1 [a]			**Model 2 [a]**			**Model 3 [a]**		
	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*
Tot Caroten*	0.78	(0.65 - 0.92)	0.004	0.82	(0.68 - 0.99)	0.03	0.82	(0.68 - 0.99)	0.04
Quartile 4	Ref			Ref			Ref		
Quartile 3	1.27	(0.78 - 2.06)	0.35	1.20	(0.73 - 1.99)	0.47	1.27	(0.77 - 2.12)	0.35
Quartile 2	1.71	(1.06 - 2.77)	0.03	1.58	(0.96 - 2.61)	0.07	1.58	(0.95 - 2.63)	0.08
Quartile 1	2.14	(1.33 - 3.45)	0.002	1.84	(1.11 - 3.07)	0.02	1.87	(1.11 - 3.14)	0.02

	3-year Incident Depressed Mood (101/591)								
	Model 1 [b]			**Model 2 [b]**			**Model 3 [b]**		
	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*
Tot Caroten*	0.97	(0.76 - 1.24)	0.81	1.01	(0.78 - 1.30)	0.94	1.02	(0.79 - 1.32)	0.88
Quartile 4	Ref			Ref			Ref		
Quartile 3	1.18	(0.64 - 2.18	0.59	1.14	(0.61 - 2.12)	0.67	1.12	(0.60 - 2.10)	0.73
Quartile 2	0.90	(0.48 - 1.70)	0.75	0.81	(0.42 - 1.55)	0.52	0.81	(0.42 - 1.55)	0.52
Quartile 1	0.97	(0.49 - 1.89)	0.92	0.90	(0.45 - 1.82)	0.77	0.86	(0.42 - 1.75)	0.68

Continued on next page

	6-year Incident Depressed Mood (78/528)								
	Model 1 [b]			**Model 2 [b]**			**Model 3 [b]**		
	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*	O.R.	95% C.I.	*P*
Tot Caroten*	0.62	(0.45 - 0.84)	0.002	0.71	(0.52 - 0.98)	0.04	0.72	(0.52 - 0.99)	0.04
Quartile 4	Ref			Ref			Ref		
Quartile 3	1.61	(0.75 - 3.43)	0.22	1.42	(0.64 - 3.13)	0.39	1.44	(0.65 - 3.22)	0.37
Quartile 2	1.73	(0.79 - 3.77)	0.17	1.42	(0.63 - 3.20)	0.39	1.50	(0.66 - 3.45)	0.33
Quartile 1	3.77	(1.77 - 8.03)	0.001	2.70	(1.20 - 6.04)	0.02	2.63	(1.16 - 6.00)	0.02

Model 1: Adjustment for age and sex.

Model 2: Model 1 + additional adjustment for BMI, physical activity, number of chronic diseases and disability.

Model 3: Model 2 + additional adjustment for (log)CRP, (log)IL-6 and (log)IL-1ra.

[a] Additionally adjusted for antidepressants use.

[b]Additionally adjusted for baseline CES-D

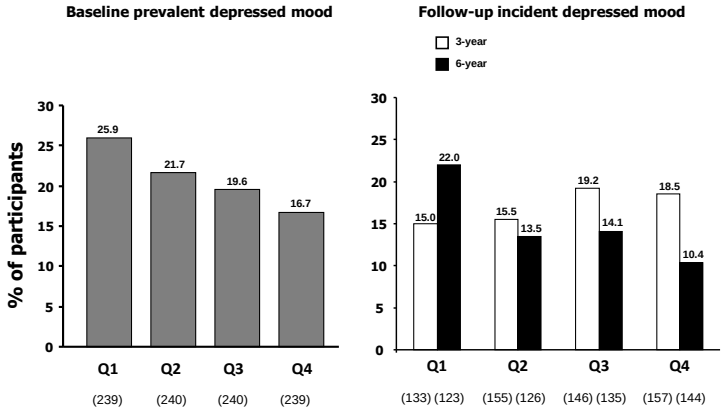
* Per SD increase; Total Carotenoids SD = 0.68 µmol/L.

O.R. Odds Ratio; C.I., Confidence Interval.

Depressed Mood: CES-D ≥20.

However, a recent randomized controlled trials on 302 Dutch elderly showed no significant effect of eicosapentaenoic acid and docosahexaenoic acid on depressive symptoms (20). Further studies are required of sufficient methodological quality, duration, and sample size to confirm these findings.

Figure 1. Proportions of participants with prevalent depressed mood at baseline and incident depressed mood at 3- and 6-year follow-ups across quartiles of total carotenoid concentrations.



Interestingly, in our study baseline plasma levels of carotenoids were not predictive of depressed mood at 3-years follow-up. We could hypothesize that the influence of carotenoids levels on the development of depressive symptoms is a slow process that takes several years. As shown in Figure 1, after the exclusion of participants already depressed at enrolment, proportions of participants with incident depressed mood across carotenoid quartiles after 3 years did not follow the same clear trend as for prevalent depressed mood at baseline. The same trend (proportions of participants with depressed mood decreased from the lowest quartile to the highest) was instead detected again after 6 years. However, the reasons for the lack of association after 3 years remain unknown, and further research is needed. Moreover, although all the single carotenoids compounds were associated with a lower risk of incident depressed mood over time, effects appeared especially strong and significant only for the combined indicator of total carotenoids. Several explanations forthis finding are possible. First of all, individual compounds may have small effects that emerge only when they are integrated. Moreover, there may be biologic interactions between the

different compounds that may be difficult to detect unless very largesamples are used. Indeed, previous study on the impact of plasma carotenoids on the health of older adults (32) commonly considered the measure of total plasma carotenoids.

