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MGAT3 mRNA: A Biomarker for Prognosis and Therapy of Alzheimer's Disease by Vitamin D and Curcuminoids

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Abstract. Practical biomarkers of Alzheimer's disease (AD) prognosis are lacking. Correspondingly, no drugs are known to decrease disease progression, although vitamin D3 has positive effects on cognition *in vivo* and 1α, 25-dihydroxyvitamin D3 (1,25 D3) on amyloid-β 1-42 (Aβ) phagocytosis *in vitro*. We have examined in a pilot study a new biomarker in peripheral blood mononuclear cells, the transcription of mRNA of β-1,4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase (*MGAT3*), the essential gene for Aβ phagocytosis. The transcription of *MGAT3* stimulated by Aβ distinguishes macrophages into Type 0 (very low *MGAT3* transcription), Type I (low *MGAT3* transcription up regulated by bisdemethoxycurcumin), and Type II (high *MGAT3* transcription down regulated by bisdemethoxycurcumin). In this pilot study of 20 AD patients and 20 control subjects, 45% patients, but only 10% control subjects, were Type 0 (p-value = 0.009). Type 0 AD patients had worse 2-year prognosis regarding loss of independence than Type I and Type II patients (p-value = 0.013). Phagocytosis of Aβ in Type I and II patients was shown to be dependent on 1,25 D3 using a specific inhibitor of the 1,25 D3-VDR activated nuclear receptor transcription factor. In a Type II patient, recovery from cognitive dysfunction related to surgical anesthesia was preceded by an improvement in phagocytosis of Aβ. The results of this pilot study suggest that the *MGAT3* Type biomarker may characterize subgroups of AD patients with different disease progression. *In vitro* results suggest that vitamin D3 supplementation might be beneficial in both Type I and II patients, whereas curcuminoids only in Type I. These results must be investigated in a large prospective study.

Keywords: Alzheimer's disease, amyloid-β, MGAT3, surgical anesthesia, vitamin D3

INTRODUCTION

A blood-based biomarker is a Holy Grail for practical diagnosis of Alzheimer's disease (AD) and mild cognitive impairment (MCI) to AD transition, but may be unattainable using plasma proteins because of the multiple mechanisms that are common to neurodegenerative diseases, such as inflammation, oxidative stress, and lipid metabolism. Ray and coworkers [1] proposed a panel of 18 plasma proteins that could distinguish AD patients from healthy controls with 89% accuracy and indicate risk of MCI to AD transition, but another study found much lower accuracy in differentiation of MCI from AD or depression [2]. Other approaches to diagnosis of MCI to AD transition are more powerful, including PET imaging with Pittsburgh Compound-B,

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which has 85–90% predictive value for MCI transition to AD [3]. Cerebrospinal fluid (CSF) biochemical biomarkers of AD, low amyloid-β (Aβ) and high phosphorylated tau, can stratify MCI patients into those with very low or high risk for future development of AD [4].

AD patients have functional defects in phagocytosis of Aβ in peripheral blood mononuclear cells (PBMCs) [5] and in autophagic-lysosomal pathways in AD fibroblasts [6] and neurons [7]. The discovery of the role of β-1,4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase (*MGAT3*) in phagocytosis of Aβ was gleaned by microarray testing of transcription in PBMCs exposed to Aβ, which showed that *MGAT3* was transcribed 327 fold higher in controls compared to patients and was indispensable for phagocytosis of Aβ [5]. Microarray testing evaluates the human transcriptome (23,000 genes in the Operon microarray [5]) compared to only a host of plasma proteins tested by plasma protein assays [1, 2]. AD patients and age-matched University-employed control subjects [8] have significant differences in phagocytosis of Aβ and transcriptional regulation of *MGAT3* [5]. The transcription of *MGAT3* stimulated by Aβ distinguishes macrophages into Type 0 (very low *MGAT3* transcription), Type I (low *MGAT3* transcription up regulated by bisdemethoxycurcumin), and Type II (high *MGAT3* transcription down regulated by bisdemethoxycurcumin).

Here, we examine the role of *MGAT3* biomarker in AD prognosis. Although currently there are no practical prognostic biomarkers, it has been noted that progression of dementia is not uniform since it was slower in patients with a "plateau" in the early stage of AD [9]. A two-year study of natural history in 686 patients found one fourth of the cohort in a relatively stable condition and 11% of patients with rapid progression but did not identify significant factors in disease progression [10]. Neuropsychological testing was also of little value for staging AD [11]. Recent studies have identified Aβ plasma level alterations in the preclinical stage or at baseline [12–15]. These alterations suggest importance of immune mechanisms for clearance of Aβ across the blood-brain barrier in disease development and progression. Thus, in AD brain tissue, monocytes are observed to traverse the blood brain barrier into the neuropil, and in a co culture of AD brain slices with mononuclear cells, normal monocytes upload and degrade Aβ, whereas AD monocytes suffer apoptosis after uploading Aβ [16]. Therefore, regulation of the endogenous innate immune system is crucial for physiological Aβ handling.

