VITAMIN *D*-TOUR

Cognition and depression: the role of vitamin D and its interplay with glucose homeostasis

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General introduction

We are getting older. In 2002, Dutch men and women reached an average age of 76 and 81 years. Recent estimates of the Dutch National Institute of Public Health and the Environment indicate an average life expectancy of 79 years in men and 83 years in women [1]. Unfortunately, old age is regularly accompanied by a rise in chronic conditions that may result in impairments and hinder an individual from active participation in daily life activities [2]. Discovering potentially modifiable environmental factors that impede the functioning of an individual is crucial to postpone or slow-down these impairments as long as possible. Vitamin D inadequacy has been identified as one of the potential environmental factors to target [3]. Associations have been suggested between vitamin D and fall incidence [4], fracture risk [5], muscle health [6], cancer [7], hypertension [8], type 2 diabetes mellitus [9], mood [10] and cognitive performance [11]. However, there are still many uncertainties regarding the causality of these associations and the underlying pathophysiology. To gain more insight in the actions of vitamin D, the research described in this thesis addresses the potential role of vitamin D in cognitive function and depression in populations aged ≥65 years, and in a sample of aged C57BL/6 mice. By exploring the hypothetical role of glucose homeostasis in the potential relationship between vitamin D and brain health also a possible underlying mechanism is addressed.

1.1. A focus on vitamin D

The vitamin D chronicle started in the early 20th century when exposure to a mercury-vapor-quartz lamp and cod liver oil appeared to be effective in the treatment of rachitis [12, 13]. Subsequently, vitamin D was identified as being the responsible agent [13]. In 1931 vitamin D_2 was chemically characterized [14], followed by vitamin D_3 in 1936 [15]. Since the 1960s knowledge on vitamin D further expanded. In 1968, Blunt and colleagues identified the inactive vitamin D metabolite, 25-hydroxyvitamin D (25(OH)D), which is at this moment considered to be the best status marker of vitamin D in blood. Nowadays it is known that skin exposure to sun, more specifically ultraviolet-B (UV) radiation, can activate the conversion of 7-dehydrocholesterol into pre-vitamin D_3 and subsequently into vitamin D_3 [16], and that most of the 25(OH)D traced in human blood is formed in response to UV-B radiation [17].

1.1.1 Vitamin D metabolism

Once vitamin D is synthesized in the skin or dietary vitamin D is absorbed in the small intestine, it is incorporated in the blood circulation and - attached to vitamin D binding protein - transported to the liver. In the liver, vitamin D is converted into 25(OH)D. To actually influence biological processes throughout our body, 25(OH)D needs to be converted in 1,25(OH)D. This conversion can take place in a variety of tissues, but primarily occurs in the kidneys. 1,25(OH)D is the active vitamin D metabolite and acts on vitamin D receptors throughout the body [3].

1.1.2 Skin synthesis

Sun exposure is considered to be the most important determinant of 25(OH)D in blood.

The exact amount of vitamin D synthesized in the skin, however, depends on many factors, including melanin pigmentation, age, time of the day, season, latitude, sunscreen use, air pollution, type of clothing worn and also gene profile [18]. In view of the scope of this thesis (i.e. populations studied mainly include Dutch older adults), the factors season, latitude and age are here described in more detail. In the Netherlands, located at a latitude of ± 52°N, the average annual vitamin D synthesis has been estimated to be around 6-7 μg vitamin D daily [19]. During the Dutch winter period, however, the quality and quantity of ultraviolet-B photons is reduced and as such the production of pre-vitamin D_3 in response to UV-B is largely absent [20]. Older age is also considered a risk factor for low 25(OH)D concentrations. This is explained by the age-dependent reduction of 7-dehydrocholesterol in the skin, and consequently a reduced capacity to produce vitamin D in response to ultraviolet-B radiation [21]. Impaired physical functioning, reduced sun exposure, and a low dietary vitamin D intake are furthermore assumed to contribute to low 25(OH)D concentrations at older age [22].

1.1.3 Dietary and supplemental vitamin D intake

After sun exposure, dietary factors, such as fatty fish (e.g. 100 grams mackerel provides 6.3 μg vitamin D_3), egg yolk (0.5 μg vitamin D_2 or vitamin D_3), mushrooms (e.g. 100 grams fresh Shiitake mushrooms provides 2.5 μg vitamin D_2), and vitamin D supplements are the second source of vitamin D [3]. The efficacy of vitamin D intake to influence 25(OH)D concentrations is assumed to depend on a variety of factors, like the body's capacity to absorb vitamin D, body composition, use of specific medication, liver and kidney function, and genetic make-up [3].

1.1.4 Dietary Reference Intakes

During the past years there has been a continuous debate on what the optimal vitamin D intake level and blood concentration should be. The Dietary Reference Intakes (DRI) formulated by the Institute of Medicine (IOM) range from an Adequate Intake (AI) of 10 μg/day in infants to a Recommended Dietary Allowance of 20 μg/day in persons aged ≥70 years [23]. The daily requirement as set by the Dutch Health Council also ranges from 10-20 μg/day, where all older adults aged ≥70 years are recommended to use a supplement containing 20 μg vitamin D per day [24].

1.1.5 Optimal 25(OH)D concentrations

According to the Dutch Health Council a 25(OH)D concentration of ≥30 nmol/L can be considered sufficient for persons aged 4-70 years [24]. This concentration is primarily based on the evidence related to bone health. Currently, the IOM concludes that persons with 25(OH)D concentrations ≥50 nmol/L are vitamin D sufficient [23]. The Endocrine Society considers a concentration of 50 nmol/L suboptimal. Based on data related to fall and fracture risk, parathyroid suppression, pathologic osteoid formation, and muscle metabolism, the Endocrine Society recommends 25(OH)D concentrations ≥75 nmol/L [25]. Whether these concentrations also beneficially relate to outcomes beyond bone health warrants further study.

1.2 A general introduction to cognitive performance, cognitive decline and dementia

1.2.1 Epidemiology

Age-related decrements in cognitive function are regularly observed. In case of pathophysiological abnormalities like Alzheimer's disease (AD), vascular dementia, Lewy body dementia or frontotemporal dementia, people are generally faced with more severe cognitive deficits. Patients gradually lose the ability to learn, recall previously learned skills, and as such their independence. Estimates suggest that worldwide more than 35 million persons are affected by dementia [26]. In about 70% of the dementia cases the diagnosis is AD [27]. Approximately 20% of the patients is diagnosed with vascular dementia [28]. In practice, however, patients often have AD pathology as well as vascular injuries [27].

1.2.2 Biological underpinnings

The aetiology of dementia and cognitive decline is complex. Necropsy studies in dementia patients have pointed towards the presence of amyloid plaques and neurofibrillary tangles, vascular damage and brain atrophy as the pathological processes to target. Amyloid plaques are made of the protein amyloid-β (Aβ). 'Normal' synaptic Aβ levels are thought to prevent neuronal hyperactivity. Too high levels of Aβ, however, can result in Aβ accumulation and the deposition of amyloid plaques. Amyloid deposits are predominantly located between brain neurons. Neurofibrillary tangles are made of the phosphorylated protein tau [27]. Tau plays an important role in the stability of axonal microtubules. However, in AD, the structure of tau is altered, resulting in the formation of neurofibrillary tangles in axonal microtubules [27]. There is increasing evidence that amyloid plaques and neurofibrillary tangles may be related to synaptic failure, and hence cognitive decline [27, 29]. Vascular dementia typically arises from problems with the cerebral blood supply resulting in infarcts and/or white matter lesions [30]. An overlapping characteristic of the different types of dementia is that they are generally accompanied by relatively severe neurodegeneration; demonstrated by thinning of the cerebral cortex, shrinkage of subcortical structures and increasing ventricle volumes [31, 32].

1.2.3 Predisposing factors

Several risk factors for cognitive decline and dementia have been identified. To date, just a few of these factors are considered unequivocally established predisposing factors, including having the apoE4 allele [33], increasing age [34], and being woman [35]. Other proposed risk factors include high blood pressure, diabetes, metabolic syndrome, high body weight, dyslipidaemia, smoking, physical inactivity, intellectual inactivity [33], high saturated fat intake [36], excessive alcohol consumption [37], and depression [38]. Studies have also suggested that specific nutrient deficiencies may relate to cognitive decline, including B-vitamins [36, 39, 40], vitamin D [11, 41], omega-3 fatty acids [36, 40], zinc [42], and selenium [43].

1.2.4 Treatment

To date, treatment options for dementia are limited. The commonly prescribed pharmacological agents mainly target the observed neurotransmitter disturbances in cognitive decline and dementia, specifically acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor (NMDA) receptor antagonists [44]. Unfortunately, the observed treatments effects are relatively small [45, 46].

1.2.5 Assessing cognitive performance, cognitive decline and dementia

To assess cognitive functioning and to acquire information on the affected brain regions in cognitive decline, neuropsychological testing can be performed, which has been defined as "(..) the intensive study of behaviour by means of interviews and standardized scaled tests and questionnaires that provide relatively precise and sensitive indices of behaviour" [47]. Numerous of these neuropsychological tests have yet been developed, targeting global cognitive function as well as more specific cognitive functions. **Table 1** provides an overview of the cognitive tests used in this thesis, their properties and a real life example. During the past decade also imaging techniques gained ground in behavioural brain research. Structural Magnetic Resonance Imaging (MRI) is one of these techniques, and provides information on amongst others total brain volume and integrity of grey matter and white matter brain structures. However, despite these relatively new techniques, still not all theories on brain function can be studied in humans. In these cases, the scientific community regularly uses mouse and rat models to gain more insight in potential behavioural and physiological processes. **Table 2** and **Figure 1** provide an overview of the behavioural tests used in the animal trial that was conducted for this thesis.

1.3 A general introduction to depression

1.3.1 Epidemiology

By affecting about 4-20% of the people at least once in their lifetime, depression is the most commonly diagnosed psychiatric disorder worldwide [48]. Estimates indicate that about 8-16% of older men and women aged ≥65 years have clinically significant depressive symptoms, and that these symptoms are more likely to occur in the oldest old [49]. Depression is frequently accompanied by a depressed mood or irritability, decreased interest or pleasure, significant weight change or change in appetite, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of guilt or worthlessness, concentration problems, indecisiveness and/or suicide [50].

1.3.2 Biological underpinnings

There are several pathophysiological hypotheses on the development of depression, including monoamine depletion, hypothalamic pituitary adrenal (HPA)-axis hyperactivity, decreased activity of specific brain regions and genetic predisposition [51]. To start with the first hypothesis, there is the monoamine hypothesis. This hypothesis posits that depression is caused by depletion of noradrenaline, serotonin and/or dopamine levels.

Table 1. Overview of the neuropsychological tests used in the human studies presented in this thesis.

Domain	Real life example	Test	Description of the test	Scoring (Interpretation: ↑/↓- score is better)	
Global cognitive performance		Mini Mental State Examination (MMSE)	Brief basic screening tool: by answering a number of questions/tasks a set of cognitive functions is assessed.	Each correct answer is 1 point, with a maximum of 30 points	↑
Attention and working memory	Memorize the steps of a recipe while cooking a meal.	Digit span forward	Repeat a sequence of digits exactly as given.	Number of correctly repeated sequences	↑
		Digit span backward	Repeat a sequence of digits in a reversed order.	Number of correctly repeated sequences	↑
Executive functioning	Despite the fact that you are in a hurry, you suppress the urge to drive through the orange traffic light.	Letter/animal fluency	Name as many words as possible within one minute (i.e. words with a specific letter (D,A,T) or animals).	Number of correct words/animals	↑
		Stroop color word test	Read 3 cards as fast as possible. Card 1: 100 color names. Card 2: 100 color patches. Card 3: 100 color names printed in a different color ink (name the color of the ink).	Time in seconds Interference: Stroop 3/((Stroop 1+Stroop 2)/2)	↓
		Trail making test A + B	TMT-A: Connect numbered circles as fast as possible in ascending order. TMT-B: Connect a sequence of numbers and letters by alternating between the two sequences, in ascending order and according to the alphabet, as fast as possible.	Time in seconds Ratio score: (TMT-B/TMT-A)	↓
		Reaction Time Task	The goal is to react as quickly and accurately as possible to a single plus-sign on a computer screen by pressing one of four keys of a computer keyboard.	Time to react (ms)	↓
Information processing speed	Ability to deal with new information, for instance when the technician informs you about the functions of the new MRI scanner.	Symbol digit modality test	A number of symbols are linked to digits. The participant has to name as many correct digits corresponding to the symbols in 90 seconds, using a symbol-digit key.	Number of correct digits	↑
		Trail making test A	TMT-A: Connect numbered circles as fast as possible in ascending order.	Time in seconds	↓
		Stroop color word test	Read 2 cards as fast as possible. Card 1: 100 color names. Card 2: 100 color patches.	Time in seconds Average score: (Stroop 1+Stroop 2)/2	↓
Episodic memory	Memorizing your shopping list.	Word learning test	Recall as many words as possible from a list of 15 words that have been read out by the researcher.	Number of correct words of trial 1 to 5	↑
		Delayed recall	Recall as many words as possible 15-20 minutes after the researcher read out the 15-word list.	Number of correct words delayed recall minus total correct words trial 5	↑
		Delayed recognition	A list of 30 words is read out loud. The participant has to identify as many words as possible from the 15-word list that has been read out earlier.	Number of correctly recognized and rejected words	↑

Table 2. Overview of the behavioural tests used in the animal study presented in this thesis.

Domain	Test	Description of the test	Parameters	Interpretation
Emotional reactivity, anxiety, locomotion, exploration	Open Field Test (OFT)	A mouse is released in an open field. Behaviour is measured for 5 minutes.	*Total distance travelled *Center time (sec)	A lengthier distance travelled/more center time suggests more exploration and less emotional reactivity.
	Elevated Plus Maze (EPM)	A mouse is released on a plus shaped apparatus with two open and two closed arms. Behaviour is recorded for 5 minutes.	*Total arm entries (freq) *Open arm entries (freq) *Open arm time (sec) *Open arm entries (%)	More total arm entries and more open arm entries suggests less emotional reactivity, more exploration, and better locomotion.
Recognition memory	Object Recognition Test (ORT)	A mouse is subjected to two test sessions of 5 minutes, which are separated by a retention interval of 1 hour. During the first session, two identical objects are presented to the mouse for 5 minutes. During the second session, the familiar object and a novel object are presented to the same mouse. The time exploring the objects is recorded.	*Exploration (sec): exploration familiar + novel object *Habituation (sec): exploration trial 1 - trial 2 *Discrimination (sec.): exploration novel - familiar object *Adjusted discrimination: discrimination / exploration	A higher adjusted discrimination index suggests a better recognition memory.
Spatial learning	Morris Water Maze (MWM)	A mouse is released in a circular pool containing an escape platform: 4 trials in a row on 5 consecutive days.	*Escape latency *Distance travelled	Shorter escape latency and shorter distance travelled imply a better spatial learning performance.
Reference memory	Probe trial of Morris Water Maze	After completion of the Morris Water Maze a mouse is again released in the pool for 60 seconds, but now without the escape platform.	*Time in target quadrant (%)	More time spent in the target quadrant (i.e. former platform location) suggests a better reference memory.

Currently, most of the prescribed antidepressants target this monoamine system [51]. Secondly, in case of depression there is often hyperactivity of the HPA-axis. In response to stress the hypothalamus starts to secrete corticotropin-releasing hormone (CRH) and vasopressin. CRH and vasopressin on their turn stimulate the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH subsequently acts on the adrenal cortex, which results in the production of glucocorticoids, mainly cortisol. In a normal situation these hormones are helpful to deal with stressful situations, where there is a negative feedback cycle in which high levels of glucocorticoids suppress the secretion of CRH and vasopressin. However, when this negative feedback cycle does not function adequately, HPA-hyperactivity may result in depression and even hippocampal atrophy [51]. Depression has furthermore been associated with an increased activity of the amygdala, orbitofrontal cortex, and a decreased activity of the subgenual cingulate and the ventral stratium [51]. Finally, neurodegeneration and vascular injury may be involved in the development of depressive symptoms [52].

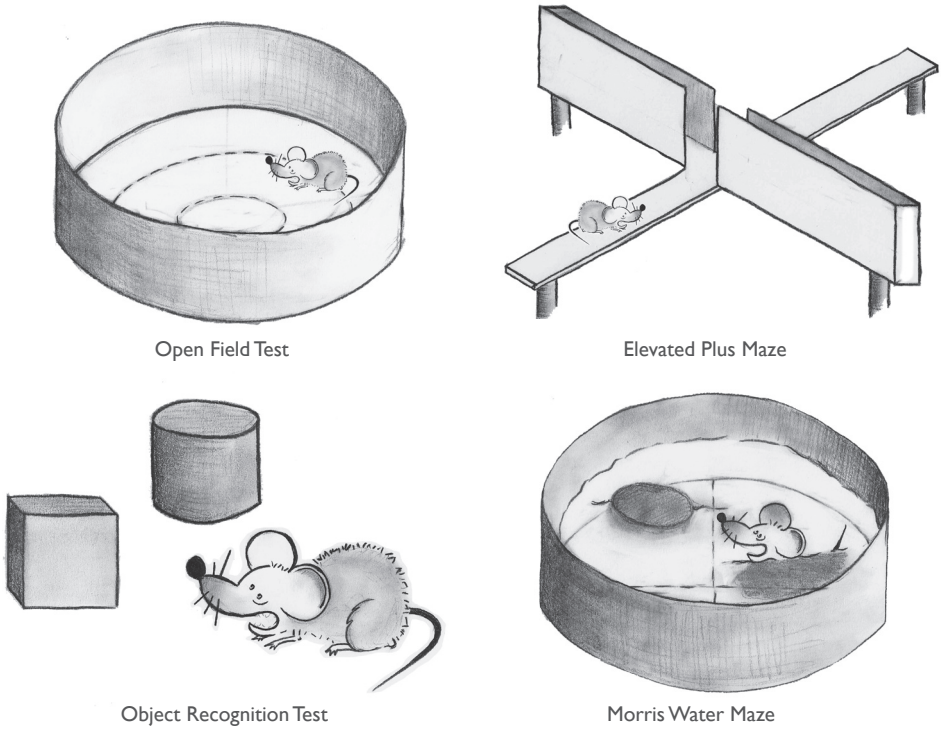

Figure 1. Visualisation of the behavioural tests used in the animal study presented in this thesis.

1.3.3 Predisposing factors

Biological, psychological and social factors have been suggested to form the basis for depression [52]. First of all, genetic predisposition may play a role in the development of depression [51]. Depression in older adults has furthermore been associated with being woman, medical

illness, functional disability, sleep disturbance, stressful life events, bereavement, being unmarried, small social network, and a poor-perceived health [52, 53]. Nutritional factors that have been associated with depression include omega-3 fatty acids [40], B-vitamins [40], vitamin D [10], zinc [54] and magnesium [55].

1.3.4 Treatment

Depression is commonly treated with antidepressants, which primarily act via the enhancement of neurotransmission in the brain by increasing the availability of serotonin and noradrenalin [49, 56]. However, for many patients treatment with anti-depressants is suboptimal (e.g. relapse), not effective and/or coincides with unwanted side-effects [57]. Alternative treatment possibilities include psychotherapy, light therapy, physical exercise and in severe depression, electroshock therapy [49].

1.3.5 Assessing depression

Epidemiological studies often use brief self-administered symptom checklists to assess mood [58, 59]. In this thesis the Geriatric Depression Scale-15 (GDS) and Centre for Epidemiologic Studies Depression scale (CES-D) were used to assess depressive symptoms, where higher scores reflect more depressive symptoms. The GDS is a depression-screening tool that has been specifically developed for population of older adults [60]. The CES-D has been specifically developed to identify persons at risk for clinical depression in a general population [61]. Many studies have attempted to estimate the cut-off value for clinical depression presenting varying results. Suggested cut-off values for clinical depression include amongst others ≥5 for the GDS [60] and ≥16 for the CES-D [62].

1.4 Vitamin D, the brain and how they relate

1.4.1 Vitamin D-mediated processes in the brain

Several in vitro studies and molecular studies have demonstrated a role of vitamin D in brain function. The clearest biological evidence for a role of vitamin D in brain function is the localization of vitamin D receptors (VDR) on various brain structures and 1,25-α-hydroxylase, the enzyme that catalyses the conversion of 25(OH)D in the active form of vitamin D, in cerebrospinal fluid [63]. Treatment with 1,25(OH)D has furthermore been shown to stimulate choline acetyltransferase activity [64], promote the synthesis of neurotrophins [65], exert anti-excitotoxic properties by decreasing L-type voltage-dependent calcium channel expression [66] and to have anti-oxidative properties by regulating glutathione concentrations [67]. In addition, Aβ-PP transgenic mice (i.e. mice that are prone to develop amyloid plaques) on a vitamin D enriched diet have been shown to have less amyloid plaque deposition, when compared to non-treated counterparts [68]. Vitamin D adequacy may furthermore prevent cerebrovascular disease by beneficially affecting several vascular risk factors [69]. The observation of a crosstalk between the glucocorticoid receptor and VDR in hippocampal cells resulted in the hypothesis that vitamin D may ameliorate the progression of neuronal atrophy that is caused by high glucocorticoid levels during depressive episodes [70].

Table 3. Summary of rodent experiments on vitamin D supplementation and behaviour: characteristics and results.

Study	Animals	Age during intervention	Intervention	Serum 25(OH)D (nmol/L)	Behavioural measures	Behavioural effect?
Altemus, et al. (1987) [73]	21 ♂ Sprague-Dawley albino rats	±3-12 wk	2 groups fed a C or D^- diet	C: 58 D^-: 6	FOFT, 8-ARMT, TMT	No
Taghizadeh, et al. (2011) [75]	43 ♂ Wistar rats	8 wk during adulthood (n.s.)	1 control group, and 3 AD groups (i.e. rats intracerebroventricularly injected with Aβ1-42) fed a C, D- or D+ diet	n.s.	MWM, MWM-P	Yes: D- ↓ spatial learning
Yu, et al. (2011) [68]	30 ♂ AβPP-PS1 transgenic mice	±3-24 wk	3 groups fed a C, D^- or D^+ diet	C: 55±5 D^-: 2±2 D^+: 96±18	MWM, MWM-P	Yes: D- ↓ spatial learning, ↓ reference memory
Groves, et al. (2013) [74]	79 ♂ C57BL/6j mice* 69 ♂ BALB/c mice*	±10-20 wk	2 C57BL/6j groups fed a C or D^- diet 2 BALB/c mice groups fed a C or D^- diet	C57BL/6j C: 65±2 D^-: 3±0 BALB/c C: 32±2 D^-: 3±0	NOFT, FOFT, EPM, HT, LDT, FST, ASRT, AAT, SIT, HPT	C57BL/6j: Yes: D- ↑ locomotion NOFT BALB/c: Yes: D-: ↑ locomotion NOFT, ↑ open arm time in EPM, ↓ locomotion FOFT and ↑ response ASRT, HPT and AAT
Byrne, et al. (2013) [76]	213 ♂ Sprague-Dawley albino rats*	±10-16 wk	2 groups fed a C or D^- diet	C: 60±2 D^-: 9±1	EPM, HT, LDT, SIT, FST, TFT, HPT, ASRT, AAT, NOFT, 5C-SRTT, 5C-CPT, DMTS	YES: D- ↑ premature responses on 5C-SRT and ↑ false alarm latency on 5C-CPT (attentional processing)

Note: n.s. denotes not specified. *Not all animals were tested in each procedure. FOFT: Familiar Open Field Test (anxiety, locomotion, exploration); 8-ARMT: 8- Arm Radial Maze Test (working and reference memory); TMT: T-Maze Test (memory and spatial learning); MWM: Morris Water Maze (spatial learning); MWM-P: Morris Water Maze-Probe trial (reference memory); NOFT: Novel Open Field Test (anxiety, locomotion, exploration); EPM: Elevated Plus Maze (anxiety and locomotion); HT: Holeboard Test (locomotion, exploration); LDT: Light/Dark Test (anxiety); FST: Forced Swim Test (mood, learned helplessness); ASRT: Acoustic Startle Response Test (startle reflex); AAT: Active Avoidance Test (avoidance learning); SIT: Social Interaction Test (social interaction); HPT: Hot Plate Test (pain response); TFT: Tail Flick Test (pain response); 5C-SRTT: 5-Choice Serial Reaction Time Task (attention and reaction time); 5C-CPT: 5-Choice Continuous Performance Test (attention and reaction time); DMTS: Delayed Match-to-Sample Task (working memory).

1.4.2 Experimental evidence from rodent experiments

Several animal studies have investigated the effect of vitamin D on behaviour and brain function (reviewed in [71, 72]). Many of these studies were conducted in vitamin D receptor knockout mice or mice that had a prenatal vitamin D deficiency. However, the behavioural effects examined in these 'early life' models may substantially differ from the effects of a vitamin D deficiency developed later in life. Studies examining the effect of an adult vitamin D deficiency on behaviour are sparse and heterogeneous in their experimental design (**Table 3**) [68, 73-76]. Due to these experimental differences - that is, the animal models used, study duration, treatment type, age of testing and testing procedures - these studies do not provide a clear-cut answer on the role of vitamin D deficiency in brain function in aging rodents.

1.4.3 Evidence from studies in older adults

Cognitive performance, cognitive decline and dementia

Up to now, there have been several human studies examining the potential relation between vitamin D and cognition [77-100]. Most of these studies have been cross-sectional and used a global screening tool, such as the MMSE [77-83, 85-87, 89-91, 93-96]. The majority of the most recent studies, however, also assessed domain-specific cognitive functions, including declarative memory, working memory, attention, information processing speed, visuospatial abilities and/or executive functioning [80, 84, 86, 88, 90, 92, 94, 95]. Some of these studies used clusters of tests to study a cognitive domain [80, 90, 92]. In the NAME study, including up to 1050 black and non-black older adults, fully adjusted models showed significant associations between serum 25(OH)D and the domains attention/processing speed (β 0.01, SE 0.003, P<0.01) and executive function (β 0.01, SE 0.003, P<0.01), but not with the domain memory (β -0.004, SE 0.004, P<0.54) [80]. In the ISAAC study, conducted among 159 men and women ≥70 years, linear regression analyses adjusted for age, sex and level of education did not point towards an association between 25(OH)D concentrations and attention (β 2.68, P=0.12), working memory (β 2.30, P=0.10) or executive function (β 1.73, P=0.27) [90]. In the ZENITH study a significant association was found between 25(OH) D status and measures of spatial working memory [92]. Prospective studies have largely focussed on tests measuring executive functioning, particularly the Trail Making Test-B (TMT-B) [86, 94, 95]. Only in the InCHIANTI study the association between 25(OH)D concentration and TMT-B performance was significant, indicating that older adults with serum 25(OH)D concentrations <25 nmol/L had a 31% increased probability of developing cognitive impairment when compared to those with concentrations ≥75 nmol/L, after an average follow-up time of 5.2 years [86]. A complete overview of the results of all the identified studies is provided in **Figure 2**. Overall, it may be concluded that results are inconclusive.

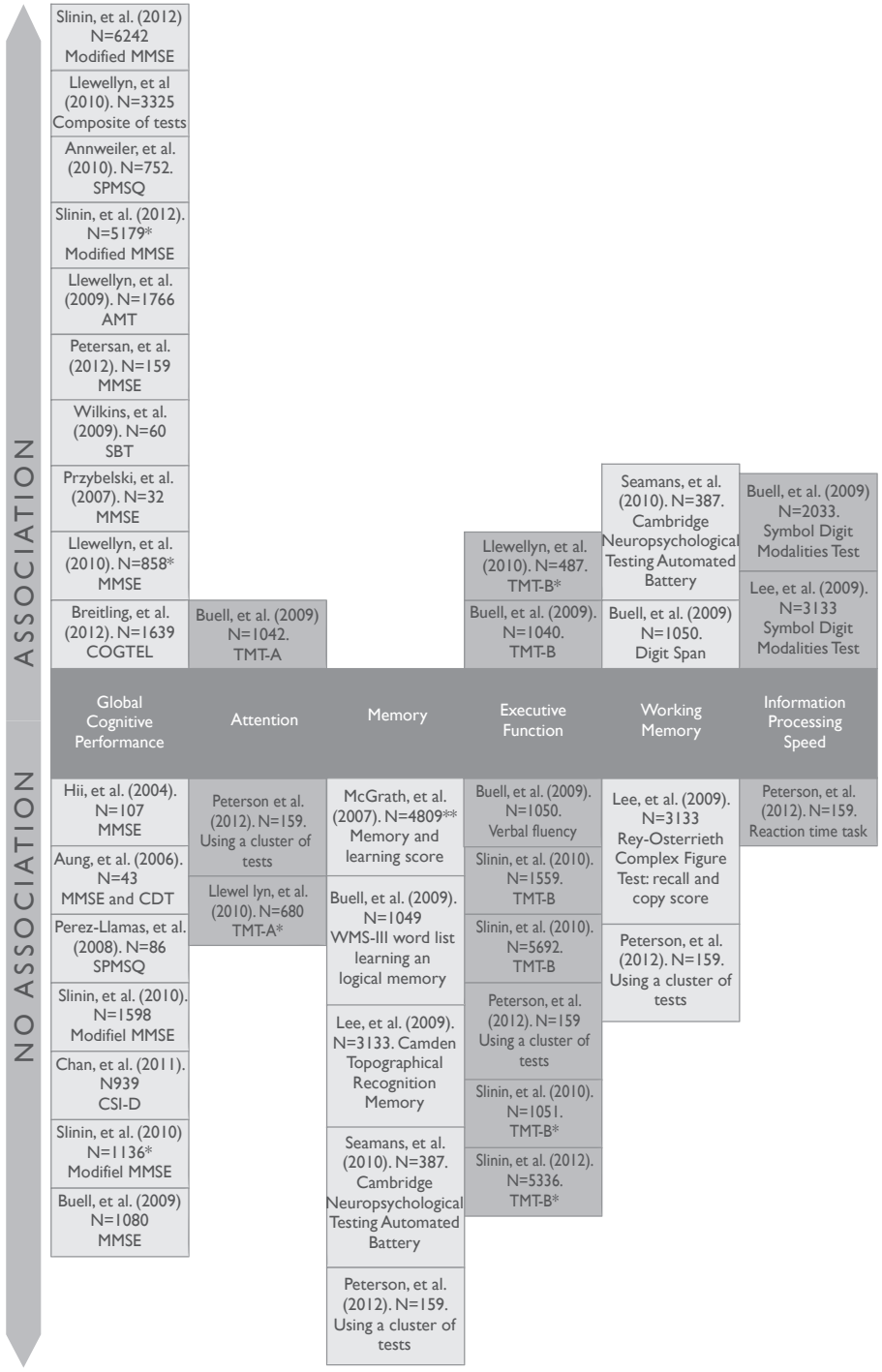

Figure 2. Schematic overview of the observational studies that examined the association between 25(OH)D concentration and cognitive performance. * Prospective study.

Depression

Six observational studies have been published that examined the association between 25(OH)D status and depression in populations aged ≥60 years [81, 101-105] (**Table 4**). Data of the Longitudinal Aging Study Amsterdam (LASA) and the Health Survey for England (HSE) both showed a significant association between lower 25(OH)D concentrations and more depressive symptoms [101, 104]. Likewise, the Os Study, including 883 Chinese men, showed an association between 25(OH)D and depressive symptoms at baseline (i.e. OR 0.46 (95% CI 0.22-0.98) in the highest 25(OH)D quartile with the lowest quartile serving as the reference), but not after 4-years of follow-up [81]. Longitudinal findings of the InCHIANTI study indicated that women with 25(OH)D concentrations <50 nmol/L were significantly more likely to report increases in CES-D scores than women with 25(OH)D concentrations ≥50 nmol/L, specifically 2.05 (0.9) and 2.19 (1.1) points higher after 3 and 6 years, respectively [103]. Accordingly, a statistically significant higher percentage of depression in persons with 25(OH)D levels <25 nmol/L, when compared to those with concentrations ≥25 nmol/L, was shown in the Older Americans Act Nutrition Program (OAANP) [102]. In a population of geriatric primary care patients those with 25(OH)D concentrations <25 nmol/L were twice as likely to be diagnosed with depression than those with 25(OH)D concentrations ≥25 nmol/L [105]. However, although these studies suggest an association between 25(OH)D concentration and depression in older adults, these associations should be interpreted cautiously as residual confounding is likely. To date, only a few well-designed RCTs have been conducted to investigate the effect of vitamin D treatment on depression, and results are uncertain [106].

1.5 Glucose homeostasis: a potential indirect pathway linking vitamin D with cognitive performance and depression?

The aetiology of cognitive decline and depression is in all probability a complex interplay between a variety of factors, and it is unlikely that vitamin D deficiency alone results in cognitive decline and depression. It has amongst others been proposed that vitamin D deficiency may render the brain more susceptible to other stressors. Glucose intolerance may be considered to be such a stressor [107, 108]. Based on this theory it is hypothesized that the magnitude of the association between vitamin D and cognitive performance/decline and depression differs between persons with a normal glucose tolerance and persons with impaired glucose tolerance. Baron and Kenny (1986) defined such a factor (glucose intolerance) that affects the strength of the association between a predictor (vitamin D) and an outcome variable (cognitive performance/depression) as being an effect modifier [109]. In several chapters in this thesis the potential modification effect of impaired glucose tolerance is examined. The hypothesis of effect modification is considered to be supported when the interaction term between the exposure (vitamin D) and the suggested modifier (glucose intolerance) is significant (**Figure 3**) [109], thus, when the magnitude of the association significantly differs between the examined groups, which are defined based on the hypothesized modifier.

Table 4. Observational studies on the association between 25(OH)D and depression in aged populations (≥60 years).

Study	Country	N	Sex	Age (yrs)	25(OH)D assay	Depression assessment	25(OH)D <50 nmol/L	Possible depression*	Association?
LASA [101]	NL	1282	♂-♀	≥65	CPBA	CES-D	48%	18%	+
InCHIANTI [103]	I	954	♂-♀	≥65	RIA	CES-D	62%	♂ 18% ♀ 42%	+ (♀)
Os study [81]	CN	883 (b) 629 (fu)	♂	≥65	RIA	GDS-15	6%	4% incidence rate during follow-up	+ (b) - (fu)
OAANP [102]	USA	159	♂-♀	μ=77	RIA	GDS-30	45%	<25 nmol/L 25% 25-50 nmol/L 12% ≥50 nmol/L 23%	+ (<25 vs. ≥25 nmol/L) - (<50 vs. ≥50 nmol/L)
HSE [104]	GB	2070	♂-♀	≥65	RIA	GDS-10	51%	25%	+ (<25 /L vs. ≥25 nmol/L) - (<50 vs. ≥50 nmol/L) - (<75 vs. ≥75 nmol/L)
Geriatric primary care patients [105]	USA	1618	♂-♀	≥60	LC-MS	HICDA	17% <60 nmol/L	43%	+

Note: HICDA: Hospital International Classification of Disease Adaptation. *No standardized measure of depression could be obtained from the studies, so the prevalence depends on the screening tool and cut-off used by the researchers of a specific study.

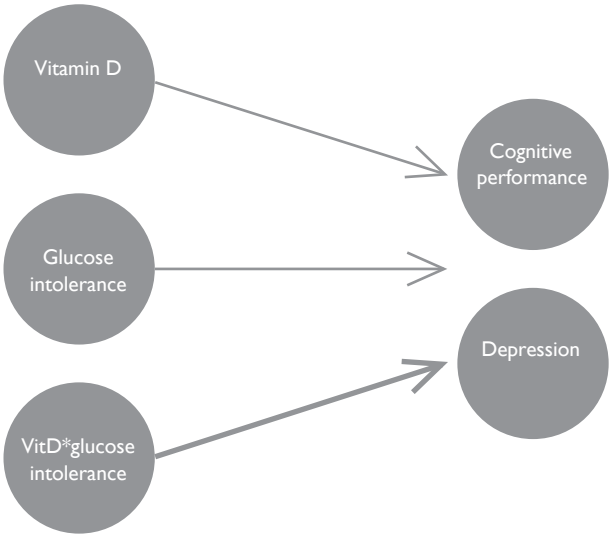

Figure 3. Visualisation of the hypothesized interaction between vitamin D and glucose homeostasis in the association with cognitive performance and depression.

However, given the proposed beneficial effects of vitamin D on insulin secretion and action [9, 110], it may also be that glucose intolerance acts as an intermediate in the pathway linking vitamin D to cognitive performance/decline and depression (**Figure 4**).

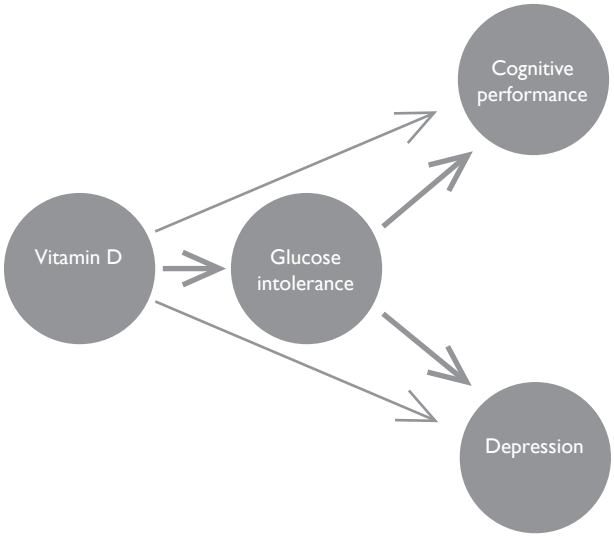

Figure 4. Visualisation of the hypothesized mediatory role of glucose homeostasis in the association of vitamin D with cognitive performance and depression.

To determine whether a factor is an actual mediating factor, several pathways have to be explored: 1) the pathway between the exposure (vitamin D) and the outcome (cognitive performance/depression); 2) the pathway between the exposure (vitamin D) and the mediator (glucose intolerance); 3) the pathway between the mediator (glucose intolerance) and the outcome (cognitive performance/depression); and 4) the pathway between the exposure (vitamin D) and the outcome (cognitive performance/depression) controlled for the hypothesized mediating factor (impaired glucose tolerance). According to Baron and Kenny (1986) a factor can be considered a mediator when it: 1) is 'influenced' by the exposure, 2) 'affects' the outcome, and 3) significantly reduces the association between the exposure and the outcome when controlled for [109].

1.6 To wrap up….

It may be clear that there is quite some scientific evidence suggesting a link between vitamin D and brain function, but the exact role of vitamin D and its specific pathway is far from well defined. To complement to the scientific literature, we conducted several studies on the possible relationship of vitamin D with cognitive performance and depression. In **chapter 2** we start off with a report of a discussion between vitamin D experts that was held to make a step towards the harmonization on the formulation of optimal intake and serum 25(OH)D concentrations across Europe. In **chapter 3** we explore how well a population of community-dwelling Dutch older adults adheres to the in chapter 2 formulated recommendations, followed by the identification of potential intervention targets. Subsequently, the cross-sectional association of vitamin D with global cognitive performance and depression is examined in an apparently healthy sample of European older adults aged 70-75 years (**chapter 4**). Next, the potential role of vitamin D in domain-specific cognitive performance and depression is studied in Dutch pre-frail and frail older adults aged ≥65 years (**chapter 5**). Associations of vitamin D with domain-specific cognitive performance (**chapter 6**), depression (**chapter 7**) and brain volume (**chapter 8**) are also explored in a relatively large group of seemingly healthy Dutch community-dwelling older adults aged ≥65 years. Finally, the results of a proof of principle study on the effect of a vitamin D deficient diet on behaviour in old C57BL/6 mice are reported (**chapter 9**). In most chapters also the potential interplay between vitamin D and glucose homeostasis is further explored. In **chapter 10** an overall reflection is given on the studies that are presented in chapter 2-9.

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2

Vitamin D. Do we get enough? A discussion between vitamin D experts in order to make a step towards the harmonisation of dietary reference intakes for vitamin D across Europe

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2

Abstract

Several groups at risk for developing vitamin D insufficiency have been identified. Accordingly, reviews indicate that a significant percentage of the population worldwide have serum 25-hydroxyvitamin D (25(OH)D) concentrations <50 nmol/L. In addition to the role of vitamin D in bone health, recent studies suggest that it may play a pivotal role in other systems, for instance the cardiovascular system, pancreas, muscle, immune system and brain. Most evidence, however, is obtained from observational studies and yet inconclusive. To exchange and broaden knowledge on the requirements for vitamin D and its effect on various health outcomes, a workshop entitled "Vitamin D Expert Meeting: Do we get enough?" was organized. Acknowledged experts, mainly European, in the field of vitamin D were brought together in order to discuss the recent advances. Despite low 25(OH)D concentrations worldwide, consensus on the definition of deficiency is not yet reached. In order to define cut-off points for vitamin D while taking into account extraskeletal health effects, randomized controlled trials (RCTs) in these fields are warranted. The experts do emphasize that there is evidence to suggest an important role for vitamin D in the maintenance of optimal bone health at all ages and that vitamin D supplementation, in most studies co-administered with calcium, reduces fracture risk in the senior population. To reach a serum 25(OH)D concentration of 50 nmol/L older adults aged ≥65 years are therefore recommended to meet a mean daily vitamin D intake of 20 µg (800 IU), which is best achieved with a supplement.

2.1 Introduction

Studies from all over the world clearly report that an inadequate vitamin D status is a global issue, both in the developed as well as in the developing world. Individuals at risk for vitamin D deficiency are infants, young children, veiled women, persons with a coloured skin, older adults and persons who live at high latitudes [1, 2]. In some studies 40% up to 100% of the older adults have been diagnosed with an insufficient 25(OH)D status [2, 3]. In hip fracture patients aged ≥65, 80% appeared to have 25(OH)D concentrations <50 nmol/L. Less than 5% reached a serum concentrations ≥75 nmol/L, considered by some as optimal fracture reduction [4, 5].

Vitamin D is best known for its role in calcium metabolism and bone health, but recent studies suggest a much broader role of vitamin D [6]. Evidence from clinical trials among older adults suggest a benefit of vitamin D supplementation in both musculoskeletal function and fall prevention, but evidence is inconclusive (reviewed in [7-9]). Vitamin D has also been associated with glucose and fat metabolism, cognitive functioning, immune function, cancer, and cardiovascular disease (reviewed in [2, 10-15]). In addition, two meta-analysis of prospective cohort studies showed that lower 25(OH)D concentrations were associated with a higher mortality risk [16, 17]. On the other hand, Zittermann and colleagues do mention that two of the studies included in their meta-analyses suggest that overabundant 25(OH)D concentrations may also increase mortality risk [16].

Although vitamin D can be obtained from food, its main source is not the diet. In fact, nutritional sources are rare and largely limited to fatty fish, such as salmon. Vitamin D is synthesized in the skin when ultraviolet-B (UV-B) radiation targets the skin at a wavelength between 290-315 nm. However, at latitudes above 33°N (all of Europe) UV-B radiation is only effective during the summer months. The effectiveness of UV-B depends furthermore on the time of the day. During the summer early morning and late afternoon UV-B radiation is also not strong enough to activate vitamin D production. Furthermore, the production of vitamin D in the skin decreases with age [18]. Skin protection, which is broadly practiced, may further reduce skin production of vitamin D [19]. Accordingly, studies suggest that in many countries 25(OH)D concentrations are <50 nmol/L, especially during the winter months (summarized in [1, 8]). Consensus on the optimal dietary intake is, however, not yet reached (**Table 1**) [8, 20-24]. Doets et al. (2008) summarized the current recommended vitamin D intakes across Europe and showed that there is a large variation in these recommendations, for instance the recommendation for Russian men ≥70 years, last updated in 1991, is set at 2.5 µg/day, whereas this is 15 µg/day in Iceland and Spain, which were updated in 2006 and 2007, respectively [25].

Circulating 25(OH)D has the longest half-life (3-6 weeks) of the vitamin D metabolites and represents both sunshine and dietary sources, and is therefore considered to be the best biomarker of vitamin D status. Although there is no unanimity yet on the optimal concentrations of serum 25(OH)D, current evidence discussed by the Institute of Medicine (IOM) suggests a serum 25(OH)D concentration >50 nmol/L as being sufficient [8]. Guidelines as provided by the International Osteoporosis Foundation (IOF) [26] and

Endocrine Society (ES) [22] suggest that for optimal fracture prevention at older age a threshold of 75 nmol/L is desirable.

Table 1. Overview of vitamin D recommendations.

	Children 0-1 yr	Children 1-2 yr	Children 2-3 yr	Children 4-10 yr	Children 11-18 yr	19-50 yr	51-60 yr	61-70 yr	>70 yr	Pregnant/ lactating (>18 yr)
Institute of Medicine (IOM) [a]	10	15	15	15	15	15	15	15	20	15
Endocrine Society [b]	10 (10-25)	15 (15-25)	15 (15-25)	15 (15-25)	15 (15-25)	15 (37.5-50)	15 (37.5-50)	15 (37.5-50)	20 (37.5-50)	20 (37.5-50)
DACH [c]	10	20	20	20	20	20	20	20	20	20
Health Council of the Netherlands [d]	10	10	10	10	10	10	10	10	20 [f]	10
Belgian Health Council [e]	10	10	10	10	10-15	10-15	10-15	10-15	15	20
Nordic Dietary Recommendations [f]	10	10	7.5	7.5	7.5	7.5	7.5	10	10	7.5

Note: All values are presented in µg/day; [a] RDA; [b] Daily requirement Endocrine Society for healthy subjects (line 1). Between brackets: recommendations Endocrine Society for subgroups at risk for vitamin D deficiency (line 2). Recommendations are mainly based on lower quality evidence, therefore they should be considered as suggestions for patient care; [c] German, Austrian and Swiss vitamin D recommendations (AI). With exception to the recommendations for young children, recommendations are for persons with an inadequate endogenous vitamin D synthesis; [d] AI for persons with inadequate endogenous vitamin D synthesis; [e] RDA; [f] Recommended daily intake (RI).