In the current study, we found evidence that inflammatory markers, in particular blood levels of IL-1ra, partially mediated the relationship between carotenoid concentrations and development of depressed mood after 6 years. In our previous study (24), we found that older persons with high plasma levels of IL-1ra had a higher risk of developing relevant depressive symptoms over time. Moreover, the association between IL-1ra and depression has been confirmed also in a recent meta-analysis (4). IL-1ra, which is considered an acute phase protein (33),is a reliable marker of IL-1 signaling network activation. IL-1ra production increases under the same inflammatory conditions that stimulate IL-1α and IL-1β,but while these molecules are produced locally, rapidly metabolized and their serum concentrations is often below the detectable, IL-1ra is produced by the liver in larger quantities and remains in the circulation for long time (34-36).The fact that inflammation mediated the relationship between plasma carotenoids and depression suggests the hypothesis of a shared biological pathway. Antioxidants, by reducing free radical concentrations, may modulate redox balance and activation of transcription nuclear factor κB (NF-kB) (7), a major transcriptional factor involved in the expression of proinflammatory cytokines, which have been shown to be associated with psychosocial stress, sickness behavior and depression (37, 38). In animal model of depression, it has been shown that NF-kB is the key mediator linking stress-induced increases in IL-1β with impaired hippocampal neurogenesis and depressive-like behaviors (39).

Different biological and behavioral pathways shouldalso be considered through which level of carotenoids may potentially influence depressive symptoms. Carotenoids, as antioxidants, play an important role in counterbalancing the age-dependent increase in oxidative stress. As people age, the central nervous system may became more vulnerable to the effect of free radicals in terms of damage and mutation of proteins, lipids and mitochondrial DNA, which in turn leads to impaired mitochondrial function and further generation of free radicals, with increased lipid peroxidation and impaired oxidative DNA repair in the nucleus (40, 41). It has hypothesized that the inability to buffer the effects of this oxidative stress may be responsible for age-relatedneuronal decrements and neurodegenerative disease (42, 43). Brain imaging studies have shown that depression is associated with structural and functional alterations of limbic and cortical structures, particularly in the hippocampus (44, 45). Antioxidants may play a preventive role in neurondamage by reducing oxidative injury through the quenching of hydroxyl radicals and reduction in lipid peroxidation (46). Furthermore, a lower level of carotenoids, considered a good index of fruit and vegetable intake, could reflect an unhealthy dietary pattern associated with overweight and obesity, which have been shown to increase the risk of developing depression through inflammation or hypothalamic-pituitary-adrenal axis dysregulation (47-49). Finally, an unhealthy dietary pattern may be associated with other lifestyle factors, such as lower level of physical activity, alcohol consumption and smoking

habit, considered risk factor for depression (50, 51). While the causal pathway from carotenoids blood levels and changes in mood has not been elucidated, it is interesting to note that the same micronutrients associated with depression have also been associated with some of the phenotypes that characterize age-related frailty, such as sarcopenia and mobility disability (7, 32, 52-56).

A limitation of the present study is the loss of participants to follow-up. Participants lost to follow-up were significantly older, more disabled and had poorer cognitive function as compared to those available for longitudinal analysis; this could limit the generalization of the findings. However, those lost at follow up had also higher depressive symptoms and lower concentrations of plasma total carotenoids at baseline. Therefore, censoring of these participants probably led to an underestimation of the relationship between carotenoids and depression. Another limitation is that depressive symptoms were evaluated by the CES-D questionnaire and the diagnosis of depression was not confirmed by a clinical psychiatric diagnosis. However, the CES-D is a commonly used scale to measure depressive symptoms and has been widely used in older population-based studies (26). Moreover DSM affective disorders are not highly prevalent among elderly persons in the community, while subsyndromal depression is more common (1, 2). Another limitation is that the study design has not allowed us to detect depressive episodes that started and remitted between subsequent follow-up visits. Finally, reverse causation should be considered. In fact, depression and stress may promote unhealthy dietary preference (57) which in turn could result in lower intake and blood concentration of carotenoids. However, when we subsequently excluded from longitudinal analyses participants who became depressed after 3 years, the association between carotenoids and incident depression after 6 years was still present.

Despite these limitations, we believe that our findings suggest that low plasma concentrations of dietary carotenoids may be considered a potential risk factor for the onset of depressive symptoms in older persons. However, further longitudinal studies are needed in order to confirm this conclusion. Moreover, since we measures dietary carotenoids, any interpretation of the findings of the present study as evidence sustaining the need for supplementation should be avoided. Attempts to translate the results from observational studies to dietary intervention trials may result in disappointing outcomes. Results from the Beta-Carotene and Retinol Efficacy Trial and the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study showed that β-carotene supplementation on participants with high risk of lung cancer was associated with an excess in risk of mortality (58, 59). Moreover, along with nutrition other physiologic and behavioral mechanisms that affect the absorption, storage, and utilization of carotenoids may influence their blood concentrations (60).

Evidence emerging from the study of the nutritional determinants of late-life depression may instead provide the rationale for intervention studies aimed to test whether improving the quality of diet may be especially effective in improving depression in older persons. Dietary patterns rich in antioxidants, such as a Mediterranenan-style diet (characterized by a high intake of fruit, vegetables and olive oil), have already been shown to be associated

with longer survival, reduced cardiovascular and cancer mortality, lower risk of chronic degenerative disease and cognitive decline (61-63). Moreover, two trials showed that nutritional interventions based on Mediterranean diet significantly reduced the levels of inflammatory markers in participants with cardiovascular risk factors (64, 65). Interestingly, two recent studies have also demonstrated that adherence to a Mediterranean-style diet could lower the risk of incident depression in a cohort of adults (66) and could buffer the inflammatory process boosted by depression among community-dwelling older persons(67). In the future dietary interventions may become a cost-effective strategy to promote healthy aging and reduce the burden of age-related depression in the population.

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Chapter 5

Serum 25-hydroxyvitamin D and depressive symptoms in older women and men

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ABSTRACT

Context: Hypovitaminosis D and depressive symptoms are common conditions in older adults.