Alterations of glycoprotein glycans have downstream effects on the target glycoproteins, contributing to a wide variety of diseases. The role of defective *MGAT3* transcription in AD was discovered by microarray analysis of Aβ-stimulated transcriptome of AD patients' PBMCs [5], and subsequently confirmed by RT-PCR [8]. Two other studies suggest a neuropathological role for *MGAT3* (a.k.a GnT-III): In AD brain, GnT-III mRNA expression was increased, possibly as a protective mechanism [17]; and expression of mutant *MGAT3* (T37/T37) in mouse brain was associated with a neuropathological phenotype [18]. The studies in AβPP transgenic mouse models overexpressing Aβ do not reproduce the dysfunction in Aβ clearance of AD patients. Whereas mouse microglia use fluid phase macropinocytosis [19], human macrophages use phagocytosis of Aβ into endosomes and lysosomes [5]. Mouse microglia are unable to phagocytize large Aβ deposits [20], whereas human macrophages become saturated with Aβ [5].

Vitamin D deficiency is associated with chronic diseases, including cancers, autoimmune, cardiovascular, and infectious diseases [21]. The secosteroid hormone 1 α,25-dihydroxyvitamin D3 (1,25 D3) regulates the transcription of 3.5% of human genes, including important genes for innate immune antimicrobial defenses, such as cathelicidin [22] and has important extranuclear immunostimulating effects [23]. Supplementation with at least 2,000 IU vitamin D3 per day is widely recommended for optimal health [21]. A preventive role of vitamin D supplementation against AD is compelling because low levels of vitamin D were associated with substantial cognitive decline in a large study of elderly population investigated over a 6-year period [24]. There are as yet no epidemiological studies of high vitamin D3 intake in prevention of AD onset or progression. Hormonally-active 1,25 D3 improves phagocytosis and degradation of Aβ by both Type I and Type II monocyte/macrophages [25] and protects neurons against Aβ toxicities related to increased intracellular calcium and decreased vitamin D receptor (VDR) and nerve growth factor [26]. A common intracellular receptor for 1,25 D3 and bisdemethoxycurcumin (BDC) was recently identified as the VDR, suggesting that VDR is essential for the recovery of Aβ phagocytosis by 1,25D3 and BDC [25].

In this pilot study, we have evaluated the transcription of *MGAT3* RNA [5] as a prognostic AD biomarker measured by the Mini-Mental State Examination (MMSE). To evaluate the immune effects of 1,25D3, we tested Aβ phagocytosis by the flow cytometric test with functioning or blocked VDR receptor.

MATERIALS AND METHODS

AD patients and controls

The blood specimens were obtained under UCLA Institutional Review Board-approved protocols from patients seen at the UCLA Neurology Clinic in Santa Monica. The diagnosis of probable AD was established by the National Institute of Neurological and Communication Disorders and Stroke/Alzheimer's Disease and Related Disorders Association criteria [27] using neurological and neuropsychological examination in every case and neuroradiological examination (MRI/MRA and PET scan with deoxyglucose) in a majority of patients. AD patients were routinely treated with a cholinesterase inhibitor and an N-methyl D-aspartate glutamate receptor inhibitor. Nutritional supplementation with vitamin D3 was recommended to all patients but was not monitored except in patient #2. As controls, professors and businessmen and caregivers to patients older than 60 years were used.

Flow cytometric assay of Aβ phagocytosis [8]

500,000 PBMCs were incubated overnight with 2 μg/ml FITC-Aβ; after PBS wash and staining with PE-CD14, FITC fluorescence was determined by flow cytometry. Monocytes were gated according to SSC/FSC and at least 5,000 cells were analyzed in FL1 (FITC) and FL2 (PE) using BD FACScan with a 488 nm argon laser and the FL1 filter 530 nm ± 15 and the FL2 585 nm ± 21. As indicated, the samples were tested with and without treatment by the specific VDR inhibitor "MK" at 10^{-7}M [23, 28] to determine the role of 1,25D3 in phagocytosis. FITC-Aβ phagocytosis was calculated as mean fluorescence intensity (MFI) in upper right corner times % cells upper right corner.