2.2 Meeting

To exchange new scientific insights on vitamin D and the resulting implications for the requirements of vitamin D, considering vitamin D's broader effect on human health, a conference entitled "Vitamin D Expert Meeting: Do we get enough?" was held on September 29th, 2011 in Ede. Acknowledged experts in the field of vitamin D were brought together in order to discuss the recent scientific advances in the field. This expert meeting was organized by Wageningen University and Research Centre in close collaboration with DSM. Invitations were primarily send to European experts. Attendees were selected according to their specialisation/health outcome and their availability at the time of the meeting. The Institute of Medicine (IOM) was invited to take US's reflections on board. The program included presentations about the Dietary Reference Intake (DRI) in North America by CJCG, global vitamin D status by ES, insights from epidemiological studies by EH, the possible relation with various metabolic processes and diseases by HAB-F, EJMF, DJL, and LCPGMdG, and the interaction between diet, sunlight and 25(OH)D by SL-N. Furthermore, the question "How to close the gap" was addressed by RB. Sessions were chaired by RB and EJMF.

2.3 The DRI for vitamin D for North America

In November 2010 the IOM presented revised DRI's for calcium and vitamin D, which were last updated in 1997 [8]. Recently, the ES published their views on the treatment and prevention of vitamin D deficiency [22]. When compared to the recommendations as set by the IOM, the ES advocates higher intake levels in pregnant and lactating women, persons with obesity and those using specific medications. According to one of the workshop participants screening all the individuals 'at risk' according to the ES guidelines would involve about 50 million individuals, just in the USA. During the meeting an overview of the similarities and differences of the two reports was given and inconsistencies were discussed. For instance, the IOM focussed its conclusions on RCTs and considers 25(OH)D concentrations ≥50 nmol/L to be sufficient for more than 97.5% of the population. Based on the effects of vitamin D on fracture and fall reduction, serum parathyroid hormone (PTH) and pathologic osteoid formation the ES advocates for a concentration ≥75 nmol/L.

The individual effect of vitamin D on fracture risk is difficult to assess, as most fracture trials that gave a higher dose vitamin D (20 μg/day) also provided calcium supplements. Of three double-blind placebo-controlled trials that provided vitamin D alone, one that gave 2500 μg in a four monthly interval for five years showed significant fracture reduction [27], one at the same dose level annually (12500 μg) suggested an increased fracture risk [28], and one that gave 7500 μg intra-muscularly showed no benefit regarding fracture risk [29]. In one meta-analysis of double-blind RCTs, the authors compared at the higher intake level (>10 μg/day) the main effect of vitamin D to the combination of vitamin D plus calcium compared to placebo, and found that both pooled effects showed significant non-vertebral fracture reduction of 20% [30].

One of the factors associated with fracture risk is PTH, as it stimulates calcium release from

bone. Low 25(OH)D concentrations have been shown to increase PTH secretion by the parathyroid glands, whereas PTH synthesis is suppressed when low 25(OH)D concentrations are restored. A recent literature review of 70 papers showed that several studies did not identify a ceiling effect for serum PTH with increasing 25(OH)D concentrations. Serum 25(OH)D concentrations varied, however, from 25-125 nmol/L and therefore no specific upper limit could be specified [31].

One large cross-sectional study investigated bone mineral density (BMD) as an endpoint for bone health with respect to 25(OH)D status in both younger and older adults and suggested a positive correlation between 25(OH)D concentrations and hip BMD with optimal concentrations occurring between 75-100 nmol/L [32]. On the other hand, intervention studies showed little increase in BMD in vitamin D-replete participants [33].

2.4 Global Vitamin D Status - The Map

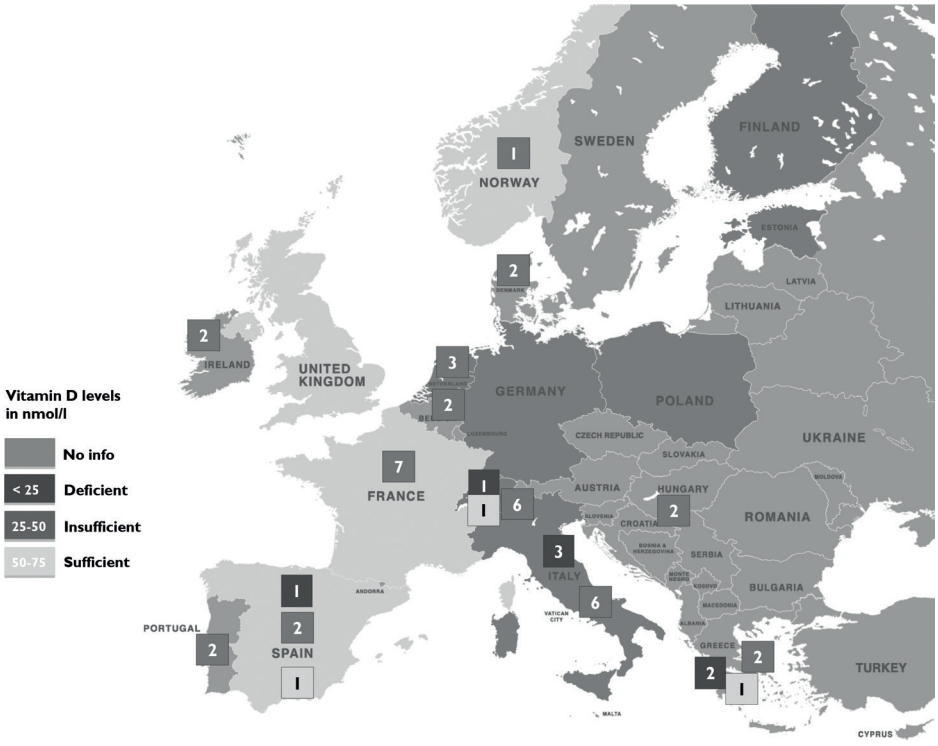

Figure 1. Serum 25(OH)D status across Europe. Based on representative data (full coloured countries) and smaller studies in older adults (number displayed in square).

Vitamin D insufficiency appears to be a common health issue all over the world. Therefore, a global map of 25(OH)D status in the different regions of the world has been launched (2012) by DSM, in partnership with the IOF [34]. During this meeting preliminary results of a European map illustrating the 25(OH)D status within different European countries was presented (**Figure 1**). A literature study resulted in a selection of 80 peer-reviewed papers. A paper was

considered eligible when it contained information on 25(OH)D status either in a representative population based study or in a representative specific age group such as postmenopausal women or older adults. For the general population mean 25(OH)D concentrations between 50-75 nmol/L were observed in Norway, France and Corsica whereas in the Netherlands, Germany, Switzerland, Finland and Estonia the serum 25(OH)D concentrations were in the range of 25-50 nmol/L, revealing insufficiency. These values are below those recommended by the IOM. Data on postmenopausal women were only available of residents of the United Kingdom and Spain, showing average concentrations of 50-75 nmol/L. Mapping of the 25(OH)D status of older adults suggest that this group is at an increased risk for low 25(OH)D concentrations.

2.5 Vitamin D and Health

2.5.1 Insights from epidemiological studies

Interest in the possible health benefits of vitamin D is rapidly growing and has led to an increasing number of papers on the association of 25(OH)D status with several non-skeletal health outcomes. The results of meta-analyses performed within various epidemiological cohort studies were presented. It is clear that environmental factors such as sunlight induced synthesis, oily fish and supplement intake are of importance. However, also genetic make-up has been shown to affect 25(OH)D status. Meta-analyses by the Sunlight Consortium demonstrated a 2.5 increased risk of vitamin D inadequacy for individuals in the top compared to lowest quartile, based on the polymorphisms of a number of genes of the vitamin D pathway, namely those coding the vitamin D binding protein (GC), 25-hydroxylase (CYP2R1) and an enzyme involved in the metabolism of 7-dehydrocholesterol (DHCR7), which is a precursor of vitamin D [35]. Epidemiological studies have provided support for an association between perinatal vitamin D supplementation and the later risk of type 1 diabetes [36], with recent genetic studies suggesting a causal relation [37]. Analyses from epidemiological cohort studies such as the 1958 British Birth Cohort, suggest a link between 25(OH)D status and a wide-range of outcomes including the metabolic syndrome [38], lung function and respiratory infections [39]. However, whether these associations are causal has yet to be demonstrated. Recent published data of an RCT in 182 patients with chronic obstructive pulmonary disease (COPD) showed no effect of vitamin D in the entire cohort, whereas COPD exacerbations were significantly reduced with vitamin D supplementation in patients with baseline 25(OH)D concentrations <25 nmol/L [40].

2.5.2 Falls – evidence from intervention studies

Most evidence suggests a favourable effect of vitamin D in relation to bone health. Therefore, also the latest studies on the possible relation of vitamin D with fall rate and fracture risk were presented during the meeting. Currently, there is disagreement regarding the interpretation of the scientific evidence on vitamin D and falls. Bischoff-Ferrari et al. (2009) state that scientific evidence clearly supports the use of vitamin D in the prevention of falling [7]. However, the IOM strongly disagrees with this point of view. According to the IOM [41] the inclusion procedure of the meta-analyses of eight double-blind RCTs (n=2426) as performed

by Bischoff-Ferrari and colleagues [7] was questionable, the statistical analyses incorrect and data inappropriately presented. Bischoff-Ferrari and colleagues did not agree on the incorrect inclusion of RCTs, but did reanalyse the data in order to account for the stochastic dependencies (correlations) [42] between the corresponding risk ratios in the multiple dosing trial by Broe and colleagues [43], and found a reduction in the odds of falling overall for dose of <17.5 μg/day versus 17.5-25 μg/day, OR 0.73 (95% confidence interval (CI) 0.62 to 0.87). The IOM re-analysed the data of this meta-analysis [8] and concluded that there is lack of sufficiently strong evidence for the formulation of DRIs for vitamin D regarding fall prevention according to their final meta-regression of RCTs showing a relative risk (RR) of 0.95 (95% CI 0.89-1.02) per 2.5 μg/day increase in vitamin D intake [8]. One of the included RCTs even shows an increased fall rate among community-dwelling women aged ≥70 years receiving a single dose of 12500 μg cholecalciferol annually for 3-5 years when compared to the placebo group, RR 1.15 with 95% CI 1.02-1.30 [28]. Also, Glendenning et al. (2011) observed in their nine month trial that 28.9% of the post-menopausal women, assigned to 3750 μg cholecalciferol every three months, experienced at least one fall, whereas this was 26.7% in the placebo group. This difference was, however, not significant (OR 1.06 with 95% CI 0.75-1.49) [44]. RCTs on vitamin D supplementation and falls with sufficient power may help to solve this disagreement.

2.5.3 Fractures – evidence from intervention studies

To date, several meta-analysis have been published on vitamin D supplementation and fracture risk, using different inclusion criteria [8, 30, 45-49]. In the latest meta-analysis of pooled participant-level data of 11 RCTs on oral vitamin D supplementation and fracture prevention, Bischoff-Ferrari and colleagues conclude their paper as follows: "High-dose vitamin D supplementation (≥20 μg/day) was somewhat favourable in the prevention of hip fracture and any non-vertebral fracture in persons ≥65 years". The authors do mention, however, that all trials with vitamin D dosages ≥20 μg/day also supplemented calcium and that therefore the effect of an actual vitamin D intake ranging from 19.8-25 μg/day without additional calcium could not be assessed [49]. Tang and colleagues (2007) reviewed evidence of calcium use and calcium in combination with vitamin D on fracture risk by including 17 trials, showing a significant risk reduction, RR 0.88 (95% CI 0.83-0.95) [48]. Separate analyses of the trials that supplemented calcium only and those supplementing calcium in combination with vitamin D shows that there is only a small and non-significant difference between the two. The authors postulate that this may be because the vitamin D dosages used - 800 IU or lower - were not high enough to be effective in reducing fracture risk. A meta-analysis performed by the US Department of Health and Human Services (UDHHS) including ten double-blinded and three open design trials (n=58,712) investigated the effect of vitamin D supplementation on total fracture risk among postmenopausal women and men aged ≥50 years and did not find a significant fracture reduction (pooled OR 0.90 with 95% CI 0.81-1.02) [46]. The authors suggest that the benefit of vitamin D may depend on additional calcium and may be primarily seen in institutionalized individuals, which is consistent with the meta-analysis of Boonen and colleagues [45]. Also in the DIPART Study,

including seven RCTs in which vitamin D or vitamin D in combination with calcium was used to prevent fractures, Hazard Ratios (HR) of 1.01 (95% CI 0.92-1.12) and 0.92 (95% CI 0.86-0.99) were found for analyses including any fracture, respectively [47]. Subgroup analyses within the analysis by the UDHHS, including four trials reporting 25(OH)D concentrations >74 nmol/L at the end of the study, showed a statistically significant decreased fracture risk for participants reaching those concentrations [46]. These results are consistent with the outcomes of the more recent meta-analysis [30], which included 12 double-blind RCTs for non-vertebral fractures (n=42,279) and eight RCTs for hip fractures (n=40,886). Significant heterogeneity for received dose of vitamin D and achieved concentration of 25-hydroxyvitamin D in the treatment group for hip and any non-vertebral fractures was found [30]. No fracture reduction was observed for a received dose of 10 µg/day or less or achieved 25(OH)D concentrations <74 nmol/L. Conversely, a higher received dose of 12-19 µg/day supplemental vitamin D reduced non-vertebral fractures by 20% (pooled RR 0.80 with 95% CI 0.72-0.89; n=33,265 from nine trials) and hip fractures by 18% (pooled RR 0.82 with 95% CI 0.69-0.97; n=31,872 from five trials). The IOM confirms a significant fracture reduction among those that reach ≥75 nmol/l in the treatment group. However, the IOM questions this finding as different assays were used to measure 25(OH)D concentrations with uncertain accuracy [8, 50, 51]. However, in an earlier meta-analysis of double-blind RCTs Bischoff-Ferrari et al. (2005) argue that despite inter-laboratory differences there would still be a similar trend between higher 25(OH)D status and fracture reduction [52]. The aforementioned differences in interpretation and other methodological differences between studies has resulted in the current discussion on optimal 25(OH)D concentrations [49, 53-57].

2.5.4 Physical performance

Human muscle tissue is also a potential target organ for vitamin D action [58]. Clinical findings in vitamin D deficiency-associated myopathy include proximal muscle weakness, diffuse muscle pain, and gait impairments such as waddling way of walking [59]. RCTs demonstrated that 20 µg/day vitamin D_3 resulted in a 4-11% gain in lower extremity strength or function [60, 61], a 9%-28% improvement in body sway in adults aged ≥65 years after 2-12 months of treatment [61, 62] and in an up to 72% reduction in the rate of falls [43]. In fact, it has been suggested that the benefit of vitamin D on fracture risk may be mediated by the effect on muscle strength and fall prevention [63]. Extending to trials among individuals with a lower risk of vitamin D deficiency and including open design trials, a meta-analysis by Stockton et al. (2011) identified 17 RCTs that tested any form of vitamin D treatment and documented a muscle strength related endpoint. The authors suggested that based on their pooled findings, vitamin D may not improve grip strength, but a benefit of vitamin D treatment on lower extremity strength could not be excluded among individuals with 25(OH)D starting concentrations of >25 nmol/L. In addition, the authors report a significant benefit among two studies with participants that started with 25(OH)D concentrations <25 nmol/L [64]. Muir and Montero-Odasso (2011) also conducted a review of RCTs on the effect of vitamin D supplementation on physical performance and showed an

effect on strength and balance, but not gait. One of the issues addressed by the authors is the possibility of selection bias [9]. The authors speculated that RCTs that excluded persons with specific medical conditions may have resulted in the attenuation of an probable relationship, as the persons with these specific conditions may be the ones experiencing the greatest benefit of supplementation with vitamin D [9]. Mechanistic studies show several pathways through which vitamin D may stimulate muscle mass and muscle strength, including amongst others the promotion of muscle cell proliferation and growth, and an increase in the diameter and the percentage of type II muscle fibres [58]. Moreover, a genomic pathway has been postulated via binding with the vitamin D receptor (VDR) in muscle resulting in de novo protein synthesis [65, 66]. However, a recent publication questions the presence of VDR in muscle tissue as the receptor could not be located in this study while using a highly specific antibody [67].

2.5.5 Insulin Resistance and Type 2 Diabetes

Based on association studies it has been suggested that there is a possible role for vitamin D in the regulation of glucose and insulin concentrations [68-71]. Vitamin D receptors and 1-α-hydroxylase have been identified in the pancreatic β-cell. Via its ability to regulate calcium fluxes, vitamin D may furthermore influence insulin release as well as insulin action. Moreover, by affecting cytokine production, vitamin D may play a beneficial role in β-cell survival and insulin sensitivity. The evidence up to now shows that although animal studies and several epidemiological cohort studies point towards a protective effect of 25(OH)D status on the development of type 2 diabetes [72], RCTs have not yet provided convincing evidence [12]. Mitri and colleagues (2011) discuss that results of observational studies have to be interpreted with caution as they are limited by the possibility of residual confounding and reverse causation. Moreover, studies often rely on only one serum 25(OH)D measurement, while 25(OH)D concentrations fluctuate during the year [12]. The authors also speculate about the inconclusive data from trials. RCTs were often small, not specifically designed to assess the effect of vitamin D on glucose outcomes and used relatively low dosages. Furthermore, it is difficult to account for exposure to other sources of vitamin D, including UV-radiation and oral intake via foods [12].

2.5.6 Cognitive functioning and dementia

A growing body of evidence implicates low serum 25(OH)D concentrations in the pathogenesis of brain dysfunction including multiple sclerosis [13] and stroke [73]. Low 25(OH)D concentrations are also associated with prevalent cognitive impairment and dementia as reviewed by Balion et al. (2012) [11]. For example, in older adults in the Health Survey for England who were severely vitamin D deficient (<25 nmol/L) an almost three times increased odds of cognitive impairment was observed when compared to those who had adequate vitamin D concentrations (>75 nmol/L) [74]. Similarly, severely deficient US older adults in the NHANES were almost four times more likely to be cognitively impaired than those with a sufficient vitamin D status (OR 3.9 with 95% CI 1.5-10.4) [75]. However, cross-sectional studies are unable to exclude the possibility that such associations are a result

of disease progression rather than being causal. Animal and in vitro experiments suggest that vitamin D is neuroprotective through several mechanisms, including vasoprotection and amyloid phagocytosis and clearance [76, 77]. Two large prospective studies go some way to establish the temporal relationship with cognitive decline. The risk of cognitive decline, as assessed with the Mini Mental State Examination (MMSE), was 60% higher in older Italian adults in the InCHIANTI study who are severely deficient when compared with those with sufficient concentrations [78]. After adjustment for age, site and season of blood drawn an OR of 1.41 (95% CI 0.61-3.28) for cognitive decline was observed when Slinin and colleagues (2010) compared older US men in the Osteoporotic Fractures in Men Study in the lowest quartile (<50 nmol/L) with those in the highest quartile (>74 nmol/L), however the association was not significant [79]. Future neuroimaging studies and randomized trials are needed to provide further information about the underlying mechanisms and the efficacy of vitamin D supplements in combating dementia.

2.5.7 Adverse health effects

There is little or no data on toxicity of high doses of vitamin D for more than one year. A safe upper intake of 250 µg/day (10,000 IU/day) based only on serum calcium data is described in the most recently published benefit - risk assessment by Bischoff- Ferrari et al. (2010) [80]. In 2010 the IOM applied a safety factor of 2.5 and defined a safe upper limit of 100 µg/day [8]. A recent cross-sectional post mortem bone histology study showed no pathologic accumulation of osteoid in a mixed German population of men and women with serum 25(OH)D concentrations >75 nmol/L although in 97.5% of the cases abnormal osteoid was only present <50 nmol/L. On the other hand, a reasonable proportion of those with 25(OH)D concentrations <25 nmol/L appeared to have normal bone mineralization, suggesting that it is not possible to directly extrapolate these results to the individual level [81]. Care has to be taken with regard to calcium intake, as a too high calcium intake may increase cardiovascular disease (CVD) risk [82]. Gallagher and colleagues (2012) observed hypercalciuria in 30% of women treated with vitamin D and a calcium intake of 1260 mg/day [83]. Moreover, an increased prevalence of nephrolithiasis was observed in the Women's Health Initiative, in which participants were assigned to 10 µg/day vitamin D in combination with 1000 mg calcium during on average 7 years [84]. At this moment, evidence for an adverse effect of high serum 25(OH)D concentrations is inadequate, possible adverse interactions with high calcium intakes may require further attention.

2.6 Diet, sunlight and 25(OH)D

The interaction between diet and sunlight exposure on 25(OH)D concentrations, functional markers of calcium metabolism, and bone health has been investigated in Asian and Caucasian women living in Surrey, Southern England, participating in the D-FINES Study. Comparing D-FINES data with data of women living in Aberdeen (North England), Macdonald and colleagues (2011) showed that the average 25(OH)D concentrations were 10 nmol/L lower during the winter period compared to women living at lower latitude [85]. Although there

was three times as much rainfall in the summer of 2007, when compared to the summer of 2005 and 2006, spring 25(OH)D concentrations in 2008 were not significantly affected. Caucasian women had consistently higher serum 25(OH)D concentrations than Asian women. Premenopausal status was associated with a 6 nmol/L higher serum 25(OH)D concentration when compared to postmenopausal status. Vitamin D intake levels were on average 2-3 μg/day and did not differ between the two ethnic groups. None of the Asian women was using vitamin D supplements at the start of the study, and exposure to sunlight appeared to be lower when compared to Caucasian women.
As vitamin D_2 and D_3, the two calciferols, display different affinities for the vitamin D binding protein, food fortification with either vitamin D_2 or D_3 may differently affect 25(OH)D status. Studies in the 1930s did not point towards a distinction, but a recent trial comparing large doses of vitamin D_2 versus vitamin D_3 1250 μg/week for 12 weeks does suggest that vitamin D_3 supplementation results in a higher increase of 25(OH)D concentrations over time [86]. Using smaller doses of D_2 and D_3 (25 μg/day), no differences in serum 25(OH)D were shown after 12 weeks of supplementation [87]. Currently, the D_2-D_3 study is conducted to further explore this dissimilarity between ergocalciferol and cholecalciferol. The study aims to compare the efficiency of 15 μg/day of both calciferols, determine whether there is a difference in effect of the calciferol when carried by either solid or fluid foods and disentangle potential underlying mechanisms. Based on aforementioned results it was suggested that dietary vitamin D intakes in the UK may be too low to significantly affect 25(OH)D status. As vitamin D only occurs in a small range of foods, food fortification may be one of the methods to increase dietary vitamin D intakes. Supplementation may, however, be necessary in specific populations like Asian people, which are at an increased risk for developing vitamin D deficiency. In future research it may be interesting to address the question whether there is a metabolic adaptation of populations accustomed to low 25(OH)D concentrations during specific periods of the year.

2.7 How to close the gap - Outlook for Europe

The meeting was concluded with a short overview of the current evidence of vitamin D recommendations and its effect on skeletal health and beyond. The main discussion among vitamin D researchers was related to the optimal 25(OH)D concentrations; do we aim for either concentrations ≥50 nmol/L or concentrations ≥75 nmol/L? The implications are that meeting a concentration of 50 nmol/L requires about 15-20 μg/day (600-800 IU), whereas meeting a concentration of 75 nmol/L requires 40-50 μg/day (1600-2000 IU) [83]. Before reaching a consensus on the optimal DRIs, participants of the meeting stated that the postulated DRIs should preferably be based on RCTs and had to be applicable to the general population. For those at an increased risk for developing inadequate 25(OH)D concentrations a more specific advice had to be formulated. Lastly, recommendations should preferably be harmonized across Europe. Subsequently, the experts concluded that up to now for most health outcomes, except bone health and risk reduction of falls (EFSA claim), there is insufficient evidence with regard to the optimum 25(OH)D concentration or vitamin

D intake. When summarizing the evidence several aspects have to be taken into account. Firstly, most RCT examining the effect of vitamin D on bone health and fall prevention co-administered calcium. The individual effect of vitamin D is therefore difficult to assess. Secondly, in vitamin D supplementation trials it is difficult to account for difference in UV-radiation, which may interact with the relations studied. Thirdly, most human studies on vitamin D and health outcomes beyond bone health were observational, using either cross-sectional or prospective data. Therefore, the possibility of reverse causation and residual confounding cannot be excluded. Fourthly, evidence from in vitro and molecular studies have to be read with caution as they often link 1,25(OH)D concentrations with specific target tissues [88], while 1,25(OH)D is not the optimal biomarker for vitamin D and showing a low correlation with 25(OH)D status [89]. Finally, pooling and comparing studies is difficult due to the lack of standardization of 25(OH)D assays [90]. Most assays used today are able to distinguish between high and low 25(OH)D concentrations, however there may be quite some variation when looking at the absolute concentrations.

2.7.1 Infants and children

Critical analysis of the current scientific evidence shows that, in children, rickets is preventable by a dose of 10 µg vitamin D each day, which is in consensus with the current guidelines for this target group in the US, UK, Belgium and the Netherlands. Therefore, the consensus dose for both infants and children was set at 10 µg/day. Difficulties in implementing these recommendations, however, include that more and more vitamin D enriched products are sold.

2.7.2 Adults and elderly

Osteoporotic fracture risk has been shown to decrease with vitamin D intakes of 12-19 µg/day [30], in combination with an adequate calcium intake. Direct and indirect evidence indicates furthermore that 25(OH)D concentrations of ≥50 nmol/L are desired and not contraindicated with regard to other health outcomes, including the optimal functioning of the parathyroid glands/PTH secretion [91, 92], calcium metabolism [93, 94] and BMD [95-97]. Moreover, valid hypotheses based on preclinical data and in vitro studies have been raised for a role of vitamin D in the development of extra-skeletal diseases, including respiratory infections, tuberculosis, multiple sclerosis, cancer, diabetes and CVD. RCTs, however, are needed to verify these results. As supplementation of 15-20 µg/day has been shown to increase 25(OH)D concentrations ≥50 nmol/L and since there is, at present, insufficient evidence supporting an amplified beneficial role of vitamin D with 25(OH)D serum concentrations >50 nmol/L, the meeting considered a vitamin D intake of 20 µg/day to be sufficient in a population of healthy adults and elderly, which is in line with the recently published DACH recommendations [20]. To date, there is insufficient data on the optimal 25(OH)D concentrations in order to achieve the maximum peak bone mass in adolescence and young adulthood, therefore no specific DRI was formulated for this age group. Issues remaining after the discussion were related to the safety level of intakes higher than 50 µg/day during a longer period, whether a higher dose of vitamin D eliminates the need for calcium

supplements and how to implement and increase the compliance to current guidelines. In conclusion, there is evidence suggesting that vitamin D - besides its established role in bone health - may contribute to reduce the risk of a variety of chronic diseases to benefit human health, but more evidence is warranted. It is generally felt that in adults a daily intake of 20 µg vitamin D is a safe dose to assure an appropriate vitamin D status.

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Attendees

Elske M Brouwer-Brolsma (Minutes secretary), Wageningen University, the Netherlands; Heike Annette Bischoff-Ferrari (Speaker), UniversitätsSpital Zurich, Switzerland; Roger Bouillon (Chair), Katholieke Universiteit Leuven; Edith JM Feskens (Chair), Wageningen University, the Netherlands; Chris J Gallagher (Speaker), Division of Endocrinology Creighton, United States of America; Elina Hypponen (Speaker), Institute of Child Health, United Kingdom; David J Llewellyn (Speaker), Institute of Biomedical and Clinical Sciences Peninsula College of Medicine and Dentistry University of Exeter Royal Devon and Exeter Hospital Barrack Road Exeter, United Kingdom; Elisabeth Stoecklin (Speaker), DSM Nutritional Products, Switserland; Susan Lanham-New (Speaker), University of Surrey, United Kingdom; Jutta Dierkes, University of Bergen, Norway; Arie K Kies, DSM Biotechnology Center, the Netherlands; Frans J Kok, Wageningen University, the Netherlands; Christel Lamberg-Allardt, University of Helsinki, Finland; Ulrich Moser, Switzerland; Henk van den Berg, the Netherlands; Stefan Pilz, Medical University of Graz, Austria; Wim H Saris, DSM Nutritional Products, the Netherlands; Natasja M van Schoor, VU University Medical Center, the Netherlands; Peter Weber, DSM Nutritional Products, Switzerland; Renger Witkamp, Wageningen University, the Netherlands; Armin Zittermann, Ruhr University Bochum, Germany; Lisette CPGM de Groot (Speaker), Wageningen University, the Netherlands.

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3

Relative importance of summer sun exposure, vitamin D intake and genes to vitamin D status in Dutch older adults: the B-PROOF study

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Abstract

Background

Sun exposure and vitamin D intake are known to have the ability to raise serum vitamin D concentrations. Despite this knowledge, the prevalence of vitamin D deficiency is high and particularly older adults are at risk. To improve the current prevention and treatment strategies, this study aims to identify determinants of vitamin D status in a population of community-dwelling Dutch older adults.

Methods

Serum vitamin D (25(OH)D) was measured in 2857 older adults aged ≥65 years. Sun exposure was assessed using a structured questionnaire. Dietary vitamin D intake and supplement use were assessed with a Food Frequency Questionnaire. Relevant single nucleotide polymorphisms (SNPs) were genotyped using the Illumina Omni-express array.

Results

Serum 25(OH)D concentrations <50 nmol/L were observed in 45% of the population, of which only 6% reported vitamin D supplement use. Higher summer sun exposure (outside daily two weeks prior to blood sampling: 66±25 nmol/L vs. not outside daily two weeks prior to blood sampling: 58±27 nmol/L, F_{633}=5.6, P=0.02) and vitamin D intake (summer: β 1.0±0.4 μg/day, P=0.02, winter: β 3.1±0.8 μg/day, P<0.0001) were associated with higher 25(OH)D concentrations. Major allele carriers of SNPs related to DHCR7, CYP24A1, GC, and CYP2R1 minor allele carriers had the highest 25(OH)D concentrations. Age, sex, BMI, education, alcohol consumption, smoking, physical activity level, self-rated health status, sun exposure (R^2=0.16), vitamin D intake (R^2=0.12) and vitamin D related genes (R^2=0.15) together explained 35% (R^2=0.35) of the variation in 25(OH)D concentrations during the summer period.

Conclusion

Of the three main determinants under study, sun exposure was the most important determinant of serum 25(OH)D, closely followed by genes and vitamin D intake. Given the low frequency of vitamin D supplement use, promoting supplement use may be an inexpensive, easy and effective strategy to fight vitamin D deficiency.

3.1 Introduction

In the Netherlands about half of the community-dwelling older adults have a vitamin D status (25(OH)D) <50 nmol/L [1] and are classified as having an insufficient vitamin D status according to recent guidelines of the Institute of Medicine (IOM) [2]. In order to tackle this issue of low 25(OH)D concentrations it is important to gain knowledge on its main determinants.

With a limited number of foods that contain vitamin D, one of the sources of vitamin D is the diet. However, vitamin D is mainly acquired through sunlight exposure, specifically ultraviolet-B radiation (UV-B), which activates the cutaneous synthesis of pre-vitamin D_3 in the skin [3]. The efficiency of sun exposure and vitamin D intake to increase 25(OH)D concentrations depends on a variety of factors, including latitude, season, air pollution, sunscreen use, skin pigmentation, age, efficiency of absorption in the gut, liver and kidney functioning, and medication use [4]. To illustrate this, at higher latitudes (i.e. >50°N) the intensity of UV-B during the winter months is assumed to be too low to activate the vitamin D synthesis in the skin [5]. It is also shown that 25(OH)D concentrations decrease with age, due to a decrease in cutaneous vitamin D synthesis [6]. Also, genetic make-up has been associated with vitamin D metabolism and variations in 25(OH)D concentrations [7]. Thus, 25(OH)D concentrations depend on a broad variety of factors ranging from environmental factors to genetics. Despite this knowledge, vitamin D deficiency is observed worldwide [8, 9], especially among older adults [10].

This study is performed to assess the prevalence of vitamin D deficiency, and to examine the associations of vitamin D intake, vitamin D supplement use, sun exposure, and genetic variance with 25(OH)D concentrations in a population of community-dwelling Dutch older men and women aged ≥65 years. Identification of the relative contribution of these factors to 25(OH)D status, might help to pinpoint important determinants in the prevention and treatment of vitamin D deficiency.

3.2 Methods

3.2.1 Participants

This cross-sectional study was performed using baseline data of the B-PROOF study; a randomized double-blind, placebo-controlled trial designed to assess the efficacy of daily oral supplementation with vitamin B_{12} and folic acid on fractures in mildly hyperhomocysteinemic (plasma homocysteine 12-50 μmol/L) older adults ≥65 years. Details of this study have been reported elsewhere [11]. Data on 25(OH)D concentrations were available of 2857 participants. Genetic information on vitamin D related genes was obtained from 2530 participants. Sun exposure was assessed in 1012 participants and vitamin D intake in 596 participants. The Medical Ethics Committee of Wageningen UR approved the study protocol and the Medical Ethics Committees of VUmc and Erasmus MC confirmed local feasibility. All participants gave their written informed consent.

3.2.2 Dietary assessment

Dieticians at the division of Human Nutrition at Wageningen University developed a 190-item Food Frequency Questionnaire (FFQ) to measure vitamin D intake and vitamin D supplement use. The questionnaire was developed based on two validated FFQs [12, 13], and updated to include vitamin D intake by means of the Dutch FFQ-TOOL™. Specifically, food items contributing to ≥0.1% of the total intake of vitamin D intake were included, which was estimated to cover 80% of total vitamin D intake based on the Dutch National Food Consumption Survey of 1998 [14].

3.2.3 Sun exposure

Habitual sunlight exposure was assessed using a questionnaire that was administered on the day of blood sampling. Data were collected on the amount of time spent outdoors and in the sun during the summer, use of sun protection and solariums, type of clothing worn during the summer, and holidays with a sunny destination in the three months prior to blood sampling.

3.2.4 Biochemical analyses

Blood samples were collected in the morning when the participants were fasted or had consumed a restricted breakfast, and collected throughout the year. Samples were stored at -80˚C until determination. Serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) [15].

3.2.5 Genotyping

DNA was isolated from buffy coats. Samples were genotyped for about 700000 SNPs using the Illumina Omni-express array, covering >90% of all common variations in the genome. SNPs selected for this study included rs12785878 (DHCR7), rs6013897 (CYP24A1), rs10741657 (CYP2R1) and rs2282679 (GC), which was based on a genome-wide association study on relations between genes and serum 25(OH)D concentrations by Wang and colleagues (2010) [7].

3.2.6 Covariates

Height was measured at baseline with a stadiometer to the nearest 0.1 cm. Weight was measured to the nearest 0.5 kg with a calibrated analogue scale, while wearing light cloths. Body Mass Index (BMI) was calculated as weight/height^2. Data on educational level (years), smoking status (never, current, former), physical activity (kcal/day) [16], and alcohol consumption (light, moderate, excessive) [17] were collected by means of questionnaires. Self-rated health was obtained from the Short-Form Health Survey (SF-12) [18]. Season of blood collection was dichotomized into summer/autumn (June-November) and winter/spring (December-May).

3.2.7 Statistical Analyses

Participants characteristics are reported as mean with standard deviation (SD), or

percentages. To compare baseline characteristics of participants with inadequate serum 25(OH)D concentrations (<50 nmol/L) with participants with adequate serum 25(OH)D concentrations (≥50 nmol/L), chi-squared tests for categorical variables and independent t-tests for continuous variables were performed. To assess the association between the total vitamin D intake and serum 25(OH)D status multiple linear regression analyses were conducted with adjustment for age, sex, BMI, years of education, alcohol consumption, smoking, physical activity and self-rated health status, stratified by season. Analyses of Covariance (ANCOVA) was used to explore the associations between sunlight exposure and serum 25(OH)D with adjustment for age, sex and BMI, and stratified by season. Associations between vitamin D related genetic make-up and 25(OH)D concentrations were tested using ANOVA. In order to further investigate the importance of summer sunlight exposure, vitamin D intake and genes to the variation in serum 25(OH)D concentrations, all three factors for serum 25(OH)D were individually and simultaneously added to the multiple linear regression model, where age, sex, BMI, years of education, alcohol consumption, smoking, physical activity and self-rated health status were included as potential covariates. In view of the seasonal variation in 25(OH)D status, only data obtained during the summer were included in the model. All tests were two-sided (P<0.05). Analyses were performed using the statistical package SPSS, version 21.0 (SPSS Inc., Chicago, IL, USA).

3.3 Results

Descriptive data of the population are shown in **Table 1**. Of 2857 Dutch men and women 45% had a vitamin D deficiency defined as a serum 25(OH)D concentration <50 nmol/L. As expected, stratification for season showed that the prevalence of vitamin D deficiency was higher when blood samples were collected during the winter/spring (63%) than summer/autumn (37%). Participants with vitamin D deficiency were more likely to be older, and woman, to have a higher BMI, and a lower physical activity level, and intake of vitamin D and alcohol. Figure 1 confirms the expected age-related differences in serum 25(OH)D.

Table 1. Descriptive statistics of 2857 Dutch men and women aged ≥65 years.

	25(OH)D <50 nmol/L	25(OH)D ≥50 nmol/L	P-value	N
25(OH)D, nmol/L	34±10	74±18	<0.0001	2857
Age, years	75.1±7.1	73.2±5.9	<0.0001	2857
Sex, n (% men)	597 (42)	831 (58)	<0.0001	2857
BMI, kg/m²	27.5±4.3	26.8±3.6	<0.0001	2842
Physical activity level, kcal/day	598±440	691±502	<0.0001	2842
Education, years	9.8±3.9	10.3±4.0	0.11	2855
Smoking, n (%)			0.01	2857
• **Never**	459 (36)	510 (32)		
• **Current**	142 (11)	135 (9)		
• **Former**	690 (53)	921 (59)		

	25(OH)D <50 nmol/L	25(OH)D ≥50 nmol/L	P-value	N
Alcohol consumption, n (%)			<0.0001	2855
• **Light**	925 (72)	998 (64)		
• **Moderate**	318 (25)	505 (32)		
• **Excessive**	46 (3)	63 (4)		
Self-experienced health, n (%)			<0.0001	2855
• **Excellent**	79 (6)	130 (8)		
• **Very good**	230 (18)	386 (25)		
• **Good**	787 (61)	874 (56)		
• **Mediocre**	183 (14)	170 (11)		
• **Poor**	11 (1)	5 (0)		
Blood sampling, n (%)			<0.0001	2857
• **December until May**	813 (63)	543 (35)		
• **June until November**	478 (37)	1023 (65)		
Vitamin D supplement use, n (%)	174 (6)	411 (14)	<0.0001	2857
Total vitamin D intake, µg/day	4.2±2.1	5.2±3.2	<0.0001	596
Vitamin D intake from foods, µg/day	4.0±1.9	4.5±2.1	0.002	596

Note: values are expressed as a mean ± SD, median (IQR) or n (%).

3.3.1 Sunlight

Figure 1 shows a clear fluctuation in serum 25(OH)D throughout the year. Additionally, ANCOVA indicates that all sunlight measures are significantly associated with serum 25(OH)D in participants that are included during the summer months, while taking into account age, sex and BMI (**Table 2**) (n=1012). Specifically, 'daily outside two weeks prior to blood sampling' (F_{633}=5.6, P=0.02), 'daily outside during summer' (F_{633}=4.9, P=0.03), 'clothing' (F_{621}=19.5, P<0.0001), 'sun holiday' (F_{631}=18.9, P<0.0001), 'sun lamps' (F_{628}=13.6, P<0.0001) and 'sunscreen use' (F_{631}=5.8, P<0.01). Associations for 'daily outside two weeks prior to blood sampling' (F_{362}=4.1, P=0.04) and 'sun lamp use' (F_{360}=11.0, P<0.01) with serum 25(OH)D were less strong, but still significant, in participants that were included during the winter months. 'Daily outside during summer' (F_{359}=1.0, P=0.32), 'clothing' (F_{355}=3.0, P=0.09), 'sun holiday' (F_{358}=4.0, P=0.05) and 'sunscreen use' (F_{359}=3.0, P=0.05) were not significant anymore during the winter.

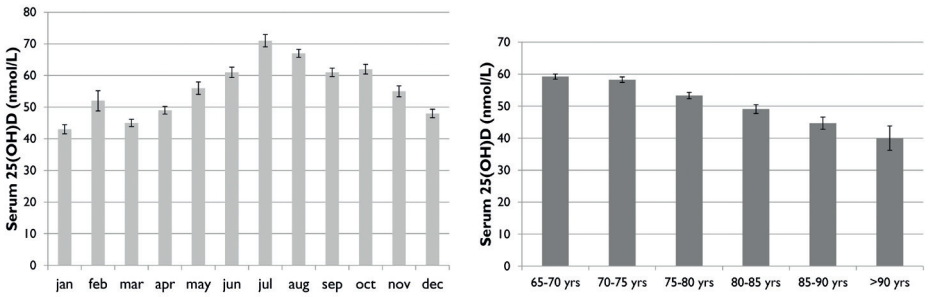

Figure 1. Serum 25(OH)D distribution (mean±SE) per month and per age group in Dutch men and women aged ≥65 years.

Table 2. Associations between sun exposure and serum 25(OH)D of 1012 Dutch men and women aged ≥65 years, stratified for season.

	Summer		Winter	
	25(OH)D	F_{df}, P-value	25(OH)D	F_{df}, P-value
Daily outside 2 weeks before blood sampling		F_{633}=5.6, P=0.02		F_{362}=4.1, P=0.04
• **No**	58±27		41±19	
• **Yes**	66±25		47±21	
Daily outside during summer		F_{633}=4.9, P=0.03		F_{359}=1.0, P=0.32
• **No**	57±25		42±20	
• **Yes**	66±25		46±21	
Clothing		F_{621}=19.5, P<0.0001		F_{355}=3.0, P=0.09
• **Long sleeved**	51±27		42±22	
• **Short sleeved**	66±25		47±20	
Sun holiday in 3 months before blood sampling		F_{631}=18.9, P<0.0001		F_{358}=4.0, P=0.05
• **No**	62±25		44±21	
• **Yes**	73±23		51±18	
Use of sunlamps		F_{628}=13.6, P<0.0001		F_{360}=11.0, P<0.01
• **No**	64±26		44±20	
• **Yes**	78±20		59±27	
Sunscreen use		F_{631}=5.8, P<0.01		F_{359}=3.0, P=0.05
• **Always**	69±24		51±22	
• **Sometimes**	67±26		45±18	
• **Never**	60±25		43±22	

Note: Serum 25(OH)D concentrations (nmol/L) are displayed as means±SD. Models are adjusted for age, sex and BMI.

3.3.2 Vitamin D intake

Mean vitamin D intake was 4.9±2.9 μg/day. Vitamin D intake was significantly associated with serum 25(OH)D; stratification for season revealed that the association between vitamin D intake and 25(OH)D status was more pronounced during winter (β 3.1±0.8, P<0.0001) than during summer (β 1.0±0.4, P=0.02). These linear regression coefficients suggest that a unit increase in vitamin D intake is associated with a 3.1 nmol/L increase in serum 25(OH)D during the winter, and with a 1.0 nmol/L increase in serum 25(OH)D during the summer.

3.3.3 Vitamin D related genetic make-up

Figure 2 shows that vitamin D status significantly differed between allele carriers for all genes, except for the CYP24A1 gene. As expected there were difference in 25(OH)D concentrations between summer/autumn and winter/spring, but differences between the alleles were reasonably comparable. The gene GC, which encodes for the protein related to vitamin D transport in the circulation, was most strongly associated with serum 25(OH)D (P<0.0001), indicating that minor allele carriers have the lowest 25(OH)D concentrations (53±20 nmol/L) and major allele carriers the highest 25(OH)D concentrations (68±25 nmol/L).

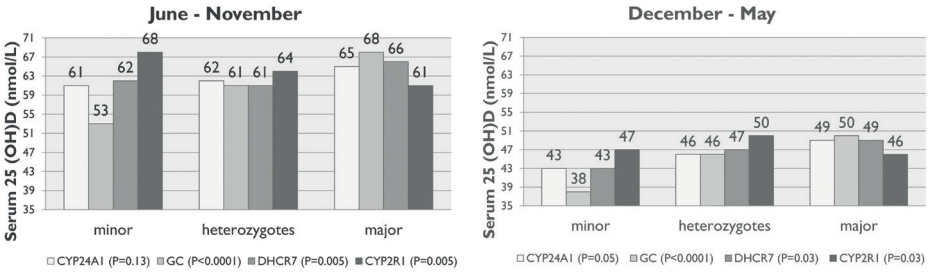

Figure 2. Differences in 25(OH)D concentrations by alleles of vitamin D related genetic make-up analysed using ANCOVA, stratified by period of blood sampling (n=2530). *Note*: June-November: Bonferroni post hoc tests indicate significant differences for DHCR7 [heterozygotes vs. major], CYP2R1 [minor vs. major] and GC [minor vs. heterozygotes, heterozygotes vs. major, minor vs. major]. December-May: Bonferroni post hoc tests indicate significant differences for DHCR7 [minor vs. major], CYP2R1 [heterozygotes vs. major] and GC [minor vs. heterozygotes, heterozygotes vs. major, minor vs. major].

Table 3. Regression analysis to assess the relative importance of vitamin D intake, sunlight exposure and vitamin D related genetic make-up, using data of 185 Dutch men and women aged ≥65 years that were obtained during the summer months.