Objective: We examined the relationship between 25-hydroxyvitamin D [25(OH)D] and depressive symptoms over a six-year follow-up in a sample of older adults.

Design: Population-based cohort study (InCHIANTI Study).

Participants: 531 women and 423 men aged 65 years and older.

Setting: Tuscany, Italy.

Main Outcome Measure: Serum 25(OH)D was measured at baseline. Depressive symptoms were assessed at baseline and at 3- and 6-year follow-ups using the Center for Epidemiological Studies-Depression Scale (CES-D). Depressed mood was defined as CES-D≥16. Analyses were stratified by sex and adjusted for relevant biomarkers and variables related to sociodemographics, somatic health and functional status.

Results: Women with 25(OH)D<50nmol/L compared to those with higher levels, experienced increases in CES-D scores of 2.1 (p=0.02) and 2.2 (p=0.04) points higher at, respectively, 3-year and 6-year follow-up. Women with low Vit-D had also significantly higher risk of developing depressive mood over the follow-up (HR=2.0, 95%CI=1.2-3.2, *p*=0.005). In parallel models, men with 25(OH)D<50nmol/L compared to those with higher levels, experienced increases in CES-D scores of 1.9 (p=0.01) and 1.1 (p=0.20) points higher at 3-year and 6-year follow-up. Men with low Vit-D tended to have higher risk of developing depressed mood (HR=1.6, 95%CI=0.9-2.8, *p*=0.1).

Conclusion: Our findings suggest that hypovitaminosis D is a risk factor for the development of depressive symptoms in older persons.The strength of the prospective association is higher in women than in men. Understanding the potential causal pathway between Vit-D deficiency and depression requires further research.

INTRODUCTION

Hypovitaminosis D is highly prevalent in older persons as a result of reduced capacity of the skin to produce vitamin D, reduced sunlight exposure due to decreased outdoor activity and reduced vitamin dietary intake (1). In older adults, Vit-D deficiency has been linked to poor health outcomes, such as fractures (2), poor physical function (3), frailty (4), sarcopenia (5), pain (6), nursing home admission (7), mortality (8) and chronic diseases, such as osteoporosis, diabetes, cancer, cardiovascular, neurodegenerative, autoimmune and infectious diseases (9-11).

Chronic depressive syndromes are also very common in older persons, especially in those affected by chronic medical illness, and strongly affect the risk of developing disability and death (12). It has been hypothesized that hypovitaminosis D may contribute to late life depression (13-15). However, only a few studies with limited sample size have examined the association between Vit-D and depression, with conflicting findings (15-19). One large population-based cohort study (20) found that the levels of 25-hydroxyvitamin D [25(OH)D] were lower in participants with minor and major depression than in controls. In this study we examined the longitudinal relationship between Vit-D and depressive symptoms over a six-year follow-up in a representative group of older adults. We hypothesized that participants with lower 25(OH)D levels at baseline would experience a steeper increase in severity of depressive symptoms and would be significantly more likely to develop clinically relevant depressed mood than those with higher 25(OH)D. Demonstrating a time sequence between Vit-D deficiency and depression would support further the hypothesis of a causal pathway that can be targeted for intervention.

METHODS AND MATERIALS

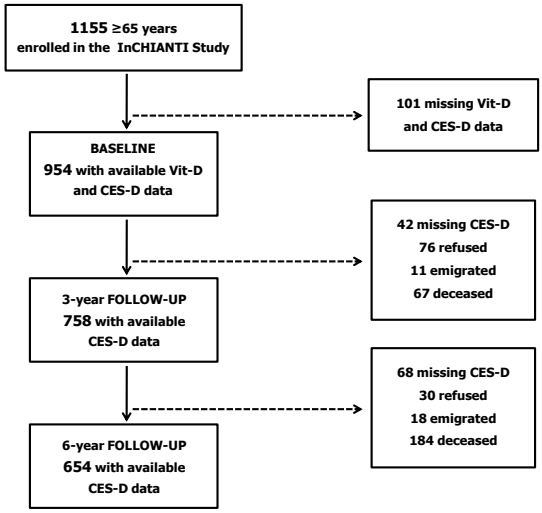
Study Population

Participants were part of the InCHIANTI (**In**vecchiare in **Chianti**, aging in the Chianti area) Study, a prospective population-based study of older persons in Tuscany (Italy) designed to investigate factors contributing to decline in mobility in later life. A description of the study rationale, design and method is given elsewhere (21). Briefly, in 1998-1999 the sample was randomly selected from two sites, Greve in Chianti and Bagno a Ripoli, using a multistage stratified sampling method. Data collection included: 1) a home interview concerning demographics, health-related behaviors, functional status and cognitive function; 2) a medical examination including several performance-based tests of physical function conducted in the study clinic; 3) 24-h urine collection and blood drawing. Participants were evaluated again at three-year (2001-2003) and six-year follow-up visits (2004-2006). All respondents received an extensive description of the study and signed an informed consent. The study protocol complies with the declaration of Helsinki and was approved by the Italian National Institute of Research and Care on Aging Ethical Committee.

The study population selection is summarized in Figure 1. Of the 1155 participants aged ≥65 years enrolled in the study, 1055 (91.3%) donated a blood sample at enrollment. The subjects who did not participate in the blood drawing were generally older and had greater

comorbidity than those who participated (23). We additionally excluded 101 participants because of missing data on Vit-D status or depressive symptoms. Among the remaining 954 participants, 758 had available data on depressive symptoms at 3-year follow-up (42 had missing data, 76 refused to participate in the survey, 11 were emigrated and 67 were deceased) and 654 had available data on depressive symptoms 6-year follow-up (68 had missing data, 30 refused, 18 emigrated and 184 deceased). Overall, 152 participants (15.9%) did not participate at both follow-up sessions. Those lost at both follow-up, as compared to participants who participated at least at one follow-up, were significantly older (80.5 vs 73.2 years), more often sedentary (38.2% vs 14.7%), reported more disabilities in activities of daily living (0.4 vs 0.1) and more comorbid chronic diseases (1.5 vs 1.2), had poorer cognitive function (Mini Mental State Examination scores 23.4 vs 25.9) and lower extremity performance (Short Physical Performance Battery scores 8.7 vs 10.7).