MGAT3 transcription by RT-PCR [5]

PBMCs (5 million) were cultured overnight without stimulation or with stimulation by Aβ (2 μg/ml) or with Aβ (2 μg/ml) and BDC (0.1 μM). RNA was isolated using the RNeasy Mini kit (Qiagen). cDNA was synthesized using the iSCRIPT cDNA Synthesis Kit (BioRad, Hercules, CA). The expression level of MGAT3 was measured using the primers designed using Primer Express (Applied Biosystems, Branchburg, NJ) by qPCR on an Opticon™ real-time PCR detector (BioRAD) and normalized to the levels of the housekeeping gene 36B4 using the IQ SYBR Green mix (BioRad) on the BioRAD Opticon Continuous Fluorescence detector (BioRAD) and analyzed with Opticon Monitor Software 1.08 (BioRAD). The relative quantities of the gene tested per sample were calculated against 36B4 using the ΔΔC (T) formula [29]. Since humans may have highly variable baseline levels of genes, the results were expressed as a ratio of log *MGAT3* RNA incubated with Aβ ± BDC and log MGAT3 RNA incubated without stimulation.

Statistical analysis

Proportions in Tables 1 and 2 were analyzed by chi-square.

Table 1
Distribution of MGAT3 macrophage types

	Mean age (years)	Type 0	Type I	Type II	Total
AD patients	80.3 ± 3.2	9 (45%)	7 (35%)	4 (20%)	20
Control subjects	71.2 ± 3.65	2 (10%)	5 (25%)	13 (65%)	20

Frequency of types is significantly different in patients vs. controls (Chi-square exact test p-value = 0.009).

Table 2
Clinical outcome of AD patients according to MGAT3 macrophage Type after a 2-year follow-up

AD type	*n*	Mean age[a]	Mean MMSE[b]	Mean duration of AD	MRI (PET)[c]	PET[d]	Living independently[e]	24 h nursing home care	Died
0	6	80 ± 3.2	24/30 ± 0.6	5.5 years	6/6	(3/6)	0	4	2
I	5	78.2 ± 3	19.6/30 ± 5.3	>8 years	5/5	(4/5)	4	0	1
II	3	87.6 ± 5.3	25/30 ± 0.6	>8 years	2/3	(1/3)	1	2	0
Total	14						5	6	3

[a] Mean age at time of the first visit (mean ± standard error of the mean).
[b] Mean MMSE at time of the first visit (mean ± standard error of the mean).
[c] Number of patients with MRI negative for other causes of dementia.
[d] Number of patients with PET consistent with AD.
[e] Difference between Type I+II vs. Type O chi-square exact test p-value = 0.013.

RESULTS

Frequency of MGAT3 types

The sample of patients included AD patients seen in 2008 at the UCLA Neurology clinic, whereas the sample of control subjects included UCLA professors and caregivers. Although this presents a selection bias due to differences in education, lifestyle, and age (patients were on the average 11 years older), the analysis provides a biochemical biomarker comparing dementia patients with cognitively normal controls. *MGAT3* types were determined according to *MGAT3* transcription (Fig. 1). Type 0 was found in 45% patients but