	β	SE	sβ	P-value
(Constant)	147.7	34.1	-	<0.0001
CYP24A1	4.1	3.1	0.09	0.18
GC	7.4	2.6	0.18	0.005
DHCR7	2.5	2.9	0.06	0.39
CYP2R1	-4.2	2.3	-0.12	0.07
Outside past 2 weeks	15.6	6.2	0.24	0.01
Outside past summer	-9.2	6.5	-0.14	0.16
Clothing worn	2.4	6.7	0.03	0.72
Sunscreen use	-2.2	2.6	-0.06	0.41
Sunlamp use	12.7	5.7	0.14	0.03
Sun holiday	3.7	4.4	0.06	0.40
Vitamin D intake	0.4	0.5	0.06	0.38

Note: The model was adjusted for age (sβ -0.21, P=0.003), sex (sβ -0.15, P=0.06), BMI (sβ -0.21, P=0.004), years of education (sβ -0.12, P=0.08), smoking (sβ -0.03, P=0.66), alcohol consumption (sβ 0.11, P=0.12), physical activity level (sβ 0.05, P=0.45) and self-experienced health (sβ -0.09, P=0.24). sβ = standardized beta.

3.3.4 Sunlight, vitamin D intake, genetic make-up and serum 25(OH)D

Finally, after considering the individual associations of sun exposure, vitamin D intake and vitamin D related genetic make-up with 25(OH)D concentrations, a multiple linear regression analysis was conducted with the data of participants that were included during summer. First of all, the individual R^2 was calculated for sunlight (n=613), intake (n=370) and genes (n=1318) with serum 25(OH)D, while taking into account age, sex, BMI, education, alcohol consumption, smoking, physical activity and self-rated health status. Vitamin D intake explained 12% of the variance in 25(OH)D concentrations, sun exposure 16%, and genes 15%. Together these factors (n=185) were able to explain 35% of the variance in 25(OH)D

concentrations as reflected by an R^2 of 0.35 (adjusted R^2: 0.27), again also age, sex, BMI, education, alcohol consumption, smoking, physical activity and self-rated health status were included in the model (**Table 3** and **Figure 3**).

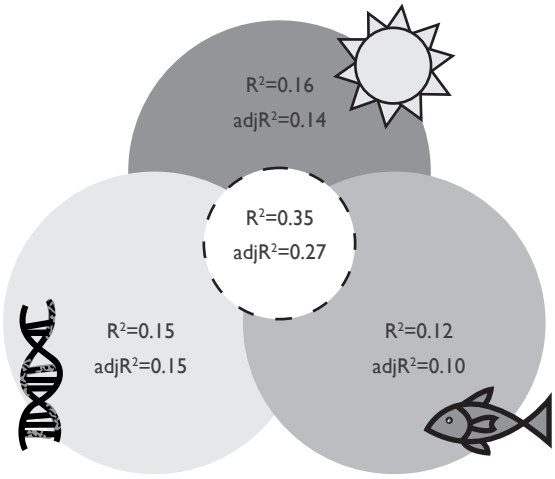

Figure 3. Explained variance per component (n=613 for sunlight exposure, n=370 for total vitamin D intake and n=1318 for genes) and for the total model using data of the people that were included during the summer months (n=185 for the total model), adjusted for age, sex, BMI, years of education, alcohol consumption, smoking, physical activity and self-rated health status.

3.4 Discussion

In this population of community-dwelling older adults living at a latitude of ±52°N, 45% of the participants had 25(OH)D concentrations <50 nmol/L. Total vitamin D intake was far below the recommended levels of 20 μg/day as set by the Dutch Health Council for adults ≥70 years [19]. Supplement use was reported by 20% of the participants. Total vitamin D intake, sunlight exposure and vitamin D related genetic make-up were all significantly associated with serum 25(OH)D. When exploring the individual contribution of the three factors to serum 25(OH)D status, while taking into account covariates, vitamin D intake explained 12% of the variance in 25(OH)D concentrations, sunlight exposure 16% and vitamin D related genetic make-up 15%. Including these three factors simultaneously, again taking relevant covariates into account, explained 35% of the variance in serum 25(OH)D.

3.4.1 Methodological considerations

In order to appreciate these findings several methodological issues of this study warrant further discussion. To the best of our knowledge, this is the first study examining the relative contribution of habitual sunlight exposure, vitamin D intake, and genetic make-up to the variation in serum 25(OH)D in community-dwelling older adults. Since participants were included throughout the year, we had the possibility to study the associations in the winter and the summer period. Unfortunately, we could not account for the potential role of medication

use or diseases known to alter the absorption and metabolism of vitamin D. However, as persons with elevated creatinine concentrations were excluded from participation in the study, we do not assume that renal dysfunction substantially influenced the associations studied. Another possible limitation is the use of a non-validated FFQ. The FFQ, however, was compiled using a validated FFQ tool. In addition, our vitamin D intake data are in line with recent vitamin D intake data of this age group that were obtained from the Dutch Food Consumption Survey of 2013 [20], suggesting that the reported vitamin D intake estimates can be considered relatively accurate. Finally, we used a very basic questionnaire to assess sun exposure, which probably resulted in an underestimation of the explained variation in 25(OH)D concentrations resulting from UV-B exposure. The use of dosimeters might have provided a more accurate estimate of UV-B exposure [21].

3.4.2 Vitamin D deficiency

Serum 25(OH)D concentrations of 50 nmol/L or higher are considered sufficient in order to prevent disturbances in calcium metabolism [2]. Recent insights also indicate that 25(OH)D deficiency may relate to cardiovascular problems, glucose homeostasis, autoimmune disease, muscle strength and cognitive function [4, 9, 22-24]. Thus, the low 25(OH)D concentrations in this population of older adults are alarming.

3.4.3 Sunlight exposure and 25(OH)D concentrations

Despite the fact that the role of sunlight exposure in the maintenance of adequate serum 25(OH)D concentrations has been shown to decrease with age [6], the surrogate markers for sun exposure obtained in this study did significantly associate with 25(OH)D status. Interestingly, serum 25(OH)D was higher among the participants that reported to use sunscreen. It may be that participants with a higher sun exposure were the same participants that felt the necessity to use sunscreen, and that due to their overall higher sun exposure these participants still had the highest 25(OH)D concentrations.

3.4.4 Vitamin D intake and 25(OH)D concentrations

Total vitamin D intake - including both dietary intake and supplement use - in this population was 4.9±2.9 μg/day. Vitamin D supplement use was reported by 20% of the population of which 6% had a 25(OH)D deficient status. Studies in southern European countries as well as Australia have reported vitamin D intakes ranging from 1.2-1.4 μg/day [25-27]. In Scandinavian countries, where vitamin D fortified products are more common, substantially higher vitamin D intake levels have been observed, ranging from 6-8 μg/day [28, 29]. The vitamin D intake in this Dutch population is far from adequate [19], which can be explained by the facts that the Dutch diet does not contain many foods that are naturally rich in vitamin D, and that fortified products are hardly available. Therefore, Dutch men and women ≥70 years are recommended to use 20 μg vitamin D daily via supplements [19]. However, based on our data and data from the Dutch Food Consumption Survey 2013 [20], it can be concluded that the adherence to this recommendation is low. Therefore, more actively promoting the vitamin D recommendation may be important to reduce the prevalence of

25(OH)D deficiency, particularly during the winter months. The dose-response analyses between vitamin D intake and serum 25(OH)D in our study showed that a µg increase in vitamin D intake associates with a 1.0 nmol/L higher serum 25(OH)D concentration during the summer/autumn, and with a 3.1 nmol/L higher serum 25(OH)D concentration during the winter/spring. This finding is in line with previous studies that showed that the magnitude of the association between dietary vitamin D intake and 25(OH)D status was stronger during winter period, than during summer period [26, 29-31].

3.4.5 Vitamin D related genetic make-up and 25(OH)D concentrations

In this population three out-of four investigated genes in the pathway of vitamin D metabolism (i.e. DHCR7, CYP2R1, and GC) were significantly associated with serum 25(OH)D concentrations. These results are in line with the findings of a genome-wide association study by Wang and colleagues (2010) [7]. Our data suggests that the major allele frequency of the DHCR7 gene is associated with a higher 25(OH)D concentration. DHCR7 encodes for the enzyme 7-dehydrocholesterol reductase. This enzyme catalyses the conversion of 7-dehydrocholesterol into cholesterol in the skin, and thus prevents that 7-dehydrocholesterol is metabolized into vitamin D. CYP2R1 minor allele frequency was also associated with higher 25(OH)D concentration. CYP2R1 encodes for the hepatic enzyme 25-hydroxylase that converts vitamin D into 25(OH)D. Higher 25(OH)D concentrations were also observed in carriers of the major allele frequency of CYP24A1 and GC. CYP24A1 encodes for an enzyme that initiates the degradation of 25(OH)D and 1,25(OH)D into calcitroic acid. GC is the major transport protein of vitamin D metabolites, such as 25(OH)D, to different target organs, tissues and cells [7].

3.4.6 Important determinants of serum 25(OH)D

Previous estimations indicate that sunlight accounts for 70-90% of the 25(OH)D supply of the body [4]. In this study, summer sun exposure (16%) was also shown to explain most of the variance in serum 25(OH)D, closely followed by genetic make-up (15%) and vitamin D intake (12%). However, larger differences between the influence of sun exposure and vitamin D intake in relation to serum 25(OH)D were expected. Assessing sun exposure is, however, challenging and measurement error is very likely to occur. The three factors, together with potential relevant covariates, explained 35% of the variance in serum 25(OH)D. When extending our findings to other studies that also calculated R^2 in order to identify determinants of serum 25(OH)D, we conclude that there are substantial differences with respect to predictors included in the models. To the best of our knowledge, this is the first study taking into account genetic factors. Gilbert and colleagues (2012) did consider the integration of genetic information in their model. However, it was concluded that this information did not improve the fit of the prediction score of the data, which explained 28% of the variation in 25(OH)D concentrations when sun exposure, vitamin D intake, anthropometrics, clinical factors, demographics, age, season, study centre and batch assay were included [32]. Other previous studies that included vitamin D intake, sun exposure, demographic and environmental factors were able to explain 21-33% of the variation in

25(OH)D status [27, 33, 34]. Studies considering vitamin D intake, demographic and environmental factors and season of blood sampling have shown an explained variation in 25(OH)D concentration ranging from 19-28% [35-37].

3.4.7 Conclusion

In summary, the findings of this study acknowledge the previously reported inadequate vitamin D intake and the relatively high prevalence of 25(OH)D deficiency in older adults. Moreover, it was shown that UV-B exposure, vitamin D intake and vitamin D-related genetic make-up substantially contribute to the variability in 25(OH)D concentrations. The high prevalence of vitamin D deficiency as well as the low intake of vitamin D supplements indicate that more effort should be undertaken to encourage the use of vitamin D supplements in order to optimize the 25(OH)D concentrations in Dutch older adults, particularly during the winter months. Moreover, given the suggested importance of genes involved in vitamin D metabolism, in combination with the on-going debate on the question whether the associations found between 25(OH)D concentrations in non-skeletal health can be considered causal [38], these results plea for studies examining associations between vitamin D related genetic make-up and the health outcomes under debate.

Acknowledgements

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4

Associations of 25-hydroxyvitamin D with fasting glucose, fasting insulin, dementia and depression in European elderly: the SENECA study

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Abstract

Purpose

The classical consequence of vitamin D deficiency is osteomalacia, but recent insights into the function of vitamin D suggest that it may play a role in other body systems as well. The objective of this study was to examine the association between serum 25-hydroxyvitamin D (25(OH)D) and markers of glucose homeostasis (n=593), global cognitive performance (n=116), and depression (n=118) in European older adults participating in the SENECA study. Moreover, we wanted to explore whether the observed associations of serum 25(OH)D with depression and global cognitive performance were mediated by fasting plasma glucose (FPG) concentrations.

Methods

Cross-sectional associations between serum 25(OH)D and FPG, fasting plasma insulin (FPI) and Homeostasis Assessment-estimated Insulin Resistance (HOMA-IR) were estimated using multiple regression analyses. Associations of 25(OH)D status with global cognitive performance (MMSE) and depression (GDS-15) were examined using Poisson regression.

Results

An inverse association was observed between serum 25(OH)D and FPG (β -0.001), indicating a 1% decrease in FPG per 10 nmol/L increase in serum 25(OH)D. However, after full adjustment for demographic factors, lifestyle factors, and calcium intake this association was not statistically significant anymore (P=0.07). Although participants with intermediate and high serum 25(OH)D concentrations showed a tendency towards a lower depression score after adjustment for demographic and lifestyle factors, RR and 95% CI: 0.73 (0.51-1.04) and 0.76 (0.52-1.11), respectively, these findings were not statistically significant.

Conclusion

An inverse association of serum 25(OH)D with depression, and FPG was observed, but these associations were not statistically significant. There was no association between serum 25(OH)D and FPI, HOMA-IR, or global cognitive performance. More studies are needed to further explore the possible role of vitamin D in the various body systems.

4.1 Introduction

Worldwide approximately 347 million people are affected by diabetes mellitus [1], which is mainly type 2 diabetes. Recent epidemiological studies suggest that diabetes patients are at increased risk of dementia [2] and depression [3]. The question whether the observed associations between these three ageing related diseases are the result of shared risk factors or specific biological mechanisms, however, remains to be solved. Vitamin D deficiency is one of the postulated links [4-8].

Vitamin D inadequacy is commonly observed in the elderly population. Restricted sun exposure, low vitamin D intake, and a decreased skin synthesis capacity may be involved in the development of vitamin D inadequacy in ageing populations. In the NHANES III study Martins and colleagues (2007) observed lower 25(OH)D concentrations in women, persons ≥60 years, and obese and diabetic participants [9]. In 1984 a study among middle-aged and elderly English men and women showed that post-prandial glucose concentrations were higher during winter than during summer [10]. Since then, evidence supporting a role for vitamin D in glucose homeostasis expanded, including amongst others the identification of vitamin D receptors (VDRs) in the human pancreatic β-cell [11], the expression of 1-α-hydroxylase enzyme in the β-cell [12], and stimulation of the expression of insulin receptors by vitamin D in vitro [13]. The presence of 1-α-hydroxylase in cerebrospinal fluid and the existence of VDRs on various brain structures [14], support the hypothesis that vitamin D may be involved in brain function.

However, while animal experiments point towards a protective effect of vitamin D with regard to development of several age-related diseases, population based studies have not yet provided conclusive evidence for the association with diabetes [4, 15], cognitive performance [16-24], and depression [6-8, 25-28]. Therefore, our objective in this European multicentre study was to examine serum 25(OH)D and the association with markers of glucose homeostasis, cognitive performance, and depression in older European men and women.

4.2 Methods

4.2.1 Participants

The study is conducted using data of older adults participating in the SENECA study; Survey in Europe on Nutrition and the Elderly, a Concerted Action [29]. Men and women aged 70-75 years who were living in preselected towns across Europe were invited for participation in the study. Towns were selected based on their representativeness of the population and social-economic structure for the whole country. Psycho-geriatric patients living in nursing homes, persons who were not fluent in the country's language, or not able to independently answer questions were excluded from participation. The total SENECA population comprised 2586 participants. Markers of glucose homeostasis and serum 25(OH)D concentrations were measured in 1989 and available for 1554 and 860 participants, respectively. Of 593 participants both serum 25(OH)D and markers of glucose homeostasis were measured. Data on cognitive performance and depression were obtained in 1993 and available for 443 and

482 participants, of whom serum 25(OH)D concentrations were measured in 116 and 118 participants, respectively. Measurements of serum 25(OH)D, markers of glucose homeostasis, cognitive performance, and depression were available of 98 (GDS) and 94 (MMSE) participants (**Figure 1**). Included in the analyses were participants from: Belgium: Hamme; Denmark: Roskilde; France: Strassbourg and Valence; Hungary: Monor; The Netherlands: Culemborg; Norway: Elverum; Switzerland: Yverdon, Burgdorf, and Bellinzona; Greece: Athens and Iraklion; Portugal: Lisbon; Spain: Madrid.

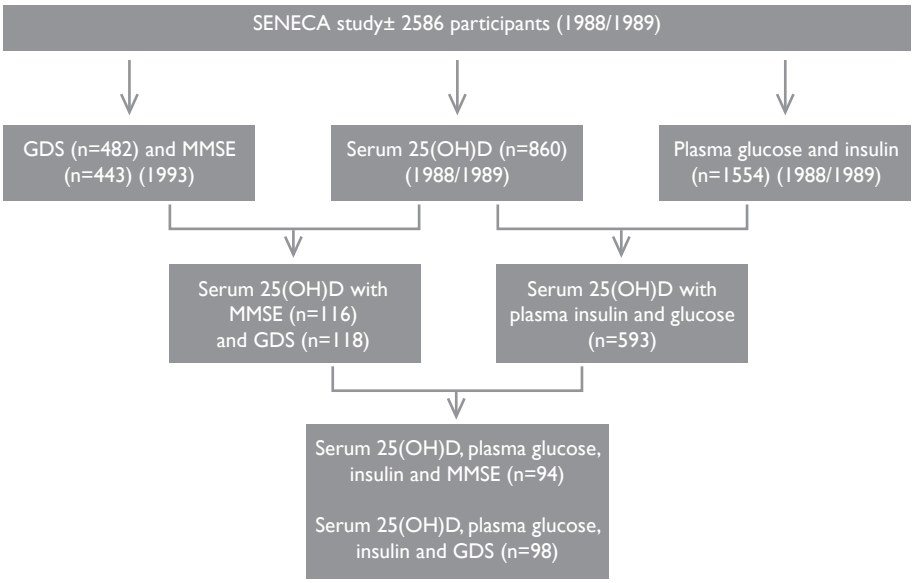

Figure 1. Flow diagram describing the data used in the analyses.

4.2.2 Cognitive performance and depression

Overall cognitive performance was assessed using the Mini Mental State Examination (MMSE). The variable for analyses was defined as the maximum MMSE score minus the MMSE score of the participant, reflecting the number of erroneous answers. Mitchell, et al. recently reviewed the accuracy of the MMSE and showed a 85.1% sensitivity and a 85.5% specificity in non-clinical community settings [30]. The 15-item Geriatric Depression Scale (GDS) was used as a screenings tool for depression. Validation with the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) revealed that 97.0% of the persons with depression (sensitivity) and 54.8% persons without depression (specificity) were correctly classified [31].

4.2.3 Biochemical analyses

Blood samples were taken between 7:30 and 9:30 AM while the participant was in a fasting state. Fasting blood samples (25 mL) were collected, and 10 mL was transferred to another tube for serum separation. A portion (0-5 mL) of each serum sample was stored at -80°C for

serum 25(OH)D determination. All blood samples were sent on dry ice to the coordinating center in Wageningen, Netherlands, and stored at -80°C. Serum 25(OH)D values were by competitive protein-binding assay (coefficients of variation: within-assay 4-7%, between-assay 7-10%) at the TNO Nutrition and Food Research Institute, Zeist, the Netherlands. As 25(OH)D concentrations may fluctuate seasonally, 25(OH)D concentration was only determined in blood samples collected between January and March 1989. Plasma glucose concentration was measured by the hexokinase method using the Hitachi 911 auto-analyser. Plasma insulin concentration was measured by using enzyme immunoassay (Boehringer-Mannheim, Mannheim, Germany) and insulin resistance was calculated using Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) from glucose and insulin concentrations [32].

4.2.4 Covariates

Information on education level (i.e. illiterate, primary, secondary or higher education), smoking status (i.e. non-smokers, former smokers or current smokers), and presence of chronic disease (i.e. hypertension, ischaemic heart disease, stroke, malignancy, arthritis, inflammatory bowel disease, respiratory problems, chronic liver disease, osteoporosis, Parkinson's disease, other) was collected using questionnaires. The Voorrips questionnaire, which is designed to assess physical activity level in older adults, was used to obtain information on habitual physical activity [33]. Participants were divided into three groups according to sex specific tertiles: low, moderate, or high physical activity level. Trained dieticians assessed dietary intake, using the dietary history method. The method consisted of a three-day record and a frequency checklist of foods, based on the meal pattern of the country and with the previous month as a reference period. Portion sizes were checked by weighing quantities of food and household measures. Intakes of nutrients and food groups were calculated in each country using local food composition tables. Food consumption data were arranged into food groups following the EUROCODE classification system [34].

4.2.5 Statistical analyses

Participant characteristics are reported as mean with standard deviation (SD), or percentages. Medians with interquartile range are reported for skewed variables. Chi-squared tests for categorical variables and one-way analysis of variance for continuous variables were performed to compare baseline characteristics over tertiles of serum 25(OH)D. Multiple regression analyses were performed to study the associations between serum 25(OH)D and fasting plasma glucose (FPG), fasting plasma insulin (FPI) and HOMA-IR, as markers of insulin resistance. FPG, FPI and HOMA-IR were not normally distributed and therefore logarithmically transformed. βs are presented as percentages (%) with 95% CI per one nmol/L increase in serum 25(OH)D. As both mental health variables followed a Poisson distribution, Relative Risks (RRs) for serum 25(OH)D with global cognitive performance [35], and depression were calculated using multiple Poisson regression, with the number of erroneous answers as outcome for global cognitive performance and the number of depressive symptoms as the outcome for depression. Participants were categorized according to tertiles

of serum 25(OH)D, using the lowest tertile as the reference category. In addition, the P-for-trend across tertiles of serum 25(OH)D was calculated. All analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol consumption, physical activity, study centre (model 2), and intake of calcium (model 3). To control for total energy intake, calcium intake was adjusted for total energy intake by using the regression residual method. The analyses were performed using the statistical package SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA).

4.3 Results

General characteristics of the study population are presented in **Table 1** and **Table 2**. The average serum 25(OH)D concentration of the total population was 37.8±20.6 and ranged from 6-141 nmol/L. Serum 25(OH)D concentrations <50 nmol/L and <75 nmol/L were observed in 79% and 94% of the participants, respectively. Participants living in the southern part of Europe were more likely to have a suboptimal 25(OH)D concentration than those living in northern countries (data not shown). Those with the highest 25(OH)D concentrations were more likely to be men (P=0.02), older (P<0.0001) and higher educated (P=0.001). Moreover, BMI (P=0.07), FPG (P=0.04), FPI (P=0.06) and HOMA-IR (P=0.05) were lower and physical activity levels (P=0.0005) and total cholesterol concentrations (P=0.001) were higher among those with the highest serum 25(OH)D concentrations. Thirty-two percent of the participants had FPG concentrations >6.0 mmol/L. FPI and FPG concentrations were lowest in those in the highest serum 25(OH)D tertile. Mean MMSE and median GDS scores of the population were 27.4±2.0 and 2.0 (IQR 3.0), respectively. As the maximum scores for the MMSE and GDS are 30 and 15, respectively, these results indicate a low prevalence of mild cognitive impairment and depressive symptoms. When compared to the total sample, persons in the mental health subsample were somewhat younger, had a lower prevalence of chronic disease, and moreover, parameters of glucose homeostasis were slightly lower.

Table 1. Characteristics of 593 European older adults of the SENECA study by tertile of serum 25(OH)D.

	T1: 6-27 nmol/L	T2: 28-42 nmol/L	T3: 43-141 nmol/L	P-value
N	204	196	193	
25(OH)D, nmol/L	19.2±5.3	34.3±4.1	61.0±18.3	<0.0001
Men, n (%)	86 (42)	98 (50)	108 (56)	0.02
Age, years	74.9±1.4	74.6±1.5	74.2±1.6	<0.0001
Body Mass Index, kg/m^{2*}	27.4±4.4	26.8±4.1	26.5±3.4	0.07
Fasting plasma glucose, mmol/L	6.2±1.9	6.1±1.6	5.8±1.5	0.04
Fasting plasma insulin, pmol/L	67.9 (46.8)	74.5 (61.3)	62.4 (43.4)	0.06
Homa-IR	1.31 (0.91)	1.45 (1.17)	1.21 (0.88)	0.05
Chronic disease present, n (%)#	166 (81)	158 (81)	144 (75)	0.24
Total cholesterol, mmol/L	6.2±1.2	6.5±1.3	6.7±1.1	0.001
Hypertension, n (%)	49 (24)	42 (21)	30 (16)	0.23
Stroke, n (%)	5 (2)	13 (7)	11 (6)	0.10

	T1: 6-27 nmol/L	T2: 28-42 nmol/L	T3: 43-141 nmol/L	P-value
Smoking status, n (%)				0.16
Non-smoking	122 (60)	106 (54)	100 (52)	
Current smoker	39 (19)	33 (17)	31 (16)	
Former smoker	43 (21)	57 (29)	62 (32)	
Physical activity level, n (%)				0.0005
Low	44 (22)	30 (15)	16 (8)	
Average	74 (36)	67 (34)	58 (30)	
High	86 (42)	99 (51)	119 (62)	
Educational level, n (%)				0.001
Primary education	122 (60)	110 (56)	102 (53)	
Secondary education	48 (23)	64 (33)	54 (28)	
Higher education	14 (7)	9 (4)	29 (15)	
Illiterate	20 (10)	13 (7)	8 (4)	
Calcium intake, mg/day*	960±433	998±424	1021±358	0.33
Alcohol intake, g/day*	0 (10)	2 (13)	1 (9)	0.17

Note: Values are expressed as a mean ± SD, median (IQR) or n (%). P-value was obtained using the X^2 test for categorical variables and one-way analysis of variance for continuous variables. *12 missing values. #Presence of chronic disease was defined as hypertension, ischaemic heart disease, stroke, malignancy, arthritis, inflammatory bowel disease, respiratory problems, chronic liver disease, osteoporosis, Parkinson's disease, others.

Table 2. Characteristics of 135 European men and women of the SENECA study per tertile of serum 25(OH)D (mental health subsample).

	T1: 7-33 nmol/L	T2: 34-52 nmol/L	T3: 53-125 nmol/L	P-value
N	50	43	42	
Age, years	73.8±1.8	73.7±1.7	73.2±1.6	0.06
MMSE-score*	27.3±2.1	26.8±2.1	27.9±1.9	0.06
GDS-score#	2.0 (3.0)	2.0 (2.5)	2.0 (2.0)	0.32
Fasting plasma glucose, mmol/L$^{\gamma}$	5.7±0.9	6.1±2.1	5.7±1.3	0.43
Fasting plasma insulin, pmol/L$^{\gamma}$	63.8 (46.3)	64.8 (57.4)	48.6 (44.3)	0.84
Homa-IR$^{\gamma}$	1.17 (0.88)	1.22 (1.01)	0.93 (0.89)	0.76
Chronic disease present, n (%)$^{\Delta}$	34 (77)	26 (68)	28 (67)	0.54
Calcium intake, mg/day$^{\pi}$	899±352	934±325	1064±360	0.07

Note: *19 missing values; #17 missing values; $^{\gamma}$21 missing values; $^{\pi}$11 missing values; $^{\Delta}$Presence of chronic disease was defined as hypertension, ischaemic heart disease, stroke, malignancy, arthritis, inflammatory bowel disease, respiratory problems, chronic liver disease, osteoporosis, Parkinson's disease, others.

Table 3 presents the associations of serum 25(OH)D with FPG, FPI and HOMA-IR, shown in percentages per nmol/L increase in serum 25(OH)D. An inverse association was observed between serum 25(OH)D and FPG (-0.1%, 95% CI -0.2-0.0), indicating a 1% decrease in FPG per 10 nmol/L increase in serum 25(OH)D. However, after adjustment for demographic factors, lifestyle factors and calcium intake, this association was not statistically significant anymore (P=0.07). Significant inverse associations were also observed for serum 25(OH)D with FPI and HOMA-IR, but these associations attenuated after full adjustment. Stratified analysis for calcium intake demonstrated a stronger association for serum 25(OH)D with

FPG among those with a high calcium intake (-0.2%, 95% CI -0.3-0.0), compared to those with a low calcium intake (0.0%, 95% CI -0.2-0.1). The interaction term, however, was not statistically significant, P=0.38.

Table 3. Associations between serum 25(OH)D and markers of glucose homeostasis of 593 men and women participating in the SENECA study, presented as % with 95% CI per nmol/L increase in serum 25(OH)D.

	Fasting plasma glucose (mmol/L)		Fasting plasma insulin (pmol/L)		HOMA-IR	
Crude	-0.1	-0.2-0$^{\Delta}$	-0.4	-0.7- 0$^{\pi}$	-0.4	-0.7- 0$^{\pi}$
Model 1*	-0.1	-0.2-0$^{\Delta}$	-0.3	-0.6-0.1	-0.3	-0.9-0.1
Model 2$^{\#}$	-0.1	-0.2-0	-0.1	-0.4-0.3	-0.1	-0.5-0.3
Model 3$^{\gamma}$	-0.1	-0.2-0	-0.1	-0.4-0.3	-0.1	-0.5-0.3

Note: *Adjusted for age and sex. $^{\#}$Adjusted for M1 and BMI, education (categorical), alcohol intake (categorical), smoking (categorical), physical activity (categorical), and study centre (categorical). $^{\gamma}$Adjusted for M2 and calcium intake (continuous) $^{\pi}$P<0.05 $^{\Delta}$P≤0.01

Data on serum 25(OH)D concentrations and depression were available of 118 participants (**Table 4**). Fully adjusted models showed that compared to the reference group, those in the middle or upper serum 25(OH)D tertile had on average a 27% (RR 0.73, 95% CI 0.51-1.04) and 24% (RR 0.76, 95% CI 0.52-1.11) (P for trend: 0.16) lower depression score, respectively. Additional adjustment for calcium intake (RR upper tertile 0.82 and 95% CI 0.59-1.14), FPG (RR upper tertile 0.88 and 95% CI 0.61-1.25) (n=83), or hypertension (RR upper tertile 0.82 and 95% CI 0.59-1.14) did not alter the direction of the results.

Table 4. Associations between serum 25(OH)D and mental health in 118 men and women participating in the SENECA study.

	T1: 0-34 nmol/L	T2: 34-52 nmol/L	T3: 52-125 nmol/L	P for trend
GDS (depression)				
Crude model (n=118)	1.0	0.78 (0.53-1.14)	0.76 (0.50-1.15)	0.05
Model 1 (n=118)*	1.0	0.80 (0.55-1.16)	0.76 (0.49-1.17)	0.05
Model 2 (n=103)$^{\#}$	1.0	0.73 (0.51-1.04)	0.76 (0.52-1.11)	0.16
Model 3 (n=103)$^{\gamma}$	1.0	0.74 (0.53-1.06)	0.82 (0.59-1.14)	0.41
MMSE (global cognitive functioning)				
Crude model (n=116)	1.0	1.19 (0.87-1.64)	0.78 (0.54-1.12)	0.04
Model 1 (n=116)*	1.0	1.19 (0.86-1.63)	0.76 (0.54-1.08)	0.04
Model 2 (n=103)$^{\#}$	1.0	1.42 (1.02-1.97)$^{\pi}$	0.92 (0.63-1.36)	0.39
Model 3 (n=103)$^{\gamma}$	1.0	1.39 (1.00-1.94)$^{\pi}$	0.94 (0.63-1.39)	0.51

Note: *Adjusted for age and sex. $^{\#}$Adjusted for age, sex, BMI, education (categorical), smoking (categorical), physical activity (categorical), alcohol intake (categorical), and study centre (categorical). $^{\gamma}$Adjusted for age, sex, BMI, education (categorical), smoking (categorical), physical activity (categorical), alcohol intake (categorical), study centre (categorical), and calcium intake (continuous). $^{\pi}$ P≤0.05

Among 116 participants of whom 25(OH)D concentrations were known and the MMSE was completed (**Table 4**), age and sex adjusted models did not show significant associations

for those in the middle or highest serum 25(OH)D group, RR 1.19 (95% CI 0.86-1.63) and RR 0.76 (95% CI 0.54-1.08), respectively. Further adjustment unexpectedly resulted in a statistically significant higher number of erroneous answers for those with intermediate 25(OH)D concentrations, RR 1.39 (95% CI 1.00-1.94). No such association was, however, observed for those with the highest 25(OH)D concentrations, RR 0.94 (95% CI 0.63-1.39). Associations did not substantially change when FPG concentrations, hypertension or depression were included in the model (RRs upper tertile 0.90 (95% CI 0.56-1.45), 1.03 (95% CI 0.69-1.55) and 0.89 (95% CI 0.58-1.35), respectively).

4.4 Discussion

In this cross-sectional population-based study in European older adults, participants with higher serum 25(OH)D concentrations tended to have less depressive symptoms. The associations observed do not support the hypothesis that higher serum 25(OH)D concentrations are associated with a better cognitive performance. Moreover, despite a modest inverse association between serum 25(OH)D and fasting plasma glucose, the hypothesized independent health benefits of serum 25(OH)D on insulin resistance could not be confirmed in this study.

Before interpreting the results, several methodological issues warrant further discussion. Firstly, blood samples were collected during the winter season and therefore reflect the lowest 25(OH)D concentrations throughout the year. Secondly, serum 25(OH)D was measured only once and may therefore not reflect long-term status. Furthermore, the debate on the most accurate method to determine serum 25(OH)D concentrations is still ongoing [36], but the competitive protein-binding (CPB) assay applied in this study might not be the most optimal method. A previous study comparing different serum 25(OH)D assays showed that CPB-assay was highly correlated with radioimmunoassay (RIA) (r=0.72) and high-performance liquid chromatography (HPLC) (r=0.69). Additionally, mean 25(OH)D concentrations as measured with CPB-assay appeared to be systematically higher compared to HPLC and RIA [37]. Therefore, CPB-assay used in our study may have overestimated the true 25(OH)D status. However, as it concerns a systematic overestimation, it will not have affected the direction of the observed associations. A strength of this study is that serum 25(OH)D samples were taken in ten countries all over Europe, collected during the same month, and analysed in one single laboratory. Therefore, seasonal variation or inter-laboratory variation cannot have affected the results. Another strength of the SENECA database is that it includes extensive information on lifestyle and dietary factors including physical activity level and calcium intake, reducing the possibility of confounding. Residual confounding, however, cannot be ruled out.

Descriptive analyses showed lower serum 25(OH)D concentrations among those with a higher BMI, lower physical activity level, and persons at older age. It may be suggested that in our population those with a higher physical activity level performed part of their exercises outdoors, which may have resulted in higher serum 25(OH)D concentrations. The decrease in serum 25(OH)D with age may be explained by the fact that the production of vitamin

D in the skin decreases while ageing [38]. Lower 25(OH)D concentrations among persons with a higher BMI has also been observed in previous studies and has been suggested to be the consequence of the storage of 25(OH)D in fat tissue, and thus not being bioavailable in serum [39].

Among individuals in the SENECA study FPG decreased with 1% when serum 25(OH)D increased with 10 nmol/L, however, this was not statistically significant after adjustment for multiple factors. No association was observed between serum 25(OH)D and FPI, or HOMA-IR. Biological evidence that vitamin D may affect markers of glucose homeostasis and the development of diabetes, is increasing [4, 40]. Previous published cross-sectional studies show, however, inconsistent results [41-46]. Among 142 Dutch men, aged 70-88 years, serum 25(OH)D was inversely associated with the AUC for both glucose and insulin, which remained significant after adjustment for BMI, skinfold thickness, alcohol consumption, smoking and physical activity [41]. High concentrations of serum 25(OH)D were furthermore positively associated with insulin sensitivity, and inversely correlated with β-cell function in a population of 126 young adults who underwent a hyperglycemic clamp experiment [44]. Moreover, inverse associations with measures of insulin resistance were observed among participants of the Framingham Heart Study [43] and NHANES III [46]. The LIPGENE study [45] and the Women's Health Initiative [42] were not able to confirm previous evidence for a possible link between serum 25(OH)D and glucose homeostasis. A recently published systematic review and meta-analyses with data of five prospective cohort studies showed that persons with 25(OH)D concentrations >62.5 nmol/L had a 43% lower risk of developing type 2 diabetes when compared to those with concentrations <35 nmol/L [15]. Mitri and colleagues (2011) also summarized the results of RCTs in this field and concluded that these trials do not yet provide definite evidence for a beneficial role of vitamin D supplementation on glycemic outcomes [15].

Despite a small sample size, this cross-sectional study showed a modest non-significant association between serum 25(OH)D and depression. Up to now, only very few population based studies examined the possible link between serum 25(OH)D and depressive symptoms, showing contradictory results. For instance, one year follow-up of 7358 middle-aged and older Americans diagnosed with a cardiovascular event, without previous depressive episode, showed that patients with a serum 25(OH)D concentration >125 nmol/L were less often depressed compared to persons with serum 25(OH)D concentrations ≤37.5 nmol/L, HR 2.70 (95% CI 1.35-5.40) [7]. Physical activity, social economic status or BMI were, however, not included as covariates. At six years of follow-up, women participating in the InCHIANTI Study with the lowest serum 25(OH)D concentrations reported significantly more depressive symptoms, compared to those in the highest tertile. It has to be mentioned that a relatively large number (42%) of the women participating in this study reported to have a depressed mood [8]. Furthermore, significantly lower serum 25(OH)D concentrations were observed among 1282 Dutch middle-aged and older adults with minor and major depressive symptoms, compared to those without depressive symptoms [6]. No clear beneficial role for vitamin D in depression was observed in population based studies among Chinese older adults and Japanese municipal officials aged 21-67 years [25-27]. In one of these studies

among Chinese older adults a significant association between 25(OH)D concentration and depression was observed at baseline, but not after four years of follow-up. The incidence rate of depression at four-years of follow-up was 4% and only 6% of the men had 25(OH)D concentrations below 50 nmol/L, which may perhaps partially explain the lack of the association observed after four years [25]. Inconsistencies between studies may also be the result of differences in the ascertainment and prevalence of depression, lack of adjustment for covariates and differences in geographical location. As persons with depressive symptoms may be less likely to go outside, associations between serum 25(OH)D and depression observed in cross-sectional studies may also be the result of reverse causality. By performing RCTs the possibility of reverse causation can be eliminated. However, up to now only very few trials studied the effect of vitamin D supplementation on mood or depression, showing conflicting findings [47-51].

While there was evidence of an inverse association between serum 25(OH)D and the number of depressive symptoms, no association was observed between serum 25(OH)D and global cognitive performance. Results of several small studies reviewed by Annweiler and colleagues (2009) [5] and more recent and larger community-based cohort studies show either no or a positive association between 25(OH)D and global cognitive performance [16, 18, 21, 23-25]. In 1766 persons ≥65 years low 25(OH)D concentrations appeared to increase the probability of experiencing cognitive impairment, particularly in men [21]. Results of the EPIDOS study point towards the same direction in a population of older women (OR 1.99, 95% CI 1.13-3.52, P=0.02), even after adjustment for iPTH, serum calcium and depression [16]. Prospective data of the InCHIANTI Study revealed that non-demented 25(OH)D deficient (<25 mmol/L) men and women were 64% more likely to experience cognitive decline, compared to those in the sufficient group (≥75 mmol/L) [23]. The MrOS study [24], NAME study [18], and Os study [25] did not observe an association between serum 25(OH)D and global cognitive function. Recently, the first RCT on vitamin D supplementation and cognitive functioning was published, which did not show an effect of a six-week treatment with 125 μg/day cholecalciferol on working memory, response inhibition or cognitive flexibility in young adults [47]. Despite the fact that we could adjust for a large number of important confounders, our sample size may not have been large enough to detect an association. Moreover, data on global cognitive performance were collected four years following the baseline measurements, which may also have affected the associations studied. Although serum 25(OH)D concentrations have been shown to decrease with age [52], two-year follow-up data of 80 frail older adults, with a mean age of 82.1 years, showed only a six nmol/L decrease in 25(OH)D concentration [53]. Therefore, we expect that only a subtle decrease in 25(OH)D concentration may have occurred during the period until the mental health indicators were measured.

In conclusion, this study showed a tendency towards an inverse association of serum 25(OH) D with FPG and depression, but not with FPI, HOMA-IR, and global cognitive performance. As the overall evidence for a role of vitamin D in glucose homeostasis, cognitive performance, and depression is still inconclusive, more prospective epidemiological studies, meta-analyses, RCTs, and mechanistic studies are warranted.

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5

Serum 25-hydroxyvitamin D is associated with cognitive executive function in Dutch pre-frail and frail elderly: a cross sectional study exploring the associations of 25-hydroxyvitamin D with glucose homeostasis, cognitive performance and depression

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Abstract

Objectives

The primary objective of this study was to explore the possible association of serum 25-hydroxyvitamin D (25(OH)D) and vitamin D intake with markers of glucose homeostasis, depression, and cognitive performance. In addition, we examined to what extent the associations between vitamin D and cognitive performance were modified or mediated by fasting plasma glucose (FPG) concentrations.

Design, Setting and Participants

Cross-sectional study using data of 127 pre-frail or frail Dutch older adults, aged ≥65 years. Frailty was defined according to the criteria of Fried and colleagues (2001). A participant was classified pre-frail when 1-2 criteria were met; frailty was classified as the presence of ≥3 criteria.

Measurements

Associations of serum 25(OH)D and vitamin D intake with markers of glucose homeostasis and domain-specific cognitive performance were examined by multivariable regression analyses. The possible association of vitamin D with depression and global cognitive performance was explored by Poisson regression.

Results

No associations were observed for serum 25(OH)D with FPG, Fasting Plasma Insulin (FPI), Homeostasis Model Assessment-estimated Insulin Resistance (HOMA-IR), or depression. In contrast, serum 25(OH)D was positively associated with executive functioning (β 0.007, P=0.01) and tended to be associated with information processing speed (β 0.006, P=0.06). FPG did not modify or mediate these associations. Vitamin D intake was not associated with cognitive performance, glucose homeostasis, or depression.

Conclusion

This cross-sectional study suggests an association of serum 25(OH)D with domain-specific cognitive performance, in particular executive functioning and possibly information processing speed, but not with FPG, FPI, HOMA-IR, or depression. Whether these associations are causal is yet to be demonstrated.

5.1 Introduction

Although vitamin D can be obtained via diet and ultraviolet-B exposure to the skin, vitamin D deficiency is a common phenomenon all over the globe [1]. Particularly older adults are at an increased risk of vitamin D deficiency as outdoor mobility, dietary vitamin D intake, and epidermal 7-dehydrocholesterol concentrations often decrease while ageing [2]. Moreover, as a consequence of diminished liver and kidney function at older age, the conversion of serum/plasma 25-hydroxyvitamin D (25(OH)D) into the biologically active form of vitamin D may also be reduced [3]. The increased risk of vitamin D deficiency among older adults, along with recent studies indicating that vitamin D may contribute to healthy ageing, has led to a rapidly growing scientific interest in this research field [4, 5]. Glucose intolerance, cognitive dysfunction and depression are three disorders that may be associated with a poor 25(OH)D status.

As reviewed by Pittas and colleagues (2007), in vitro and molecular studies have shown the existence of vitamin D receptors (VDR) and 1-α-hydroxylase in pancreatic β-cells [6]. In addition, several direct and indirect mechanisms for a possible relationship of vitamin D with insulin secretion and glucose tolerance have been suggested, namely via the activation of insulin receptor expression, the regulation of intra- and extracellular calcium concentrations and the down-regulation of cytokine generation [6]. Also a number of human population-based cohort studies and RCTs examined the possible association between 25(OH)D concentrations and glucose homeostasis [6-21]. Mitri and colleagues (2011) reviewed these RCTs and prospective cohort studies, and concluded that more research is warranted to understand the role of vitamin D in the development and progression of type 2 diabetes [7].

The most evident neurobiological indication for a potential role of vitamin D in brain function is the localisation of VDRs on various brain structures and the presence of 1-OH-ase in cerebrospinal fluid. Moreover, molecular and in vitro studies (as reviewed in [22, 23]) have shown that the biologically active form of vitamin D beneficially affects the synthesis and degradation of several neurotrophins, specifically the nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and glial cell line-derived neurotrophic factor (GDNF) [24]. Vitamin D administration may also prevent too high intraneuronal calcium influxes by down-regulating L-type voltage-sensitive calcium channel (L-type VSCCs) expression, and have a beneficial effect on neurotransmission by increasing the activity of choline acetyltransferase. Several animal studies confirm the potential effect of vitamin D, by showing that mice lacking VDRs demonstrate an increased anxiety level [25], inferior nest building [26] and impaired motor performance [27]. The possible role of 25(OH)D status in cognitive performance [8, 28-34] and depression [8, 30, 35-42] has also been studied in several human population-based studies. Results, however, are still inconclusive. In a recent review, Eyles and colleagues (2012) summarized the neurobiological evidence and stated that there is data showing that vitamin D may play a role in brain function, but that there is still insufficient and inconsistent data from epidemiological studies and well-designed RCTs in humans to confirm this potential role of vitamin D [22].

Besides a potential unfavourable role of vitamin D deficiency in cognitive decline and

depression, glucose intolerance has also been implicated as a risk factor for cognitive decline and depression [43]. The potential effect of vitamin D on glucose homeostasis, concurrently with the higher rates of depression and cognitive decline among diabetes patients, might therefore suggest an indirect role of vitamin D in brain health. Accordingly, associations of vitamin D with cognitive decline and depression may be partially mediated by glucose intolerance. Alternatively, considering that diabetes patients may be more predisposed to experience cognitive decline and depression, a modification effect of glucose intolerance may also be observed.

With vitamin D deficiency as a joint risk factor for glucose intolerance and cognitive decline, concurrently with studies indicating that diabetes patients may be at an increased risk of cognitive decline, this study aimed to further explore the possible interrelatedness between vitamin D, glucose homeostasis and brain function. Hence, we investigated the possible associations of serum 25(OH)D, vitamin D intake, and sun exposure with markers of glucose homeostasis, various domains of cognitive performance, and depression in Dutch prefrail and frail older adults. In addition, to get more insight in the underlying mechanism linking vitamin D with cognitive performance, we also investigated whether fasting plasma glucose concentrations played a mediatory role in this association. Finally, we explored whether participants with an impaired glucose tolerance showed more benefit of optimal vitamin D concentrations, compared to those with a normal glucose tolerance.