Figure 1. Flow chart of study population selection.



Vitamin D status

Vitamin D status was measured at baseline by assessing circulating levels of 25(OH)D, which is the combined product of cutaneous synthesis from solar exposure and dietary sources. Morning, fasting blood samples were collected after a 15-minute rest. Aliquots of serum were stored at -80°C and never thawed before analysis. Serum 25(OH)D was measured by radioimmunoassay (RIA kit; DiaSorin, Stillwater, Minn). Intra- and inter-assay coefficients of variation (CVs) were 8.1% and 10.2%, respectively. The assay consists of a two-step procedure (23): the first step involves a rapid extraction of 25(OH)D and other

hydroxylated metabolites with acetonitrile; following extraction, the treated sample is then assayed using an equilibrium radioimmunoassay procedure that uses a 25(OH)D specific antibody.Although there is no formal consensus on the optimal levels of 25(OH)D, Vit-D insufficiency is often defined as a 25(OH)D level of less 50 nmol/L (24). Only 25 participants (2.6%) of the sample, 22 women and 2 men, were taking vitamin supplements.

Depressive symptoms

Depressive symptoms were assessed at baseline and at the 3 and 6 year follow-up visits using the Center for Epidemiological Studies-Depression Scale (CES-D) (25). The CES-D is a 20-item self report scale, ranging from 0 to 60. The CES-D has been shown to have good psychometric properties in assessing depressive symptoms in older adults (26), also in an Italian sample (27). A score ≥16 is generally considered to represent clinically relevant "depressed mood" (25).

Covariates

The following covariates assessed at baseline were selected:age, gender, education (years), smoking habit (current/former/non-smoker), alcohol use (<30 vs ≥30 g/day), Mini Mental State Examination (MMSE) score, body mass index (BMI), season of data collection (winter, spring, summer and fall), number of prescribed and non-prescribed drugs, use of antidepressants and vitamin D supplements coded according to Anatomical Therapeutic Chemical (ATC) classification system. Level of physical activity in the previous 12 months was classified as sedentary/light/moderate-high (28). Number of ADL (0-6) and IADL (0-8) disabilities was defined as self-report of inability or needing personal help in performing any basic or instrumental activities of daily living (29). Total number of chronic diseases (heart failure, coronary heart disease including angina and myocardial infarction, stroke, chronic obstructive lung disease, hypertension, diabetes, cancer, dementia and hip arthritis) was calculated as a global marker of poor physical health; diseases were ascertained according to standardized, pre-established criteria and algorithms based upon those used in the Women's Health and Aging Study (30) using information on self-reported history, pharmacological treatments, medical exam data and hospital discharge records. The same method was used to ascertain osteoporosis. The Short Physical Performance Battery (SPPB) (0-12, higher scores indicate better performance) was used to assess lower extremity function using a standard protocol as described elsewhere (31). Energy and vitamin D daily dietary intake were collected by the food-frequency questionnaire created for the European Prospective Investigation on Cancer and nutrition (EPIC) study, previously validated in the InCHIANTI population (32). Creatinine clearance was calculated using the Cockcroft-Gault formulaadjusted for 1.73 body surface area (BSA) calculated according to the DuBois and Dubois formula (33). Serum creatinine for this calculation was measured using a standard Jaffe method (Roche Diagnostics, GmbH, Mannheim, Germany). Serum intact parathyroid hormone (PTH) was measured with a two-site immunoradiometric assay kit (N-tact PTHSP;

DiaSorin); intra- and inter-assay CVs were less than 3.0% and 5.5%, respectively. PTH levels were dichotomized at the median (high vs low; median=22.2 pg/mL).

Statistical Analyses

Variables were reported as percentage, or means ± standard deviation (SD). All analyses were stratified by sex due to sex differences in 25(OH)D levels. Differences in baseline characteristics were tested according to 25(OH)D tertiles and 25(OH)D <50 nmol/L versus ≥50 nmol/L. Associations of 25(OH)D levels with changes in CES-D scores over time while accounting for correlation of repeated CES-D measures, were analyzed by generalized estimating equations (GEE) with an unstructured covariance (34). In all models, 25(OH)D status was coded as an indicator variable with higher level as the reference group. Appropriate 25(OH)D level–by-time interaction terms were included in the model to compare rates of change in CES-D scores between participants with different baseline serum 25(OH)D at each follow-up point. All models were adjusted for covariates that showed an association with 25(OH)D at baseline with a level of significance of $p < 0.1$ plus season of data collection. Next, Cox proportional hazards model were fit to compare risk of developing depressed mood over the follow-up period by Vit-D status. In these analyses, among the 954 participants available at enrollment, 298 subjects with prevalent depressed mood at baseline were excluded; in addition 17 subjects who did not participate at both follow-up sessions and who did not die during the follow-up period were also excluded. Thus the study sample for time-to-event analyses consisted of 639 subjects. Participants whosurvived without developing depressed mood were censored at the date of the last follow-up; those who died without developing depressed mood were censored at the time of their death. Hazard Ratios (HRs) and 95% Confidence Intervals (CIs) were used to compare rates of depressed mood across 25(OH)D levels. Multivariable analyses were initially adjusted for age and baseline CES-D score, then additionally adjusted for the previously selected covariates that were significantly related to the outcome. The analyses were repeated in a subset of healthy participants with no ADL disabilities and with SPPB≥9 at enrollment. Finally, an adjusted Cox proportional hazards model was fit including a baseline 25(OH)D level-by-sex interaction term, to test whether associations were consistent across gender. All analyses were performed using SAS (v. 8.2, SAS Institute, Inc., Cary, NC) with a statistical significance level set at $P<0.05$.