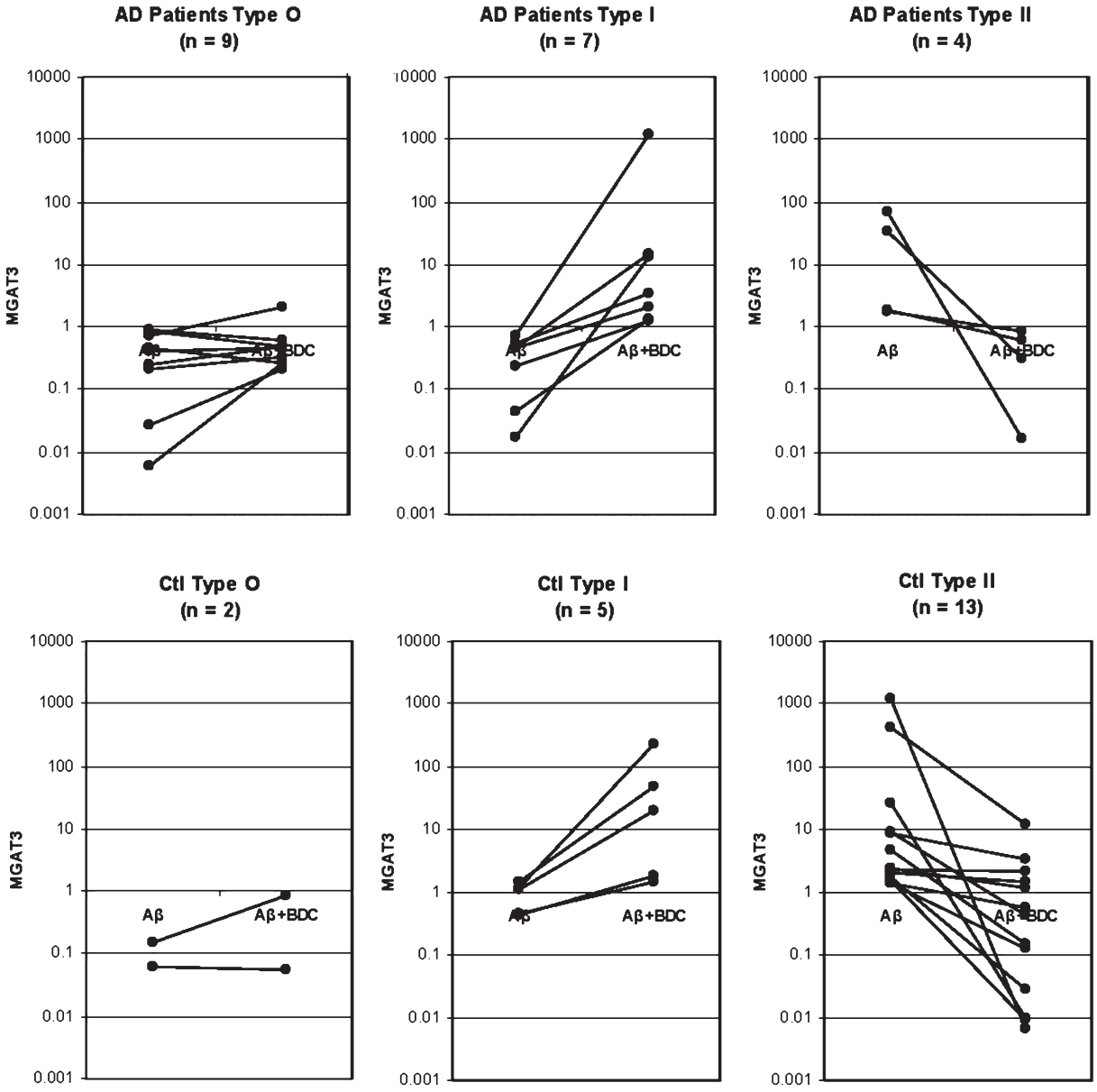

Fig. 1. *MGAT3* macrophage Types 0, I, and II in patients and controls according to *MGAT3* mRNA transcriptional responses in PBMCs. Type 0 patients are inhibited in transcription of Aβ-stimulated *MGAT3* mRNA at baseline and after BDC stimulation; Type I patients are inhibited in transcription of Aβ-stimulated *MGAT3* mRNA at baseline but increase transcription after BDC stimulation; Type II patients are not inhibited in transcription of Aβ-stimulated *MGAT3* mRNA at baseline but are inhibited after BDC stimulation. Note that 45% control subjects have Type II responses, 25% Type I responses and 10% Type 0 responses. Vertical axis indicates *MGAT3* transcription after stimulation (with Aβ or Aβ + BDC) versus *MGAT3* transcription not stimulated [5].

in only 10% control subjects (Table 1, $p = 0.009$). To demonstrate the stability of the *MGAT3* type (*MGAT3* RNA stimulated by Aβ/*MGAT3* RNA stimulated by Aβ and BDC), the same control subject was tested over a one-year period with the results: 410/12 = 34.1; 40/0.008 = 5,000; 117/12 = 9.7; 52/0.002 = 26,000, i.e., the *MGAT3* type II (ratio > 1) was unchanged. However, prospective *MGAT3* testing of patients was not done.

The relation between *MGAT3* Type and mean fluorescence intensity (MFI) of Aβ phagocytosis is shown in Fig. 2. Low and high *MGAT3* transcription separated Type I and Type II patients but both showed low Aβ phagocytosis (<500 MFI U). All control subjects except one showed positive Aβ-stimulated *MGAT3* transcription; however, the professors had good Aβ phagocytosis, whereas caregivers had low Aβ phagocytosis (<500 MFI U), as previously reported [8].