5.2 Methods

5.2.1 Participants

Analyses were performed using baseline data of 127 participants of the ProMuscle study, which was originally designed to study the effect of protein supplementation in combination with or without progressive exercise training on muscle function and muscle mass [44, 45]. Eligibility was defined as ≥65 years, and being prefrail or frail. Frailty was defined according to the criteria from Fried and colleagues (2001) as having unintentional weight loss, weakness, self-reported exhaustion, slow walking speed, and/or low physical activity level [46]. A participant was classified as prefrail when 1-2 of the aforementioned criteria were met, while frailty was defined as the presence of ≥3 or more criteria. Participants were excluded if they had fasting plasma glucose (FPG) concentrations ≥7.0 mmol/L, cancer, chronic obstructive pulmonary disease (COPD), renal failure, or if they had participated in any structured exercise training program in the past two years. The Wageningen University Medical Ethical Committee approved the study and all participants gave their written informed consent.

5.2.2 Cognitive performance and depression

The 20-item Centre for Epidemiological Studies Depression scale (CES-D) was used as a screening tool for depression [47]. Overall cognitive performance was assessed using the Mini Mental State Examination (MMSE) [48]. The variable for the analyses was defined as the maximum MMSE score minus the MMSE score of the participant, reflecting the number of erroneous answers. Domain-specific cognitive performance was measured using an extensive

cognitive test battery, performed by well-trained research assistants, and according to a strict protocol. The cognitive test battery included the Word Learning Test (WLT) direct recall, decayed recall, and recognition to measure episodic memory [49]; Wechsler digit span forward and backward test to determine attention and working memory [50]; Trail Making Test-A (TMT-A) and Trail Making Test-B (TMT-B) to assess information processing speed and concept shifting interference [51]; Stroop Colour-Word Test to determine selective attention and susceptibility to behavioural interference [52]; and the Verbal Fluency test [53], and Reaction Time Task (RT) [54, 55] to measure executive functioning. Except for the RT, all tests were performed in the afternoon. RT was assessed during the morning, in fasted state, by the computerized finger precueing task.

5.2.3 Biochemical analyses

Blood samples were collected at baseline while participants were in a fasting state. EDTA containing tubes were centrifuged at 1000g at 4˚C for 10 minutes and serum tubes were centrifuged 90 minutes after the blood collection at 1000g at 18˚C for 30 minutes. Aliquots of plasma and serum were frozen in liquid nitrogen and stored at -80˚C until further analysis. Plasma glucose concentrations were analysed with a COBAS FARA analyser (Uni Kit III; Roche, Basel, Switzerland). Insulin was analysed by radioimmunoassay (Insulin RIA Kit; LINCO Research Inc, St Charles, MO). Serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS), which was performed at the Endocrine Laboratory of the VU University Medical Centre. Serum 25(OH)D was released from its binding protein(s) and a deuterated internal standard (IS: 25(OH)D3-d6) was added. Samples were extracted and analysed by XLC-MS/MS (a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA)). Method characteristics: limit of qualification 4.0 nmol/L; intra-assay coefficient of variation (CV) <6% and inter-assay CV <8% for 3 concentrations between 25 and 180 nmol/L.

5.2.4 Dietary intake

Three-day food records were used to obtain dietary intake data. Trained dieticians gave oral and written instructions about recording the type of foods and estimation of portion sizes in household measures. At a second visit, food records were checked for completeness. Where necessary additional information was obtained about unclear items or amounts; household measures were used as an example to improve the estimation of portion sizes. Days of recording were randomly assigned so that all days of the week, including weekend days, were equally represented. Dietary intake data were coded (type of food and amount) and energy and nutrient intakes were calculated using a food calculation system (BAS nutrition software, 2004, Arnhem, the Netherlands), in which the Dutch food composition database of 2006 was included [56].

5.2.5 Sun exposure

Sun exposure was assessed six months following baseline blood sampling using a questionnaire

containing eight items about the amount of time spend outdoors and in the sun during the summer, use of sun protection and solariums, type of clothing worn during the summer, holidays with a sunny destination in the past three months and skin colour.

5.2.6 Covariates

Height was measured at baseline with a wall-mounted stadiometer to the nearest 0.1 cm. Weight was measured in a fasted state to the nearest 0.1 kg with a calibrated digital scale (ED-6-T; Berkel, Rotterdam, The Netherlands). Subsequently, Body Mass Index (BMI) was calculated as weight/height^2. Information on education level (i.e. primary, secondary or higher education), smoking status (i.e. non-smoker or current smoker), medical history and presence of chronic disease (i.e. kidney disease, liver disease, cardiovascular disease, muscle disease, and hip- or knee replacement) was collected using questionnaires. Blood pressure was measured in the morning after 10 minutes of rest in supine position with an Omron HEM-907 (Lake Forest, IL, USA) device. Habitual physical activity was quantified using a triaxial accelerometer (ActiGraph GTX3, 2009, Pensacola, FL) worn on the hip for one week. Change of acceleration per second and epochs of 60 seconds were used. After seven days, data were uploaded for analysis and analysed using the MAH/UFFE analyser, version 1.9.0.3 (MRC Epidemiology Unit, Cambridge, UK). Data files that did not meet ten hours of monitoring per day on at least five days as well as files that included periods of >100 minutes without activity were excluded from the analysis. Season was entered as a dichotomous variable, based on unpublished serum 25(OH)D data obtained from the B-PROOF study, a large intervention study conducted at the Division of Human Nutrition of Wageningen University [57]. Blood samples collected in June-November were defined as summer/autumn; samples collected in December-May were defined as winter/spring.

5.2.7 Potential effect modifiers and intermediates

Serum 25(OH)D may be related to glucose homeostasis and brain function. Moreover, diabetes patients have been suggested to be at an increased risk of cognitive decline. Hence, we hypothesized that the association between vitamin D and cognitive performance may be more beneficial in persons with glucose intolerance/diabetes, compared to those with a normal glucose tolerance. Additionally, we hypothesized that the association between vitamin D and cognitive performance may be mediated by glucose homeostasis. Therefore, we examined the potential modifying as well as the intermediary role of fasting plasma glucose in the association between serum 25(OH)D and cognitive performance.

5.2.8 Statistical Analyses

Characteristics of the study population are reported using the mean with standard deviation (SD), or as percentages. Medians with interquartile range were used to report skewed variables. Chi-squared tests for categorical variables and one-way analysis of variance for continuous variables were performed to compare baseline characteristics over tertiles of serum 25(OH)D. Multivariable regression analyses were performed to study associations of serum 25(OH)D with fasting plasma glucose, fasting plasma insulin, HOMA-IR and domain-specific cognitive

performance. Multivariable regression analyses were also used to explore the associations between markers of glucose homeostasis and domain-specific cognitive performance. To compare the results of the individual cognitive tests and to limit the number of dependent variables, crude test scores were clustered into compound Z-scores for four neuropsychological domains: Episodic memory, Attention and working memory, Information processing speed, and Executive functioning. In formula form: episodic memory = (WLT total immediate recall + WLT decayed recall + WLT recognition)/3; attention and working memory = (Digit Span forward + Digit Span backward)/2; information processing speed = (Average Stroop card 1 and 2 + -TMT-A + -RT uncued)/3; executive functioning = (Stroop ratio + Verbal Fluency + -TMT- ratio + -RT finger-cued + -RT hand-cued + -RT neither-cued)/6. WLT decayed recall was calculated as the number of words recalled after approximately 15 minutes following the fifth session of the WLT minus the number of words recalled during the fifth session of the WLT. Stroop ratio was calculated by the following: Stroop card 3/ ((Stroop card 1 + Stroop card 2)/2). Accuracy and speed-accuracy trade-off (SATO) were calculated to take into account errors made during the Stroop Test. Accuracy = (maximum right answers – amount of errors / maximum right answers). SATO = accuracy / time needed to complete the task [58]. TMT-ratio was calculated by TMT-B/TMT-A. As the MMSE-score and the CES-D score followed a Poisson distribution, Relative Risks (RRs) for serum 25(OH)D and global cognitive performance and depression were calculated using multivariable Poisson regression, with the number of erroneous answers as the outcome for global cognitive functioning and the number of depressive symptoms as an outcome for depression [59]. This RR corresponds to the probability of developing depression or global cognitive dysfunction in participants with either intermediate or highest 25(OH)D concentrations in this population compared to participants with the lowest 25(OH)D concentrations. Participants were categorized according to tertiles of 25(OH)D status, using the lowest tertile as the reference category. All analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol consumption, physical activity level and season of blood sampling (model 2). Spearman and Pearson correlation analyses were performed to obtain correlations coefficients between sunlight exposure, vitamin D intake, 25(OH)D concentrations, domain-specific cognitive performance and measures of glucose homeostasis. All analyses were performed using the statistical package SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA).

5.3 Results

Participants of the ProMuscle Study were on average 79 years old and serum 25(OH)D concentrations were lower with increasing age (P=0.03) (**Table 1**). The mean serum 25(OH)D concentration was 54 nmol/L; 17% of the population had serum 25(OH) D concentrations <30 nmol/L and 53% <50 nmol/L. Only 23% of the participants had serum 25(OH)D concentrations ≥75 nmol/L. The mean vitamin D intake was 4.6 μg/ day, with a 25th percentile of 2.5 μg/day and a 75th percentile of 5.8 μg/day. The mean FPG concentration was 5.3 and ranged from 4.4-6.5 mmol/L. FPI concentrations ranged from 5.1-45.1 μU/ml with a median concentration of 17.5 μU/ml (P=0.01). FPI and

HOMA-IR (P=0.04) were lowest in persons with the highest serum 25(OH)D status. Calcium intake increased across serum 25(OH)D tertiles (P=0.04). In this frail population 59% of the persons in the top serum 25(OH)D tertile had one or more chronic diseases, which was 55% and 42% in the lowest and intermediate tertile (P=0.25), respectively.

Table 1. Characteristics of 127 Dutch prefrail and frail older adults per tertile of serum 25(OH)D.

	T1 13-38 nmol/L	T2 38-65 nmol/L	T3 65- 163 nmol/L	P-value
N	40	43	44	
Men, n (%)	16 (40)	18 (42)	16 (36)	0.29
Age, years	81.4±7.8	78.7±7.8	77.0±7.3	0.03
Body Mass Index, kg/m²	27.8±4.4	28.0±3.8	26.8±4.8	0.42
Fasting plasma glucose, mmol/L	5.25±0.50	5.28±0.44	5.24±0.48	0.90
Fasting plasma insulin, µU/ml	17.35±6.18	20.96±7.46	16.98±6.67	0.01
Homa-IR	4.1±1.8	5.0±2.1	4.1±1.9	0.04
Chronic disease present, n (%)	22 (55)	18 (42)	26 (59)	0.25
Systolic Blood Pressure, mmHg	152±22	144±22	144±23	0.21
Diastolic Blood Pressure, mmHg	75±9	75±9	74±9	0.76
Serum creatinine, mmol/L	76.6±15.6	74.5±13.1	70.9±15.6	0.21
Smoking status, n (%)				0.07
Non-smoker	35 (88)	43 (100)	40 (91)	
Smoker	5 (12)	0 (0)	4 (9)	
Physical activity, counts/min	91±62	136±75	184±112	0.0001
Educational level, n (%)				0.26
Primary education	1 (3)	3 (7)	3 (7)	
Secondary education	28 (70)	20 (46.5)	27 (61)	
Higher education	11 (28)	20 (46.5)	14 (32)	
Vitamin D intake, µg/day	3.2 (1.7-5.7)	3.9 (2.5-5.0)	4.4 (3.0-7.3)	0.21
Calcium intake, mg/day	921±343	1029±322	1135±476	0.04
Alcohol intake, g/day	4.5 (0-12.2)	6.3 (0.1-19.7)	7.0 (0-20.2)	0.35
MMSE score	27.5±2.3	27.7±2.2	27.8±2.2	0.81
Attention and working memory, z-score	-0.16±0.80	0.00±0.86	0.14±0.99	0.31
Executive functioning, z-score	-0.15±0.83	-0.07±0.74	0.25±0.62	0.04
Information processing speed, z-score	-0.22±0.77	-0.03±0.87	0.24±0.74	0.05
Episodic memory, z-score	-0.04±0.76	-0.11±0.58	0.10±0.64	0.36
CES-D score	5.55±5.33	7.42±4.79	7.77±6.77	0.17

Note: Values are expressed as a mean ± SD, median with Q1-Q3 or n (%); Chi-squared tests for categorical variables and one-way analysis of variance for continuous variables were performed to compare baseline characteristics over tertiles of serum 25(OH)D; Chronic disease: defined as kidney disease, liver disease, cardiovascular disease, muscle disease, hip- or knee replacement; MMSE: Mini Mental State Examination; CES-D: Centre for Epidemiological Studies Depression scale; Missing values: physical activity (n=21), executive function (n=9), information processing speed (n=8), fasting plasma insulin, fasting plasma glucose and Homa-IR (n=3), and blood pressure and creatinine (n=1).

Vitamin D intake was not significantly correlated with serum 25(OH)D, *r*=0.09 (P=0.30). However, significant correlations were found for higher serum 25(OH)D concentrations with more sunbathing behaviour during the summer months and less sunscreen use, *r*=0.18 (P=0.05) and *r*=-0.36 (P<0.0001), respectively. Higher 25(OH)D concentrations tended to be correlated with the type of clothing worn during the summer/autumn months, *r*=0.16 (P=0.09).

5.3.1 Vitamin D and markers of glucose homeostasis

Table 2 presents the regression coefficients of serum 25(OH)D and vitamin D intakes with FPG, FPI and HOMA-IR. After full adjustment no significant associations were observed for serum 25(OH)D or vitamin D intake with FPI, FPG, or HOMA-IR. Correlation analyses did show modest associations for sunbathing behaviour during the summer months with FPG, *r*=0.17 (P=0.08) and type of clothing worn with FPI and HOMA-IR, *r*=0.18 (P=0.06) and *r*=0.16 (P=0.08), respectively.

Table 2. Associations of serum 25(OH)D and vitamin D intake with markers of glucose homeostasis of 127 prefrail and frail older adults participating in the ProMuscle Study.

	Fasting plasma glucose (mmol/L), β±SE		Fasting plasma insulin (μU/mL), β±SE		HOMA-IR, β±SE	
	25(OH)D	Vitamin D intake*	25(OH)D	Vitamin D intake*	25(OH)D	Vitamin D intake*
Crude model	-0.002±0.002 (P=0.30)	0.03±0.01 (P=0.06)	-0.021±0.02 (P=0.36)	0.15±0.20 (P=0.46)	-0.006±0.006 (P=0.36)	0.05±0.06 (P=0.38)
Model 1	-0.002±0.002 (P=0.12)	0.02±0.01 (P=0.15)	-0.034±0.02 (P=0.14)	0.06±0.20 (P=0.77)	-0.010±0.006 (P=0.14)	0.02±0.06 (P=0.71)
Model 2	-0.001±0.002 (P=0.39)	0.01±0.01 (P=0.37)	-0.016±0.02 (P=0.51)	0.10±0.21 (P=0.65)	-0.004±0.007 (P=0.57)	0.02±0.06 (P=0.72)

Note: Model 1: Adjusted for age and sex. Model 2: Adjusted for age, sex, BMI, education, alcohol intake, smoking, physical activity and season. *Analyses for vitamin D intake are not adjusted for seasonal influences. Crude model and model 1: n=126 (FPI) and n=124 (FPG and HOMA-IR). Model 2: n=106 (FPI) and n=104 (FPG and HOMA-IR).

5.3.2 Vitamin D and cognitive performance

Compared to participants in the lowest serum 25(OH)D tertile, persons in the intermediate and top tertile scored higher on the MMSE. This finding was, however, not significant (**Table 3**). Associations between serum 25(OH)D and various cognitive domains are displayed in **Table 4**. Fully adjusted models showed significantly better performance in tasks involving executive functioning per 1 nmol/L increase in serum 25(OH)D, β 0.007 (P=0.01). Serum 25(OH)D tended to be associated with information processing speed, β 0.006 (P=0.06). When the models were expanded to capture the impact of depression or calcium, β's did not significantly change (data not shown). Moreover, within the domain executive functioning an association was observed for serum 25(OH)D with the reaction time task (neither cued: β -2.86, P=0.01; finger cued: β -2.71, P=0.01; hand cued: β -2.86, P=0.01). Serum 25(OH)D also showed a significant association with the reaction time task uncued (β -2.58, P=0.01), a task related to the domain information processing speed. A modest association

was observed for serum 25(OH)D with digit span (β 0.02, P=0.07), within the domain attention and working memory. Scores on the word learning tests indicated that with every 11 nmol/L increase in serum 25(OH)D one word more could be memorized (P=0.008) (**supplementary Table**). Vitamin D intake was not associated with any of the domains of cognitive functioning (**Table 4**). There was a significant correlation between type of clothing worn during the summer months (i.e. less skin covered) and information processing speed, *r*=0.21 (P=0.03), and a modest correlation with attention and working memory, *r*=0.17 (P=0.06). Neither sunscreen use nor sunbathing behaviour was correlated with any of the cognitive domains (data not shown).

Table 3. Associations between serum 25(OH)D and mental health of 127 prefrail and frail older adults participating in the ProMuscle Study, Relative Risk with 95% CI.

	Low serum 25(OH)D (<34 nmol/L)	Moderate serum 25(OH)D (34-52 nmol/L)	High serum 25(OH)D (52-125 nmol/L)	P for trend
CES-D (depression)				
Crude model	1.0	1.34 (0.94-1.90)	1.40 (0.95-2.07)	0.0004
Model 1	1.0	1.31 (0.92-1.88)	1.33 (0.87-2.02)	0.005
Model 2	1.0	1.34 (0.89-2.04)	1.47 (0.91-2.40)	0.001
MMSE (global cognitive performance)				
Crude model	1.0	0.93 (0.62-1.38)	0.87 (0.58-1.31)	0.79
Model 1	1.0	1.00 (0.68-1.47)	0.98 (0.65-1.47)	0.96
Model 2	1.0	0.83 (0.55-1.24)	0.78 (0.51-1.20)	0.77

Note: Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, and season; Crude model and model 1: n=127. Model 2: n=106.

5.3.3 Vitamin D, cognitive performance and the interplay with glucose homeostasis

The βs for serum 25(OH)D and domain-specific cognitive performance did not significantly change when glucose was entered into the fully adjusted model, showing that associations between serum 25(OH)D and cognitive performance in this population were not mediated by glucose concentrations. The association between serum 25(OH)D and information processing speed, however, did become statistically significant after adjustment for glucose, β 0.006 (P=0.05). Stratifying the data for those with low and high glucose concentrations did not suggest a clear modification effect.

5.3.4 Vitamin D and depressive symptoms

Data on the association between serum 25(OH)D concentrations and depression can be found in **Table 3**. Neither serum 25(OH)D, nor vitamin D intake (data not shown), was associated with the number of depressive symptoms observed in this frail population. In addition, no significant correlations between sunlight exposure and the number of depressive symptoms were observed.

Table 4. Associations of serum 25(OH)D and vitamin D intake with domain-specific cognitive performance in prefrail and frail older adults participating in the ProMuscle Study, β±SE.

	Total		'Low' FPG (<5.20 mmol/L)[b]	'High' FPG (≥5.20 mmol/L)[b]
	25(OH)D, nmol/L	Vitamin D intake, µg/d[a]	25(OH)D	25(OH)D
Attention and working memory				
Crude	0.006±0.003 (P=0.06)	-0.007±0.03 (P=0.78)	0.004±0.003 (P=0.19)	0.005±0.005 (P=0.17)
Model 1	0.005±0.003 (P=0.08)	-0.013±0.03 (P=0.62)	0.004±0.003 (P=0.26)	0.007±0.006 (P=0.23)
Model 2	0.006±0.004 (P=0.11)	-0.02±0.03 (P=0.46)	0.006±0.004 (P=0.17)	0.008±0.008 (P=0.31)
Executive functioning				
Crude	0.007±0.002 (P=0.003)	0.002±0.02 (P=0.94)	0.007±0.003 (P=0.04)	0.010±0.004 (P=0.01)
Model 1	0.006±0.002 (P=0.01)	-0.01±0.02 (P=0.65)	0.005±0.003 (P=0.11)	0.009±0.004 (P=0.03)
Model 2	0.007±0.003 (P=0.01)	-0.05±0.02 (P=0.06)	0.006±0.004 (P=0.12)	0.007±0.005 (P=0.17)
Information processing speed				
Crude	0.007±0.003 (P=0.01)	0.03±0.02 (P=0.24)	0.007±0.004 (P=0.07)	0.01±0.004 (P=0.02)
Model 1	0.006±0.003 (P=0.03)	0.02±0.02 (P=0.47)	0.006±0.004 (P=0.14)	0.008±0.004 (P=0.05)
Model 2	0.006±0.003 (P=0.06)	0.01±0.03 (P=0.59)	0.006±0.004 (P=0.16)	0.004±0.005 (P=0.42)
Episodic memory				
Crude	0.003±0.002 (P=0.14)	0.009±0.02 (P=0.65)	0.003±0.003 (P=0.25)	0.004±0.004 (P=0.24)
Model 1	0.002±0.002 (P=0.47)	-0.002±0.02 (P=0.93)	0.003±0.003 (P=0.49)	0.002±0.004 (P=0.67)
Model 2	0.002±0.002 (P=0.28)	-0.03±0.02 (P=0.17)	0.003±0.003 (P=0.30)	0.001±0.004 (P=0.74)

Note: Model 1: Adjusted for age and sex; Model 2: Adjusted for model 1 and BMI, education level, alcohol, smoking, physical activity, and season; [a]Analyses for vitamin D intake are not adjusted for seasonal influences; [b]Low and high FPG concentrations were based upon the median split; Crude model and model 1: n=127 (AWM, EM), n=118 (EF) and n=119 (IPS). Model 2: n=106 (AWM, EM) and n=99 (EF, IPS).

5.4 Discussion

In this population of older adults, serum 25(OH)D was positively associated with domain-specific cognitive performance; more specifically executive functioning. A near significant association was observed for serum 25(OH)D with information processing speed.

To our knowledge this is the first study exploring the association between vitamin D and cognitive performance in a prefrail and frail elderly population. A recent study showed that executive function and information processing speed were compromised in frail elders when compared to non-frail elders [60]. Therefore, investigating the association between vitamin D with cognitive performance in this specific population is an important strength of this study. Other strengths include the use of various measures of vitamin D exposure (i.e. serum 25(OH)D, vitamin D intake and sunlight exposure), the use of LC-MS/MS to quantify the 25(OH)D concentration, the broad variety of domain-specific cognitive tests used and the ability to adjust for a large number of potential confounders. However, also some limitations need to be considered, such as the relatively small sample size, cross-sectional design and the fact that sun exposure was assessed six months following the baseline measurements. Another limitation of this study is the exclusion of participants with FPG concentrations

>7.0 mmol/L, which may explain the null finding with regard to serum 25(OH)D and markers of glucose homeostasis in this study.

Whereas associations of serum 25(OH)D with diabetes, cognitive performance, and depression are already somewhat more explored, vitamin D intake and its association with cognitive performance [61, 62], depression [63], and glucose homeostasis [64-66] has only been studied in a limited number of observational studies. In this study we were not able to detect any association between vitamin D intake and the aforementioned outcome measures. Moreover, correlations between vitamin D intake and serum 25(OH)D appeared to be low. One of the explanations for these findings may be the low vitamin D intake levels and the narrow range in vitamin D intake in our population, which in turn may be explained by the fact that food fortification with vitamin D in the Netherlands is not broadly practiced and vitamin D-rich products are often not part of the daily menu.

In this study, we did not observe an association between serum 25(OH)D and vitamin D intake with indicators of glucose homeostasis, specifically FPG, FPI, or HOMA-IR. Already in 1984 a study among English men and women showed higher postprandial glucose concentrations during the winter period [67]. Baynes and colleagues (1997) observed an inverse association between 25(OH)D and the Area Under the Curve (AUC) for both insulin and glucose among elderly Dutch men [68]. However, reviews discussing cross-sectional studies [6], prospective studies [7] as well as RCTs [7] showed that there is no consensus as to what extent vitamin D is involved in glucose homeostasis.

In this study we observed a statistically significant association between serum 25(OH)D and cognitive executive performance, which was assessed using tasks related to response inhibition, cognitive flexibility, and mental shifting. Smaller studies as well as larger community-based cohort studies investigated the association of 25(OH)D status with global cognitive performance [8, 29-31, 33] and domain specific cognitive performance (**Table 5**) [28, 32, 34, 69-71].

Table 5. Summary of cross-sectional and prospective studies examining the association between serum 25(OH)D and domain-specific cognitive performance in populations of older adults.

Study	Population	Design	Domain specific cognitive tests	Main findings
NAME Study *Buell, et al. (2009)*	377 black and 703 non-black older adults, 65-99 years	Cross-sectional	Trail Making Test- A and B Digit Symbol Test Matrix Reasoning WAIS-III Block Design WMS-III word list learning and logical memory Digit span Verbal fluency	Higher 25(OH)D concentrations were associated with a better performance on: TMT-B (β = -0.73, P<0.02) TMT-A (β = -0.49, P<0.03) Digit Symbol Test (β = 0.19, P<0.001) Matrix reasoning (β = 0.04, P <0.02) Block Design Test (β = 0.07, P<0.04)

Study	Population	Design	Domain specific cognitive tests	Main findings
InCHANTI Study *Llewellyn, et al. (2010)*	858 men and women, ≥65 years	Prospective	Trail Making Test- A and B	Higher 25(OH)D concentrations were associated with a better performance on TMT-B. RR 1.32 with 95% CI 1.03-1.51 for those with serum 25(OH)D concentrations <25 nmol/L with RR 1.0 for those with concentrations ≥75 nmol/L (P for trend over quartiles 0.04).
MrOS Study *Slinin, et al. (2010)*	1564 men, ≥65 years	Cross-sectional / prospective	Trail Making Test- B	No significant associations.
European Male Ageing Study *Lee et al. (2009)*	3133 men, 40-79 years	Cross-sectional	Rey-Osterrieth Complex Figure test Camden Topographical Recognition Memory Digit-Symbol Substitution Test	Higher 25(OH)D concentrations were associated with a better performance on the Symbol Digit Test, β 0.15 per 10 nmol/L (P<0.01).
NHANES III *McGrath, et al. (2007)*	≥60 years: 4809 men and women	Cross-sectional	Memory and learning score	Significant difference between serum 25(OH)D quintiles: Wald test *F*=3.38, P=0.02. However, those with the highest serum vitamin D concentrations had the lowest scores.
ZENITH Study *Seamans, et al. (2010)*	387 men and women, 55-87 years	Cross-sectional	Cambridge Neuropsychological Testing Automated Battery, including assessments of spatial working memory, pattern recognition memory, spatial span, reaction time and match-to-sample tests	Serum 25(OH)D was significantly associated with spatial working memory: Tertile 1 (ref) vs. Tertile 3 - between errors β 5.39 (P=0.03), strategy β 2.36 (P=0.02) and total errors β 5.36 (P=0.04).

Annweiler and colleagues (2012) reviewed the current literature on serum 25(OH)D and domain-specific cognitive performance and speculated that vitamin D may be particularly related to executive function, more specifically mental shifting and information updating impairments. Moreover, it was postulated that vitamin D deficiency is predominantly associated with dysfunction of the frontal-subcortical neuronal circuits [72]. This finding for executive function is in line with our findings and also with results of other studies [32, 69, 70], but not all [71]. In the NAME study participants with higher 25(OH)D concentrations completed the TMT-B significantly faster, β -0.73 (P<0.02) [69]. TMT-B results also showed a significant higher probability of experiencing cognitive decline among older adults with 25(OH)D concentrations <25 nmol/L in the InCHIANTI Study, when compared

to those with concentrations of ≥75 nmol/L, RR 1.32 (95% CI 1.03-1.51) [70]. Serum 25(OH)D was also associated with spatial working memory as assessed with the Cambridge Neuropsychological Testing Automated Battery among 387 Europeans aged ≥55 years [32]. In contrast, no association was found between serum 25(OH)D and TMT-B performance in the MrOS study [71]. Information processing speed was also significantly faster among those with higher 25(OH)D concentrations in the NAME study, as shown by the results from the TMT-A and symbol digit test, β -0.49 (P<0.03) and β 0.19 (P<0.001), respectively [69]. Moreover, 25(OH)D status was associated with a higher score on the symbol digit test in the European Male Ageing Study, β 0.15 per 10 nmol/L (P<0.01) [34]. Also, serum 25(OH)D and visuospatial function were positively associated in the NAME study, as shown by significant results on matrix reasoning and block design test [69]. In the NHANES III, learning and memory, as assessed by one single test, was significantly worse among US older adults with the highest 25(OH)D concentrations [73].

RCTs examining the effect of vitamin D supplementation on cognitive performance in older adults are rare, of small sample size, and had a relatively short intervention period [74, 75]. Corless and colleagues (1985) supplemented 64 geriatric patients with 225 μg/day vitamin D_2 or a placebo. Next, cognitive performance was assessed at 10 (n=64), 24 (n=38) and 40 (n=25) weeks. Statistical analyses did not reveal a significant effect of vitamin D_2 supplementation at any of the time points [74]. Stein and colleagues (2011) conducted an RCT with 32 participants, which were assigned to either 150 μg vitamin D_2 daily or 25 μg vitamin D_2 daily. After eight weeks of supplementation no statistical differences in cognitive performance were observed between those receiving the high dose of vitamin D and those receiving the low dose of vitamin D [75].

In the first published MRI-study on vitamin D and cerebrovascular pathology Buell and colleagues (2010) found an association between serum 25(OH)D and large vessel infarcts, but also with all-cause dementia, white matter hyperintensity volume, and white matter hyperintensity severity [76]. Previous studies also indicate an association of diabetes with vascular factors and cognitive performance [43]. Therefore, diabetes may be considered an intermediate in the association between serum 25(OH)D and cognitive performance. Alternatively, diabetes may also be considered a potential effect modifier in the association between serum 25(OH)D and cognitive performance, as vitamin D supplementation may directly as well as indirectly promote cognitive performance. In the current study we did not observe a mediation effect or a modification effect of FPG concentrations in the association between serum 25(OH)D and domain-specific cognitive performance. However, it may be that a potential modification effect of FPG in our study was masked owing to the small range in glucose concentrations. Three other studies also investigated the potential role of diabetes in the association between vitamin D and domain-specific cognitive performance [31, 34, 70], of which only the NHANES III study observed partial mediation by the presence of diabetes mellitus [31].

Last, neither dietary vitamin D nor serum 25(OH)D was associated with depression, as measured by CES-D. Whereas some studies showed results that are in line with our data [40, 77], others observed a tendency of lower depression rates among those with higher serum

25(OH)D concentrations [30, 36-39, 78]. RCTs showed inconsistent results as well [79-83], which may, amongst others, be explained by methodological differences and differences in responsiveness to vitamin D supplementation.
Previous studies have suggested a possible relationship between low 25(OH)D concentrations and extraskeletal health. This study also suggests an association between serum 25(OH)D and cognitive performance. However, we did not observe associations of vitamin D with glucose homeostasis or depression, nor did we observe a potential mediatory or modifying role of glucose homeostasis in the association between serum 25(OH)D and cognitive performance. Yet, more studies are warranted in order to confirm our findings and to define disease specific cut-off points for serum 25(OH)D and dietary reference intakes for cognitive function, depression, and glucose homeostasis.

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Supplementary material

sTable. Association between serum 25(OH)D and individual cognitive tests in 127 prefrail and frail older adults.

	β	P-value
Information Processing Speed		
Stroop average		
Crude model	0.002	0.05
Model 1	0.002	0.10
Model 2	0.002	0.26
Trail Making Test A		
Crude model	0.081	0.18
Model 1	0.063	0.30
Model 2	0.113	0.14
Reaction Time Task uncued		
Crude model	-2.56	0.004
Model 1	-2.08	0.02
Model 2	-2.58	0.01
Executive function		
Stroop ratio		
Crude model	0.000	0.65
Model 1	0.000	0.95
Model 2	0.000	0.94
Verbal Fluency Score		
Crude model	0.05	0.02
Model 1	0.06	0.06
Model 2	0.06	0.09
Trail Making Test ratio		
Crude model	-0.002	0.26
Model 1	-0.002	0.32
Model 2	-0.002	0.32
Reaction Time Task neither cued		
Crude model	-3.03	0.002
Model 1	-2.51	0.01
Model 2	-2.86	0.01
Reaction Time Task finger cued		
Crude model	-2.98	0.003
Model 1	-2.34	0.01
Model 2	-2.71	0.01
Reaction Time Task hand cued		
Crude model	-2.78	0.004
Model 1	-2.34	0.01
Model 2	-2.86	0.01

Trail Making Test B		
Crude model	-0.059	0.32
Model 1	-0.061	0.30
Model 2	-0.067	0.30
Episodic Memory		
Word Learning Test- Total immediate recall		
Crude model	0.102	0.003
Model 1	0.069	0.03
Model 2	0.089	0.008
Word Learning Test - Delayed recall		
Crude model	-0.007	0.20
Model 1	-0.007	0.24
Model 2	-0.005	0.45
Word Learning Test - Delayed recognition		
Crude model	0.010	0.20
Model 1	0.005	0.52
Model 2	0.005	0.48
Attention and Working Memory		
Digit Span forward		
Crude model	0.009	0.10
Model 1	0.009	0.13
Model 2	0.007	0.34
Digit Span backward		
Crude model	0.011	0.08
Model 1	0.010	0.12
Model 2	0.014	0.08

Note: Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, BMI, education, alcohol intake, smoking, physical activity and season; Numbers for model 2: n=106 digit span and letter fluency, n=102 reaction time task, n=101 Stroop, n=103 trail making test, n=106 word learning test.

6

Cognitive performance: is there an interplay between serum vitamin D and glucose homeostasis?

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Abstract

Background

Vitamin D inadequacy is frequently observed all over the globe and has been hypothesized to be a modifiable risk factor for cognitive decline. Vitamin D may directly influence cognitive decline, but the effect may also be indirect, for instance through changes in glucose homeostasis. As glucose homeostasis may also affect cognitive decline independent of vitamin D, interactions between vitamin D and glucose homeostasis may be observed. Therefore, we studied the cross-sectional interplay of vitamin D with glucose homeostasis in the association with domain-specific cognitive performance.

Design

25-hydroxyvitamin D (25(OH)D), glucose and insulin concentrations were measured. Cognitive performance was assessed with an extensive cognitive test battery in 776 (3 domains) up to 2722 (1 domain) Dutch older adults aged ≥65 years. Prevalence Ratio's (PRs) were calculated to quantify the association between serum 25(OH)D and cognition; poor performance was defined as the worst 10% of the distribution of the cognitive scores.

Results

Higher serum 25(OH)D was associated with better attention and working memory, PR 0.49 (95% CI 0.29-0.83) for the third serum 25(OH)D tertile, indicating a 51% lower probability of being a poor performer when compared to participants in the lowest tertile. Beneficial trends were shown for serum 25(OH)D with executive function and episodic memory. Serum 25(OH)D was not associated with plasma glucose and plasma insulin. Plasma insulin modified the association between serum 25(OH)D and executive function (P for interaction: 0.001), suggesting that the improvement in executive function with high 25(OH)D concentrations is stronger in participants with higher plasma insulin than in those with lower plasma insulin concentrations.

Conclusion

Higher 25(OH)D concentrations were significantly associated with better attention and working memory performance. This study does not show an interplay between serum 25(OH)D and glucose homeostasis in the association with cognitive performance.

6.1 Introduction

Aging is a generally known key risk factor for dementia. However, the exact underlying pathophysiological mechanisms still need to be unraveled. Nutritional factors may play a role; vitamin D is one of the nutrients under study [1].
Several biological studies have provided evidence for a role of vitamin D in cognitive performance [2-4]. The discovery of vitamin D receptors (VDRs) and 1-OH-ase, the enzyme that catalyses the conversion of the biological inactive form of vitamin D in the biological active form, in brain tissue are probably the two most consistent findings supporting a potential vitamin D effect. The potential favourable effect of vitamin D on cognitive performance may also be partially explained by its effect on glucose homeostasis. Serum vitamin D has in fact been associated with glucose homeostasis [5], and an impaired glucose tolerance has been associated with an increased risk of cognitive decline [6].
So far, results from observational studies exploring the association between vitamin D and cognition in humans have been equivocal [1, 7-16]. Four studies adjusted their final analyses for a marker of glucose homeostasis [9, 10, 15, 16]; one suggests a modest role for diabetes. Unfortunately, more detailed studies examining the influence of glucose homeostasis in the association between serum 25(OH)D and cognitive performance are lacking.
Therefore, we studied the association between serum 25(OH)D and domain-specific cognitive performance in Dutch older adults aged ≥65 years, with a specific interest for the potential mediating and modifying role of glucose homeostasis.

6.2 Methods

6.2.1 Participants

This cross-sectional study was performed using baseline data of the B-PROOF study; a randomized, double blind, placebo-controlled trial designed to assess the effect of daily oral supplementation of vitamin B_{12} and folic acid on fractures in mildly hyperhomocysteinemic (plasma Hcy 12-50 μmol/L) older adults ≥65 years; details have been reported previously [17]. The Medical Ethics Committee of Wageningen UR approved the study protocol and the Medical Ethics Committees of VUmc and Erasmus MC confirmed local feasibility. All participants gave written informed consent. For the current analyses, only data of participants with serum 25(OH)D measurements have been used (n=2857) (**supplementary Figure 1**).

6.2.2 Cognitive performance

Cognitive performance was assessed according to a standard protocol, by a well-trained staff. The Mini-Mental State Examination (MMSE) was administered to assess overall cognitive status [18]. An extensive neuropsychological test battery covered four cognitive domains: attention and working memory (n=787), executive functioning (n=776), information processing speed (n=797) and episodic memory (n=2722).
The Rey Auditory Verbal Learning Test (RAVLT) was used to measure immediate and delayed recall, as well as recognition for word lists, as indices of episodic memory [19]. RAVLT

decayed recall was calculated as the number of words recalled approximately 15 minutes after the fifth session of the RAVLT minus the number of words recalled at the fifth session of the RAVLT. The Digit Span forward and backward from the Wechsler Adult Intelligence Test (WAIS-III) measures attention and working memory, respectively [20]. Trail Making Test (TMT) part A measures information processing speed and part B assesses concept-shifting interference as part of executive functioning [21]. Stroop Color-Word Test determines selective attention and inhibition as part of executive functioning [22]. To control for the effect of motor speed on TMT and Stroop performance, we calculated interference measures, specifically TMT-ratio (TMT B/TMT A) and Stroop (Stroop3/((Stroop1+Stroop2)/2)). The Symbol Digit Modalities Test (SDMT) measures information processing speed [23]. Letter Fluency - using three letters - measures response generation as part of executive function [24]. The letter fluency score was composed of the sum score of the three letters. To compare the results of the individual cognitive tests and to limit the number of dependent variables, raw test scores were clustered into compound scores for the four neuropsychological domains. Data of baseline measurements were used as norm data to create individual Z-scores (Z-score = (score test - mean baseline)/SD baseline) and the following domains in formula form were created: Episodic memory = ($Z_{\text{RAVLT total immediate recall}} + Z_{\text{RAVLT decayed recall}} + Z_{\text{RAVLT recognition}}$)/3, Attention and working memory = ($Z_{\text{Digit Span forward}} + Z_{\text{Digit Span backward}}$)/2, Information processing speed = ($-Z_{\text{Stroop mean I and II}} + -Z_{\text{Trail making test part A}} + Z_{\text{Symbol Digit Modalities Tes}_t}$)/3, Executive functioning = ($-Z_{\text{Stroop interference}} + -Z_{\text{Trail making ratio}} + Z_{\text{Verbal Fluency}}$)/3. Cognitive performance was dichotomized: a participant belonging to the worst 10% of cognitive performers was defined as being a poor performer. The other 90% of the population was defined as 'normal' performer [25].

6.2.3 Biochemical analyses

Blood samples were drawn in the morning when the participants were fasted or had consumed a restricted breakfast. Samples were stored at -80˚C until determination. Serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) [26]. Plasma glucose concentrations were analysed using a hexokinase method (Gluco-quant, Roche Diagnostics). Plasma insulin concentrations were determined using an immunometric assay (ADVIA Centaur immunoassay system, Siemens Medical Solutions Diagnostics).

6.2.4 Covariates

Height was measured at baseline with a stadiometer to the nearest 0.1 cm. Weight was measured to the nearest 0.5 kg with a calibrated analogues scale. Body Mass Index (BMI) was calculated as weight/height^2. Data on education level (i.e. primary, secondary or higher education), smoking status (i.e. non-smoker or current smoker), physical activity (kcal/day) [27] and alcohol consumption (i.e. light, moderate, excessive) [28] were collected by means of questionnaires. The 15-item Geriatric Depression Scale (GDS) was used as a screening tool for depression. Scores ≥5 are indicative of a depressive episode [29]. Season was based on the month of blood sampling. Season of blood collection was dichotomized into summer (June–November) and winter (December–May).

6.2.5 Statistical Analyses

Participant characteristics are reported as mean with standard deviation (SD), or percentages. Medians with interquartile range (IQR) were used to report skewed variables. Cox proportional hazards analysis with robust error variance was conducted to calculate Prevalence Ratios (PRs) for domain-specific cognitive performance per serum 25(OH)D tertile, using the lowest tertile as the reference group. By assigning a constant risk period to all participants in the study, the obtained hazard ratio can be considered as a prevalence ratio (PR) [30]. This PR corresponds to the probability of being defined as a poor cognitive performer in participants with moderate or high serum 25(OH)D concentrations, compared to participants with low serum 25(OH)D concentrations. Analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol consumption, habitual physical activity, and season of blood sampling (model 2).

The dose-response of the association between serum 25(OH)D with plasma glucose and plasma insulin was explored by restricted cubic spline regression, and multiple linear regression analyses. Plasma glucose and plasma insulin were not normally distributed and therefore logarithmically transformed. All analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol consumption, habitual physical activity, and season of blood sampling (model 2).

Cox proportional hazards analysis was used to evaluate the associations of plasma glucose and plasma insulin with domain-specific cognitive performance by estimating prevalence ratios as suggested by Barros and Hirataka (2003) [30]; results are presented per 0.1 and 5 unit increment in plasma glucose and plasma insulin, respectively. Analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol consumption, habitual physical activity (model 2), and depression.

Mediation of the association between serum 25(OH)D and cognitive performance by glucose homeostasis was examined by studying the association 1) between serum 25(OH)D and glucose homeostasis, 2) between glucose homeostasis and domain-specific cognitive performance, and 3) by adding plasma glucose and plasma insulin, independently, to fully adjusted Cox proportional hazards models that were used to explore the associations between serum 25(OH)D and domain-specific cognitive performance. Moreover, the interaction between serum 25(OH)D and glucose homeostasis in its association with cognitive performance was tested. In case of significant findings, data were also presented stratified. Analyses were performed using the statistical package SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA). Spline regression analyses were performed using R version 2.15.

6.3 Results

Table 1 shows the population characteristics. Participants of the subsample who participated in more extensive cognitive performance assessment were on average 72.6±5.8 years of age, and had a median MMSE score of 29 (IQR: 28-30). Mean serum 25(OH)D concentration was 60±26 nmol/L. Thirty-seven percent of the participants had serum 25(OH)D concentrations below the recommended concentration of 50 nmol/L [31]. Mean plasma

glucose concentration was 5.8±1.4 mmol/L and median plasma insulin concentration was 66 pmol/L (IQR: 41-127). Participants with the lowest 25(OH)D concentrations were more likely to be older (P<0.0001), have a higher BMI (P=0.002), a lower physical activity level (P=0.006), more depressive symptoms (P=0.009), and more likely to be included in the study during the winter/spring months (<0.0001).

Table 1. Population characteristics, overall and by tertile of serum 25(OH)D.

	Total population	Cognition subsample	Cognition subsample, by tertile of serum 25(OH)D			P-value
			T1 <47 nmol/L	T2 47-70 nmol/L	T3 >70 nmol/L	
N	2857	846	280	286	280	
25(OH)D, nmol/L	56±25	60±26	33±10	59±7	89±17	<0.0001
Sex, men (%)	1459 (50)	500 (58)	149 (53)	174 (61)	172 (61)	0.09
Age, years	74.1±6.5	72.6±5.8	74.1±6.6	72.1±5.2	71.4±4.7	<0.0001
Body Mass Index, kg/m²	27.1±4.0	27.2±3.9	27.5±4.4	27.6±3.8	26.5±3.3	0.002
Glucose, mmol/L	-	5.8±1.4	5.7±1.3	5.9±1.5	5.8±1.6	0.50
Insulin, pmol/L	-	66 (41-127)	71 (44-133)	68 (44-136)	61 (38-105)	0.23
Smoking, n (%)						0.56
Non	989 (34)	267 (31)	80 (29)	89 (31)	92 (33)	
Current	281 (10)	87 (10)	34 (12)	29 (10)	23 (8)	
Former	1649 (56)	503 (59	166 (59)	168 (59)	165 (59)	
Physical activity, kcal/day	650 (343-826)	576 (344-862)	496 (289-785)	581 (382-874)	625 (386-894)	0.006
Education, n (%)						0.68
Primary	1547 (53)	368 (43)	122 (44)	127 (44)	113 (40)	
Secondary	615 (21)	190 (22)	63 (23)	65 (23)	59 (21)	
Higher	757 (26)	299 (35)	95 (34)	94 (33)	108 (39)	
Alcohol intake, n (%)						0.02
Light	1966 (67)	556 (65)	201 (72)	171 (60)	174 (62)	
Moderate	839 (29)	272 (32)	68 (24)	105 (37)	98 (35)	
Excessive	112 (4)	29 (3)	11 (4)	10 (3)	8 (3)	
MMSE score, range 0-30	29 (28-30)	29 (28-30)	29 (27-30)	29 (28-30)	29 (28-30)	0.16
GDS-15 score, range 0-15	1.0 (0-2.0)	1.0 (0-2.0)	1.0 (0-2.0)	1.0 (0-2.0)	0 (0-2.0)	0.009
Blood sampling from June - November	1531 (52)	557 (65)	145 (52)	191 (67)	216 (77)	<0.0001

Note: MMSE=Mini-Mental State Examination. GDS-15=Geriatric Depression Scale-15. T=Tertile. P-value refers to differences between tertiles. Missing (n): BMI (16), plasma glucose (11), plasma insulin (10), physical activity (16), alcohol intake (2), MMSE (14), GDS-15 (19).