RESULTS

The study sample included 531 women (55.7%) and 423 men (44.3%) with average (±SD) age of 75.0 (±7.1) and 73.6 (±6.5) years, respectively. Prevalence of depressed mood was 42% in women and 18.0% in men. As shown in Figure 1, 74.6% of women and 50.4% of men had serum 25(OH)D<50 nmol/L ($p <.0001$). Overall, 72.2% of participants with depressed mood and 60.0% of those without depressed mood at baseline had levels of 25(OH)D<50 nmol/L ($p =0.0003$). Table 1 describes the characteristics of participants for the total baseline sample and according to 25(OH)D tertiles (tertile 1: <31.7 nmol/L; tertile

2: ≥31.7 to <53.9 nmol/L; tertile 3: ≥53.9 nmol/L). Participants with low levels of 25(OH)D were older, had a higher number of chronic diseases, were more likely to be disabled and sedentary, had lower SPPB scores and were more likely to have participated in the data collection during winter. Men with low 25(OH)D also had low energy dietary intake. Furthermore, women with low 25(OH)D were more likely to take antidepressants, had lower BMI and tended to have higher PTH, lower MMSE scores and less years of educations. At baseline, men and women in the higher 25(OH)D tertiles tended to have less depressive symptoms than those in the higher tertiles, although differences across tertiles were not statistically significant.

Figure 2. Serum 25(OH)D status at baseline in men, women and in participants with and without depressed mood. Depressed mood: Center for Epidemiological Studies-Depression Scale (CES-D) score ≥16.

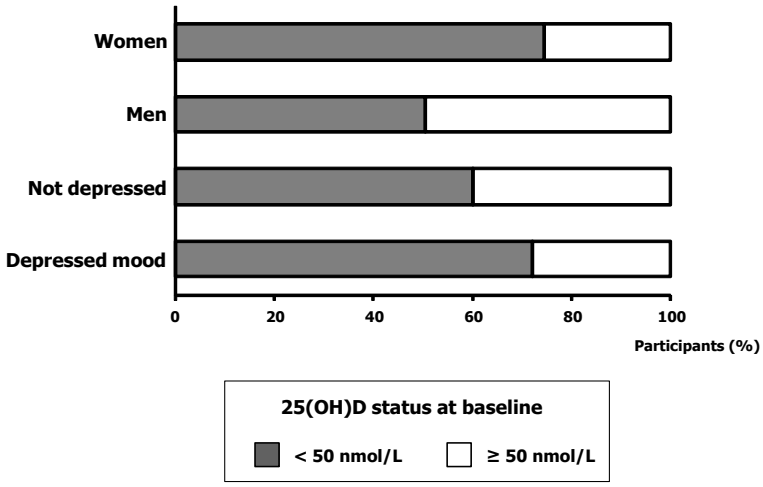


Table 1. Characteristics of the study population at baseline.

		25(OH)D							
		Men				Wonen			
	Tot sample	**Tertile 1**	**Tertile 2**	**Tertile 3**		**Tertile 1**	**Tertile 2**	**Tertile 3**	
	(n=954)	(n=84)	(n=136)	(n=203)	*p**	(n=232)	(n=184)	(n=115)	*p**
Age *(yrs)*	74.4±6.9	77.1±7.4	73.9±6.0	72.0±5.9	<.0001	77.2±7.5	74.1±6.6	72.0±5.7	<.0001
Educat. *(yrs)*	5.5±3.3	5.8±3.7	6.2±3.7	6.6±3.5	0.79	4.6±2.8	4.6±2.8	5.5±2.8	0.08
Alcohol*(≥3/d)*	15.2	21.4	30.9	36.5	0.68	1.3	3.3	1.7	0:59
Smoking					0.77				0.87
non smoker	58.7	32.1	25.0	30.5		84.1	83.2	77.4	
former smok	26.9	50.0	52.9	46.8		6.9	9.8	12.2	
current smok	14.4	17.9	22.1	22.7		9.1	7.1	10.4	
MMSE scores	25.3±3.2	25.1±3.2	25.9±2.7	26.1±2.8	0.86	24.1±3.8	24.9±3.1	25.9±3.0	0.10
BMI *(Kg/m^2)*	27.5±4.1	27.2±3.4	26.9±3.2	27.1±3.3	0.54	27.8±4.8	28.5±4.8	27.2±3.8	0.03
Physical activ					<.0001				0.001
low	18.6	27.4	14.0	3.5		32.8	22.8	8.7	
medium	75.7	69.1	76.5	85.7		65.5	74.5	84.4	
high	5.8	3.6	9.6	10.8		1.7	2.7	7.0	
CES-D score	12.7±8.8	11.3±9.2	9.2±6.6	9.1±6.6	0.5	15.4±8.9	15.5±9.6	14.4±8.9	0.74
No. of ADL	0.1±0.5	0.3±0.8	0.1±0.4	0.04±0.4	0.03	0.2±0.8	0.04±0.3	0.01±0.1	0.02
No. of IADL	0.6±1.5	1.1±1.9	0.4±1.3	0.2±0.9	0.005	1.2±2.1	0.4±1.2	0.01±0.1	0.007
No. of drugs	2.2±2.0	2.5±2.1	2.0±2.1	1.9±2.0	0.5	2.6±2.1	2.2±1.9	2.0±1.7	0.65
Antidep. use	4.4	6.0	2.9	2.5	0.59	7.3	4.9	1.7	0.03
Vit D suppl	2.6	2.4	0.0	0.5	0.38	3.5	3.3	7.0	0.25