6.3.1 Vitamin D and cognitive performance

Unadjusted models showed that low serum 25(OH)D was associated with a higher probability of a poor performance on all four cognitive domains (**Table 2**). After full adjustment only the association of serum 25(OH)D with attention and working memory remained significant,

showing that participants in the upper serum 25(OH)D tertile had a 51% lower probability of being a poor performer. For associations with executive function and episodic memory a non-significant trend remained. Specifically, for executive function, people in the second and third tertile had a 53% lower (PR 0.47 (95% CI 0.27-0.80) and borderline non-significant 39% lower (PR 0.61 (95% CI 0.35-1.06)) probability of a poor performance. A borderline significant association was observed for episodic memory, PR 0.75 (95% CI 0.55-1.02) for the upper serum 25(OH)D tertile. Additional adjustment for depression did not alter these results.

Table 2. Associations of serum 25(OH)D with domain-specific cognitive performance as shown by Prevalence Ratio's (PRs) with 95% Confidence Interval (CI), continuously and by tertile of 25(OH)D.

	Prevalence Ratio's by tertile of serum 25(OH)D (nmol/L)			
WUR (total population)	**T1: 4-47 (4-42)**	**T2: 47-70 (42-65)**	**T3: 70-194 (65-194)**	**P for trend**
AWM, n (cases)*	263 (38)	262 (27)	262 (18)	
Crude model	1.0	0.69 (0.44-1.09)	0.46 (0.27-0.78)	0.005
Model 1	1.0	0.70 (0.44-1.11)	0.47 (0.27-0.81)	0.008
Model 2	1.0	0.72 (0.45-1.14)	0.49 (0.29-0.83)	0.008
EF, n (cases)*	254 (40)	260 (17)	262 (19)	
Crude model	1.0	0.39 (0.23-0.67)	0.43 (0.26-0.72)	0.004
Model 1	1.0	0.46 (0.27-0.79)	0.53 (0.31-0.91)	0.04
Model 2	1.0	0.47 (0.27-0.80)	0.61 (0.35-1.06)	0.13
IPS, n (cases)*	260 (32)	267 (29)	270 (19)	
Crude model	1.0	0.86 (0.54-1.37)	0.56 (0.33-0.95)	0.04
Model 1	1.0	1.35 (0.84-2.15)	0.95 (0.56-1.62)	0.95
Model 2	1.0	1.44 (0.91-2.27)	1.01 (0.59-1.74)	0.86
EM, n (cases)*	896 (108)	918 (96)	908 (70)	
Crude model	1.0	0.87 (0.67-1.12)	0.64 (0.48-0.85)	0.002
Model 1	1.0	1.02 (0.78-1.31)	0.76 (0.57-1.02)	0.07
Model 2	1.0	0.99 (0.76-1.29)	0.75 (0.55-1.02)	0.07

Note: *Participants were characterized as poor cognitive performers when cognitive test scores fell below the 10th percentile. Model 1 is adjusted for age and sex. Model 2 is adjusted for age, sex, BMI, education, alcohol consumption, smoking, physical activity, season of blood sampling, and centre. None of the PRs substantially changed after additional adjustment for depression, glucose or insulin. AWM=Attention and Working Memory. EF=Executive Function. IPS=Information Processing Speed. EM=Episodic Memory. WUR: Wageningen subsample.

6.3.2 Vitamin D and glucose homeostasis

Tests for non-linearity of the restricted cubic splines showed that the dose-response association of serum 25(OH)D status with log-transformed plasma glucose and plasma insulin could be considered linear (**supplementary Figure 2**). Subsequently, multiple linear regression analyses showed significant associations for serum 25(OH)D with plasma insulin after adjustment for age and sex, β -0.003 (P=0.02). However, in the fully adjusted regression analysis this association attenuated and became non-significant, β -0.001 (P=0.31).

6.3.3 Glucose homeostasis and domain-specific cognitive performance

In fully adjusted models only a significant association between plasma glucose and attention and working memory was observed, PR 1.01 (1.00-1.02) per 0.1 unit increase in plasma glucose, which roughly suggests a 10% higher probability of being a poor performer with every unit increase in plasma glucose. Plasma glucose and insulin did not significantly associate with any of the other cognitive outcome measures (data not shown).

6.3.4 Is there an interplay of serum 25(OH)D and glucose homeostasis?

The above-mentioned findings on (1) serum 25(OH)D and cognitive performance, (2) serum 25(OH)D with glucose homeostasis, (3) glucose homeostasis and cognitive performance, (4) and the association between serum 25(OH)D and cognitive performance adjusted for either plasma glucose or plasma insulin, imply that there is no mediation effect by glucose homeostasis in the associations between serum 25(OH)D and domain-specific cognitive performance (**Figure 1**). Exploration of a potential modification effect of plasma glucose and plasma insulin (5) showed a significant interaction of plasma insulin with serum 25(OH)D for the domain executive function (P=0.001), indicating a stronger association between serum 25(OH)D with executive function when having high plasma insulin concentrations compared to having low plasma insulin concentrations (**Figure 1 and Figure 2**). In line, a modest trend towards a modification effect was also shown for plasma glucose on the association of serum 25(OH)D with executive function, suggesting that the association between high

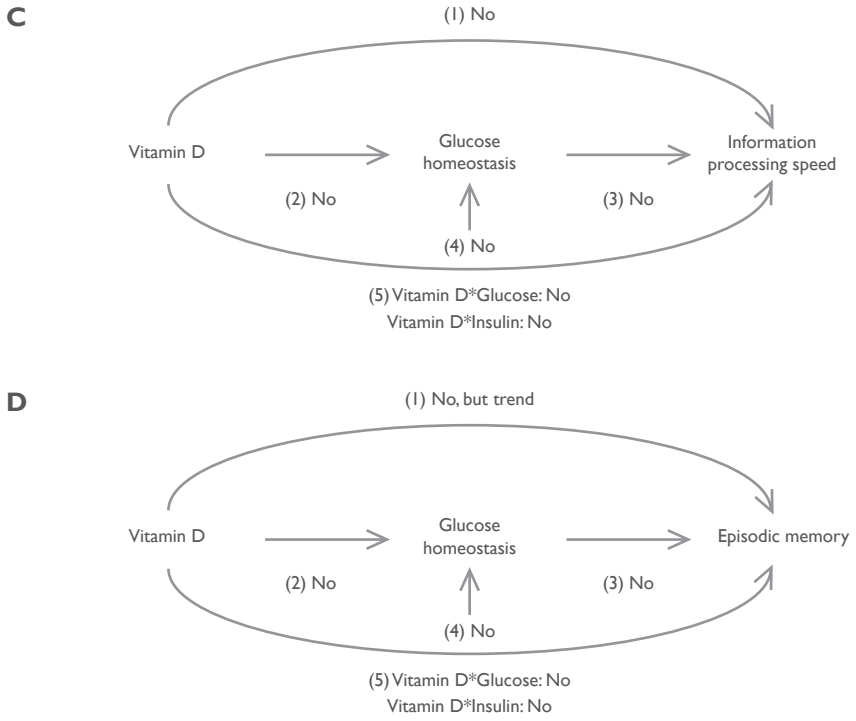

Figure 1. Overview of the associations studied. Associations of (1) serum 25(OH)D with cognitive performance, (2) serum 25(OH)D with glucose homeostasis, (3) glucose homeostasis and cognitive function, (4) serum 25(OH)D and cognitive function that were adjusted for either plasma glucose or plasma insulin, (5) interactions between vitamin D*plasma glucose and vitamin D*plasma insulin in association to cognitive performance.

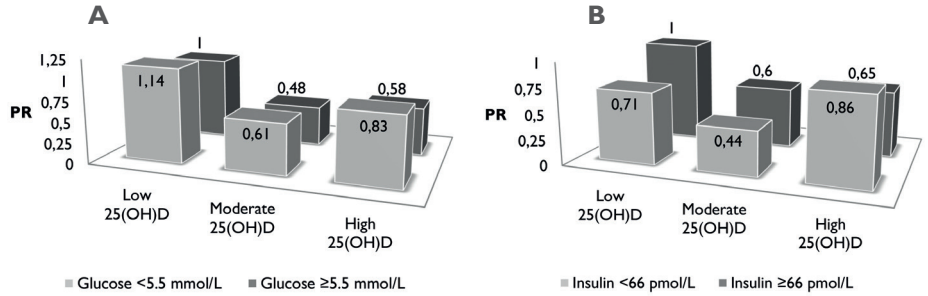

Figure 2. Associations of serum 25(OH)D with the domain executive function, stratified for (A) glucose and (B) insulin. *Note*: Associations are shown by Prevalence Ratio's (y-axis). Participants were characterized as poor cognitive performers when cognitive test scores fell below the 10th percentile.

25(OH)D concentrations and executive function is stronger in participants with high plasma glucose concentrations compared to those with low plasma glucose concentrations (*P for interaction: 0.11*). Finally, a modest trend was observed for a modification effect of plasma glucose in the association of serum 25(OH)D with attention and working memory (*P for interaction: 0.12*).

6.4 Discussion

In this study, serum 25(OH)D was associated with attention and working memory. Borderline significant trends for associations of serum 25(OH)D with executive function and episodic memory were observed. There was not convincing evidence for an interplay between vitamin D and glucose homeostasis in these associations.

Already several studies investigated the association between 25(OH)D status and global cognitive performance [1, 7], but only some focussed on specific cognitive domains [8-15]. In line with our results on serum 25(OH)D and attention and working memory, participants of the NAME study (n=1069) [8] and the ZENITH study (n=387) [12] also performed better on tasks related to working memory with increasing 25(OH)D concentrations. In the ISAAC study (n=159) [11], a trend towards an association with the domain working memory was observed. We furthermore observed a trend towards an association between serum 25(OH)D and executive function. Four other studies also provided evidence for a potential role of serum 25(OH)D in executive function [8, 10, 13, 15]. Data of the MrOS (n=1559) [14] and the ISAAC study [11] did not support an association between vitamin D and executive function. The association between serum 25(OH)D and episodic memory has been studied before, but none of these studies observed a trend association as shown in our study [8, 9, 11, 12, 32]. McGrath and colleagues showed in the NHANES III (n=4809) that, in contrast to their hypothesis, older adults with higher serum 25(OH)D concentrations had worse memory and learning scores after adjustment for age, sex, ethnicity and physical activity [32]. We did not observe an association between serum 25(OH)D and information processing speed, which is in line with results of the ZENITH study [12]. In contrast, the European Male Ageing study [9] and the NAME study [8] have shown significant associations of serum 25(OH)D with information processing speed tasks. Trials examining the effect of vitamin D on cognitive performance are scarce and most of them were suboptimal, having a small sample size, being relatively short term, or lacking a control group [33-36].

Mechanistic studies suggest a role for vitamin D in the production of neurotrophins and neurotransmitters, inflammation, oxidative stress, and excitotoxicity, which may influence neurogenesis, neurotransmission, synaptic plasticity as well as neuronal survival [4]. Based on previous neuropsychological findings in observational studies, Annweiler and colleagues (2012) proposed that low vitamin D concentrations may be particularly associated with executive dysfunction, and as such with dysfunction of frontal-subcortical neuronal circuits [37]. In our study a significant association was shown between serum 25(OH)D and attention and working memory; trends were observed for executive function and episodic memory. Explanations for these findings may lie in the fact that conceptually, the domains executive function and working memory overlap, as reflected in strong correlations between tasks of working memory and executive function (r=0.97) [38], and that executive function and working memory by themselves may determine episodic memory task performance [38]. Due to this conceptual overlap between the specific brain functions [38] it is challenging to identify what specific brain regions are affected in case of vitamin D deficiency.

To further explore these underlying mechanisms linking vitamin D to cognitive performance

we explored the potential modification and mediation effect of markers related to glucose homeostasis in the association between serum 25(OH)D and cognitive performance. It was shown that plasma glucose or plasma insulin did not mediate the pathway between serum 25(OH)D and cognitive performance. Our analyses did reveal a significant interaction of serum 25(OH)D with plasma insulin on the domain executive function. To the best of our knowledge, this is the first study examining the role of glucose homeostasis in this detail. Four other studies adjusted for diabetes or surrogate markers of diabetes in the association between serum 25(OH)D and cognitive performance [9, 10, 15, 16]; only the NHANES III data suggested that diabetes may be involved [16].

Our participants were mainly community-dwelling Caucasian men and women with a slightly elevated homocysteine concentration (≥12 μmol/L). Even though a substantial proportion of the Dutch older adults have such an elevated concentration [39], our findings may not be generalizable to other populations. Another limitation of this study is the cross-sectional design. We observed that vitamin D concentrations were higher in persons with a better attention and working memory. Consequently, we suggest that the lower vitamin D concentrations may result in a poorer attention and working memory. However, it is also plausible that participants with a poor attention and working memory were less likely to go outside and that low 25(OH)D concentrations are the result of the poor cognitive performance observed. Another limitation is that participants were allowed to consume a restricted breakfast. Therefore, the plasma glucose and plasma insulin concentrations measured cannot be considered completely fasting. Consequently, associations in this study with plasma glucose and plasma insulin as the outcome may have been biased towards the null. Associations with plasma glucose and plasma insulin as the exposure may be underestimated. Strengths of this study are the extensive cognitive test battery used, its large sample size, the possibility to adjust for a large number of potential confounders, and the possibility to further explore the potential role of glucose homeostasis in the association between 25(OH)D and cognitive performance.

In all, we conclude that this study points towards a link between serum 25(OH)D and cognitive performance, specifically attention and working memory. This study did not provide convincing evidence for an interplay between vitamin D and glucose homeostasis in association with cognitive performance. The fact that we mainly observed non-significant trends for the associations between serum 25(OH)D and cognitive performance may be because our participants were too young and healthy to show a clear clinical manifestation of cognitive problems. While in our population brain regions may already function less efficiently, it is likely that this decreased efficiency is compensated for via the activation of alternative brain networks [40]. In future studies it might therefore be interesting to further explore this potential 'compensatory' mechanism by including brain activity measurements, for instance by using near-infrared spectroscopy, functional Magnetic Resonance Imaging, or EEG.

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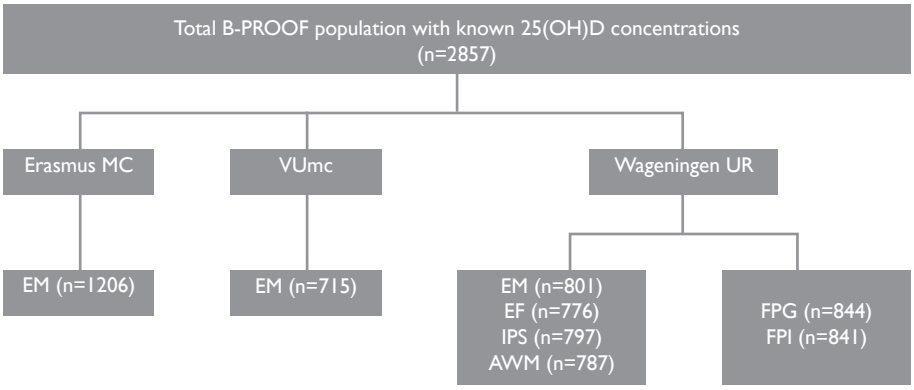
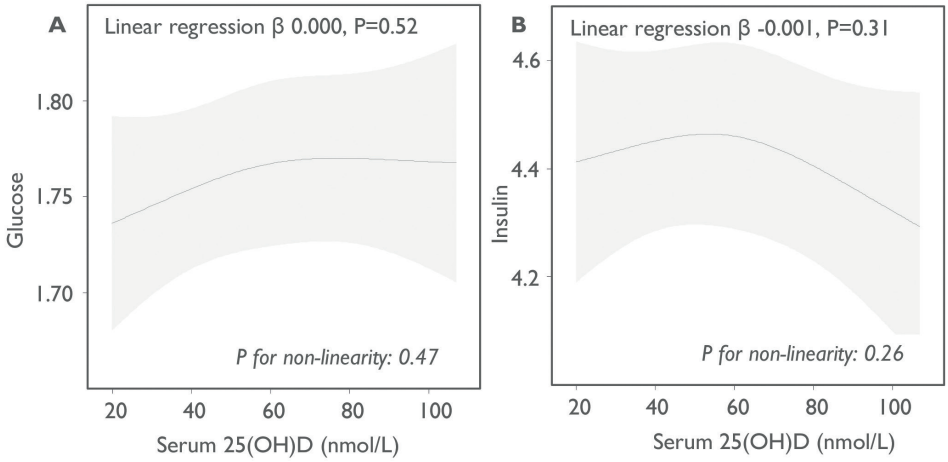
Supplementary material



sFigure 1. Flow diagram describing the data used for the statistical analyses.



sFigure 2. Associations of serum 25(OH)D with glucose and insulin concentrations. *Note:* Associations were adjusted for age, sex, BMI, education, alcohol consumption, smoking, physical activity and season of blood sampling. Knots were at 30, 58 and 91 nmol/L.

7

Vitamin D-epressed: Low vitamin D status is associated with more depressive symptoms in Dutch older adults

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van Wijngaarden JP
van der Zwaluw NL
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van Dijk SC
Swart KMA
Enneman AW
Ham AC
van Schoor NM
van der Velde N
Uitterlinden AG
Lips P
Feskens EJM
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Abstract

Background
The existence of vitamin D receptors in the brain point to a possible role of vitamin D in brain function. We examined the association of vitamin D status and vitamin D related genetic make-up with depressive symptoms among 2839 Dutch older adults aged ≥65 years.

Methods
Serum 25-hydroxyvitamin D (25(OH)D) was measured and seven 'vitamin D SNPs' were selected. Depressive symptoms were measured with the 15-point Geriatric Depression Scale. Results were expressed as the Relative Risk (RR) of the score of depressive symptoms by quartiles of 25(OH)D concentration or the number of affected alleles, using the lowest quartile or minor allele group as the reference.

Results
A clear cross-sectional and prospective association between serum 25(OH)D and the depressive symptom score was observed. Fully adjusted models indicated a 22% (RR 0.78, 95% CI 0.68-0.89), 21% (RR 0.79, 95% CI 0.68-0.90) and 18% (RR 0.82, 95% CI 0.71-0.95) lower score of depressive symptoms in people in the 2nd, 3rd and 4th 25(OH)D quartile, when compared to people in the 1st quartile (P for trend <0.0001). After two-years of 15 μg/day vitamin D supplementation, similar associations were observed. Serum 25(OH)D concentrations did not significantly interact with the selected SNPs.

Limitations
Serum 25(OH)D concentrations at two-years of follow-up were not measured.

Conclusion
Low serum 25(OH)D was associated with a higher score of depressive symptoms. No interactions between 25(OH)D concentrations and vitamin D related genetic make-up were observed. In view of the probability of reverse causation we propose that the association should be further examined in prospective studies as well as in randomized controlled trials.

7.1 Introduction

Globally almost 350 million people are affected by depression [1]. Depression is regularly accompanied by a reduced quality of life, a variety of co-morbidities, and a higher mortality rate [2]. The high prevalence of depression [1], its possible consequences [2], plus the unwanted side-effects [3] that often accompany the use of anti-depressive medication emphasize the need for preventive measures.

One of the factors that has been suggested to beneficially influence mood and depression is sun exposure [4]. Sun exposure, more specifically exposure to ultraviolet-B radiation, may amongst others positively reduce depressive symptoms by activating the vitamin D synthesis in the skin. Mechanistic studies on brain function support this hypothetical pathway [5, 6]. Furthermore, as low vitamin D concentrations have been associated with diabetes [7], and diabetes with depression [8], vitamin D may also indirectly affect the prevalence of depressive symptoms by influencing glucose homeostasis. In addition, it has been postulated that vitamin D inadequacy may make the brain more susceptible to neurobiological triggers, like diabetes. Thus, both interaction and modification effects by diabetes may be observed when examining the association between vitamin D and depression.

One of the groups at risk for a vitamin D inadequacy are older adults, which may be explained by their reduced skin capacity to synthesize vitamin D, reduced outdoor activities and decreased dietary intake. To our knowledge, seven observational studies investigated the potential association between 25(OH)D concentrations and depression in populations aged ≥60 years [9-15]. Six studies observed significant associations [11-15], indicating that persons with higher 25(OH)D concentrations had a lower probability of having higher scores of depressive symptoms when compared to those with lower 25(OH)D concentrations. Even though most of these studies plea for vitamin D, it needs to be emphasized that there is considerable heterogeneity between studies due to differences in study design, populations, sample sizes, the covariates adjusted for and the method to quantify depression. Moreover, specific pathways clarifying the association between serum 25(OH)D and depressive symptoms in these populations have not been investigated. Thus, more evidence is warranted.

In the current study we investigated the cross-sectional association between serum 25(OH)D and the score of depressive symptoms in a large sample of older adults. To further elucidate the effect of temporality, we also explored the association between baseline 25(OH)D concentrations and the score of depressive symptoms after two years of 15 μg/day vitamin D_3 supplementation. In addition, interactions between 25(OH)D concentrations and seven vitamin D related SNPs were examined, specifically DHCR7, CYP2R1, CYP24A1, GC, TaqI/BsmI, ApaI and Cdx2. Finally, to further investigate the potential underlying mechanisms, also the potential modification and mediation effects of self-reported diabetes were studied.

7.2 Methods

7.2.1 Participants

This study was performed using data of the B-PROOF study; a randomized, double-blind, placebo-controlled trial designed to assess the efficacy of two-year daily oral supplementation of vitamin B_{12} and folic acid on fractures in mildly hyperhomocysteinemic (plasma homocysteine 12-50 μmol/l) older adults ≥65 years; details have been reported previously [16]. Given the known beneficial effect of vitamin D on bone health, 15 μg/day vitamin D_3 was added to both placebo and treatment tablets. The Medical Ethics Committee of Wageningen UR approved the study protocol and the Medical Ethics Committees of VUmc and Erasmus MC confirmed local feasibility. All participants gave written informed consent. At baseline 25(OH)D status and depression score data was available of 2839 participants; after two-years of follow-up data for 2544 participants was available. See **Figure 1** for a detailed overview of the participant flow.

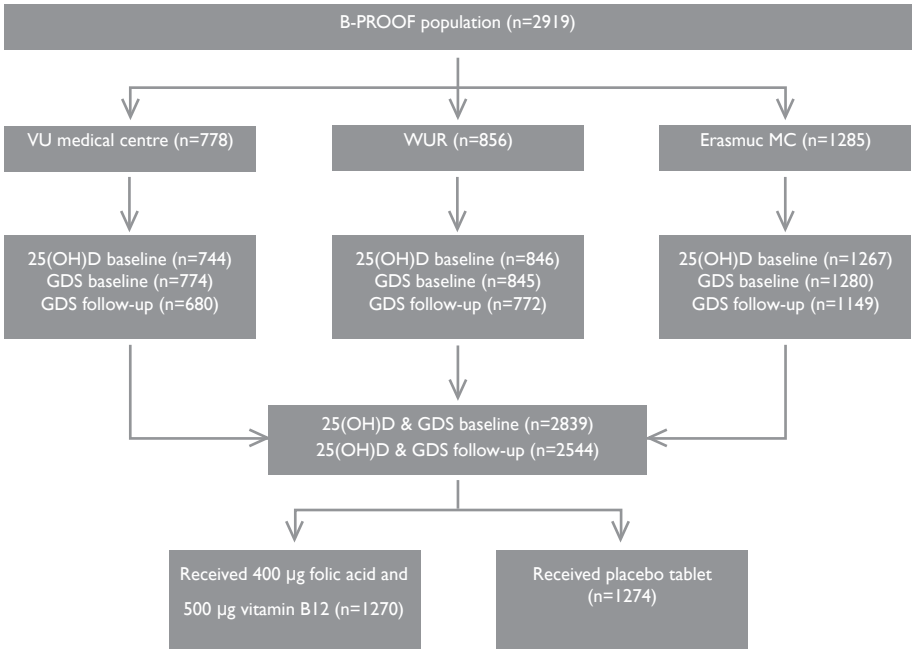

Figure 1. Flowchart of B-PROOF study data used for the cross-sectional and prospective analyses on 25(OH)D concentrations and depression scores. *Note:* GDS indicates Geriatric Depression Score-15 questionnaire.

7.2.2 Biochemical analyses and genotyping

Blood samples were drawn in the morning when the participants were fasted or had consumed a restricted breakfast. Samples were stored at -80˚C until determination. Serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) [17]. DNA was isolated from buffy coats. Samples were genotyped for about 700000 SNPs using the Illumina Omni-express array, covering

>90% of all common variation in the genome. SNPs selected for this study included SNPs affecting vitamin D synthesis (rs12785878 [DHCR7] and rs10741657 [CYP2R1]), SNPs affecting vitamin D metabolism (rs6013897 [CYP24A1] and rs2282679 [GC]) and vitamin D receptor (VDR) SNPs (rs731236 [TaqI], rs1544410 [BsmI], rs7975232 [ApaI] and rs11568820 [Cdx2]).

7.2.3 Mental Health

The 15-item Geriatric Depression Scale (GDS) is a widely used self-report questionnaire, which is designed to measure to number of depressive symptoms in a population of older adults [18]. Scores can range from 0-15 points, where higher scores are indicative of more depressive symptoms. Scores ranging from 5-7 are suggestive of mild depression; scores ranging from 8-9 are indicative of moderate depression [18].

7.2.4 Covariates

Height was measured at baseline with a stadiometer to the nearest 0.1 cm. Weight was measured to the nearest 0.5 kg with a calibrated analogue scale. Body Mass Index (BMI) was calculated as weight/height^2. Data on education level (i.e. primary, secondary or higher education), smoking status (i.e. no, current, former), physical activity (kcal/day) [19], and alcohol consumption (i.e. light, moderate, excessive) [20] were collected by means of questionnaires. Diabetes diagnosis was ascertained by asking participants whether they had diabetes (questionnaire). Season was based on the month of blood sampling and dichotomized in summer/autumn (June-November) and winter/spring (December-May).

7.2.5 Statistical Analyses

Participants characteristics are reported as mean with standard deviation (SD), or as percentages. Medians with interquartile range were used to report skewed variables. Restricted cubic splines were used to estimate the dose-response for serum 25(OH)D with the score of depressive symptoms. The association between serum 25(OH)D and the score of depressive symptoms was further explored using multiple Poisson regression, resulting in Relative Risks (RRs) for 25(OH)D status with the score of depressive symptoms. This RR corresponds to the probability of reporting a higher score of depressive symptoms when allocated to the second, third or fourth quartile of this population compared to participants allocated to the first quartile (i.e. those with the lowest 25(OH)D concentrations). Analyses were adjusted for age, sex (model 1), BMI, education, smoking, alcohol intake, physical activity, season of blood sampling, center (model 2), and diabetes (model 3). Follow-up analyses were additionally adjusted for treatment.

Poisson regression was also used to test whether self-reported diabetes and vitamin D SNPs were associated with the score of depressive symptoms. Associations between self-reported diabetes and the score of depressive symptoms were adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, and center.

Cox proportional hazards regression was applied to examine the associations between serum 25(OH)D and self-reported diabetes. By assigning a constant risk period to all participants

in the study, the obtained hazard ratio can be considered as a prevalence ratio (PR) [21]. This PR corresponds to the probability of having diabetes when allocated to the second, third or fourth serum 25(OH)D quartile, compared to participants allocated to the first serum 25(OH)D quartile (i.e. lowest serum 25(OH)D concentrations). These analyses were adjusted for age, sex (model 1), BMI, education, smoking, physical activity, alcohol intake, season of blood sampling, and center (model 2).
Finally, Poisson regression was conducted to examine potential interactions between serum 25(OH)D and self-reported diabetes, and serum 25(OH)D and vitamin D related SNPs, in the association with the depressive symptom score. All analyses were performed using the statistical package SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA), except for the restricted cubic spline regression analysis, which was performed in the program R.

7.3 Results

Population characteristics are presented in **Table 1**. Participants were on average 74.1±6.5 years old and had a mean serum 25(OH)D concentrations of 56±25 nmol/L. 45% of the participants had a 25(OH)D concentration <50 nmol/L, 7% (baseline) / 8% (follow-up) reported ≥5 depressive symptoms, and 10% indicated to have diabetes. Over quartiles of serum 25(OH)D, significant differences were observed for age, sex, BMI, smoking habits, alcohol consumption, physical activity level, season of blood sampling, the score of depressive symptoms, self-reported diabetes, DHCR7 and CYP2R1.

Table 1. Characteristics of Dutch older adults participating in the B-PROOF study per quartile of serum 25(OH)D in nmol/L.

	Q1 <36.7	Q2 36.7-53.4	Q3 53.4-71.7	Q4 >71.7	P-value
N*	711	718	712	716	
25(OH)D, nmol/L	26±7	45±5	62±5	89±15	<0.0001
Sex, number of men (%)	306 (43)	365 (51)	383 (54)	374 (52)	0.0002
Age, years	76.1±7.4	73.7±6.3	73.7±6.1	72.7±5.7	<0.0001
Body Mass Index, kg/m²	27.7±4.6	27.3±3.9	27.3±3.7	26.3±3.5	<0.0001
Smoking status, n (%)					0.02
Non-smoker	266 (37)	240 (33)	232 (33)	231 (32)	
Smoker	83 (12)	72 (10)	66 (9)	56 (8)	
Former smoker	362 (51)	406 (57)	414 (58)	429 (60)	
Physical activity, kcal/day	544±385	681±504	679±539	693±451	<0.0001
Educational level, n (%)					0.07
Primary education	393 (55)	309 (54)	386 (54)	347 (48)	
Secondary education	142 (20)	151 (21)	155 (22)	152 (21)	
Higher education	176 (25)	177 (25)	171 (24)	217 (30)	
Alcohol intake, n (%)					0.001
Light	512 (72)	509 (71)	446 (63)	456 (64)	
Moderate	168 (24)	188 (26)	239 (34)	228 (32)	
Excessive	29 (4)	21 (3)	27 (3)	32 (4)	
GDS score at baseline	1 (3)	1 (2)	1 (2)	1 (2)	<0.0001
(% GDS score ≥5)	(11)	(5)	(6)	(6)	

	Q1 <36.7	Q2 36.7-53.4	Q3 53.4-71.7	Q4 >71.7	P-value
GDS score 2-years follow-up (% GDS score ≥5)	1 (3) (11)	1 (2) (5)	1 (2) (9)	1 (2) (7)	<0.0001
Self-reported diabetes, n (%)	79 (14)	55 (10)	54 (10)	43 (8)	0.01
Blood sampling in summer, n (%)	224 (32)	324 (45)	429 (60)	524 (73)	<0.0001
Vitamin B_{12} and folic acid treatment, n (%)	372 (52)	359 (50)	329 (46)	373 (52)	0.07
TaqI/BsmI (n=2555)					
• **0 affected alleles, n (%)**	108 (18)	113 (18)	110 (17)	127 (20)	0.75
• **1 affected alleles, n (%)**	285 (48)	315 (49)	316 (49)	318 (49)	
• **2 affected alleles, n (%)**	206 (34)	211 (33)	173 (34)	198 (31)	
ApaI (n=2555)					
• **0 affected alleles, n (%)**	137 (23)	141 (22)	155 (24)	134 (21)	0.55
• **1 affected alleles, n (%)**	299 (50)	328 (51)	302 (46)	316 (49)	
• **2 affected alleles, n (%)**	163 (24)	170 (27)	192 (30)	193 (30)	
Cdx2 (n=2555)					
• **0 affected alleles, n (%)**	21 (4)	23 (4)	25 (4)	25 (4)	0.99
• **1 affected alleles, n (%)**	180 (30)	201 (31)	193 (30)	200 (31)	
• **2 affected alleles, n (%)**	398 (66)	415 (65)	431 (66)	418 (65)	
DHCR7 (n=2555)					
• **0 affected alleles, n (%)**	50 (8)	59 (9)	49 (7)	37 (6)	0.004
• **1 affected alleles, n (%)**	261 (44)	252 (40)	232 (36)	233 (36)	
• **2 affected alleles, n (%)**	288 (48)	328 (51)	368 (57)	373 (58)	
CYP2R1 (n=2555)					
• **0 affected alleles, n (%)**	112 (19)	97 (15)	98 (15)	116 (18)	0.01
• **1 affected alleles, n (%)**	251 (42)	307 (48)	337 (52)	318 (49)	
• **2 affected alleles, n (%)**	236 (39)	235 (37)	214 (33)	209 (33)	
CYP24A1 (n=2555)					
• **0 affected alleles, n (%)**	35 (6)	25 (4)	25 (4)	25 (4)	0.06
• **1 affected alleles, n (%)**	218 (36)	198 (31)	226 (35)	199 (31)	
• **2 affected alleles, n (%)**	346 (58)	416 (65)	398 (61)	419 (65)	
GC (n=2555)					
• **0 affected alleles, n (%)**	75 (13)	62 (10)	55 (8)	20 (3)	<0.0001
• **1 affected alleles, n (%)**	234 (39)	278 (43)	252 (39)	215 (33)	
• **2 affected alleles, n (%)**	290 (48)	299 (47)	342 (53)	408 (64)	

Note: Values are expressed as mean±SD, median (IQR) or n (%). Chi-squared tests for categorical variables, and one-way analysis of variance for continuous variables were performed to compare baseline characteristics over quartiles of 25(OH)D. *Drop-out after 2-years of follow-up: Q1 (n=94), Q2 (n=75), Q3 (n=74) and Q4 (n=52).

Restricted cubic spline regression showed a modest dose-response association between serum 25(OH)D and the score of depressive symptoms, leveling off around 60-65 nmol/L (**Figure 2**). The test for non-linearity indicated that the association does not follow a linear tendency (*P for non-linearity: 0.04*). Fully adjusted Poisson regression (model 2) subsequently showed a 22% (RR 0.78, 95% CI 0.68-0.89), 21% (RR 0.79, 95% CI 0.68-0.90) and 18% (RR 0.82, 95% CI 0.71-0.95) lower score of depressive symptoms in people in the second, third and fourth quartile of serum 25(OH)D, respectively, when compared to people in the first quartile (**Table 2**). Prospective analyses using baseline serum 25(OH)D and depression data

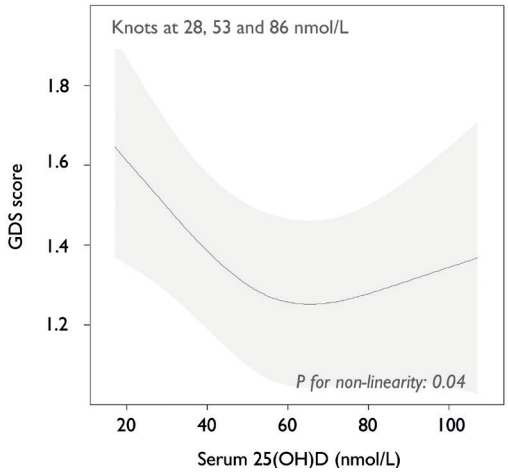

Figure 2. Cross-sectional association between serum 25(OH)D and the number of depressive symptoms.

obtained after two years of vitamin D supplementation with 15 µg of per day showed 26%, 18% and 21% less depressive symptoms in people in the second, third and fourth quartile of serum 25(OH)D, when compared to people in the first quartile (model 2). Additional adjustment for treatment group did not substantially alter the results. Associations between vitamin D-related genetic make-up and the score of depressive symptoms were non-significant (**supplementary Table 1**). Moreover, no significant interactions between serum 25(OH)D and any of the vitamin D-related SNPs were observed in association to the score of depressive symptoms.

Table 2. Associations between serum 25(OH)D at baseline with the number of depressive symptom score at baseline and after 2-years of vitamin D supplementation with 15 µg/day, Relative Risks (95% CI).

	Q1	Q2	Q3	Q4	P for trend
25(OH)D Status (nmol/L)	**<36.7**	**36.7-53.4**	**53.4-71.7**	**71.7>**	
Baseline, n	704	714	709	712	
Crude model (n=2839)	1.0	0.69 (0.60-0.79)	0.69 (0.61-0.79)	0.68 (0.60-0.78)	P<0.0001
Model 1 (n=2899)	1.0	0.76 (0.66-0.87)	0.77 (0.67-0.88)	0.78 (0.68-0.90)	P<0.0001
Model 2 (n=2880)	1.0	0.78 (0.68-0.89)	0.79 (0.68-0.90)	0.82 (0.71-0.95)	P<0.0001
Model 3 (n=2183)	1.0	0.71 (0.61-0.83)	0.73 (0.62-0.86)	0.83 (0.70-0.98)	P=0.0001
2-year follow-up, n	610	639	635	660	
Crude model (n=2544)	1.0	0.66 (0.58-0.76)	0.74 (0.64-0.85)	0.68 (0.59-0.78)	P<0.0001
Model 1 (n=2544)	1.0	0.72 (0.64-0.84)	0.82 (0.71-0.95)	0.78 (0.67-0.90)	P<0.0001
Model 2 (n=2531)	1.0	0.74 (0.64-0.84)	0.82 (0.71-0.95)	0.79 (0.68-0.92)	P<0.0001
Model 3 (n=1996)	1.0	0.69 (0.59-0.79)	0.78 (0.66-0.93)	0.76 (0.64-0.89)	P<0.0001

Note: Model 1 is adjusted for age and sex. Model 2 is adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, season of blood sampling, and center. Model 3 is adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, season of blood sampling, center, and self-reported diabetes.

As vitamin D inadequacy has been suggested to be a potential modifiable risk factor for diabetes [7], and diabetes patients have been shown to be at an increased risk of depression [8], also the potential mediation and modification effect of diabetes was examined. First of all, the association between serum 25(OH)D and self-reported diabetes was explored, showing strong associations over quartiles of serum 25(OH)D. Namely, crude models showed an up to 42% lower probability of having diabetes in the upper quartile, PR 0.58 (95% CI 0.41-

0.82). In the full-adjusted model the associations attenuated, which was mainly attributable to the inclusion of BMI, the PR was 0.77 (0.52-1.14) in the upper quartile (**Table 3**). Full-adjusted model that did not include BMI, which was investigated because BMI might be an intermediate in the association between serum 25(OH)D and diabetes, indicated a 43% lower probability of having diabetes in the upper 25(OH)D quartile. Second, we studied the possible association between self-reported diabetes and the score of depressive symptoms, showing that participants with diabetes (n=231) reported a 17% higher score of depressive symptoms than participants without diabetes (n=2024) after full adjustment, RR 1.17 (95% CI 1.00-1.38) (**Table 4**). Mediation was further examined by extending the model of serum 25(OH)D and the score of depressive symptoms with self-reported diabetes, showing that the observed associations were even slightly strengthened. Finally, including the interaction term serum 25(OH)D*diabetes did not point towards a modification effect in the association with the score of depressive symptoms (*P for interaction=0.82*).

Table 3. Associations between serum 25(OH)D and self-reported diabetes, PRs (95% CI).

25(OH)D Status (nmol/L)	n	Q1 4.1-36.7	Q2 36.7-53.4	Q3 53.4-71.7	Q4 71.7-193.6	P for trend
Diabetes, n (cases)		569 (79)	560 (55)	543 (54)	536 (43)	
Crude model	2208	1.0	0.71 (0.51-0.98)	0.72 (0.52-0.99)	0.58 (0.41-0.82)	0.003
Model 1	2208	1.0	0.68 (0.49-0.95)	0.69 (0.50-0.96)	0.56 (0.39-0.80)	0.002
Model 2	2191	1.0	0.80 (0.57-1.11)	0.81 (0.57-1.16)	0.77 (0.52-1.14)	0.20

Note: Model 1 is adjusted for age and sex. Model 2 is adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, season of blood sampling, and centre.

Table 4. Associations of self-reported diabetes with the number of depressive symptom score at baseline, analysed with Poisson regression resulting in Relative Risks (95% CI).

	Self-report diabetes	
	No (n=2024)	**Yes (n=231)**
Crude model	1.0	1.22 (1.04-1.43)
Model 1	1.0	1.25 (1.07-1.46)
Model 2	1.0	1.17 (1.00-1.38)

Note: Model 1 is adjusted for age and sex. Model 2 is adjusted for age, sex, BMI, education, smoking, physical activity, alcohol intake, season of blood sampling, and centre.

7.4 Discussion

This study showed a clear cross-sectional association between serum 25(OH)D and the score of depressive symptoms as reported by the Geriatric Depression Scale-15. This association remained after two years of vitamin D supplementation. No significant associations between vitamin D-related genetic make-up and the score of depressive symptoms were observed, nor did we observe significant interactions between vitamin D SNPs and 25(OH)D concentrations. There was also no evidence for a potential mediation or modification effect by the presence of self-reported diabetes.

7.4.1 A broader perspective

Several other observational studies also examined the association between serum 25(OH)D and depression in populations ≥60 years [9-15]. A study in 1282 Dutch men and women aged 65-95 years from the Longitudinal Aging Study Amsterdam observed that persons in the highest 25(OH)D quartile had a lower probability of depression as measured with the Center for Epidemiological Studies Depression Scale (CES-D) (β −1.33 P=0.03) when compared to the lowest quartile [12]. The Os Study, including 883 Chinese men ≥65 years, also showed a beneficial association between 25(OH)D status and depression with an OR for depression of 0.46 (95% CI: 0.22-0.98, P for trend=0.004) in the highest quartile after adjustment for age, BMI, education, physical activity, number of activities of daily living, diet quality index, smoking, alcohol consumption, season, number of chronic diseases, cognitive performance, and serum (ln)PTH concentration [11]. Another large study that observed a beneficial association between 25(OH)D status and depression was the Health Survey for England, which used data of 2070 men and women aged ≥65 years [15]. To the best of our knowledge, none of the aforementioned studies accounted for gene profiles. We did, but we did not observe any association between vitamin D SNPs and the score of depressive symptoms, or interactions between 25(OH)D concentrations and vitamin D SNPs. Kuningas and colleagues (2009) also explored associations between several vitamin D-related SNPs and observed an association between ApaI and depressive symptoms in 563 Dutch Caucasian older adults [22]. However, as we did not observe any association between the vitamin D SNPs, we do consider the possibility that it is not a higher 25(OH)D concentration that is responsible for a lower score of depressive symptoms, but that the observed association is for instance explained by reverse causation or another factor that we could not control for in our analyses. Randomized Controlled Trials (RCTs) can provide more conclusive evidence on the direction of the association. However, to date, RCT results are inconclusive. It has been argued that shortcomings may be the reason for the indecisive evidence, as several studies did not measure 25(OH)D concentrations, included participants with high 25(OH)D concentrations or used a relatively low dose of vitamin D [23].

7.4.2 Underlying mechanisms

A low vitamin D concentration may predispose to depression through several biological pathways. Vitamin D has amongst others been linked to an increase in serotonin production [24] and a decrease in glucocorticoid-induced hippocampal cell death [25]. Vitamin D has also been hypothesized to play a role in the synthesis of neurotrophins, the production of acetylcholine and glutathione, and the down-regulation of L-type voltage-sensitive calcium channel expression [6]. Vitamin D may also indirectly fight depressive symptoms via its proposed anti-inflammatory effect [26]. To further explore the underlying mechanisms, we investigated the possible role of diabetes. Due to a variety of disease-related stress factors, diabetes patients are considered to be at increased risk for developing depression [8]. With that, low serum 25(OH)D concentrations may also predispose to glucose intolerance [7]. Thus, mediation as well as modification effects could be expected to be present. Nevertheless, fully adjusted models did not support an association between serum 25(OH)D and self-

reported diabetes. In addition, adding self-reported diabetes to the model on serum 25(OH)D and depression did not attenuate the association observed. Interaction analyses furthermore indicated that there was no difference in the score of depressive symptoms when having diabetes or not. All in all, we therefore conclude that our data do not support a mediation or modification effect of diabetes in the vitamin D-depression pathway.

7.4.3 Limitations

A well-known limitation of observational studies is that it is not possible to say something about causality, specifically, is the so-called exposure an actual risk factor, or is it merely a consequence of the disease? Fortunately, we had the possibility to further explore this association in a prospective fashion, showing that two years of vitamin D supplementation with 15 μg/day did not change the association as observed at baseline. Interpreting these findings, however, is quite challenging, especially because 25(OH)D concentrations were not measured after two years of follow-up. It may be suggested that vitamin D supplementation did not beneficially affect depressive symptoms, and that the association is explained by an unknown other factor that is strongly correlated with 25(OH)D status. On the other hand, it may also be that participants in the lowest quartile did reach higher 25(OH)D concentrations, but that these concentrations were still not high enough to decrease the probability of having a higher score of depressive symptoms relative to the other groups. Another limitation of this study is that data were obtained from the B-PROOF study, an intervention trial examining the impact of vitamin B_{12} and folic acid on a variety of outcomes, which may have interfered with our prospective analyses. However, adjusting the associations between 25(OH)D concentrations and depressive symptoms after two-years of supplementation for treatment group did not substantially alter the associations. This study is also limited by the fact diabetes diagnosis is based on self-report. Strengths of this study were the large study population, the possibility to study the association after two years of vitamin D supplementation, the opportunity to adjust for important confounders, as well as the possibility to investigate the role of diabetes and SNPs that have been linked to vitamin D synthesis, metabolism and vitamin D receptor expression.

7.4.4 Conclusion

Our data support previously reported cross-sectional associations between higher 25(OH)D concentrations and a decreased probability of reporting depressive symptoms. Our subsequent finding that two years of vitamin D supplementation does not translate into a shift towards less depressive symptoms, however, raises concern about the causality of the association. No mediation or modification effect by diabetes was observed. To define the direction of the link between serum 25(OH)D and depression, there is a continuous need for prospective studies as well as well-designed RCTs.

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Supplementary material

sTable. Associations between vitamin D related genetic make-up at baseline with the number of depressive symptom score at baseline and after 2-years of vitamin D supplementation with 15 µg/day, shown as Relative Risks (95% CI).

	Continuous	Minor allele carriers	Heterozygotes	Major allele carriers
Baseline (n=2555)				
DHCR7	0.00±0.04 (P=0.99)	1.0	0.96 (0.78-1.17)	0.98 (0.80-1.19)
CYP2R1	-0.02±0.04 (P=0.52)	1.0	1.01 (0.87-1.18)	0.96 (0.82-1.13)
CYP24A1	0.03±0.05 (P=0.58)	1.0	0.93 (0.71-1.22)	0.98 (0.75-1.28)
GC	-0.07±0.04 (P=0.11)	1.0	0.93 (0.77-1.14)	0.87 (0.72-1.06)
TaqI / BsmI	0.01±0.04 (P=0.75)	1.0	1.03 (0.88-1.21)	1.03 (0.87-1.22)
ApaI	-0.01±0.04 (P=0.79)	1.0	1.03 (0.91-1.17)	0.98 (0.85-1.14)
Cdx2	0.01±0.05 (P=0.79)	1.0	1.01 (0.79-1.32)	1.02 (0.79-1.32)
2-year follow-up (n=2307)				
DHCR7	0.04±0.05 (P=0.35)	1.0	1.02 (0.81-1.28)	1.07 (0.86-1.35)
CYP2R1	-0.02±0.04 (P=0.68)	1.0	1.03 (0.88-1.20)	0.98 (0.83-1.15)
CYP24A1	-0.04±0.05 (P=0.46)	1.0	0.96 (0.71-1.29)	0.92 (0.69-1.24)
GC	-0.04±0.04 (P=0.36)	1.0	0.83 (0.69-1.01)	0.85 (0.71-1.03)
TaqI / BsmI	0.03±0.04 (P=0.51)	1.0	1.02 (0.87-1.20)	1.06 (0.89-1.25)
ApaI	-0.04±0.04 (P=0.27)	1.0	0.97 (0.85-1.12)	0.92 (0.78-1.08)
Cdx2	-0.02±0.05 (P=0.75)	1.0	0.87 (0.65-1.15)	0.88 (0.67-1.17)

8

Higher serum 25(OH)D and lower plasma glucose are associated with a higher grey matter volume in Dutch community-dwelling older adults

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Abstract

Aim

The aim of this study was to investigate the cross-sectional association between vitamin D status and brain volume, and to examine whether surrogate markers of glucose homeostasis modify this association.

Methods

Vitamin D status (25(OH)D), glucose and insulin were measured. Measures of total brain volume, grey matter volume, and white matter volume were obtained by MRI in 218 community-dwelling older adults aged ≥65 years. Associations of 25(OH)D status, glucose, and insulin concentrations with brain volume were evaluated using linear regression analyses.

Results

After full adjustment, higher 25(OH)D concentrations and lower glucose concentrations were associated with more grey matter volume, β 0.20±0.08 (P=0.02) and β -3.26±1.59 (P=0.04), respectively. No association of 25(OH)D status or insulin concentration with total brain volume or white matter volume was observed. There was no evidence for a mediation or modification effect of plasma glucose in the association between 25(OH)D status and brain volume.

Conclusion

Higher 25(OH)D status and lower glucose concentrations are associated with more grey matter volume, but not with white matter volume or total brain volume.

8.1 Introduction

Despite great efforts an effective anti-Alzheimer drug has not yet been developed [1]. Therefore, identification of factors that may prevent or slow-down the development of Alzheimer's Disease (AD) is important. Vitamin D is proposed as being one of those potential preventive factors [2]. Support for this hypothesis originated from pre-clinical studies showing that vitamin D beneficially influences several cerebrovascular risk factors, synaptic plasticity, synthesis of neurotransmitters and neurotrophins, and slows down synaptic and neuronal loss [2].

Vitamin D may also be indirectly associated with brain functioning, with glucose homeostasis acting as a mediator. Specifically, on a mechanistic level, vitamin D has been shown to promote pancreatic β-cell function as well as insulin action [3]. Several observational studies support these potential effects by showing beneficial associations between 25(OH)D concentrations and fasting glucose, insulin, glucose clearance as well as type 2 diabetes [3, 4]. There is also considerable evidence that maintenance of glucose concentrations within a healthy range is beneficial to preserve cognitive function [5, 6]. Furthermore, it has been hypothesized that vitamin D inadequacy may be particularly harmful in the presence of a second neurobiological trigger [7-9]. As glucose intolerance may be such a neurobiological trigger, we therefore also hypothesized that markers of glucose homeostasis may act as modifiers in the association between vitamin D and brain volume.

Several human studies provided evidence for a potential role of vitamin D in the maintenance of cognitive function. Meta-analyses indicate that participants with concentrations ≥50 nmol/L have better global cognitive performance [10], episodic memory, information processing speed, mental shifting abilities and information updating ability than those with 25(OH)D concentrations <50 nmol/L [11]. Intervention studies on vitamin D and cognitive function in older adults are sparse, and those that have been conducted had a small sample size, were of short duration, not blinded and sometimes even without placebo group [12-18]. The only randomized controlled trial (RCT) examining the effect of vitamin D, independent of calcium, was conducted in young adults and did not point towards a beneficial role in cognitive performance [17].

Cognitive performance has been associated with the rate of brain atrophy as measured with MRI [19, 20]. Via studying grey and white matter loss, MRI may also be a valuable tool to get more insight in the association between 25(OH)D concentration and brain function. The use of MRI is a relatively new area in vitamin D research and up to now only a few studies explored the associations between vitamin D and measures of brain volume [21]. As there is a large heterogeneity in study populations and outcome measures in these particular studies, and because results are inconclusive [21], more evidence is needed. We therefore investigated associations of serum 25(OH)D with total brain volume, grey matter volume, and white matter volume in Dutch older adults. The second aim of this study was to explore the potential synergism of 25(OH)D concentrations with surrogate markers of glucose homeostasis in the association with brain volume.

8.2 Methods

8.2.1 Participants

This observational study was performed using data of the B-PROOF study; a randomized double blind placebo-controlled trial that was designed to assess the efficacy of daily oral supplementation of vitamin B_{12} and folic acid on bone fractures in mildly hyperhomocysteinemic (12-50 μmol/L) older adults aged ≥65 years. Details have been reported elsewhere [22]. The Medical Ethics Committee of Wageningen UR approved the study protocol and all participants gave their written informed consent. For the current data-analyses only data of participants with MRI data have been used (n=218) (**Figure 1**).

8.2.2 Biochemical analyses

Blood samples were drawn in the morning when participants were fasted or had consumed a restricted breakfast. Samples were stored at -80˚C until determination. Serum 25(OH)D in baseline samples was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) [23]. Glucose concentrations were analysed using a hexokinase method (Gluco-quant, Roche Diagnostics). Insulin concentrations were determined using an immunometric assay (ADVIA Centaur immunoassay system, Siemens Medical Solutions Diagnostics).

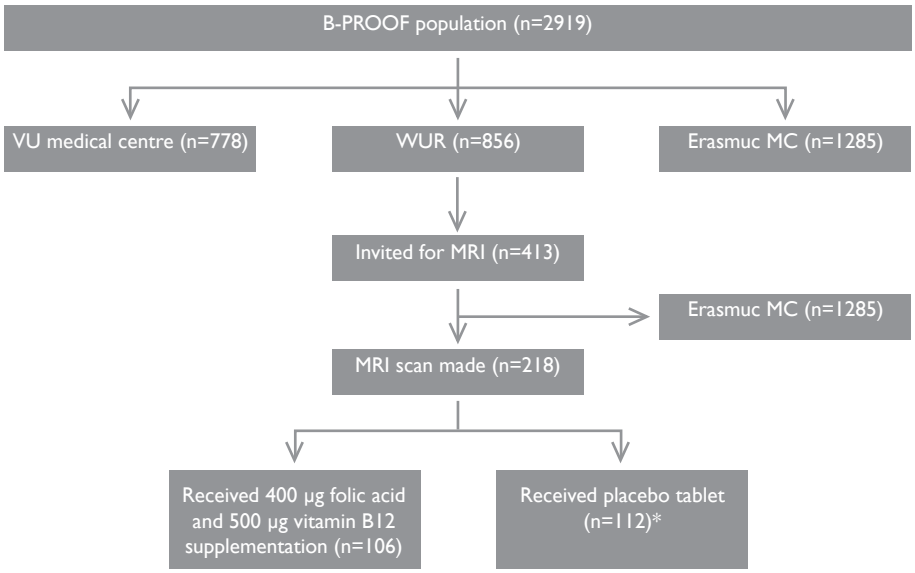

Figure 1. Flowchart of participants that were enrolled in the MRI measurements of the B-PROOF study.
Note: *One participant was excluded from the analyses because of unknown 25(OH)D status.

8.2.3 MRI

Cranial volumetric MRI scans were made two years after the baseline measurements, at Hospital Gelderse Vallei (Ede, the Netherlands) on a 3-Tesla Siemens Magnetom Verio

(Siemens, Erlangen, Germany), with a 32-channel head coil. We analysed the T_1-weighted scan (MPRAGE, repetition time=2300ms, echo time=3.0ms, inversion time=900ms, 9º flip angle, field of view=256x256mm, 192 sagittal slices, voxel size= 1mmx1mmx1.3mm OF 1x1x1mm), acceleration factor (GRAPPA)=2. The voxel-based morphometric (VBM8) toolbox within SPM8 (Wellcome Department of Imaging Neuroscience, London, UK, http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) and FSL-VBM v6.0 (FMRIB Software Library, Oxford, UK, http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM) were used for segmentation [24]. T_1-weighted images were reoriented to match the standard template images in FSL-VBM. VBM8 spatially normalizes participants' brain images to a standard space and then to automated segments of grey matter, white matter, and cerebrospinal fluid, using a unified tissue segmentation approach [25]. Grey and white matter volumes were summed to calculate total brain volume. Grey matter, white matter and cerebrospinal fluid measures were summed to compute intracranial volume.

8.2.4 Covariates

Height was measured at baseline with a stadiometer to the nearest 0.1 cm. Weight was measured to the nearest 0.5 kg with a calibrated analogue scale. Body Mass Index (BMI) was calculated as weight/height^2. Data on education level (i.e. primary, secondary or higher education), smoking status (i.e. non-smoker or current smoker), physical activity (kcal/day) [26], and alcohol consumption (i.e. light, moderate, excessive) [27] were collected by means of questionnaires. The 15-item Geriatric Depression Scale (GDS) was used as a screening tool for the number of depressive symptoms [28]. The Mini-Mental State Examination (MMSE) was used to assess global cognitive status [29]. Season of blood collection was divided into summer/autumn (June–November) and winter/spring (December–May).

8.2.5 Statistical Analyses

Participant characteristics are reported as mean with standard deviation (SD), or percentages. Medians with interquartile range (IQR) were used to report skewed variables. Characteristics are shown for the total population, stratified according to the cut-of values for vitamin D deficiency that are currently used by the Institute of Medicine (IOM) [30], and stratified for low and high plasma glucose concentrations. Multiple regression analyses were performed to explore the associations between serum 25(OH)D and brain volume. All analyses were adjusted for intracranial volume (crude model), crude model + age, sex (model 1); model 1 + BMI, education, smoking, alcohol consumption, habitual physical activity (model 2); and model 2 + the number of depressive symptoms (model 3).

Multiple regression analysis was also conducted to examine associations between plasma glucose, plasma insulin and brain volume. Again, all analyses were adjusted for intracranial volume (crude model), crude model + age, sex (model 1); model 1 + BMI, education, smoking, alcohol consumption, habitual physical activity (model 2); model 2 + the number of depressive symptoms (model 3).

Effect modification by plasma glucose was examined by stratifying the data for low and high glucose concentrations (median split), and by testing for interaction. Mediation was

examined by adding plasma glucose, independently, to fully adjusted regression models for 25(OH)D concentrations and brain volumes. Alpha was set at 0.05 and two-tailed analyses were performed. Analyses were performed using the statistical package SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA).

8.3 Results

Participant characteristics are presented in **Table 1**. Participants were on average 72±6 years old, had a 25(OH)D concentration of 61±23 nmol/L, BMI of 27.6±4.2 kg/m^2, and used 669±422 kilocalories for habitual physical activities. Fifty-seven percent of this population was men, 35% had a degree in higher education, 76% was included in the study during the summer/autumn. The median score on the MMSE was 29, suggesting that this population is cognitively healthy. No remarkable differences in population characteristics were observed for participants with 25(OH)D concentrations <50 nmol/L and those with concentrations ≥50 nmol/L. Participants with glucose concentrations ≥5.6 mmol/L, however, were more likely to be men, smoker, to have a higher BMI, lower educational level, and to be included during the winter/spring months, than participants with glucose concentrations <5.6 mmol/L.

8.3.1 Serum vitamin D and brain volumetric measures

Linear regression analyses showed that 25(OH)D status was significantly associated with grey matter volume (β 0.25, SE 0.09, P=0.005) and total brain volume (β 0.26, SE 0.11, P=0.02) after adjustment for intracranial volume, but not with white matter volume (**Table 2**). The association between 25(OH)D status and grey matter remained after adjustment for intracranial volume, age, sex, BMI, education, alcohol, smoking, physical activity, season and depressive symptoms (β 0.20, SE 0.08, P=0.02), indicating that one unit increase in 25(OH)D concentration is associated with 0.20 mL (0.01%) more grey matter volume. The association between 25(OH)D status and total brain volume was not significant anymore after full adjustment.

Table 1. Participant characteristics of 217 Dutch older adults.

	Total population	Serum 25(OH)D <50 nmol/L (n=71)	Serum 25(OH)D ≥50 nmol/L (n=146)	Plasma glucose <5.63 mmol/L (n=110)	Plasma glucose ≥5.63 mmol/L (n=107)
Serum 25(OH)D, nmol/L	61±23	36±10	73±18*	63±26	59±20
Age, years	72±6	72±6	71±6	71±6	72±5
Sex, n (%)					
Men	124 (57)	39 (54)	85 (59)	55 (50)	69 (64)*
Women	94 (43)	33 (46)	59 (41)	56 (50)	38 (36)
Body Mass Index, kg/m^2	27.6±4.2	27.4±4.4	27.7±4.0	26.7±3.7	28.5±4.4*
Educational level, n (%)					
Primary	90 (41)	28 (39)	60 (42)	45 (41)	45 (42)*
Secondary	52 (24)	21 (29)	31 (21)	20 (18)	32 (30)
Higher	76 (35)	23 (32)	53 (37)	46 (41)	30 (28)

	Total population	Serum 25(OH)D <50 nmol/L (n=71)	Serum 25(OH)D ≥50 nmol/L (n=146)	Plasma glucose <5.63 mmol/L (n=110)	Plasma glucose ≥5.63 mmol/L (n=107)
Alcohol intake, n (%)					
Light	147 (67)	54 (75)	93 (64)	81 (73)	66 (62)
Moderate	61 (28)	16 (22)	45 (31)	25 (23)	36 (33)
Excessive	10 (5)	2 (3)	8 (5)	5 (4)	5 (5)
Smoking history, n (%)					
Non-smoker	64 (29)	19 (26)	45 (31)	41 (37)	23 (22)*
Smoker	15 (7)	6 (8)	9 (6)	3 (3)	12 (11)
Former smoker	139 (64)	47 (65)	92 (63)	67 (60)	72 (67)
Physical activity level, kcal/day	669±422	610±418	698±422	671±428	667±417
Glucose level, mmol/L	5.9±1.3	5.8±1.2	5.9±1.3	5.1±0.5	6.7±1.3*
Number of depressive symptoms	1 (1)	1 (1)	1 (1)	0 (1)	1 (2)
Season, n (%)					
December-May	52 (24)	24 (33)	28 (19)*	17 (15)	35 (33)*
June-November	166 (76)	48 (67)	118 (81)	94 (85)	72 (67)
MMSE score	29 (2)	29 (2)	29 (2)	29 (2)	29 (2)
Grey matter volume, mL (% of ICV)	574±56 (42.2)	567±57 (41.5)	578±55 (42.2)	572±58 (42.3)	576±58 (41.6)
White matter volume, mL (% of ICV)	493±61 (35.9)	492±62 (36.0)	493±61 (35.9)	489±59 (36.0)	497±64 (35.8)
Total brain volume, mL (% of ICV)	1067±108 (78.1)	1060±109 (77.5)	1071±108 (78.1)	1062±104 (78.3)	1073±112 (77.4)
Receiving vitamin B_{12} and folic acid supplement prior to MRI measurement, n (%)	106 (49)	38 (53)	68 (47)	54 (49)	52 (49)

Note: Comparing participants with vitamin D concentrations ≤50 nmol/L with participants with vitamin D concentrations ≥50 nmol/L using the Chi-square test in case of categorical variables or the Student's t-test in case continuous variables. Variables are displayed as means ± standard deviations or medians (quartile range). *Serum 25(OH)D ≥50 nmol/L group is significantly different from serum 25(OH)D <50 nmol/L group (P<0.05), or Glucose level <5.63 mmol/L group is significantly different from Glucose level ≥5.63 mmol/L (P<0.05). ICV: Intracranial volume.

Table 2. Linear regression analyses for serum 25(OH)D with absolute brain volume (mL), β (SE).

	Total brain volume	Grey matter volume	White matter volume
Crude model	0.26 (0.11), P=0.02	0.25 (0.09), P=0.005	0.01 (0.08), P=0.93
Model 1	0.12 (0.08), P=0.13	0.18 (0.08), P=0.02	-0.06 (0.08), P=0.43
Model 2	0.13 (0.08), P=0.11	0.19 (0.08), P=0.02	-0.06 (0.08), P=0.47
Model 3	0.14 (0.08), P=0.09	0.20 (0.08), P=0.02	-0.06 (0.08), P=0.46

Note: Model 1: adjusted for intracranial volume, age and sex. Model 2: adjusted for intracranial volume, age, sex, BMI, education, alcohol intake, smoking, physical activity, and season. Model 3: adjusted for intracranial volume, age, sex, BMI, education, alcohol intake, smoking, physical activity, season and the number of depressive symptoms.

8.3.2 Glucose homeostasis and brain volumetric measures

Higher glucose concentrations were associated with less grey matter volume (**Table 3**), but not with total brain volume or white matter volume. In the fully adjusted model, one unit increase in glucose was associated with -3.26 mL (P=0.04) less grey matter volume. After further adjustment for serum 25(OH)D, this association became even stronger, β -5.53 mL, SE 1.57, P=0.03. No association between glucose and total brain volume, or white matter volume was observed.

Table 3. Linear regression analyses for plasma glucose and insulin with absolute brain volume (mL), β (SE).

	Total brain volume		Grey matter volume		White matter volume	
	Plasma glucose	**Plasma insulin**	**Plasma glucose**	**Plasma insulin**	**Plasma glucose**	**Plasma insulin**
Crude model	-3.80 (2.0), P=0.06	0.01 (0.02), P=0.37	-4.60 (1.7), P=0.006	0.02 (0.01), P=0.13	0.80 (1.5), P=0.59	-0.01 (0.01), P=0.62
Model 1	-2.70 (1.54), P=0.08	-0.00 (0.01), P=0.76	-3.96 (1.50), P=0.009	0.01 (0.01), P=0.21	1.26 (1.42), P=0.38	-0.01 (0.01), P=0.32
Model 2	-2.54 (1.62), P=0.12	-0.00 (0.01), P=0.83	-3.45 (1.60), P=0.03	0.02 (0.01), P=0.20	0.91 (1.51), P=0.55	-0.01 (0.01), P=0.26
Model 3	-2.38 (1.63), P=0.15	-0.00 (0.01), P=0.85	-3.26 (1.59), P=0.04	0.02 (0.01), P=0.19	0.88 (1.52), P=0.56	-0.01 (0.01), P=0.23

Note: Model 1: adjusted for intracranial volume, age and sex. Model 2: adjusted for intracranial volume, age, sex, BMI, education, alcohol, smoking, and physical activity. Model 3: adjusted for intracranial volume, age, sex, BMI, education, alcohol intake, smoking, physical activity, and the number of depressive symptoms.

Table 4. Linear regression analyses for serum 25(OH)D in nmol/L with absolute brain volumetric measures (mL), stratified for low and high glucose concentrations in mmol/L, β (SE).

	Total brain volume		Grey matter volume		White matter volume	
	Glucose <5.63 (n=110)	**Glucose ≥5.63 (n=107)**	**Glucose <5.63 (n=110)**	**Glucose ≥5.63 (n=107)**	**Glucose <5.63 (n=110)**	**Glucose ≥5.63 (n=107)**
Crude model	0.38 (0.12), P=0.002	-0.01 (0.19), P=0.96	0.27 (0.10), P=0.01	0.19 (0.16), P=0.22	0.11 (0.10), P=0.24	-0.21 (0.14), P=0.15
Adjusted model	0.20 (0.10), P=0.04	0.17 (0.16), P=0.31	0.23 (0.10), P=0.02	0.29 (0.16), P=0.07	-0.03 (0.10), P=0.76	-0.12 (0.15), P=0.39
P for interaction	P=0.45		P=0.86		P=0.32	

Note: Adjusted model: adjusted for intracranial volume, age, sex, BMI, education, alcohol intake, smoking, physical activity and season. Interaction is tested for the adjusted model.

8.3.3 Interplay between serum vitamin D and glucose homeostasis

Incorporating glucose in the full-adjusted models for 25(OH)D status and grey matter did not support the hypothesis of mediation, β 0.20 mL, SE 0.08, P=0.02. Nor did stratification for low and high glucose concentrations (**Table 4**) or interaction analyses point towards an interplay between serum 25(OH)D with plasma glucose in the association with grey matter volume, white matter volume, or total brain volume, *P- for interaction* 0.86, 0.32 and 0.45, respectively.

8.4 Discussion

This study shows that higher 25(OH)D concentrations and lower plasma glucose concentrations are associated with more grey matter volume in a population of cognitively healthy Dutch older adults. Markers of glucose homeostasis did not mediate or modify the association between 25(OH)D and brain volume.

8.4.1 Serum vitamin D and brain volumetric measures

Previous studies have shown that in the normal aging brain, grey matter loss predominates over white matter loss [31]. In this study, one unit increase in serum 25(OH)D was associated with 0.20 mL (0.01%) more grey matter volume, but not with white matter volume, or total brain volume. Our results are in line with previous pre-clinical research that indicates that the active form of vitamin D, calcitriol, may support neuronal survival, neurogenesis and synaptogenesis [2], and with previous observational studies that show associations between higher 25(OH)D concentrations and better cognitive performance [8, 10, 32-39]. The earliest studies on brain volume were conducted in rats and showed that a prenatal vitamin D deficiency resulted in larger total brain volumes, larger lateral ventricles [40, 41], reduced cortical thickness and increased cell proliferation [40]. As recently reviewed, studies evaluating the association between serum 25(OH)D and brain volumetric measures in humans are sparse [21], but suggest that lower vitamin D concentrations are associated with larger ventricle volumes [42], more vascular pathologies [43] and a higher rate of cortical thinning [44]. In addition, higher plasma 25(OH)D concentrations have been associated with larger volumetric measures of white matter, amygdala, thalamus and anterior cingulate gyrus in 28 patients referred to a memory clinic [45]. Conversely, several other studies did not observe associations between 25(OH)D concentrations and total brain volume [43, 46], grey matter volume [46], or volume measures of the hippocampus [43], amygdala [43], parahippocampal gyrus [43], and temporal horn [42]. Overall, the number of studies examining associations between 25(OH)D status and brain volume are sparse and generally had relatively small sample sizes, differed largely in type of population studied, and the particular brain volumes examined. Hence, as yet, no firm conclusions can be drawn based on these data.

8.4.2 Glucose homeostasis and brain volumetric measures

Previous research has shown that an impaired glucose tolerance may lead to advanced protein glycation, oxidative stress, increased secretion of amyloid, decreased breakdown of amyloid, microvascular brain complications, brain infarcts an brain atrophy [6, 47]. In line, there is substantial evidence that glucose intolerance is associated with cognitive decline. Our findings add that higher glucose concentrations associate with less grey matter, which has also been observed by others [48-50], and hence confirm that increased glucose concentrations may play a role in the pathogenesis of cognitive decline and dementia.

8.4.3 Interplay between serum vitamin D and glucose homeostasis

In this population, higher 25(OH)D concentrations and lower glucose concentrations were associated with more grey matter volume, but glucose concentrations did not seem to modify or mediate the association between 25(OH)D status and grey matter volume. The hypothesis that glucose homeostasis could (partially) explain or modify the association between serum 25(OH)D and brain volume has not yet been extensively studied. To the best of our knowledge, four studies examined whether the association between 25(OH)D status and cognitive function was influenced when a marker of glucose homeostasis was added [8, 33, 34, 51], which showed either no change [8, 33, 34] or a partial attenuation of the association between serum 25(OH)D concentrations and cognitive performance [51].

8.4.4 Methodological aspects

Several methodological issues of the present study warrant further discussion. Firstly, our results are limited due to the observational study design. Secondly, participants were selected to have a mildly elevated homocysteine concentration. As approximately 50% of the Dutch older adult population has a mildly elevated homocysteine concentration [52], this selection criterion limits the generalizability of these results to the whole Dutch population of older adults. Thirdly, glucose and insulin concentrations presented in this study cannot be considered fasting as participants were allowed to consume a light breakfast prior to blood sampling. Therefore, participants may have been misclassified as having 'high' glucose concentrations, due to the consumption of a light breakfast, even though they did not have an impaired glucose tolerance. Finally, MMSE data indicate that this study included cognitively healthy older adults, which may have reduced our ability to detect associations between serum 25(OH)D or plasma insulin and white matter volume and total brain volume. Strengths of this study include the possibility to control for several potential covariates and the possibility to explore the role of glucose homeostasis.

8.4.5 Conclusion

This study showed that higher 25(OH)D concentrations and lower plasma glucose concentrations are associated with more grey matter volume. No associations between 25(OH)D concentrations and total brain volume or white matter volume were observed. Our data do not indicate that the association between serum 25(OH)D and grey matter volume is mediated or modified by glucose concentrations. Future prospective studies and well-designed randomized controlled trials are warranted to examine whether vitamin D treatment could play a significant role in slowing down the progress of brain atrophy.

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9

No role for vitamin D or a moderate fat diet in aging induced cognitive decline and emotional reactivity in C57BL/6 mice

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Abstract

Background
Epidemiological studies have shown associations between vitamin D, cognitive performance, depression, and glucose homeostasis in older adults. Causal evidence, however, is still lacking.

Objective
The objective of this study was to investigate the importance of vitamin D in the prevention of emotional disturbances and cognitive decline in aging C57BL/6 mice, with pre-diabetes type II as potential effect modifier.

Methods
Mice were exposed to one of four diets from 10-24 months of age: low fat vitamin D adequate (LFD), low fat vitamin D deficient (LF), moderate fat vitamin D adequate (MFD), and moderate fat vitamin D deficient (MF). The MFD/MF diet was applied to induce a condition resembling pre-diabetes type II. Behaviour was assessed twice in the same group of mice at 6-8 and at 22-23 months of age using the Open Field Test (OFT), Elevated Plus Maze (EPM), Object Recognition Test (ORT) and the Morris Water Maze (MWM).

Results
We successfully induced a vitamin D deficiency in the LF/MF mice. Moreover, fasting glucose and fasting insulin concentrations were significantly higher in MFD/MF mice than in LFD/LF mice. A significant aging effect was observed for most behavioural parameters. A MF(D) diet was shown to delay or prevent the age-related increase in emotional reactivity in the EPM. No effect of vitamin D or vitamin D*fat on behavioural outcomes was observed.

Conclusion
Aging significantly affected emotional reactivity and cognitive performance. Although other studies have shown effects of vitamin D on emotional reactivity and cognitive performance in mice, these findings could not be confirmed in aged C57BL/6 mice in this study.

9.1 Introduction

Aging is a universally accepted key risk factor for cognitive decline. Recent studies also suggest that specific nutritional factors may be linked with brain function, and as such with cognitive decline [1] and depression [2]. Since pharmacological treatment of cognitive decline and dementia is currently far from effective, knowledge on the effect of modifiable dietary factors may bring us a step closer towards preventive solutions. Vitamin D inadequacy is one of the suggested modifiable factors for brain dysfunction [3]. Currently, large numbers of mostly older adults have a vitamin D inadequacy [4]. Therefore, preventing and/or treating vitamin D inadequacy may be a relevant, easy and cost-effective strategy to improve long-term brain functioning. Although several observational studies in humans support the notion of a beneficial link between vitamin D and cognitive performance [5-10] and depression [11-13], other studies do not (for cognition: [14, 15], for depression: [10, 16, 17]). Moreover, evidence for a causal relationship from human intervention trials is still limited (for cognition: [18-22], for depression: [23-27]).

Studies using rodents as subjects offer more opportunities than human studies to investigate causal relationships between vitamin D and brain functions. However, in most studies conducted up to now, mice were already vitamin D deficient prenatally or had an inactivated vitamin D receptor gene [3]. These models do not mimic the most common clinical condition as observed in humans. In humans vitamin D inadequacy usually slowly develops over time, mainly because the ability of the human skin to synthesize vitamin D under the influence of ultraviolet-B exposure decreases while aging. Thus, alike age-related depression and cognitive decline, vitamin D inadequacy in humans often manifests at older age. To our knowledge there has not yet been a study that examined the impact of long-term dietary vitamin D deficiency on brain functions in aging C57BL/6 mice; a model that more closely resembles the clinical condition of vitamin D inadequacy in older adults, and in which the physiological consequences and co-existing pathologies can be studied better.

Various pathways for a role of vitamin D in brain function have been suggested. It has been shown that vitamin D stimulates the production of several neurotrophins and acetylcholine, reduces oxidative stress, increases phagocytosis and clearance of amyloid β peptide by macrophages [3, 28], and interacts with glucocorticoid receptors in hippocampal cell-lines [29]. The exact physiological mechanisms underlying possible effects of vitamin D on brain function, however, are far from clear. Indirect mechanisms have also to be taken into account. There is evidence that vitamin D contributes to the maintenance of glucose homeostasis [30]. In accordance, diabetes patients tend to be more prone to develop cognitive deficits than non-diabetes patients [31]. Therefore, it has been suggested that vitamin D might indirectly affect brain function via this metabolic pathway. Glucose intolerance in mouse models is frequently studied by exposing mice to a high fat load. A 60% fat load has for instance been shown to result in rapid weight gain and increased fasting glucose and insulin concentrations, and insulin insensitivity as measured in glucose tolerance tests [32]. This extremely high fat load, however, also adversely affects life expectancy of mice and does not reflect the fat load of humans consuming fatty meals. Therefore, the physiological consequences of long-term

dietary fat exposure probably can be better studied in a mouse model with a moderate fat load.

The aim of our study was to investigate the effect of long-term adult vitamin D deficiency on emotional reactivity and cognitive decline in aging C57BL/6 mice. The potential role of glucose homeostasis in the hypothesized relation between vitamin D and brain function was examined by studying the effect in mice exposed to a low fat (LF) diet (nine En% from fat) or a moderate fat (MF) diet (20 En% from fat). Emotional reactivity was assessed in the Open Field Test (OFT) and the Elevated Plus Maze (EPM). Cognitive performance and cognitive decline were measured in the Object Recognition Test (ORT) and the Morris Water Maze (MWM).

9.2 Materials and methods

9.2.1 Ethical approval

The experiment was conducted according to the institutional and national guidelines for the care and use of animals. The Local Committee for Care and Use of Laboratory Animals at Wageningen University approved the experiment (code number: drs-2010123). All effort was taken to minimize the number of animals used and their suffering. By contacting researchers working in the same field of study, effort was taken to optimally use the tissues of the animals subjected to this experiment.

9.2.2 Animals

Seventy-six male C57BL/6j RJ mice were purchased from Janvier Laboratories (Le Genest Saint Isle, France). At arrival at the animal facility the mice were five months of age and did not show signs of illness or behavioural abnormalities. Until eight months of age all mice received a standard chow diet, containing nine En% from fat (low fat diet). From eight months of age onwards, following the completion of the baseline behavioural tests, 46 mice were randomly allocated to a 20 En% fat diet (moderate fat diet) to provoke a diet-induced impaired glucose homeostasis. The remaining 30 mice continued on the low fat diet. At the age of ten months, mice in the low fat group and the moderate fat group were rank ordered according to body weight and fasting glucose concentrations, and subsequently randomly assigned to either a vitamin D adequate diet or a vitamin D deficient diet: 1) Low fat group receiving a AIN-93W diet, nine En% fat (LFD) (n=15); 2) Low fat group receiving a vitamin D deficient AIN-93W diet, nine En% fat (LF) (n=15); 3) Moderate fat group receiving a 20 En% fat diet (MFD) (n=23); 4) Moderate fat group receiving a vitamin D deficient 20 En% moderate fat diet (MF) (n=23). Mice were weighed once every two weeks. As sensorimotor assessment has been shown to be an effective tool to predict health-related drop-out [33], general health was assessed every three months using the Horizontal Wire Test, Balance Rod Test and Open Field test. At the end of the study, at an age of 24 months, the mice were sacrificed and macroscopically examined for pathologies. A laboratory veterinarian monitored the study. The timeline of the study is depicted in Figure 1.

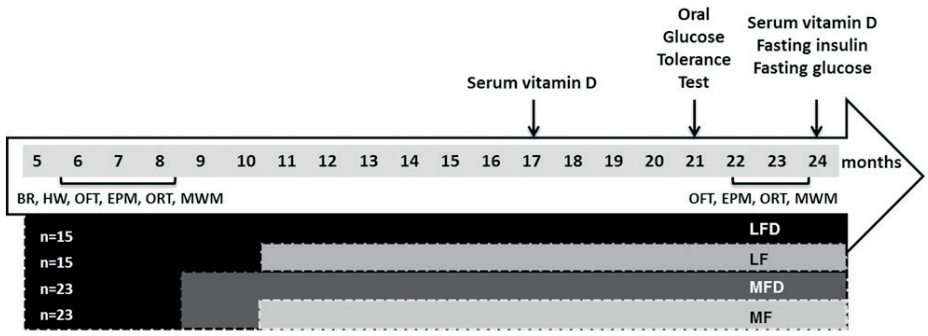

Figure 1. Study design. *Note*: Mice arrived at the laboratory at an age of five months and were fed a standard low fat diet. From eigth months of age onwards 46 mice were randomly allocated to a moderate fat diet to provoke a diet-induced impaired glucose homeostasis. At the age of ten months, mice in the low fat group and the moderate fat group were randomly assigned to either a vitamin D adequate diet or a vitamin D deficient diet. LFD: low fat diet with vitamin D. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

9.2.3 Housing and test conditions

In order to monitor the dietary intake and to prevent large differences in body weight within groups, animals were housed individually. Makrolon® type II cages were cleaned every two weeks and contained standard woodchip bedding material. Tissues served as cage enrichment. Room temperature and humidity were regulated at approximately 21°C and 50%, respectively. Mice were held on a reversed 12-hour light/dark cycle. Lights were off from 6:00 AM till 6:00 PM. During the mice's active period red lights were turned on and music was played as a background noise to conceal human activities in the lab. During the inactive phase, from 6.00 PM – 6.00 AM, there were no activities in the lab and the music was turned off.

9.2.4 Diet

Food and tap water were available at libitum. Food was weighed and refreshed every two weeks. Low fat groups were fed a Wageningen variant (AIN-93W) of the AIN-93M diet until they were ten months old. The AIN-93M diet is generally accepted as the 'golden standard' for adult maintenance [34]. AIN-93W is almost similar to AIN-93M diet, with the exception that the soybean oil in AIN-93M diet has been partially replaced by palm oil, in order to create a diet that is more equivalent to a Western style diet. MF(D) animals were fed an AIN-93W diet until they were eight months old; from eight to ten months of age, all MF animal were switched to an AIN-93W with 20 En% from fat. At the age of ten months, half of the mice in the low-fat group (LFD) and moderate fat group (MFD) were switched to a vitamin D deficient regimen (LF and MF) containing 0 IU vitamin D_3 per gram (Figure 1). The exact diet composition of the different diets can be found in the supplementary material (eTable 1).

9.2.5 Biochemical parameters

Glucose homeostasis was assessed via measuring fasting blood glucose concentrations, fasting plasma insulin concentrations, and an oral glucose tolerance test (OGTT). Fasting glucose

and insulin concentrations prior to the OGTT were determined in samples obtained after a 5-6 hour fast. Blood samples were collected by cutting 1-2 mm of tissue from the tail tip and then gently massaging the tail. Glucose concentrations were obtained from whole blood samples and measured using Accu-Chek hand-held whole-blood glucose devices (Roche Diagnostics, Almere, The Netherlands). Plasma insulin was determined using a mouse insulin ELISA kit (ALPCO Diagnostics, Salem NH, United States). Following the fasting glucose measurement mice received 5 µl of 20% glucose solution per gram body weight via a feeding needle in order to determine the glucose response in the OGTT. Blood samples were taken at 15, 30, 45, 60, 90 and 150 minutes following the glucose load. Fasting glucose and fasting insulin concentrations at the day of sacrifice were determined in arterial blood collected by heart puncture using a syringe with EDTA, after 11±1 hours of fasting. During the procedure mice were anesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). Serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS), which was performed at the Endocrine Laboratory of the VU University Medical Center (Amsterdam, the Netherlands). Serum was incubated with a deuterated internal standard (IS: 25(OH)D_3- d6), where after 25(OH)D was released from its binding protein(s). Samples were extracted and analysed by XLC-MS/MS, a Symbiosis online Solid Phase Extraction system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp, Milford, MA). Serum 25(OH)D measurements require relatively large sample volumes, specifically 150 µl per sample. Therefore, the 25(OH)D concentrations in blood from 17-month-old mice had to be estimated in pooled samples, which consisted of equal volumes (25µl) of six randomly selected mice per group. At the moment of the sacrifice, larger blood samples could be obtained from each mouse, and as such the 25(OH)D concentrations could be determined for each individual mouse.

9.2.6 Behavioural testing

Setting

To reduce stress due to handling animals were regularly handled after arrival in the lab. One month after arrival, at that time mice were six months of age, behavioural parameters were measured pre-treatment. The final behavioural measurements started when the mice were 22-month-old. Behavioural tests were performed according to the expected burden of the specific tests, starting with the least stressful tasks: OFT, EPM, ORT and finishing with the MWM. The day before the start of the tests, mice were transported to the test room, which was about five meters away from their 'home' room. Mice were allowed to habituate to their new environment for approximately 14 hours before behavioural testing started. The 'home' and 'test' room were similar with respect to the conditions, specifically lighting, temperature and humidity. During the testing phase, mice were housed in the test room. Testing mice occurred in randomized order, started one hour after the beginning of the dark period and was performed under red light conditions. The test equipment was cleaned with a damp tissue and dried with a towel after each mouse.

Open Field Test

The circular OFT, which is widely used to assess both sensorimotor functioning and emotional reactivity [35], was made of grey polyvinyl chloride (PVC) with a 78 cm diameter. The surface of the OFT was subdivided in three zones (periphery, middle and center) with a width of 13 cm each. Mice were always started from the same point along the wall of the open field with their head pointing in the direction of the wall. Behaviour during a five minute trial was recorded by a video camera. Total distance moved and the number of times entering the center were quantified using the software system Ethovision 3.1. (Noldus IT, Wageningen, the Netherlands).

Elevated Plus Maze

The EPM is another validated tool to measure the degree of emotionality [35]. The EPM was made of grey PVC and consisted of two open and two closed arms; length 28 cm, width 5 cm, height closed arms 15 cm. In the center of the four arms there was a squared platform of 5x5 cm. The animal was placed in the center of the EPM facing an open arm, having the experimenter in the back. Subsequently behaviour was recorded during five minutes and analysed using Ethovision 3.1. The number of open arm entries, the time spent in the open arms, the percentage of open and closed arm entries and the total number of arm entries were key measures in this task.

Object Recognition Test

Recognition memory was assessed by the ORT [36]. The arena of the ORT consisted of a circular field - diameter 48 cm - made of transparent PVC. Objects were placed in a symmetrical position about 12 cm from the wall, at a distance of 24 cm from each other. Mice were placed in the arena at a standardized point along the wall of the arena always facing the wall in front of the experimenter. The first part of the procedure consisted of two adaptation days, in which the mice were allowed to explore the empty apparatus twice a day for 5 minutes per session. The second part of the ORT included the test day. On the test day, the animal underwent two sessions, separated by a retention interval of one hour. In the first session the animal was exposed to two identical objects during five minutes. In the second session the animal was again placed in the arena for five minutes with a familiar object from the first session and a novel object. The time spent exploring the two objects during the first and second session were manually registered using a non-commercial software program “Object Recognition Task” version 2. Two sets of objects were used, specifically small white stones and yellow Lego bricks. Objects met the following requirements: they had a comparable height and volume, were too heavy to be replaced by a mouse, were easy to clean, and there was no possibility for a mouse to hide under or climb on the objects. Habituation, exploration and discrimination were the main parameters measured in this task [36]. Habituation was defined as the difference in exploration time between the two sessions on the same day, namely exploration time in trial one minus exploration time in trial two. Exploration was defined as the time spent exploring both the familiar and new object, namely exploration of object one plus exploration of object two. Discrimination was defined as the

discrimination between the new and familiar object, that is time spent in exploring the novel object minus the time spent in exploring the familiar object. Based upon these measures we finally calculated the discrimination index in which we adjusted for total exploration time, namely discrimination divided by the total exploration time at both objects.

Morris Water Maze

The MWM test was conducted to measure spatial learning [37]. The MWM was a circular pool made of grey PVC with a diameter of 110 cm. The escape platform consisted of grey PVC, had a diameter of nine cm and was invisibly hidden at approximately one cm beyond the water surface at the north quadrant of the pool. Extra maze cues were placed at two of the nearby walls of the testing room. To avoid the negative effects of a decrease in body temperature, the water was kept at a constant temperature of 24-26°C. Water was colored with non-toxic white paint in order to obtain a clear contrast between the water and the mice and to mask the platform position. Mice performed four trials during five daily acquisition sessions. In every trial the mouse started from a different starting position, which was predetermined by randomization. As soon as an animal succeeded to reach the escape platform within the cut-off time of 60 seconds, it was allowed to stay on it for 15 seconds. Thereafter, it was removed from the pool and put back in its cage were it was allowed to rest for 15 seconds, after which the animal was again released into the pool. This procedure was repeated four times on each test day. When an animal did not find the escape platform within the cut-off time, it was placed on the escape platform by hand and stayed on it for 15 seconds. To avoid possible olfactory tracks the water was stirred after a mouse completed the test session. Moreover, in order to prevent a decrease in body temperature during testing, the cage of the animals was placed on a rodent warming pad with a temperature of approximately 37°C for ten minutes. In addition, all mice received a new, dry tissue. MWM performance was recorded and analysed using Ethovision 3.1.

Morris Water Maze - Probe trial

After completing the fifth testing day in the MWM, a probe trial was conducted in which we assessed reference memory [37]. Mice were released into the pool, without the escape platform, starting in the south quadrant. During 60 seconds we measured the time spent in each quadrant using Ethovision 3.1. Subsequently, we calculated the percentage of time spent in the quadrant that initially contained the escape platform.

9.2.7 Statistical analyses

Analysis of Variance (ANOVA) was used to test whether vitamin D adequate and vitamin D deficient mice differed with respect to their serum 25(OH)D concentrations. Similarly, ANOVA was performed to test for differences in blood glucose concentrations or plasma insulin concentrations between LF(D) and MF(D) mice. Aging effects were tested using a paired sample t-test, comparing the results of all mice tested pre-treatment with the results of all mice after treatment. Kaplan–Meier survival analysis and Mantel Cox log-rank test were used for survival comparisons between the four treatment groups. ANOVA was used to study

potential main effects of vitamin D and the type of fat diet and its interaction on the absolute change in behaviour in the OFT, EPM, ORT and the MWM. Linear Mixed Models were used to assess whether there was a learning curve over test days in the MWM. A P-value of <0.05 was used to determine statistical significance. All statistical analyses were performed using SPSS Statistics v19 (SPSS Inc. Chicago, IL).

9.3 Results

9.3.1 Survival and dropout

With survival rates of 87%, 80% and 83% in the LFD, LF and MFD groups respectively, no obvious survival differences were observed between these groups. With a survival rate of 59%, MF mice did notably though not significantly differ from the LFD (X^2=3.0, P=0.08), LF (X^2=1.2, P=0.27) and MFD (X^2=2.6, P=0.11) mice (Figure 2). When reviewing the data of the mice that dropped-out during the study and the mice that showed pathologies at necropsy, the most frequently observed abnormalities were liver pathologies, followed by abnormal coagulating glands and kidney abnormalities (eFigure 1 and eFigure 2). Since the pathologies were expected - and in many cases also observed - to interfere with the performance in the behavioural tests, the results presented below are based upon the analyses conducted with the data of the mice without any sign of underlying illness (n=33), specifically LFD (n=10), LF (n=9), MFD (n=8) and MF (n=6).

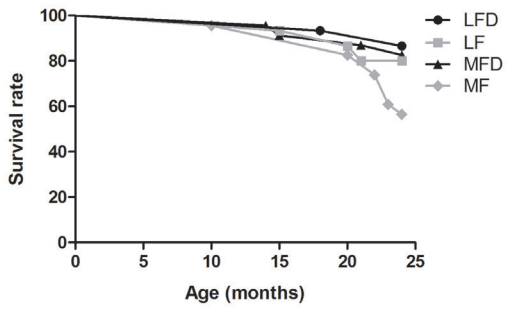

Figure 2. Survival per month. *Note*: Survival rates were 87%, 80%, 83% and 59% in the LFD, LF, MFD and MF groups, respectively. MF mice showed a substantial lower survival rate than the LFD, LF and MFD mice. Though, survival in MF mice did not significantly differ from the LFD (X^2=3.0, P=0.08), LF (X^2=1.2, P=0.27) or MFD mice (X^2=2.6, P=0.11). LFD: low fat diet with vitamin D. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

9.3.2 Body weight development

Mean body weight of the mice in all treatment arms increased until the time of sacrifice at 24 months of age. As intended, the mice on the MFD/MF diet gained more weight than mice receiving a LFD/LF diet. MF mice were on average less heavy when compared to MFD mice (Figure 3A).