Continued on next page

		25(OH)D							
		Men				Wonen			
	Tot sample	**Tertile 1**	**Tertile 2**	**Tertile 3**		**Tertile 1**	**Tertile 2**	**Tertile 3**	
	(n=954)	(n=84)	(n=136)	(n=203)	*p**	(n=232)	(n=184)	(n=115)	*p**
N chron dis.	1.2±1.0	1.4±1.1	1.3±1.0	1.1±0.9	0.045	1.3±1.0	1.1±0.9	1.0±0.8	0.027
Osteoporosis	19.6	11.9	13.2	13.3	0.46	30.2	19.0	23.5	0.36
SPPB score	10.1±2.8	9.4±3.3	10.8±2.3	11.2±1.8	0.002	8.8±3.5	10.2±3.3	10.5±2.1	0.007
En intk *(Kcal/day)*	1929.4±561.6	1941.2±526.9	2214.7±533.7	2262.7±537.1	0.006	1718.0±451.1	1703.7±472.7	1778.4±543.9	0.51
Vi D intk *(mcg/day)*	1.8±0.9	1.8±0.8	2.0±0.8	2.0±2.8	0.54	1.7±0.8	1.7±0.9	1.8±1.2	0.48
Crea clear *(ml/min)*	65.5±18.9	64.1±19.1	69.6±17.8	73.2±17.6	0.93	58.2±17.9	62.8±19.6	66.1±17.5	0.9
High PTH	50.2	60.9	31.3		0.5	60.9	31.3		0.08
Season					0.74				0.11
winter	24.1	33.3	27.9	20.2		28.9	23.4	11.3	
spring	14.1	3.6	19.9	15.3		10.8	20.1	9.6	
summer	23.3	9.5	7.4	29.1		19.0	24.5	48.7	
fall	38.6	53.6	44.9	35.5		41.4	32.1	30.4	

Variables were reported as percentage or means±standard deviation as appropriate.

*Based on age-adjusted general linear model or logistic regression as appropriate.

25(OH)D, 25-hydroxyvitamin D; SD, Standard Deviation; MMSE, Mini Mental State Examination; BMI, Body Mass Index; CES-D, Center for Epidemiological Studies-Depression Scale; ADL, Activities of Daily Living; IADL Instrumental Activities of Daily Living; SPPB, Short Physical Performance Battery; PTH, parathyroid hormone. 25(OH)D: Tertile 1 <31.7 nmol/L; Tertile 2 ≥31.7, <53.9 nmol/L; Tertile 3 ≥53.9 nmol/L. high PTH: >22.2 pg/mL

Table 2. Adjusted associations between 25(OH)D levels and CES-D scores.

	Men						Women					
	Baseline		**Follow up1**		**Follow up2**		**Baseline**		**Follow up1**		**Follow up2**	
	β (SE)	*p**	β (SE)	*p**	β (SE)	*p**	β (SE)	*p**	β (SE)	*p**	β (SE)	*p**
25(OH)D												
Tertile 3	Ref		Ref		Ref		Ref		Ref		Ref	
Tertile 2	-1.02 (0.7)	0.14	1.91 (0.9)	0.03	1.32 (0.8)	0.12	0.65 (1.1)	0.55	1.13 (1.0)	0.26	1.73 (1.2)	0.16
Tertile 1	0.04 (1.0)	0.97	2.13 (1.2)	0.06	0.74 (1.5)	0.63	-0.53 (1.1)	0.63	2.20 (1.0)	0.03	2.50 (1.3)	0.05
≥ 50 nmol/L	Ref		Ref		Ref		Ref		Ref		Ref	
< 50 nmol/L	-0.64 (0.7)	0.35	1.91 (0.8)	0.01	1.07 (0.8)	0.20	0.001 (1.0)	0.9	2.05 (0.9)	0.02	2.19 (1.1)	0.04

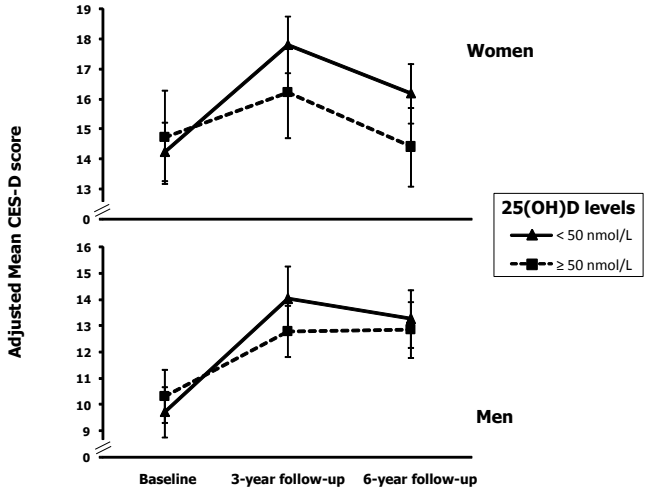
All analyses adjusted for age, education, MMSE score, physical activity, BMI, ADL and IADL disabilities, use of antidepressants, number of chronic diseases, SPPB score, energy intake, high PTH and season of data collection.

25(OH)D, 25-hydroxyvitamin D; SE, Standard Error.

25(OH)D: Tertile 1 <31.7 nmol/L; Tertile 2 ≥31.7, <53.9 nmol/L; Tertile 3 ≥53.9 nmol/L.