9.3.3 Serum 25(OH)D concentrations and glucose homeostasis

Serum 25(OH)D analyses at the age of 17 months (data not shown) and at 24 months (Figure 3B) showed a clear vitamin D deficiency in mice receiving vitamin D deficient diets (F_{32}=388.1, P<0.0001). Moreover, a significant interaction between vitamin D and a moderate fat diet in relation to serum 25(OH)D was observed, indicating that mice on a

moderate fat diet had higher serum 25(OH)D concentrations than mice on a low fat diet (F_{32}=14.1, P=0.001). In addition, serum 25(OH)D of mice receiving the vitamin D adequate diet reached in most cases concentrations ≥50 nmol/L. At sacrifice, mice fed a moderate fat diet exhibited higher fasting glucose concentrations (11.4±0.7 mmol/L vs. 9.3±0.7 mmol/L, F_{32}=4.1, P=0.05) and higher fasting plasma insulin concentrations (1.9±0.2 ng/mL vs. 1.5±0.1 ng/mL, F_{31}=4.4, P<0.05) than their low fat counterparts (Figure 3C and Figure 3D). OGTTs conducted three months before sacrifice did not show a difference in glucose response between the four groups (Figure 3E and Figure 3F).

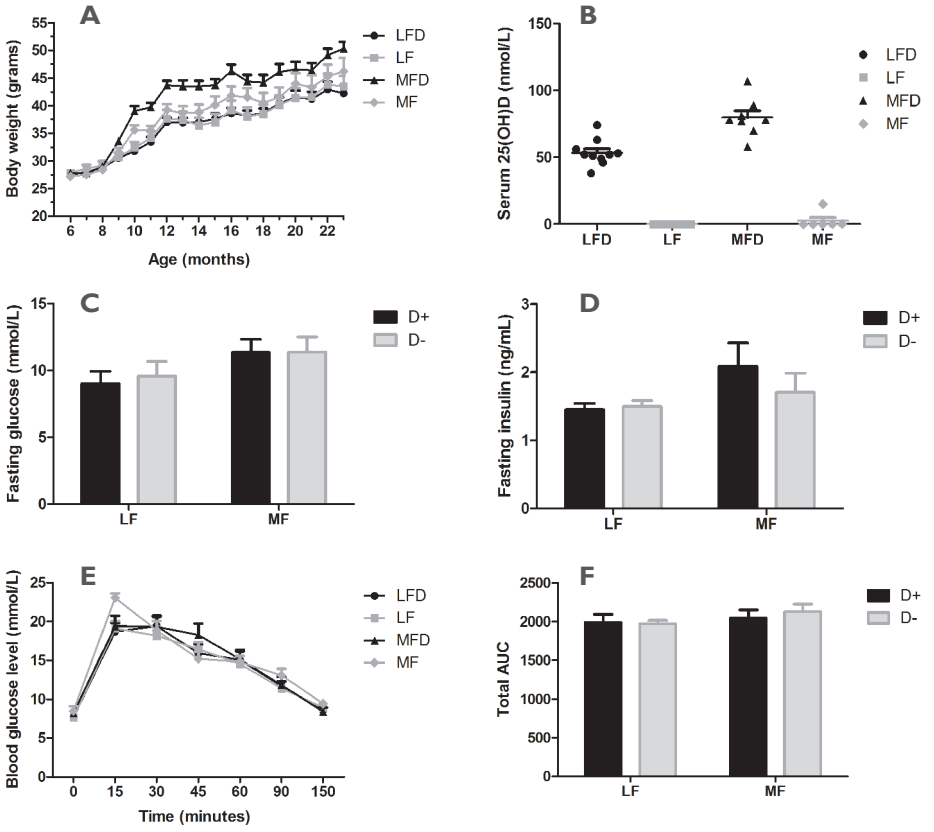

Figure 3. Body weight development and biochemical characteristics at 21-24 months of age. *Note*: (A) Body weight development of the mice during the study, as shown by the mean with SEM. (B) Serum 25(OH)D concentrations at 24 months old. (C) Fasting plasma glucose concentrations at 24 months old. (D) Fasting plasma insulin concentrations at 24 months old. (E) Oral Glucose Tolerance Test (OGTT) results at 21 months old; blood samples were taken at 15, 30, 45, 60, 90 and 150 minutes following the glucose load of 5 µl of 20% glucose solution per gram body weight. (F) Area Under the Curve for OGTT data.

9.3.4 Aging in relation to behaviour

When comparing the overall mean performance at young age with the overall mean performance at old age, it is concluded that aging significantly affected behavioural parameters related to exploration and emotional reactivity. At old age, mice travelled significantly less far during a five minute trial in the OFT (t_{32}=4.5, P<0.0001), and spent significantly more time in the center of the OFT (t_{32}=-2.7, P=0.01) (Figure 4A, Figure 4B and Table 1). In the EPM, aging significantly decreased the time spent in the open arms (t_{32}=3.7, P=0.001), the percentage of open arm entries (t_{32}=6.0, P<0.0001) and the number of open arm entries (t_{32}=6.4, P<0.0001) (Figure 5A, Figure 5B and Table 1). In addition, recognition memory, as assessed in the ORT, significantly decreased when comparing mice's performance at young age with the performance at old age (t_{32}=3.5, P=0.001; Figure 6 and Table 1). With respect to the MWM, when comparing the Area Under the Curve (AUC) for the escape latency and distance moved to platform at young age with the AUC at old age, a significant effect was observed for the distance moved to the platform (t_{32}=-2.1, P=0.05), but not for the escape latency (t_{32}=-0.31, P=0.76) (Figure 7A, Figure 7B, Figure 7C, Figure 7D and Table 1). At old age, mice spent significantly more time in the target quadrant during the probe trial when compared to their performance at young age (t_{32}=-6.1, P<0.0001) (Figure 8 and Table 1).

9.3.5 Vitamin D and impaired glucose homeostasis in relation to behaviour

Exploration and emotional reactivity

In the OFT, there was no significant effect of vitamin D, the type of fat diet or the interaction between the two on the total distance moved or the time spent in the center (Figure 4A, Figure 4B and Table 1). There was also no significant effect of vitamin D or an interacting effect of vitamin D with fat on EPM performance. Fat did significantly affect emotional reactivity as assessed with the EPM, showing a larger decrease in the percentage of open arm entries in LF(D) than MF(D) mice (absolute change ± SEM in MF(D) vs. LF(D) mice: -8±5 vs. -23±2, $F_{1,29}$=9.72, P=0.004) (Figure 5A, Figure 5B and Table 1). Accordingly, the total number of arm entries decreased less in the MF(D) than LF(D) mice (absolute change ± SEM in MF(D) vs. LF(D) mice: -5±2 vs. -11±2, $F_{1,29}$=5.31, P=0.03), which was mainly attributable to the MFD group that showed a decrease of -2±2 vs. -8±3 in MF mice. Moreover, the decrease in the percentage of open arm entries tended to be lower in vitamin D deficient mice than in mice receiving a vitamin D adequate diet (absolute change ± SEM in LF/MF vs. LFD/MFD mice: -25±11 vs. -41±24, $F_{1,29}$=3.21, P=0.08).

Recognition memory, spatial learning and reference memory

We did not observe significant effects of vitamin D, fat or the interaction between vitamin D and fat on the adjusted discrimination index in the ORT, but there was a tendency towards an vitamin D by fat interaction ($F_{1,29}$=3.83, P=0.06) (Figure 6 and Table 1). Our treatments did also not significantly affect spatial learning (Figure 7A-D and Table 1) or reference memory, assessed in the probe trial (Figure 8 and Table 1) as assessed in the MWM. Though, the figures of the MWM do suggest a weak tendency for a beneficial effect of vitamin D.

Specifically, the absolute increase in time needed to locate the platform tended to be larger in vitamin D deficient mice than in vitamin D adequate mice (absolute change±SEM in vitamin D adequate vs. vitamin D deficient: -7±7 vs. 10±7, $F_{1,29}$=2.96, P=0.10).

Table 1. Behavioural test results at the age of 6-8 and 22-23 months.

		Pre-treatment	22-23 months	22-23 months	Time#	Vitamin D*Fat*	Vitamin D*	Fat*
Open Field Test	**Total distance travelled, cm**				*t*=4.5, P<0.0001	*F*=0.08, P=0.78	*F*=0.03, P=0.88	*F*=0.33, P=0.57
	LFD	3737±110	3052±129	3026±216				
	LF			2926±170				
	MFD			3018±210				
	MF			3328±525				
	Center time, sec				*t*=-2.7 P=0.01	*F*=0.12 P=0.74	*F*=0.02 P=0.89	*F*=0.20 P=0.66
	LFD	9±1	13±1	14±1.6				
	LF			14±2.1				
	MFD			12±3.3				
	MF			11±2.6				
	Open arm time, sec				*t*=3.7 P=0.001	*F*=1.67 P=0.21	*F*=0.88 P=0.36	*F*=3.48 P=0.07
	LFD	61±6	34±4	25±8				
	LF			38±5				
	MFD			32±8				
	MF			45±13				
Elevated Plus Maze	**Open arm entries, %**				*t*=6.0 P<0.0001	F=0.20 P=0.66	F=3.21 P=0.08	F=9.72 P=0.004
	LFD	35±2	19±2	13±2				
	LF			21±2				
	MFD			19±4				
	MF			26±6				
	Open arm entries, freq				*t*=6.4 P<0.0001	*F*=0.13 P=0.72	*F*=0.17 P=0.68	*F*=15.8 P<0.0001
	LFD	12±0.8	5±0.6	3.3±0.6				
	LF			5.1±0.6				
	MFD			5.3±1.3				
	MF			7.5±2.3				
	Total arm entries, freq				*t*=5.7 P<0.0001	*F*=0.05 P=0.83	*F*=1.82 P=0.19	*F*=5.31 P=0.03
	LFD	34±1.3	26±0.9	26±2				
	LF			25±1				
	MFD			26±2				
	MF			26±3				

		Pre-treatment	22-23 months	22-23 months	Time#	Vitamin D*Fat*	Vitamin D*	Fat*
Object Recognition Test	**Adjusted discrimination index**				*t*=3.5 P=0.001	*F*=3.83 P=0.06	*F*=0.21 P=0.65	*F*=0.17 P=0.68
	LFD	0.35±0.03	0.21±0.04	0.31±0.08				
	LF			0.11±0.06				
	MFD			0.19±0.10				
	MF			0.21±0.07				
Morris Water Maze	**Area under the Curve for escape latency**				*t*=-0.31, P=0.76	*F*=0.69, P=0.41	*F*=2.96, P=0.10	*F*=0.08, P=0.79
	LFD	87±4	88±3	83±6				
	LF			85±8				
	MFD			95±6				
	MF			94±8				
	Area under the Curve for distance travelled				*t*=-2.1, P=0.05	*F*=0.62, P=0.44	*F*=3.55, P=0.07	*F*=0.06, P=0.80
	LFD	1264±57	1448±64	1362±106				
	LF			1418±158				
	MFD			1484±96				
	MF			1585±166				
	% in target quadrant during probe trial				*t*=-6.1, P<0.0001	*F*=0.56, P=0.46	*F*=2.01, P=0.17	*F*=0.53, P=0.47
	LFD	41±2	56±2	60±5				
	LF			57±5				
	MFD			55±2				
	MF			49±5				

Note: Values displayed are absolute mean with standard error (SEM). #Degrees of freedom: 32. *Degrees of freedom: 29.

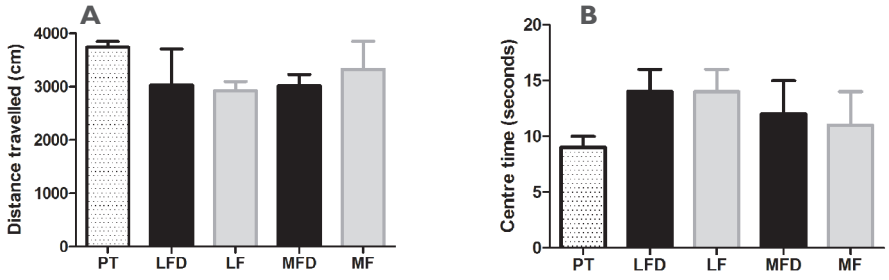

Figure 4. Open Field Test (OFT) performance during a 5-minute trial at 6 (PT) and 22 months old (LFD, LF, MFD, MF). Specifically, the total distance travelled and the time spend in the center of the OFT, displayed by the mean with SEM. *Note*: PT: pre-treatment. LFD: low fat diet with vitamin D. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

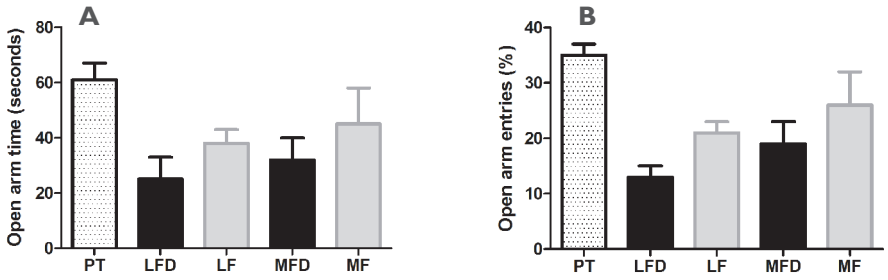

Figure 5. Performance in the Elevated Plus Maze (EPM) during a five minute trial at 6 (PT) and 22 (LFD, LF, MFD, MF) months old. Specifically, the total time spend in the open arm and the percentage of open arm entries, displayed by the mean with SEM. *Note*: PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

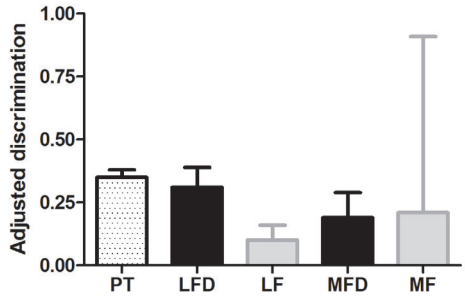

Figure 6. Object Recognition Test (ORT) performance at 6 (PT) and 22 (LFD, LF, MFD, MF) months old, displayed by the mean with SEM of the adjusted discrimination index. *Note*: Discrimination was defined as the discrimination between the new and familiar object, specifically time spent in exploring the new object minus the time spent in exploring the old object. Subsequently, this discrimination index was adjusted for total exploration time, namely discrimination divided by the total exploration time. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

Accordingly, a similar pattern was observed when studying the distance travelled before locating the platform (absolute change ± SEM in vitamin D adequate vs. vitamin D deficient: 31±119 vs. 345±123, $F_{1,29}$=3.55, P=0.07) and reference memory in the probe trial of the MWM (absolute change ± SEM in vitamin D adequate vs. vitamin D deficient: 18±4 vs. 12±3, $F_{1,29}$=2.01, P=0.17).

9.4 Discussion

9.4.1 Main findings

Cognitive performance and emotional reactivity of mice at the age of 22-23 months - as assessed with the ORT, MWM, OFT and the EPM - significantly differed from the performance of the same mice at the age of 6-8 months. Furthermore, a significant effect of the type of fat diet was observed on emotional reactivity in the EPM, suggesting that a moderate fat diet may prevent or delay the age-related increase in emotional reactivity. This study did not provide evidence for an effect of adult vitamin D deficiency on the behavioural indices in 22-23 months old C57BL/6 mice.

9.4.2 Our study in a broader perspective

Laboratory studies have suggested a number of pathways via which vitamin D may beneficially affect brain function [3, 28]. When looking into the current literature on studies in rodents, it can be concluded that even though the results are mixed [38-42] the majority points towards a favorable effect of vitamin D on behaviour [39-42]. When comparing these studies, however, it is clear that there are large differences between the applied study designs. Three out of five rodent studies were conducted in rats [38, 39, 41] and two in mice [40, 42]. Whereas four studies intervened by means of a modified dietary regimen [38, 40-42], one study intervened with a subcutaneous injection of 42 IU 1,α25(OH)D3 per kg [39]. There was also a large variety in the behavioural tests applied. Also physiologically there were major differences between the models studied. The age of the behavioural assessment varied from 3-20 months, and the duration of the intervention varied from 2-20 weeks. Moreover, in two of the studies that observed a vitamin D effect, a very particular neuropathology was induced, specifically amyloid plaques deposition [41, 42]. Thus, the mechanisms underlying the behavioural deficiencies or changes may differ substantially between the used models, and therefore the potential molecular "targets" for vitamin D are model-dependent. As already mentioned previously, in this study the aim was to examine the effect of long-term vitamin D deficiency on emotional reactivity and cognitive performance in 'normal' aging C57BL/6 mice. In view of the often relatively slowly decreasing vitamin D status with aging in humans, we believe that our model more closely resembles the clinical condition of vitamin D

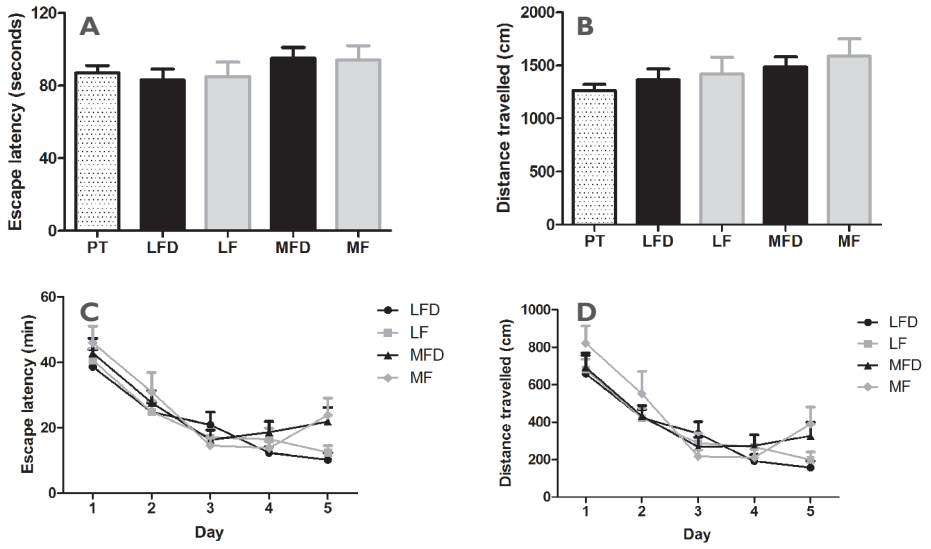

Figure 7. Morris Water Maze (MWM) performance at 7 (PT) and 23 (LFD, LF, MFD, MF) months old. Performance was assessed by means of four trails during five daily acquisition sessions; the mean with SEM of each daily session is displayed in the figure. *Note:* (A) Area under the Curve (AUC) for escape latency. (B) AUC for distance travelled to platform. (C) Learning curve for escape latency at 23 months old. (D) Learning curve for distance travelled to platform at 23 months old. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

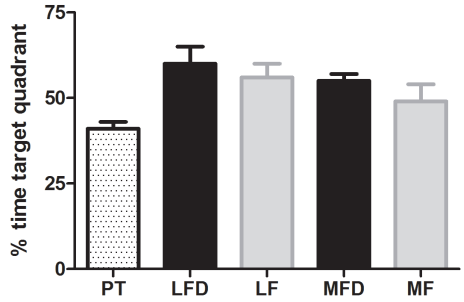

Figure 8. Probe trial performance at 7 (PT) and 23 (LFD, LF, MFD, MF) months old. *Note*: Bar graph shows the percentage of time spent in the target quadrant during a 1-minute trial at the end of the fifth test day. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

inadequacy and natural aging in older adults than the models that have been described previously. However, in future studies the design could be optimized further, for instance by reducing the vitamin D content of the food gradually. Such a gradual decrease may even more closely mimic the development of vitamin D inadequacy as observed in human aging. Groves and colleagues examined the effect of a 10-week vitamin D-deficient diet in 20-week-old C57BL/6 mice [40], which we consider physiologically most comparable to our design. The findings in these 20-week-old C57BL/6 mice were in line with the current results, specifically no effect of vitamin D was found with regard to behaviour in the EPM, a familiar OFT, the hole-board test, the light/dark test, the forced swim test, or the social interaction test. Vitamin D deficient C57BL/6 mice did show signs of hyperlocomotion in an unfamiliar OFT when compared to their vitamin D adequate counterparts. More robust vitamin D effects were observed in the BALB/c strain, showing that deficient mice spent more time on the open arms of the EPM and responded faster to heat, sound and shock [40]. However, as 20-week-old mice cannot yet be considered aged and the period of an absolute vitamin D deficiency in these mice was relatively short, Groves and colleagues did not specifically target the aging mechanisms related to brain function like we did. This study, however, does suggest that BALB/c mice may be more susceptible to vitamin D deficiency than C57BL/6 mice, which might advocate the use of BALB/c mice instead of C57BL/6 in future behavioural studies on the effect of vitamin D deficiency. When extending our comparison with the literature by looking at the behavioural tests used, we saw that three of the five previous studies assessed spatial learning using the MWM [39, 41, 42]. All suggested an effect of vitamin D on spatial learning. We also assessed spatial learning in the MWM. Our data only suggest a weak tendency for a vitamin D effect in the MWM.

9.4.3 Methodological considerations

Aging

Our longitudinal study showed that age was an important determinant of behaviour. Two of these findings warrant clarification. First of all, the finding that old mice spent more time in the center of the OFT. This may be explained by the fact that, in view of health monitoring throughout the trial, mice had already been exposed to the OFT for five times before the final measurements. Thus, whereas the open field at six month of age was a novel open field,

at old age the open field had become familiar to the mice, resulting in habituation and hence less signs of emotional reactivity. Secondly, although of borderline significance, our results showed that the distance travelled towards the platform in the MWM was longer in old mice than in young mice, and that probe trial performance improved with age. Stronger age effects might have been expected. As shown by previous longitudinal studies, carry-over effects may occur when administering the MWM at multiple time points [43, 44]. This may explain the modest age effect observed in the MWM in this study. Guidi and colleagues also showed that once the reference memory task is learned, animals remain capable to successfully complete the task, irrespective of age [43]. As the MWM with varying platform locations did appear to be sensitive to detect subtle cognitive changes [43], using this modified version may refine future studies.

Vitamin D deficiency

This study did not provide evidence for a significant effect of adult vitamin D deficiency on measures of emotional behaviour (EPM and OFT) or cognitive performance (ORT and MWM) in aged mice. Neither did we observe an effect of the type of fat diet in combination with vitamin D on emotional reactivity, learning and memory. In order to substantiate our conclusion that vitamin D does not seem to play a major role in learning, memory and emotional behaviour in aging C57BL/6 mice at the age of 22-23 months, it should be evident that the vitamin D status differed sufficiently between the treatment groups. Serum 25(OH)D measurements in pooled samples collected at seven months after the initiation of the vitamin D deficient diet indicated that the concentrations in the deficient group were below the detection limit of the assay. Serum 25(OH)D concentrations in the adequate groups were around 55 nmol/L, which is considered to be an adequate concentration in humans [45]. Measurements in individual blood samples collected at sacrifice showed that serum 25(OH)D concentrations were still very low or non-detectable in the deficient mice. Thus, these results provide evidence that mice had an absolute vitamin D deficiency for at least six months preceding the final behavioural tests, which to our knowledge has never been done before. Based upon these data we argue that the difference in vitamin D intake between the adequate and deficient group was large enough to show a potential effect of vitamin D on brain function and behaviour. Since we did observe weak tendencies for a vitamin D effect in the MWM, it might also be postulated that we did not observe an effect of the treatments on cognitive decline because brain functions were still too well preserved at 22-23 months of age - mice as old as 18-24 months are considered to be comparable to humans at the age of 56-69 years [46] - resulting in behavioural differences between treatment groups that were too small to detect.

Impaired glucose homeostasis

Another aim of this study was to examine the role of an impaired glucose homeostasis in the potential relationship between vitamin D and behaviour. Byrne and colleagues postulated that the effect of a vitamin D deficiency might be stronger in the presence of a second neurobiological stressor as Parkinson's disease or brain ischemia [47]. However, we have to

conclude that in our study the effect of vitamin D deficiency on behavioural outcomes was not stronger in mice with an impaired glucose homeostasis compared to mice with a normal glucose tolerance. Our data did suggest that mice with an impaired glucose homeostasis did not have an age-related increase in emotional reactivity, whereas mice with a normal glucose homeostasis did. We do not have an explanation for this unexpected finding. To induce an impaired glucose homeostasis, a subsample of mice in this study were exposed to a moderate fat load - a diet where 20% of energy was provided by fat - to mimic the relatively slow development of glucose intolerance as often observed at older age in humans. Thus, to reflect a more relevant physiological state, the fat load fed in this study was deliberately lower than the high fat loads used in previous short-term studies that provoked diet-induced diabetes [32]. The moderate fat load appeared to be potent enough to induce a higher weight gain and modest differences in fasting glucose and insulin concentrations, which indicates a state of impaired glucose homeostasis in MF(D) mice. As there was no significant difference in glucose response in the OGTT, it cannot be concluded that the moderate fat diet resulted in an impaired glucose clearance. This profile of metabolic changes combined with elevated body weight in MF(D) mice mimics a human state which can be described as pre-diabetic. However, we feel that the achieved pre-diabetic state was not as strong as we aimed at. To increase the impact of a moderate fat diet, future studies might consider the use of a diet with a higher fat content, specifically a diet containing 25 En% from fat. However, the rapid increase in body weight following the switch to the moderate fat diet as observed in our study, does imply that caution is warranted. Besides the fact that a too extensive weight gain may result in early dropouts, it may also become a limiting factor in the behavioural assessment.

Other factors that may have interfered with the relations studied

As the cognitive and emotional tests involve a clear physical component, it might be argued that the results of the behavioural tests are influenced by sensorimotor impairments and as such influenced the internal validity of this study. Another factor that may have been involved in the relationships studied is hypocalcaemia. Specifically, vitamin D deficiency may lead to hypocalcaemia, which has been linked with neuromuscular as well as neuropsychiatric symptoms [48]. Unfortunately, serum calcium concentrations have not been measured in this study. However, tests of sensorimotor performance and muscle strength - specifically the Horizontal Wire Test, the Balance Rod, the Open Field Test as well as ex vivo muscle measurements [49] - did not point towards differences between the treatment groups (data not shown). Nor did we observe large differences in anxiety related behaviour between vitamin D deficient and vitamin D adequate mice.

Novelty

During the past decade interest in vitamin D has grown enormously [50]. As vitamin D receptors are located on many tissues throughout the body including the brain, vitamin D is hypothesized to target a variety of bodily tissues. Likewise, a large number of epidemiological observational studies have shown associations linking vitamin D not only to brain health but also to cardiovascular disease, fat metabolism, muscle function, and glucose homeostasis

[50, 51]. A major issue in many of these human studies, however, is the possibility of reverse causation and residual confounding. In randomized clinical trials (RCTs) these factors are less likely to disturb the potential relationships studied, and from these studies the effects of vitamin D seem to be less clear-cut than they appear from observational studies. A very recent review summarized the data of RCTs that studied the effect of vitamin D supplementation on a variety of non-skeletal health outcomes in humans, showing no clear vitamin D effect [50]. Based on their findings the authors speculate that the discrepancy between observational and intervention studies in humans may indicate that low 25(OH)D status is rather a marker of ill health than a risk factor of disease. In view of these previous RCTs, our mouse study can be regarded as a proof of principle experiment, albeit with a negative result. Thus, despite the fact that there are aspects of our study that can be debated, it should be realized that this study is unique in its kind. It is the first study that investigated the effect of a one-year vitamin D deficient diet, and an absolute verified vitamin D deficiency for at least six months, on various behavioural parameters related to emotional reactivity and memory in natural aging mice.

9.4.4 Conclusion

This study showed a clear aging effect on various measures of emotional behaviour and cognition in male C57BL/6 mice at 22-23 months of age. No large differences in behavioural phenotype, as assessed with the OFT, EPM, ORT and MWM, between vitamin D adequate mice and vitamin D deficient mice were observed.

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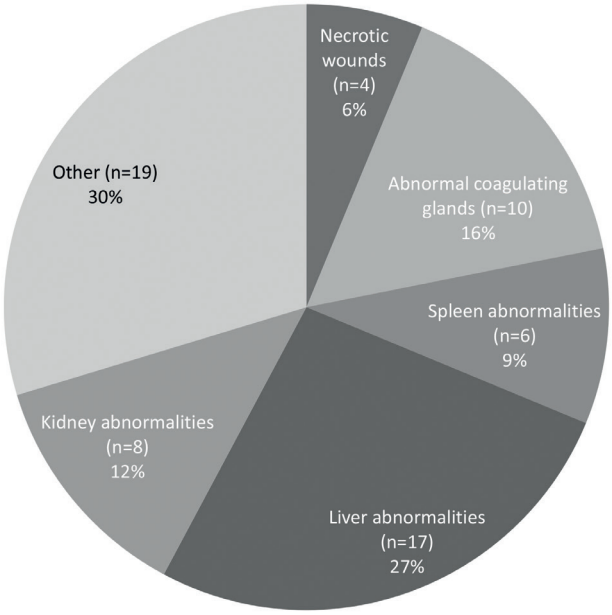
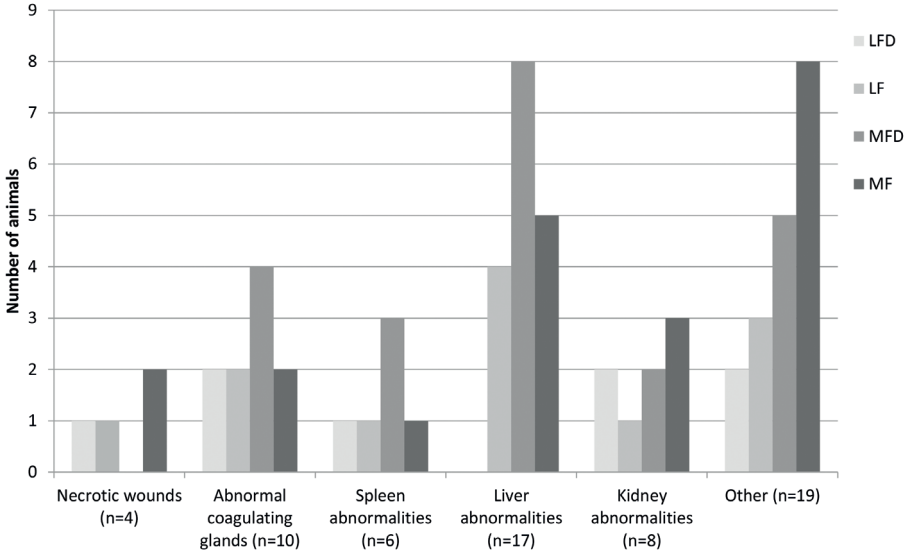
Supplementary material



sFigure 1. Overview of observed pathological abnormalities during the study (dropout) and at the sacrifice. *Note*: In total 64 pathological abnormalities were observed. In total 43 mice were affected by one or more pathological abnormalities and therefore excluded from the analyses.



sFigure 2. Pathological abnormalities during the study (drop-out) and at the sacrifice, stratified according to treatment group.

sTable 1. Diet composition of the 4 diets.

Ingredients*	LFD	LF	MFD	MF
Protein (gram%)	12	12	12	12
Carbohydrate (gram%)	72	72	64	64
Fat (gram%)	4	4	12	12
Casein, lactic	140	140	140	140
L-Cystine	1.8	1.8	1.8	1.8
Corn Starch	447.9	447.9	367.9	367.9
Maltodextrin	100	100	100	100
Dextrose	72.8	72.8	72.8	72.8
Sucrose	100	100	100	100
Cellulose	50	50	50	50
Soybean oil	22	22	40	40
Palm oil	18	18	80	80
Mineral mixture	35	-	35	-
Mineral mixture without vitamin D	-	35	-	35
Vitamin mixture	10	-	10	-
Vitamin mixture without vitamin D	-	10	-	10

Note: Nutrients are displayed in grams, unless specified otherwise.

10

General discussion

Parts of this chapter have been accepted for publication in Current Opinion in Clinical Nutrition and Metabolic Care as: Vitamin D and cognition in older adults: an update of recent findings. Brouwer-Brolsma EM, de Groot CPGM.

To contribute to the current state of knowledge, the aim of this PhD thesis was to study the role of vitamin D, and its potential interplay with glucose homeostasis, in association with cognitive performance and depressive symptoms. Whereas some of our studies suggest a potential role for vitamin D in the prevention of cognitive impairment and depressive symptoms, others do not. Moreover, our data do not provide evidence for an interplay between vitamin D and glucose homeostasis in the association with cognitive performance and depressive symptoms. In the following paragraphs it will be addressed how these findings fit with the existing literature, whether there is coherence between the different levels of evidence, whether the evidence-base on vitamin D and brain health is convincing enough to be incorporated once revising the vitamin D recommendations, and what can be done to further expand our understanding in this research field. The argumentation in this chapter will start off with a paragraph recapping the current vitamin D recommendations, the underlying scientific base for these recommendations and some figures related to the adherence to these recommendations by older adults.

10.1 Telling figures on vitamin D deficiency

10.1.1 Current recommendations

In **chapter 2** we summarized the outcomes of a vitamin D expert meeting (Ede, 2011) that aimed to make a step towards the harmonization of the dietary reference intakes for vitamin D across Europe [1]. Following the exchange of recent scientific insights on vitamin D relating to a variety of disciplines, it was concluded that the evidence supporting a role for vitamin D in bone health is convincing. In addition, it was acknowledged that a substantial body of observational evidence suggests that vitamin D may be beneficial for other body systems (e.g. muscle, glucose homeostasis, brain, respiratory system). However, trial evidence confirming the vitamin D effects on these outcomes was considered insufficient to take into account in the revision of the vitamin D recommendations. Furthermore, it was concluded that a 25(OH)D concentration of 50 nmol/L can be considered sufficient with respect to bone health, and that current evidence is insufficient to conclude that there is an amplified beneficial effect of 25(OH)D concentrations that exceed 50 nmol/L. Considering the results of dose-response studies on vitamin D, a vitamin D intake of 20 µg/day was assumed to assure a 25(OH)D concentration within the optimal range. The outcomes of this expert meeting were in line with those proposed by the Institute of Medicine (IOM) [2], but are considered conservative by other vitamin D experts [3]. Since 2011 the scientific evidence on vitamin D and its possible relation to health effects beyond bone health substantially increased. Whether this evidence has implications for the current policy will be discussed in paragraph 10.5.

10.1.2 Are the current recommendations met by older adults?

In **chapter 3** we explored how well a community-dwelling sample of Dutch older adults met the recommendations as set by the vitamin D experts. It was observed that 45% of a population of Dutch community-dwelling older adults, aged ≥65 years, had a vitamin D concentration

<50 nmol/L. Mean daily vitamin D intake, including dietary sources and supplemental vitamin D intake, was 4.9±2.9 µg/day. Only 20% of this population reported the use of vitamin D supplements, of which 70% had an adequate vitamin D status. Telling figures, one would say, and they do not stand-alone. The Longitudinal Aging Study Amsterdam reported a similar prevalence of 25(OH)D inadequacy, namely 46% [4]. With 79% and 53%, even higher percentages of vitamin D deficiency were observed in a population of European older adults (chapter 4) and pre-frail/frail Dutch older adults (chapter 5). Moreover, our findings on vitamin D intake are in line with results of the Dutch Food Consumption Survey that was conducted in community-dwelling older adults from 2010-2012. This survey showed a median total vitamin D intake of 4.6 µg/day in men and 4.1 µg/day in women; 18% of the men and 26% of the women reported the use of vitamin D containing supplements [5]. The current level of vitamin D intake, however, may be underestimated [6]. Namely, foods may not only contain vitamin D_2 and vitamin D_3 but also 25(OH)D, a compound that food consumption tables do not yet account for. A recent study suggests that taking into account 25(OH)D in animal-based foods may result in vitamin D intake estimates that are about 1.7-2.9 µg/day higher than the current estimates [6]. Nevertheless, vitamin D intakes and serum vitamin D concentrations are low in a large part of the population and thus action is warranted.

10.2 Evaluating the scientific evidence on vitamin D and brain health in older adults

As concluded by several recommendations setting bodies [2, 3, 7-10] there is convincing evidence that vitamin D is important for optimal bone health. However, more evidence is needed before conclusions can be drawn on the potential role of vitamin D in other body systems, including brain function.

10.2.1 Vitamin D mediated brain processes

Up to now, several biological studies provided evidence that links vitamin D with brain function, and new evidence continues to be added. As summarized in chapter 1 many pre-clinical studies have reported immunohistochemical, biochemical or molecular biological findings supporting a role of vitamin D in brain function. Recent evidence adds to this that vitamin D may reduce age-related tau hyperphosphorylation [11], nitrosative protein damage [12], formation of Aβ oligomers [13], and increase amyloid clearance [13]. The active vitamin D metabolite, calcitriol, has also been shown to rebalance inflammation and promote Aβ phagocytosis in peripheral blood mononuclear cells (PBMCs) of Alzheimer's Disease (AD) patients [14]. Higher 25(OH)D concentrations have furthermore been associated with increased levels of $Aβ_{1-42}$ in cerebrospinal fluid (CSF) in a cognitively compromised group, including patients with Mild Cognitive Impairment (MCI) and AD [15]. In line, vitamin D binding protein has been linked with the inhibition of the aggregation and oligomerization of $Aβ_{1-42}$, and attenuation of Aβ-induced neuronal death [16]. In addition to these direct pathways, there are also speculations on indirect pathways. Vitamin D has for instance been associated with several cardiovascular risk factors [17, 18] that have been postulated to

adversely affect brain function [19, 20]. Thus, it may well be that vitamin D affects the brain via direct and indirect pathways, including non-vascular and vascular pathways.

10.2.2 Neuropsychological evidence

Global cognitive performance

Data presented in **chapter 4** (SENECA Study) [21] and **chapter 5** (ProMuscle Study) [22] of this thesis did not show an association between higher 25(OH)D concentrations and better global cognitive performance as assessed with the Mini Mental State Examination (MMSE). Our data are in contrast with a recent meta-analysis, which shows that participants with 25(OH)D concentrations ≥50 nmol/L score on average 1.2 MMSE points higher than participants with 25(OH)D concentrations <50 nmol/L [23]. A very reasonable explanation for the discrepancy between our findings and the findings of the meta-analysis is a lack of power in our studies. Whereas the analyses conducted in chapter 4 and 5 included only 116 and 127 participants, the meta-analysis was conducted with data of 2749 participants [23]. Moreover, although widely used and accepted, the MMSE was originally developed to quantify relatively severe cognitive deficits like dementia. As the MMSE scores in the SENECA study and the ProMuscle study were relatively high, a low prevalence of dementia is assumed. Therefore, the MMSE may not have been sensitive enough to detect cognitive differences. Given the relative small sample size and the supposed low prevalence of cognitive impairment, it is not expected that an updated meta-analysis including the two studies presented in chapter 4 and 5 would demonstrate substantially different results. Domain-specific cognitive tests have a higher sensitivity to detect cognitive differences than global cognitive screening tools, particularly in seemingly healthy populations. During the past decade, the research field has therefore gradually shifted from global tasks towards domain-specific tasks.

Domain-specific cognitive performance

In **chapter 5**, it is shown that higher 25(OH)D concentrations linearly associate with better executive function and tend to associate with a better information processing speed, reported as a cluster of multiple tests, in pre-frail and frail older adults aged ≥65 years [22]. Results in **chapter 6** indicate that apparently healthy older adults with the upper serum 25(OH)D quartile have a 51% lower probability of belonging to the 10% of the population with the worst attention and working memory as compared to those with the lowest 25(OH)D concentrations. Data also suggest a beneficial role for 25(OH)D concentrations in executive function and episodic memory, but these associations are not statistically significant. Recently, the first meta-analysis was published that evaluated the performance of participants with lower versus higher 25(OH)D concentrations on tests related to the domains episodic memory, information processing speed, attention and working memory, and executive function in older adults [24]. When comparing the performance of participants with lower and higher 25(OH)D concentrations, modest differences were observed with respect to episodic memory [24]. Moreover, persons with higher 25(OH)D concentrations completed

the Trail Making Test-A (information processing speed) 4 seconds faster and the Trail Making Test-B (mental shifting abilities) 13 seconds faster, and performed significantly better on tasks related to information updating than persons with lower 25(OH)D concentrations [24]. Prospective data furthermore indicated a 1.25 higher odds of mental shifting impairments among people with lower 25(OH)D concentrations [24]. To date, trial evidence is sparse. The only randomized controlled trial (RCT) conducted up to now included younger adults and did not provide evidence for a vitamin D effect [25]. Evidence from the Women's Health Initiative trial adds that 10 μg/day vitamin D_3 in combination with 1000 mg calcium versus placebo does not beneficially affect verbal memory, visual memory, mental updating or incidence of mild cognitive impairment (MCI) or dementia [26]. Supplements often contain both vitamin D as well as calcium, in view of their interplay in relation to bone health. However, it has been suggested that the joint administration of vitamin D and calcium may be controversial when studying cognitive performance [27], as high serum calcium concentrations have been associated with a faster rate of cognitive decline in a population of Dutch older adults [28].

Dementia

None of the studies presented in this thesis were conducted in patients with dementia. However, several other research groups did perform studies on vitamin D in Alzheimer's Disease (AD) populations. Meta-analysis of seven case-control studies, including 357 AD cases and 648 controls, showed that 25(OH)D concentrations were substantially lower in AD cases (-1.4 SD) [29]. Studies including older participants were more likely to observe significant associations than studies including younger participants. Therefore, it was proposed that the null-findings in the 'young-old' may be explained by the fact that these 'young-old' had 25(OH)D concentrations that still met the physiological needs of the brain, while this may not have been the case in the 'older-old' [29]. Furthermore, comparing untreated AD patients with AD patients treated with acetylcholinesterase inhibitors revealed that untreated AD patients had significantly lower 25(OH)D concentrations than treated AD patients [30]. However, despite these data it cannot be taken for granted that the observed associations are causal. In contrast to the hypothesized direction (i.e. low 25(OH)D concentrations adversely affect the pathological process in AD), low 25(OH)D concentrations may also be the result of altered outdoor behaviour, dietary intake, intestinal absorption and/or metabolism in AD. This last aspect can for instance be illustrated by a recent pre-clinical study that revealed that β-amyloid could increase calcitriol degradation and suppress VDR expression and as such alter vitamin D metabolism [31].

Depression

Estimates suggest that about 8-16% of the community dwelling older adults will face a depression at least once in their life [32]. These numbers are in line with the estimates in the populations studied in this thesis, namely 16% (**chapter 4**), 8% (**chapter 5**) and 7% (**chapter 7**). It has been suggested that the prevalence of depression may be lower in people with higher 25(OH)D concentrations. However, two small observational studies presented

in this thesis did not point towards a significant association between serum 25(OH)D and depression. More specifically, in the SENECA study (chapter 4) we observed a trend towards less depressive symptoms in participants with higher 25(OH)D concentrations (n=103) [21]. In the ProMuscle study (chapter 5) an opposite trend was observed (n=106) [22]. Cross-sectional data of the B-PROOF study, including almost 3000 participants, provided more robust evidence, indicating an association between higher 25(OH)D concentrations and less depressive symptoms (chapter 7).

So, how do these findings fit with the current literature? In chapter 1 an overview is given of the different observational studies that have been conducted on the association between 25(OH)D concentrations and depression in populations of older adults. Based on this overview it can be concluded that the findings of the SENECA study and the B-PROOF study are more in line with the literature than the findings of the ProMuscle study.

Recently, also several meta-analyses of observational studies [33, 34] and RCTs [35-37] have been published on this topic. Ju et al. (2013) calculated that a 25 nmol/L increase in serum 25(OH)D lowers the odds of having depression by 4% (pooled OR using data of 11 cross-sectional studies: 0.96 (95% CI 0.94-0.99). The analysis with data of five prospective studies provided an OR of 0.92 (95% CI 0.87-0.98) for a 25 nmol/L increase in serum 25(OH)D [34]. Subgroup analyses of prospective studies furthermore indicated a stronger risk reduction in participants aged ≥60 years (-10%) than in those <60 years (-4%) [34]. Although methodological disparities limit the comparison of the magnitude of the association, the results by Ju et al. point in the same direction as the meta-analysis conducted by Anglin and colleagues [33]. Schaffer and colleagues analysed data of seven RCTs, showing a non-significant overall mean difference in depression score of -0.14 (95% CI -0.33 to 0.05) in favour of vitamin D supplement use [36]. Limiting the analyses to the two RCTs including participants with clinically significant depressive symptoms showed a significant vitamin D effect, specifically a mean difference in depressive symptoms of -0.69 (95% CI -1.19 to -0.01) [36]. Sensitivity analyses including studies that had the lowest vitamin D concentrations at baseline did not suggest a stronger effect in this subgroup [36]. Spedding also conducted a systematic review and meta-analysis of RCTs and concluded that the methodological quality of the trials was high, but that eight out of 15 RCTs were limited due to shortcomings [37], more specifically, not measuring 25(OH)D concentrations, ineffective vitamin D dosing, high 25(OH)D concentrations at baseline, and/or vitamin D treatment that decreased 25(OH)D concentrations [37].

A factor that can be added to this list of points for attention for future studies is the assessment of depression. In many studies, including the studies reported in this thesis, self-report screening tools were used to identify the presence and severity of depressive symptoms. Validation studies report a satisfactory sensitivity and specificity of these questionnaires, but there are some drawbacks accompanying these tools. Specifically, higher scores indicate higher number of depressive symptoms, but some items relate to problems that may also exist in the absence of depression. For instance, the Centre for Epidemiological Studies Depression Scale (CES-D), used in chapter 5, also contains items on somatic problems (e.g. appetite and sleep) [38, 39]. Hence, non-differential misclassification may have occurred, resulting in an

underestimation of the associations observed. It has been proposed that the identification of depression using a structured clinical diagnostic interview or clinical diagnosis may demonstrate stronger estimates [34]. On the other hand, the GDS-15 used in chapter 4 and 7 of this thesis, does not contain items on somatic concerns and was furthermore specifically designed to distinguish depressive symptoms from problems related to cognitive decline and dementia [39, 40]. Future studies could be refined, for instance by collecting data on depressive symptoms in combination with data of biomarkers for depression [41]. Several promising candidate biomarkers for depression have been suggested, for instance serum brain-derived neurotrophic factor concentrations, hippocampal volume obtained with structural neuroimaging, and glucose metabolism in limbic system as can be measured with a PET-scan [41]. It needs to be emphasized, however, that none of these candidate biomarkers are sufficiently tested and validated to serve as a diagnostic biomarker [41].
Overall it may be concluded that the current evidence is inconclusive, and as Spedding (2014) highlights there is a continuous need for well-designed studies to further elucidate the effect of vitamin D in depression and mood.

10.2.3 Evidence related to brain volume

Neuropsychological assessment has been the most informative method to differentiate between different types of cognitive deficits for ages. However, the field is evolving and due to imaging studies we now know that virtually all the cognitive domains relate to the functioning of one or more brain regions. In **chapter 8** we showed associations between higher 25(OH)D concentrations, lower glucose concentrations and larger grey matter volumes. Although the current literature includes only a few studies that examined associations between 25(OH)D concentrations and structural MRI measures of the brain, these studies appraised a variety of brain structures, ranging from total brain volume to volumes of specific brain areas or structures (reviewed by Annweiler and colleagues [42]). High 25(OH)D concentrations have been linked with less vascular pathologies [43] and smaller ventricle bodies [44], but not with volumetric differences of total brain volume, hippocampus, amygdala and parahippocampal gyrus [43] or temporal horn volume [44]. Plasma 25(OH)D was beneficially associated with white matter volume, and several structures located at the medial temporal lobe (i.e. amygdala, thalamus and anterior cingulate gyrus) among 28 people with memory complaints [15]. In addition, a study among 92 healthy persons, aged 63±9 years, showed a higher atrophy rate of the prefrontal cortex in those with lower 25(OH)D concentrations as compared to those with higher 25(OH)D concentrations [45]. In 264 patients with multiple sclerosis, associations were observed between more abundant sun exposure and more total brain volume and grey matter volume [46]. Interestingly, the association remained after adjustment for 25(OH)D concentrations, which may insinuate that it is not vitamin D but another factor resulting from sunlight exposure that is responsible for the neuroprotective effect.

10.2.4 Neuropsychological evidence versus structural MRI data

In chapter 5, we observed an association between 25(OH)D status and executive functioning. In chapter 6, 25(OH)D status was associated with attention and working memory. Tendencies

towards associations were observed for 25(OH)D status with information processing speed (chapter 5), executive function (chapter 6) and episodic memory (chapter 6).
As vascular dementia is generally accompanied by deficits in executive functioning (i.e. mental flexibility, shifting and response inhibition) and modest recall deficits in episodic memory [47], our findings may suggest the involvement of a vascular pathway. Neuroimaging studies in vascular dementia usually show cerebrovascular pathologies (i.e. infarcts and/or white matter lesions), which may affect both grey and white matter [47, 48]. We observed associations of lower plasma glucose and higher serum 25(OH)D concentrations with more grey matter volume (chapter 8), but no associations between markers of glucose homeostasis and white matter volume were observed (chapter 8). We did not yet quantify the degree of cerebrovascular disease, or volumes of specific brain structures. To get more insight in the underlying pathways additional analyses should therefore focus on measures of white matter hyperintensity volume and grade, large vessel infarcts and small vessel infarcts.
AD is primarily a disease of grey matter, and in the first stage predominantly the memory, language and visuospatial abilities are affected [49]. Therefore, the association of 25(OH)D status with episodic memory and grey matter volume may also imply a pre-clinical stage of AD. To further explore this potential pathway, additional analyses should be conducted on specific regional brain volumetric measures that are involved in memory, like hippocampal volume. In addition, it may be interesting to explore associations between 25(OH)D concentrations and molecular biomarkers like plasma Aβ. Nevertheless, it needs to be stressed that the predictive value of these blood-based biomarkers for AD is still under investigation [50].

10.2.5 Where the shoe pinches: challenges in observational vitamin D research

Methodological issues are a common topic of debate in vitamin D research. One of the main reasons for lack of strong evidence is related to the fact that many studies up to now have been observational, particularly cross-sectional. Here it is where we start talking about the chicken and the egg, and what came first. Are low vitamin D concentrations the cause of the health issues observed, or do the health issues result in altered behaviours like outdoor activities and dietary habits? Insufficient adjustment for known and unknown confounders is another acknowledged issue in observational studies, resulting in residual confounding. Residual confounding may also have occurred in the studies described in this thesis, for instance resulting from the fact that we did not collect extensive data on the presence of chronic diseases. As many important methodological aspects have already been discussed above and in the previous chapters, they will not be discussed here. Some aspects, however, need some more attention and will be discussed below.

Measurement of 25(OH)D concentrations

Tens of vitamin D metabolites have been identified in the circulation [51]. Although vitamin D biomarker research is an on-going process [52, 53], 25(OH)D is currently considered as being the most optimal biomarker of vitamin D availability in the circulation [52]. There is quite some debate on the most optimal method to measure this marker [54]. The

Competitive Protein Binding Assay, as used in chapter 4, that was developed in the 70's is at present labelled as having an unacceptable performance [54]. Nowadays, immunoassays are frequently used, which are considered to perform satisfactory. However, it has also been reported that these assays may have a stronger binding affinity to $25(OH)D_3$ than to $25(OH)D_2$, which may result in misclassification, particularly in populations with a high vitamin D_2 intake [54]. LC-MS/MS (chapter 3, 5, 6, 7, 8, 9) seems to be equipotent to $25(OH)D_3$ and $25(OH)D_2$ and is currently considered to be the most optimal method to determine serum vitamin D status [54]. However, LC-MS/MS has its limitations as well (i.e. cross-reacting with 3-epi-$25(OH)D_3$ [55] and less standardized procedures between laboratories [54]). Thus, determining serum vitamin D status remains challenging and continues to be refined. Differences between 25(OH)D assays hamper the comparison across studies, and may also hamper the interpretation of multicentre studies that determined vitamin D status at various locations and using different assays. A strength of the multicentre studies reported in this thesis is therefore that 25(OH)D concentrations were analysed in one central lab facility (chapter 3, 5, 6, 7, 8) using the same analysis method and protocol.

Seasonal variation

Blood 25(OH)D concentrations are known to fluctuate throughout the year, especially at higher latitudes. Therefore, studies collecting samples throughout the year may be biased by seasonal variation [56]. Most of the data collected for the research presented in this thesis were collected throughout the year (chapter 3, 5, 6, 7, 8). However, we accounted for this seasonal variation by incorporating season of blood sampling in the statistical model, which is a commonly used approach. In case of studying vitamin D in categories (e.g. insufficient, adequate, surplus), one can also account for seasonal variation by creating season-specific or month-specific categories. Wang and colleagues (2009) compared the two aforementioned methods (i.e. adjustment for season by incorporating season in the model versus creating season-specific quartiles) with the true long-term vitamin D concentration. It was shown that using quartiles created without taking into account seasonal variation, but adjusted for season or month of blood collection, occasionally results in a modest bias away from the null. The use of season-specific or month-specific categories, however, did not result in bias away from the null and successfully reduced bias toward the null [56]. However, it was also emphasized that the approach of season-specific categories is less effective when there are large variations in 25(OH)D concentrations. For instance when blood samples are collected throughout the year, as in most of the studies presented in this thesis.

Vitamin D, what else?

As suggested in paragraph 10.2.3, it may also be that the association between 25(OH)D status and brain function is explained by other factors that are tightly correlated with 25(OH)D status, but not captured by the measurements in our studies. Specifically, besides the fact that sunlight activates vitamin D synthesis, there may also be other processes that are activated by sunlight exposure. UV has for instance been shown to exert immunomodulary effects that are assumed to be independent of vitamin D [57]. In addition, UV-A has been shown to increase

nitric oxide concentrations [58, 59], which might be beneficial for neurotransmission as long as concentrations do not become too high. (Sun)light also plays an important role in the regulation of the circadian rhythm, which relates to the production of melatonin and noradrenaline [60]. A disturbed circadian rhythm has furthermore been linked with learning and memory deficits in hamsters [61]. In addition, similarly with diet, it cannot be expected that factors like vitamin D come in isolation. For instance, fish is a dietary source that is naturally high in vitamin D, but fish also contains zinc, selenium and omega-3 fatty acids that may be beneficial for brain function [62-64]. Unravelling whether the observed associations are actually caused by vitamin D or by one or more closely associated factors will be extremely difficult.

Power

Recently, several meta-analyses have been published. Although these analyses included relatively few studies, they are important. As the effect of vitamin D, if present, on cognitive performance/decline and depression will probably be subtle, combining the results of multiple studies may provide datasets that are more powerful to detect potential associations. Nevertheless, future meta-analyses can be improved. Many published meta-analyses, including those summarized previously, are based on data that are available from published reports, or summary data provided by the researchers of the individual studies. Consequently, data are often heterogeneous (e.g. in population, sample size, confounders taken into account, assessment method of the outcome). Therefore, collecting and combining the individual patient data from different studies may result in more detailed analyses and consequently a more balanced interpretation of the outcomes [65]. For instance, analyses could be stratified for sex, and if data are available, also for markers of glucose homeostasis.

Experimental proof

Many of the aforementioned methodological aspects can be captured in RCTs. However, RCTs in vitamin D and mental health research are challenging. Preferably, one would like to test the effects in persons that are vitamin D deficient. However, given the recognized role of vitamin D in bone health, not supplementing participants with a known vitamin D deficiency (i.e. control group) may be considered unethical. Another question relates to what dose of vitamin D can be expected to be effective. Additionally, what should be the minimal trial duration and during which months should the trial be conducted? To exclude sunlight effects, a trial should preferably be conducted during the winter. However, the period to induce a supplementation effect is probably longer than one winter season. We should furthermore thoroughly think about the method to assess cognitive decline and depression. Are the currently used methods sensitive enough to capture the effect of a single nutrient? Are there other, more sensitive, outcome measures available? To further study the potential vitamin D effect, independent of the commonly experienced limitations in human studies, we therefore conducted a proof of principle study in aging C57BL/6 mice (chapter 9). In the next paragraph the validity of this animal model is discussed.

10.3 Mice as a model for cognitive decline and emotional reactivity: how good are they?

In **chapter 9** the effect of a long-term vitamin D deficiency in male C57BL/6 mice was studied. Mice were fed a vitamin D adequate or a vitamin D deficient diet for one-year, starting at the age of 10 months (±38 years in humans) [66]. At 22/23 months of age (±64/69 years in humans) [66] no difference in emotional reactivity, recognition memory, spatial learning and reference memory was observed between the vitamin D adequate and vitamin D deficient group. Whether the lack of effect in our study has to be contributed to the nonexistence of a causal relationship between vitamin D and brain health, and/or methodological issues, can be debated. When discussing this study, there is one question that always comes up: "Using animal species as a model for human aging, how valid is that?" Undeniably, the best model to study human disease is of course the human itself. Due to ethical as well as practical issues, however, the human is not always included in the list with optional models. In view of this knowledge and as 96% of the genes of men and mice show major resemblances [67], using a mouse model may actually be a good solution to get a better understanding of human disease processes. Obviously, physiological differences between mice and men do exist and as such it is not all moonlight and roses. Van der Staay (2006) wrote: "It is the task of the scientist to identify shortcomings of animal models, to refine them, and to develop tools that allow a better understanding of the human condition. (…) It is only by identifying the weaknesses and errors of models that improvements can be made." [68] Therefore, this paragraph will provide a critical evaluation of the mouse model used in chapter 9, guided by the model evaluation approach as proposed by Van der Staay, Arndt and Nordquist [69].

10.3.1 Reproducibility

Reproducibility refers to the comparability of a model for the disease being modelled, obtained from independent experiments that are similar in methodology and biology. Multiple experiments indicate that C57BL/6 mice have a relatively long life-span (e.g. mean age of death of males: 26.6 months [70]) and a reasonably low tumour incidence up to the third life year [70-72], which suggests that C57BL/6 may be a suitable strain for aging research. In addition, several studies have shown age-related impairments in spatial learning as measured in the Morris Water Maze (MWM) [73-76] and decreases in explorative activity as measured by Open Field Test (OFT) and Elevated Plus Maze (EPM) performance [77, 78]. Studies on the effect of aging on recognition memory, as assessed with the ORT, are less definite [73, 78] and it has been suggested that effects may only be detectable in the oldest-old [78]. Thus, already several experiments showed that age-related cognitive impairment and emotional reactivity can be observed in aging C57BL/6 mice.

10.3.2 Internal validity

With respect to the internal validity there are two important confounding factors of behavioural assessment that need to be considered, specifically motor performance and vision. With respect to motor performance it can be reported that in vivo and ex vivo muscle

measurements in this study did not point towards significant differences between treated and non-treated animals, and therefore it is considered unlikely that differences in motor performance affected the internal validity. Another factor that may have confounded the behavioural assessment is vision [79]. Although vision was not quantified in our study, no differences in the behavioural outcomes were observed between treatment groups. Hence, we do not assume that vision confounded the results of our study. Nevertheless, for future studies the assessment of vision is recommended.

10.3.3 Face validity

The next question that should be raised is how well the model used in chapter 9 mimics the behavioural phenotype observed in cognitive decline and depression in humans. Comparing behavioural features of depression and cognitive decline in humans with behavioural dysfunction in mice is challenging, and for some aspects even impossible (e.g. mood, feelings of worthlessness, concentration problems). Nevertheless, several characteristics of cognitive decline and depression in humans have been observed, including age-related working memory deficits [76, 80], impairments in spatial memory/executive functioning [73, 74, 76, 81, 82] and increases in anxiety/depressive symptoms [81, 83]. Thus, face validity has been shown for some of the characteristics of cognitive impairment and depression, but not for all. However, as argued by others, given the condition being modelled, requiring complete face validity is unrealistic [69, 84].

10.3.4 Predictive validity

With regard to the predictive validity of an animal model Van der Staay, Arndt and Nordquist (2009) refer to: " (…) the extrapolation of the effect of a particular experimental manipulation from one species to other species (…)" and "(...) the ability of a drug screening or an animal model to correctly identify the efficacy of a putative therapeutic" [69]. As already addressed in chapter 1, effective treatment for cognitive decline/dementia is lacking. To date, pharmacological treatment in cognitive decline/dementia is symptomatic and not curative. Nevertheless, several preclinical studies have shown that the commonly prescribed acetylcholinesterase inhibitors [85-87] and NMDA-antagonist memantine [88] in AD patients also improve cognitive performance in C57BL/6 mice. Depression is commonly treated with antidepressants, which act to increase the availability of serotonin and noradrenalin in the brain. Several studies have shown that C57BL/6 mice are also responsive to antidepressants as serotonin reuptake inhibitors and selective-noradrenaline reuptake inhibitors [89-91]).

10.3.5 Construct and aetiological validity

The next aspect of attention in the evaluation of the validity of the animal model is the construct and aetiological validity. In view of this criterion two aspects are highlighted: 1) the similarity in the potential role of vitamin D in brain functioning in rodents and humans, and 2) the similarity in mechanisms of age-related cognitive decline and depression as observed in rodents and humans.

With respect to the first aspect, although evidence is sparse, it seems that there are several important similarities in vitamin D metabolism/action in rodents and humans. Specifically, resemblances have been shown in VDR distribution in the brain, circulating concentrations of vitamin D binding protein and apoptotic properties of calcitriol on brain glial cells. Additionally, alike humans, rodents have the ability to synthesize vitamin D under the influence of ultraviolet radiation (reviewed in [92, 93]).
Several parallels in the aetiology of cognitive decline and depression of normal aging mice and humans have been suggested as well, including impaired connectivity [76, 94-96], morphological changes [49, 95] and homology in regions involved in stress, fear and depression (e.g. amygdala, hypothalamus, hippocampus) [97, 98]. However, it is also known that the normal ageing C57BL/6 mouse, as used in chapter 9, does not spontaneously develop plaques and tangles [95]. Hence, the proposed effect of vitamin D to prevent or slow-down plaque and tangle formation [13, 14] is not reflected in our model. Several transgenic mouse models for studying Aβ – and/or tau pathologies have been developed [95], which could be used to address this specific hypothesis in future studies. A comparative study of three inbred mice strains, furthermore, indicates that BALB/c and C3H:He mice tend to be more susceptible to chronic stress than C57BL/6 mice. Hence, these mouse strains may be more suitable to study chronic stress-related pathology like depression than the C57BL/6 strain [99].
Although many researchers consider construct validity to be the most important criterion to evaluate validity, it needs to be emphasized that animal models usually selectively model only a subset of the disease symptomatology. It is probably impossible to create a (rodent) model that captures all the known features of a specific pathological process. In addition, the assessment of construct validity of the aging vitamin D deficient C57BL/6 mouse model is restricted by the fact there are still many unidentified aetiological factors. Thus, although the current evidence suggests important similarities, there is insufficient evidence to determine the construct validity of the aging vitamin D deficient C57BL/6 mouse model.

10.3.6 External validity

External validity refers to whether the experimental results can be generalized across populations, and species. Only a few other studies investigated the impact of a vitamin D deficiency developed later in life on behaviour in rodents. In a recent study on adult vitamin D deficiency in 16 to 20-week-old Sprague-Dawley rats - receiving a vitamin D deficient diet from 10 weeks of age onwards - it was concluded that animals did not exhibit major impairments in behaviour, but subtle vitamin D effects seemed to be present as observed in tasks of attentional processing [100]. Another study that investigated the effect of a vitamin D deficient diet for 10 weeks showed behavioural and neurochemical changes in 20-week old vitamin D deficient BALB/c mice, but no convincing patterns in 20-week old C57BL/6 mice [101]. Several human studies also examined the effect vitamin D on cognitive processes and depression, but - as already summarized earlier in this chapter - the results are inconclusive.
Overall we conclude that the current evidence-base is insufficient to judge the validity of the model used in chapter 9. However, as no notable effects of a long-term vitamin D deficiency

on various measures of emotional behaviour and cognition were observed in aged male C57BL/6 mice, this study does suggest that there is no causal relationship between adult vitamin D deficiency and cognitive decline and depression in C57BL/6 mice.

10.4 Vitamin D and the brain: A role for glucose homeostasis?

As mentioned in paragraph 10.2.1, vascular risk factors may play a role in the link between vitamin D and brain function, including glucose intolerance and diabetes [19, 102]. Pre-clinical studies and epidemiological studies have associated hyperglycaemia and hyperinsulinaemia with macrovascular and microvascular brain changes, increased Aβ secretion, decreased Aβ degradation, insufficient energy supply of neurons and significant alterations in glucose metabolism in the brain [19, 102].

10.4.1 Is there effect modification by the degree of glucose tolerance?

We postulated that vitamin D deficiency makes the brain more vulnerable to other stressors [29, 100], like glucose intolerance and diabetes. Byrne and colleagues illustrated this hypothesis by referring to a study on vitamin D and post-stroke effects. Mice that had a low vitamin D concentration pre-stroke appeared to have a slower and worse recovery after being 'hit' by an experimentally induced stroke than animals that had a vitamin D adequate status [100]. However, this 'second hit' hypothesis was not supported by the research presented in this thesis. Namely, stratification by low and high fasting plasma glucose did not point towards substantial differences in cognitive function in Dutch pre-frail and frail older adults (chapter 5) [22], or in a population of Dutch older adults with mildly elevated homocysteine concentrations (chapter 6). Also, no interaction with glucose homeostasis could be established in the association between 25(OH)D concentration and the number of depressive symptoms (chapter 8). It needs to be emphasized, however, that the population size in chapter 5 was relatively small to conduct stratified analyses. Moreover, data on glucose tolerance used in chapter 6 were not obtained completely fasting, as participants were allowed to consume a restricted breakfast. In addition, in chapter 9 no interaction was observed between a long-term adult vitamin D deficiency and a pre-diabetic metabolic state in relation to cognitive decline and emotional reactivity in aged C57BL/6 mice. Data in chapter 9, however, are limited by the fact that we only induced a modest glucose intolerance, which may not have been sufficient to adversely affect brain function in such a way that it is measureable with a behavioural test battery. To the best of our knowledge, only one small cross-sectional study can be added to our findings. In this study higher 25(OH)D concentrations were significantly associated with lower cognitive function in 165 type 2 diabetes patients (<71 years), independent of age, sex and years of education [103]. However, as this study did not include non-diabetics, the interpretation with respect to a potential interaction between vitamin D and type 2 diabetes is limited.

10.4.2 Is there mediation by the degree of glucose tolerance?

Secondly, as vitamin D has been proposed to beneficially influence glucose intolerance, and

glucose intolerance and diabetes are considered to be a potential risk factor for cognitive decline/dementia and depression, we also hypothesized that an impaired glucose tolerance serves as an intermediate in the pathway between vitamin D and brain health. The research in this thesis, however, did not provide evidence to support this second hypothesis on the interplay between vitamin D and glucose homeostasis. This potential mediation effect of glucose homeostasis in the association between vitamin D and mental health has been explored in three other observational studies. Data of the European Male Aging Study [104] and the InCHIANTI Study [105] did also not support a potential mediation effect of glucose in the association between 25(OH)D concentrations and cognitive performance. In the NHANES study there seemed to be partial mediation by the presence of diabetes mellitus, as incorporation of diabetes in the model slightly attenuated the association [106].
Thus, evidence for an interaction or mediation effect by glucose homeostasis in the association between vitamin D and mental health can be considered sparse and limited due to several methodological constraints. Given the biological plausibility of an interplay between vitamin D and glucose intolerance, concomitantly with the limited human studies up to now, it can be concluded that further studies on this aspect are needed.

10.5 Vitamin D: hype or high potential?

In the first paragraph of this discussion an overview is given of the recommendations that emerged from an expert meeting that took place in 2011 in Ede [1]. Meanwhile, the scientific evidence substantially increased and several reviews and meta-analyses on non-skeletal health outcomes have been published [107]. The previous paragraphs have shown that several studies point towards a beneficial effect of vitamin D in relation to brain health. Whether the current evidence-base is sufficient to conclude that vitamin D is causally related to cognitive decline and depression can be evaluated using nine criteria for causality as proposed by Bradford Hill (1965) [108]. Below these criteria will be used to summarize the conclusions that can be drawn from the evidence resulting from this thesis, as well as the emerging evidence provided by colleagues in the field.

10.5.1 Bradford-Hill's criteria for causality

Strength of the association
The cross-sectional association between 25(OH)D and global cognitive performance has been widely examined. A recent meta-analysis suggests a potential beneficial role for vitamin D in global cognitive performance, as assessed with a brief screening tool (MMSE) (paragraph 10.2.2). In addition, despite the limited number of studies, observational evidence on vitamin D and domain-specific cognitive performance is promising, especially for tasks related to executive function (paragraph 10.2.2). Also several studies, but not all, have shown cross-sectional associations between 25(OH)D and depression in older adults (paragraph 10.2.2). Large heterogeneity in methodologies, however, limit the comparison of the magnitude of the associations observed across these studies.

Consistency of the evidence
Whereas several observational studies point towards associations between vitamin D and cognitive performance, and depressive symptoms, others do not. Thus, observational data are inconclusive, and therefore the current evidence-base does not reach a sufficient level of consistency yet.

Specificity
The etiology of cognitive decline/dementia and depression is undoubtedly complex, and it is unlikely that these conditions are the result of one single factor. Most observational studies accounted for a substantial number of confounding factors. However, the occurrence of residual confounding cannot be excluded.

Temporality
Reverse causation is a commonly raised concern in the field of vitamin D research. Besides the possibility that vitamin D deficiency precedes the onset of functional disabilities, it may well be that functional limitations precede the development of vitamin D deficiency. To date, prospective studies and RCTs examining the link between 25(OH)D concentrations/vitamin D and cognitive performance and depression are sparse. Therefore, the possibility that the observed associations are the result of an artefact resulting from reverse causation rather than a true biological link cannot be rejected yet.

Biological gradient
Most observational studies that observed an association between 25(OH)D status and mental health do suggest a dose-response gradient, where higher 25(OH)D concentrations associated with a better cognitive performance, less depressive symptoms and larger brain volumes.

Biological plausibility
Up to now, numerous studies provided biological evidence linking vitamin D with brain function, and new evidence continues to be added (paragraph 10.2.1). Hence, these studies do suggest a role for vitamin D in brain functioning.

Experimental evidence
Trials examining the effect of vitamin D on cognitive performance (paragraph 10.2.2), and depression (paragraph 10.2.2) in populations of older adults are sparse and the 'biological quality' of these trials has been questioned. Hence, much more evidence is warranted before we can judge on the causality of the association between vitamin D and cognition.

Coherence
As shown in this thesis, several approaches can be used to study brain health, like pre-clinical research (paragraph 10.2.1), neuropsychological research (paragraph 10.2.2) and imaging research (paragraph 10.2.3). Mechanistic studies support a role of vitamin D in brain processes.

Whereas several observational studies on 25(OH)D concentrations that studied either cognitive function, depression or brain volumetric measures show promising associations, others do not support that evidence. Only a few animal and human trials examined the effect of low vitamin D concentrations on cognitive function; results are inconclusive.

Analogous evidence

As already discussed previously (paragraph 10.2.5), it is likely that 25(OH)D status is correlated with other factors that result from exposure to sunlight, and these correlated factors may be beneficial for brain functioning independent of the vitamin D synthesis in the skin. As vitamin D obtained from dietary sources does also not come in isolation, for vitamin D intake the same idea applies. Studies aiming to untangle the effect of vitamin D from other potentially correlated factors are therefore warranted.

10.5.2 Conclusions

Thus, it can be concluded that the evidence-base to recommend vitamin D supplementation in order to enhance cognitive performance, slow down cognitive decline and brain atrophy, or prevent/counteract depression is currently insufficient to justify the revision of the vitamin D recommendations. However, given the established role of vitamin D in bone health and the high prevalence of vitamin D deficiency, we do call for dietary strategies to increase vitamin D intake, particularly during the winter months. Although there are probably a limited number of options to counteract vitamin D deficiency in the general population, several dietary strategies have been put forward, that is promoting the intake of food products that are naturally high in vitamin D, increasing the number of foods fortified with vitamin D, and encouraging supplemental vitamin D intake [109].

10.6 Future perspectives

Thus, founded on the current evidence-base it can be concluded that there is a continuous need for well-designed prospective observational studies and RCTs. We all know that this is easier said than done. Throughout this discussion several suggestions for refinement of studies have been given; they are summarized in the box on the next page. In short, the prevalence of vitamin D deficiency is high, which is concerning given the known effects of vitamin D on bone health. It should be examined how the effectiveness of strategies aiming to ensure an adequate vitamin D status can be improved. In addition, in the design of future studies, we must pay more attention to the biology of the associations studied. For instance, trials in populations that have an adequate vitamin D status, or do not show any variation in outcome measures studied, are unlikely to provide conclusive evidence. Furthermore, prospective studies and trials are needed to replicate previous data and subsequently, individual patient data meta-analyses should provide us with the cherry on the pie. We also need to continue research on potential underlying mechanisms. This could for instance be via including parameters that are related to vascular brain health and by including biomarkers of Alzheimer pathology, and depression. To adequately address vitamin D related mechanisms that

cannot be studied in humans, (aging) models of vitamin D deficiency should be developed, systematically evaluated, and, if considered valuable, further refined.

Suggestions for future research

- The high prevalence of vitamin D deficiency calls for research on effective approaches to ensure an optimal vitamin D status.
- More evidence is warranted to establish whether the current vitamin D recommendations cover the optimal concentrations needed for brain health. Thus, there is a continuous need for prospective and well-designed randomized controlled trials.
- To provide more conclusive data, results of individual studies should be combined in individual patient data meta-analyses.
- Dose-response analyses (observational) and designs (trials) are warranted to establish the optimal 25(OH)D concentrations for brain health (if causality exists).
- In the design of trials one should thoroughly consider the target population. For instance: What should be the maximum 25(OH)D concentration at the time of inclusion? Is the expected variation in the level of cognitive performance and/or the prevalence of depressive symptoms in the population sufficient to expect/measure an effect?
- Although diagnostic biomarkers of cognitive impairment and depression are lacking, the use of biomarkers may be helpful to corroborate neuropsychological findings and to generate hypotheses on the underlying pathways.
- It might also be interesting to further explore the vascular hypothesis in the association between vitamin D and brain health, for instance by including measures of white matter hyperintensities and infarcts.
- To further explore the potential role of vitamin D in AD pathology, it may be valuable to include measures on for instance hippocampal volume and CSF/blood-based biomarkers like Aβ.
- Examining associations between common single-nucleotide polymorphisms (SNPs) related with 25(OH)D concentrations and disease outcomes highlights another potential interesting field of study (chapter 3 and chapter 7).
- To further study the underlying vitamin D pathways in brain function, currently used animal models should be critically evaluated for their relevance. For example, for the C57BL/6 mouse the question whether there is sufficient comparability in the vitamin D mediated mechanisms when compared to 'the human being' is still largely unanswered.
- Finally, as vitamin D status may be closely linked with other factors resulting from sunlight or diet it may be valuable to think about study techniques to disentangle these factors.

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Summary

According to recent estimations approximately 35.6 million people have dementia worldwide. Globally, 350 million people experience one or more depressive episodes during their life. As the therapeutic options for dementia and depression are limited, these conditions form a major challenge for public health and society. More and more researchers have initiated research on potential preventive factors for dementia and depression, including the potential effects of nutritional factors. The aim of this PhD-project was to study the role of vitamin D and its potential interplay with glucose homeostasis, in the development of cognitive decline and depression, using epidemiological data as well experimental animal data.

Chapter 2 recapitulates a debate between vitamin D experts that was organized to make a step towards the harmonization on the formulation of optimal vitamin D intake levels and serum 25(OH)D concentrations across Europe. It was concluded that based on the current evidence-base 25(OH)D concentrations ≥50 nmol/L are sufficient with respect to optimal bone health. For health outcomes beyond bone health evidence was considered insufficient to formulate optimal levels. In order to achieve and maintain a 25(OH)D concentration ≥50 nmol/L, older adults aged ≥65 years were recommended to adhere to a vitamin D intake of 20 μg/day.

Chapter 3 shows that there is a high prevalence of 25(OH)D inadequacy in a population of Dutch older adults that participated in the B-PROOF study (n=2857), namely 45% had 25(OH)D concentrations <50 nmol/L. Mean vitamin D intake was 4.9±2.9 μg/day and only 20% of the participants reported to use vitamin D containing supplements. Exploration of the determinants of 25(OH)D status showed significant associations between vitamin D 'raising' SNPs (n=2530), higher sun exposure (n=1012), vitamin D intake (n=596) and higher 25(OH)D concentrations. Including all the potential relevant and measured predictors in one model explained 35% of the variance in 25(OH)D status (R^2=0.35).

In **chapter 4** the associations between 25(OH)D status and global cognitive performance (n=116), depressive symptoms (n=118), and surrogate markers of glucose intolerance (n=593) were evaluated using data of European adults aged 70-75 years. None of the associations reached significance.

Studying the potential role of vitamin D in domain-specific cognitive performance and depression in 127 Dutch pre-frail and frail older adults aged ≥65 years (**chapter 5**), showed an association between 25(OH)D concentration and executive functioning, and a tendency towards an association with information processing speed. Stratification for 'low' and 'high' fasting glucose concentrations did not suggest an interaction between vitamin D and glucose homeostasis in the association with domain-specific cognitive performance. Moreover, adding fasting glucose or insulin did not substantially influence the associations between 25(OH)D status and domain-specific cognitive performance, and hence a mediation effect of glucose homeostasis was considered unlikely.

We furthermore observed associations of 25(OH)D status with attention and working memory (n=787) (**chapter 6**), depression (n=2839) (**chapter 7**) and grey matter volume of the brain (n=217) (**chapter 8**) in a population community-dwelling Dutch older adults aged ≥65 years. Again, these studies did not provide evidence that the associations were modified or mediated by glucose intolerance. However, it should be emphasized that glucose intolerance in these three chapters was defined sub-optimally, specifically using blood samples that may have been collected in a non-fasting state, or by using self-reported diabetes data. Hence, the mediation and interaction effects should be interpreted cautiously.

Finally, **chapter 9** shows the results of a proof of principle study on the effect of a long-term vitamin D deficiency on cognitive decline and emotional reactivity in old C57BL/6j mice. Modest tendencies were shown for a relation between vitamin D and spatial learning, but these tendencies did not reach significance. Vitamin D deficiency did not affect recognition memory, spatial memory or emotional reactivity. Mice that received a higher dietary fat load, which was given to induce an impaired glucose tolerance, did not respond differently to a vitamin D deficiency than mice that received a low fat diet did.

Overall, it is concluded that the evidence for an effect of vitamin D on cognitive performance/decline, depression or brain volume is insufficient to formulate disease specific cut-off values for vitamin D intake or 25(OH)D status. However, given the high prevalence of 25(OH)D concentrations <50 nmol/L we do call for a more active promotion of the current vitamin D intake recommendations.

Samenvatting

Volgens recente schattingen zijn er wereldwijd ruim 35 miljoen mensen met dementie. Daarbij maken circa 350 miljoen mensen minimaal eens in hun leven een depressieve periode door. De behandeling van dementie richt zich momenteel met name op symptoombestrijding; een curatieve behandeling is helaas nog niet voor handen. Depressies kunnen effectiever behandeld worden, maar deze behandeling gaat regelmatig gepaard met vervelende bijwerkingen en/of treedt er op termijn toch weer recidief op. Onderzoek naar factoren die een bijdrage kunnen leveren aan de preventie van deze aandoeningen is daarom belangrijk. Het doel van dit promotieproject was om te onderzoeken of vitamine D - ook wel bekend als de zonlichtvitamine - van belang kan zijn voor de preventie van dementie en depressie. Ten tweede wilden we weten of glucose homeostase een rol speelt in deze veronderstelde associaties. Om meer inzicht te krijgen in deze processen hebben we in het kader van dit promotietraject zowel epidemiologisch als dierexperimenteel onderzoek uitgevoerd.

Hoofdstuk 2 geeft een overzicht van de resultaten van een bijeenkomst van vitamine D experts. Het doel van deze bijeenkomst was om inzicht te krijgen in de huidige wetenschappelijke stand van zaken omtrent de rol van vitamine D in relatie tot gezondheid en ziekte, en om te bediscussiëren of het op basis van deze informatie mogelijk is om een gezamenlijke Europese richtlijn op te stellen aangaande de optimale vitamine D inname en vitamine D waarde in het bloed. De experts stelden dat er voldoende wetenschappelijk bewijs is om te concluderen dat een serum/plasma vitamine D niveau van ≥50 nmol/L van belang is voor de botgezondheid. Voor andere gezondheidsuitkomsten, zoals bijvoorbeeld spierfunctie, cognitieve functie en diabetes, is er onvoldoende wetenschappelijk bewijs om een afkapwaarde te definiëren. Om een serum/plasma vitamine D niveau van ≥50 nmol/L te bereiken en te behouden luidt het advies voor ouderen met een leeftijd van ≥65 jaar om te streven naar een vitamine D inname van 20 µg/dag.

In **hoofdstuk 3** zien we dat 45% van een Nederlandse ouderen populatie (n=2857) een serum vitamine D niveau heeft dat onder de 50 nmol/L grens ligt. De gemiddelde vitamine D inname in deze populatie was 4.9±2.9 µg/dag; slechts 20% van de deelnemers rapporteerde gebruik te maken van supplementen die vitamine D bevatten. Deze onderzoeksgegevens toonden verder aan dat het serum vitamine D niveau significant hoger is in deelnemers met bepaalde vitamine D gerelateerde single nucleotide polymorphisms (SNPs) (n=2530), een hogere mate van zonlichtblootstelling (n=1012) en een hogere vitamine D inname (n=596). Met behulp van regressie analyse met alle mogelijk relevante en gemeten determinanten van vitamine D status bleken we 35% van de variatie in vitamine D status te kunnen voorspellen (R^2=0.35).

In **hoofdstuk 4** zijn de mogelijke associaties tussen vitamine D status en cognitief functioneren (n=116), depressieve symptomen (n=118) en markers voor glucose tolerantie (n=593) onder de loep genomen. Voor deze analyses is gebruikgemaakt van data afkomstig van een Europees ouderen cohort (leeftijdsrange: 70-75 jaar) genaamd de SENECA studie. Geen van de associaties in deze studie behaalde het statistische significantieniveau.

Bij het bestuderen van de associatie tussen vitamine D en domein specifieke cognitieve functie in een groep van 127 kwetsbare Nederlandse mannen en vrouwen ≥65 jaar (**hoofdstuk 5**), bleek serum vitamine D wel significant geassocieerd te zijn met het cognitieve functioneren, namelijk het executief functioneren. Een bijna significante associatie werd geobserveerd tussen serum vitamine D en informatieverwerkingssnelheid. Stratificatie duidde niet op belangrijke verschillen in de associaties tussen serum vitamine D en cognitief functioneren bij mensen met 'lage' of 'hoge' nuchtere glucose waarden, hetgeen suggereert dat verschillen in glucose homeostase geen of weinig invloed hebben op de bestudeerde associaties in deze populatie. Ook wanneer nuchter glucose en nuchter insuline als co-variabele werden toegevoegd aan het statistische model had dit geen of slechts een geringe invloed op de uitkomsten van dit onderzoek, hetgeen verder suggereert dat deze variabelen niet fungeren als belangrijke intermediairs in de bestudeerde associaties.

Vitamine D status bleek ook significant geassocieerd te zijn met het cognitieve domein aandacht en werkgeheugen (n=787) (**hoofdstuk 6**), depressie (n=2839) (**hoofdstuk 7**) en het grijze stof volume van de hersenen (n=217) (**hoofdstuk 8**) in een groep Nederlandse ouderen in de leeftijdscategorie van 65 jaar en ouder. Ook in deze studie was er geen bewijs dat de associaties substantieel beïnvloed werden door verschillen in glucose homeostase. Het moet echter wel genoemd worden dat glucose homeostase in deze onderzoeken niet op een optimale wijze gedefinieerd is; ofwel is er gebruik gemaakt van zelfgerapporteerde data, ofwel is er gebruik gemaakt van bloedmonsters die mogelijk zijn afgenomen terwijl mensen een licht ontbijt geconsumeerd hadden. Het mag daarom duidelijk zijn dat we deze gegevens met betrekking tot de rol van glucose homeostase met enige voorzichtigheid dienen te interpreteren.

Tot slot, in de studie gepresenteerd in **hoofdstuk 9** is onderzocht of een algehele en langdurige vitamine D deficiëntie invloed heeft op de cognitieve achteruitgang en emotionaliteit van oude C57BL/6j muizen. De conclusie van dit onderzoek is dat vitamine D geen tot weinig invloed lijkt te hebben op cognitieve achteruitgang en emotionaliteit in deze dieren. Mogelijkerwijs heeft vitamine D enigszins een effect op het ruimtelijke leervermogen, maar de geobserveerde trends waren niet statistisch significant. Muizen gevoed met een gematigd vet dieet (20 En% vet), met als doel het induceren van een glucose intolerantie, reageerden cognitief en emotioneel niet anders op het vitamine D deficiënte dieet dan de met laag vet (9 En% vet) gevoede muizen.

Samenvattend kan geconcludeerd worden dat er momenteel onvoldoende bewijs is om te kunnen stellen dat vitamine D van belang is voor de gezondheid van ons brein. Het is daarom nog te vroeg om ziekte specifieke afkapwaarden te formuleren voor vitamine D in relatie tot cognitieve functie en depressie. Echter, gezien de hoge prevalentie van serum vitamine D waarden <50 nmol/L en de bewezen rol van vitamine D in relatie tot de botgezondheid, adviseren we wel om meer aandacht te besteden aan de promotie van de huidige vitamine D aanbevelingen.

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About the author

Curriculum Vitae

Elske was born on the 3th of April 1985 in Harlingen, the Netherlands. After completing high school at the Simon Vestdijk Franeker/Harlingen, she moved to Groningen to study Nutrition and Dietetics at the Hanze University for Applied Sciences, which she completed in July 2007. During this four year *BSc* program Elske was amongst others involved in a study on the effectiveness of weight loss programs. Next to that she worked as a dietician at the homecare organisation "Zuid-West Friesland" (The Netherlands) and the Academic Medical Center Paramaribo (Surinam). From 2007-2009 Elske participated in the *MSc* program Epidemiology and Public Health at Wageningen University. During her *MSc* she was involved in a project within the Rotterdam Study on physical activity and coronary artery calcification, and she completed the program with a 4-month internship at the Julius Center Utrecht where she studied the association of fish consumption and omega-3 fatty acids with cardiovascular and cerebrovascular health. Thereafter, Elske worked 6 months for "Stichting Grasland Wetenschappen" on a project that aimed to examine the role of milk- and dairy consumption in relation to a broad variety of health outcomes. End 2009 she applied for a PhD position at Wageningen University, the Netherlands, of which the results are presented in this thesis. Currently, Elske is working as a nutritional researcher at the Division of Human Nutrition of Wageningen University on a tender project for the European Food Safety Authority with the aim to collect and analyse published scientific information as preparatory work for the setting of dietary reference values for vitamin D.

List of publications

- **Brouwer-Brolsma EM**, Vaes A, van Wijngaarden JP, van der Zwaluw NL, Sohl E, van Dijk SC, van Schoor NM, van der Velde N, Uitterlinden AG, Lips P, Feskens EJM, Dhonukshe-Rutten RAM, de Groot LC. Relative importance of summer sun exposure, vitamin D intake and genes to vitamin D status in Dutch older adults: the B-PROOF study.
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- **Brouwer-Brolsma EM**, Dhonukshe-Rutten RAM, van Wijngaarden JP, van der Zwaluw NL, in 't Veld PH, Wins S, Swart KMA, Enneman AW, Ham AC, van Dijk SC, van Schoor NM, van der Velde N, Uitterlinden AG, Lips P, Kessels RPC, Steegenga WT, Feskens EJM, de Groot LC. Cognitive performance: is there an interplay between serum vitamin D and glucose homeostasis?
- **Brouwer-Brolsma EM**, Dhonukshe-Rutten RAM, van Wijngaarden JP, van der Zwaluw NL, in 't Veld PH, Sohl E, van Dijk SC, Swart KMA, Enneman AW, Ham AC, van Schoor NM, van der Velde N, Uitterlinden AG, Lips P, Feskens EJM, de Groot LC. Vitamin D-epressed: Low vitamin D status is associated with more depressive symptoms in Dutch older adults.
- **Brouwer-Brolsma EM**, van de Rest O, de Groot LC. Vitamin D and the association with cognitive performance, cognitive decline, and dementia. Book chapter in Diet and Nutrition in Dementia and Cognitive Decline, 1st Edition, 2014. In press.
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Overview of completed educational activities

Discipline specific activities

- Conference: Nutrition for the Ageing Brain (Milan, Italy, 2014)
- Conference: Vitamin D and Human Health (London, United Kingdom, 2014)
- Conference: AgeingResearch@NL (Rotterdam, The Netherlands, 2013)
- Conference: Gezonde voeding, gezond ouder worden (Wageningen, The Netherlands, 2013)
- Course: Synapses, Neurons and Brains, www.coursera.org, 2013
- International Conference on Nutrition and the Brain (Washington, United States, 2013)
- Alzheimer's Association International Conference (Boston, United States, 2013)
- Workshop: Introduction Canoco (Wageningen, The Netherlands, 2013)
- 11th Dutch Endo-Neuro-Psycho Meeting (Lunteren, The Netherlands, 2013)
- International Conference on Aging & Cognition (Dortmund, Germany, 2013)
- Masterclass: Longitudinal data analysis (Wageningen, The Netherlands, 2013)
- Masterclass: Analysis in R (Wageningen, The Netherlands, 2012)
- Conference: Annual NWO nutrition days (Deurne, The Netherlands, 2011)
- Vitamin D expert meeting (Ede, The Netherlands, 2011)
- 4th International Congress on Prediabetes and the Metabolic Syndrome (Madrid, Spain, 2011)
- Course: Epigenesis & Epigenetics (Wageningen, The Netherlands, 2011)
- Course: Nutrition and Lifestyle Epidemiology (Wageningen, The Netherlands, 2010)
- Masterclass: Linear and logistic regression (Wageningen, The Netherlands, 2010)
- Course: Laboratory animal science, article 9 (Wageningen, The Netherlands, 2010)

General activities

- Course: Career perspectives (Wageningen, The Netherlands, 2013)
- Course: Scientific Writing (Wageningen, The Netherlands, 2013)
- Course: Wet- en regelgeving van klinisch onderzoek (Ede, The Netherlands, 2011)
- Course: Supervising MSc-students (Wageningen, The Netherlands, 2011)
- Workshop: How to write a world-class paper (Wageningen, The Netherlands, 2011)
- Workshop Mindmapping (Wageningen, The Netherlands, 2011)
- NWO talent day: onderhandelen en netwerken (Utrecht, The Netherlands, 2010)
- VLAG PhD-week (Venlo, The Netherlands, 2010)

Optionals

- Preparation research proposals
- Literature study program Oldsmobiles/Paperclip/EPIC, 2010-2014, Wageningen
- PhD study tour, Mexico / United States, 2011

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