GEE models adjusted for age, education, MMSE score, physical activity, ADL and IADL disabilities, BMI, use of antidepressants, number of chronic diseases, SPPB score, energy intake, high PTH and season of data collection, were fit in order to compare average changes in depressive symptoms over time across baseline 25(OH)D levels (Table 2). Women in tertile 1 and 2, as compared to those in the highest tertile, experienced increases in CES-D scores of, respectively, 2.2 (SE=1.0,p=0.03) and 1.1 (SE=1.0,p=0.26) points higher after three year, and 2.5 (SE=1.3,p=0.05) and 1.7 (SE=1.2,p=0.16) points higher after six years. Similarly, men in tertile 1 and tertile 2, as compared to those in the highest tertile, experienced increases in CES-D scores of 2.1 (SE=1.2,p=0.06) and 1.9 (SE=0.9,p=0.03) points higher after three year, and 0.7 (SE=1.5,p=0.63) and 1.3 (SE=0.8,p=0.12) points higher after six years. Analogous results were obtained when 25(OH)D was dichotomized using a cut-off threshold of 50 nmol/L (Table2, Figure 3). Among women, the three-year and six-year average adjusted increases in CES-D scores were respectively 2.1 (SE=0.9,p=0.02) and 2.2 (SE=1.1,p=0.04) points higher for women with 25(OH)D< 50 nmol/L compared to those with levels ≥ 50 nmol/L. Among men, the three-year and six-year average increases were, respectively, 1.9 (SE=0.8,p=0.01) and 1.1 (SE=0.8,p=0.20) points higher for those with 25(OH)D< 50 nmol/L compared to those with levels ≥ 50 nmol/L, although the differential change in CES-D score according to 25(OH)D level was not statistically significant.

Figure 3. Center for Epidemiological Studies-Depression Scale (CES-D) scores during 6 years of follow-up according to baseline 25(OH)D levels in women and men. Estimated Means and 95% Confidence Intervals (CIs) are adjusted for age, education, MMSE score, physical activity, BMI, ADL and IADL disabilities, use of antidepressants, number of chronic diseases, SPPB score, energy intake, high PTH and season of data collection.



Lower baseline serum level of 25(OH)D were also associated with higher probability of developing depressed mood during the follow-up (Table 3). Of the 298 women and 342 men who were free of the depressed mood at baseline, 130 women (43.6%) and 70 men (20.5%) developed depressed mood. After adjustment for age, baseline CES-D, ADL disabilities, use of antidepressants, number of chronic diseases, SPPB, high PTH and season of data collection, women in the lowest tertile of 25(OH)D had an higher hazard (HR=2.6,95%CI=1.4-4.6, p=0.002) of developing depressed mood during 6 years of follow-up, as compared to those in the highest tertile. Similarly, men in the lowest tertile, as compared to those in the highest tertile, had a higher hazard (HR=2.0,95%CI=1.0-4.0, p=0.07) of developing depressed mood, although the association was not statistically significant. Analogous results were obtained using the cut-off of 50 nmol\L; HR for new depression for women and men with lower level of 25(OH)D, as compared to those with higher levels, were respectively 2.0 (95%CI=1.2-3.2, p=0.005) and 1.6 (95%CI=0.9-2.8, p=0.1). To obtain a picture of the effect of Vit-D status free of the possible confounding effect of disability or poor physical functioning, we performed additional analyses restricted to a subset of 535 healthy participants with no ADL disabilities and SPPB≥9 at baseline. Again, we found that women (HR=2.1,95%CI=1.3-3.5, p=0.005) and men (HR=1.5,95%CI=0.8-2.7, p=0.2) with low levels of 25(OH)D had higher risk of developing depressed mood compared to those with higher levels (Table 3).

To better interpret the difference in the strength of the association between 25(OH)D level and depression in women and men, we included a "25(OH)D status-by-sex" interaction term in a fully adjusted Cox regression model predicting depression in the whole study sample. The interaction term was not statistically significant suggesting that the nature of association between 25(OH)D and depression is substantially similar in the two sexes.

Table 3. Adjusted Risk of Depressed Mood according to baseline 25(OH)D levels.

	Risk of Depressed Mood											
	Men						Women					
n = 639	**Model 1 ***			**Model 2 ****			**Model 1 ***			**Model 2 ****		
	H.R.	95% C.I.	*P*	H.R.	95% C.I.	*P*	H.R.	95% C.I.	*P*	H.R.	95% C.I.	*P*
25(OH)D												
Tertile 3	Ref			Ref			Ref			Ref		
Tertile 2	1.05	(0.60 - 1.85)	0.87	1.03	(0.55 - 1.90)	0.93	1.57	(0.92 - 2.66)	0.097	1.72	(0.98 – 3.00)	0.06
Tertile 1	1.74	(0.95 - 3.18)	0.07	1.96	(0.96 – 4.00)	0.07	2.15	(1.30 - 3.57)	0.003	2.56	(1.41 – 4.64)	0.002
≥ 50 nmol/L	Ref			Ref			Ref			Ref		
< 50 nmol/L	1.62	(0.99 - 2.64)	0.05	1.61	(0.92 - 2.82)	0.10	1.87	(1.22 - 3.57)	0.004	1.97	(1.22 - 3.17)	0.005
n = 535 Participants with no ADL disabilities and SPPB ≥ 9 at baseline												
Tertile 3	Ref			Ref			Ref			Ref		
Tertile 2	0.96	(0.53 - 1.75)	0.9	0.97	(0.51 - 1.83)	0.92	1.66	(0.93 - 2.97)	0.09	1.75	(0.96 – 3.20)	0.07
Tertile 1	1.6	(0.81 - 3.16)	0.17	1.67	(0.76 - 3.68)	0.21	2.36	(1.36 - 4.12)	0.002	2.90	(1.53 – 5.50)	0.001
≥ 50 nmol/L	Ref			Ref			Ref			Ref		
< 50 nmol/L	1.47	(0.87 - 2.48)	0.15	1.46	(0.81 - 2.65)	0.21	2.01	(1.26 - 3.21)	0.004	2.09	(1.25 - 3.49)	0.005

*Adjusted for age and baseline CES-D.

**Adjusted for age, baseline CES-D, ADL disabilities, use of antidepressants, number of chronic diseases, SPPB, high PTH and season of data collection.

25(OH)D, 25-hydroxyvitamin D; H.R. Hazard Ratio; C.I., Confidence Interval.

Depressed mood: CES-D ≥16. 25(OH)D: Tertile 1 <31.7 nmol/L; Tertile 2 ≥31.7, <53.9 nmol/L; Tertile 3 ≥53.9 nmol/L.

DISCUSSION

Using data from a population-based study of older persons, we found evidence of a prospective independent association between circulating levels of 25(OH)D and depressive symptoms. Participants with low 25(OH)D serum levels experienced a grater increase in depressive symptoms over six years of follow-up. Moreover,among participants free of clinically relevant depressive symptoms at baseline, a higher risk of developing clinically relevant depressive symptoms over time was found for those with low serum 25(OH)D. In the baseline sex-stratified analysis, men and women with higher 25(OH)D levels tended to have lower depressive symptoms, although the difference was statistically significant only in analyses that included both men and women.

It has been hypothesized (14) the association between Vit-D and depression may be difficult to capture in cross sectional analyses because clinically detectable mood disorders may take many years to develop.

Relatively few cross-sectional epidemiologic studies have evaluated the relationship between Vit-D and depression in older adults, and results have been mixed. In one study (16) comparing 40 individuals with mild Alzheimer's disease with 40 non-demented persons, all over 60 years of age, subject with low 25(OH)D were significantly more likely to have a mood disorder, although the mean depressive features score did not vary by Vit-D status. In the Longitudinal Aging Study Amsterdam (20), depression symptoms as measured by CES-D scores was significantly associated with 25(OH)D. Moreover, mean 25(OH)D levels in participants with major depressive disorder and those with minor depression were comparable and 14% lower than those of non-depressed participants. More recently, a large cross-sectional study (19) of older adults in China found no associations between 25(OH) levels depressive symptoms as assessed by CES-D.

Different mechanisms through which Vit-D may potentially influence brain functions have been proposed (10-12). First of all, Vit-D may have a direct neuroregulatory activity. Vitamin D receptors (VDR) and 25-Hydroxyvitamin D3 1-alpha-hydroxylase, the cytochrome P450 which catalyzes the hydroxylation of calcidiol to calcitriol (the bioactive form of Vit-D) are widely distributed throughout the central nervous system (35). VDR gene polymorphisms in humans have been associated with cognitive impairment and depressive symptoms (36). Furthermore, Vit-D regulates the expression of important neurotrophic factors which affect neurotransmission and synaptic plasticity (11). Moreover, Vit-D has been shown to be neuroprotective, notably by inducing the synthesis of calcium-binding proteins or by antioxidant mechanisms (10-12). Finally, the immunomodulatory activity of Vit-D has been related to recent evidence that inflammation may play a causal role in depression: Vit-D has been shown to down-regulate inflammatory mediators, such as nuclear factor kappa B (NFkB), which have been linked to sickness behavior, psychosocial stress and depression (11,37).

Sex differences in the relationship between 25(OH)D and depression could be attributable to different factors. One explanation for the weaker association between 25(OH)D and depression in men could be the smaller number of men with low levels of

25(OH)D. Furthermore, in our study sample, women were older and those with lower 25(OH)D showed specific characteristics (use of antidepressants, low BMI, MMSE and years of education and high PTH) commonly associated with depression (12,19).

Our study has both strengths and limitations. A major strength of this study is the use of a large population-based sample with measured serum 25(OH)D, which is the best clinical indicator of Vit-D body store levels, and the longitudinal design. An important limitation of our study is the loss of participants to follow-up. Participants lost to follow-up were significantly older, more disabled, had poorer cognitive function and more chronic diseases compared to those available for longitudinal analysis; this could limit the generalization of the findings. Another limitation of this study is that depressive symptoms were evaluated by the CES-D questionnaire and the diagnosis of depression was not confirmed by a clinical psychiatric diagnosis. However, the CES-D is a commonly used scale to measure depressive symptoms, has been widely used in older population-based studies and has been shown to substantially converge with physician ratings of depression (26). In addition, because of the long intervals between follow-up visits, we could not detect depressive episodes that started and remitted between subsequent visits. Finally, residual confounding in studies of Vit-D and depression should be considered. A variety of factors are associated with Vit-D, including age, physical activity, disability and chronic diseases such as osteoporosis, diabetes, cancer, cardiovascular, neurodegenerative, autoimmune and infectious diseases (1-11). Many of these factors are also associated with depression in older age (12). In the present study, lower levels of 25(OH)D were associated with more disabilities and comorbidities and could have resulted in more incident unfavorable health events, which in turn could have increased depressive symptoms; even though our analysis was adjusted for an extensive array of potential confounders, we cannot exclude the hypothesis that some critical variable was not measured. In other terms, we cannot exclude that the association between Vit-D and depressive symptoms in this study could be still partially explained by residual confounding. However, the association between 25(OH)D and depressed mood remained significant after the selection of a subset of healthy participants with no disabilities and high physical function as measured by SPPB, a strong predictor of nursing home admission, disability in self-care tasks and mobility, and death among older adults (31).

Despite limitations, we believe that our findings provide evidence of a prospective association between low Vit-D levels and the onset of depressive symptoms in older persons over time. Such evidence is not sufficient to conclude with certainty that there is a causal connection. However, our findings in conjunction with recent pre-clinical studies that confirmed the strong biological activity of Vit-D on brain function (11,12,16) suggests the hypothesis that normalization of Vit-D levels may positively contribute to the successful treatment of depression in older persons.

Hypovitaminosis D is highly prevalent throughout the world in the elderly (1). Potentially modifiable determinants of Vit-D status, such as consumption of Vit-D rich food, fortification of foods, use of dietary supplements and habits related to sun exposure, have

been identified (9,38). Prevention of Vit-D deficiency in the elderly may become in the future a strategy to prevent the development of depressive mood in the elderly (39) and avoid its deleterious consequences on health (12). In addition, normalization of Vit-D levels may be part of any depression treatment plans in older patients. These hypotheses should be tested in appropriately designed, randomized, controlled trials.

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