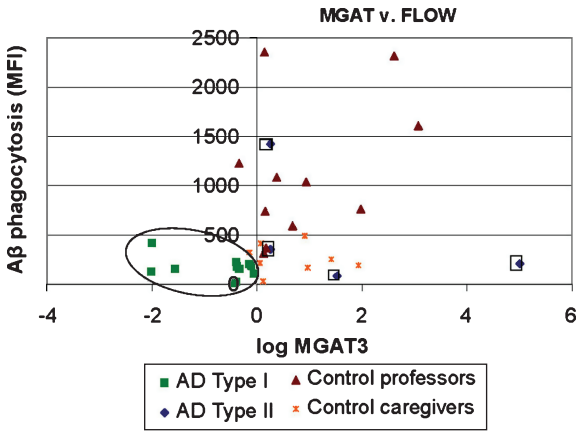

Fig. 2. Relation between *MGAT3* transcription and macrophage phagocytosis of Aβ. The graph shows the relation between Aβ phagocytosis (mean fluorescence intensity (MFI) Units) and *MGAT3* transcription. Type II patients (diamonds) and Type I patients (squares) had low Aβ phagocytosis (<450 MFI Units) except one Type II patient. Control subject professors had excellent phagocytosis and all except one good *MGAT3* transcription. Control subject care givers (>60 years old) had low phagocytosis (<450 MFI Units) but good *MGAT3* transcription.

Progression of AD is slower in Type I and Type II patients than in Type 0 patients

We studied prospectively a two-year course of 14 AD patients whose *MGAT3* type was ascertained at the first visit. The enrolled patients were Type II ($n = 3$), Type I ($n = 5$), and Type 0 ($n = 6$). Type II and Type I patients had characteristics suggesting worse prognosis than Type 0: Type II patients were older, Type I and II patients had lower MMSE score and longer duration of AD than Type 0 (Table 2). Despite these confounders predicting more favorable course for Type 0, after the 2-year follow-up, Type 0 patients had the worst outcome (none living independently; two died) in comparison to Type II patients (one of 3 living independently) and Type I patients (four of 5 living

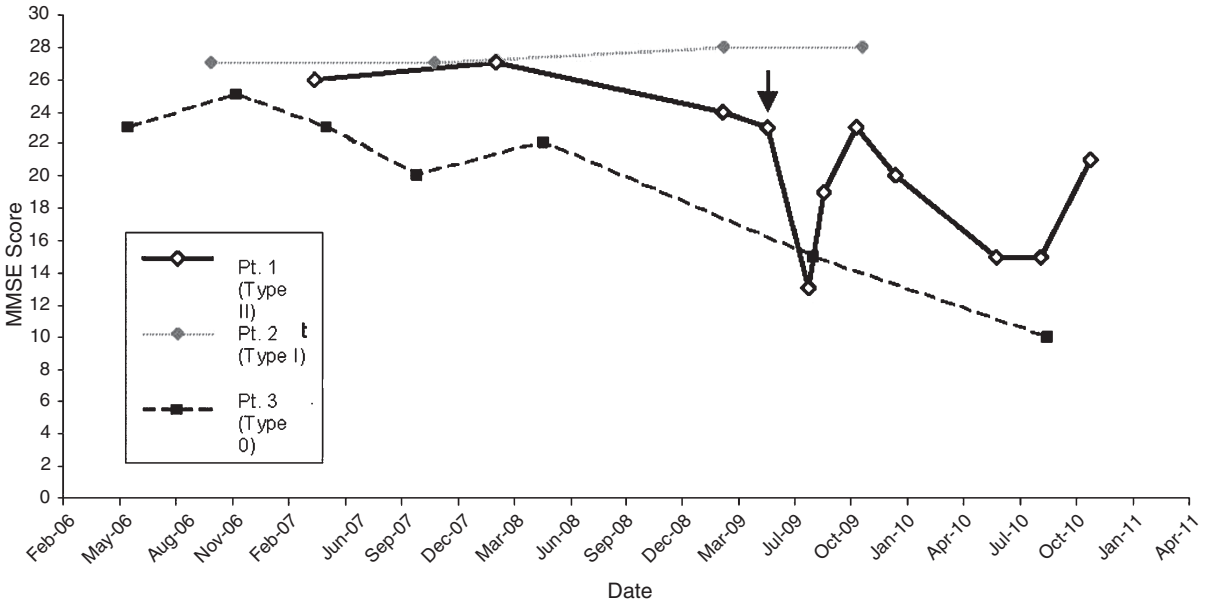

Fig. 3. Decline of cognitive function in some Type I, Type II, and Type 0 patients. A patient Type II had relative preservation of MMSE score; Type I patient had severe decline after surgery but considerable recovery; Type 0 patient showed rapid decline.

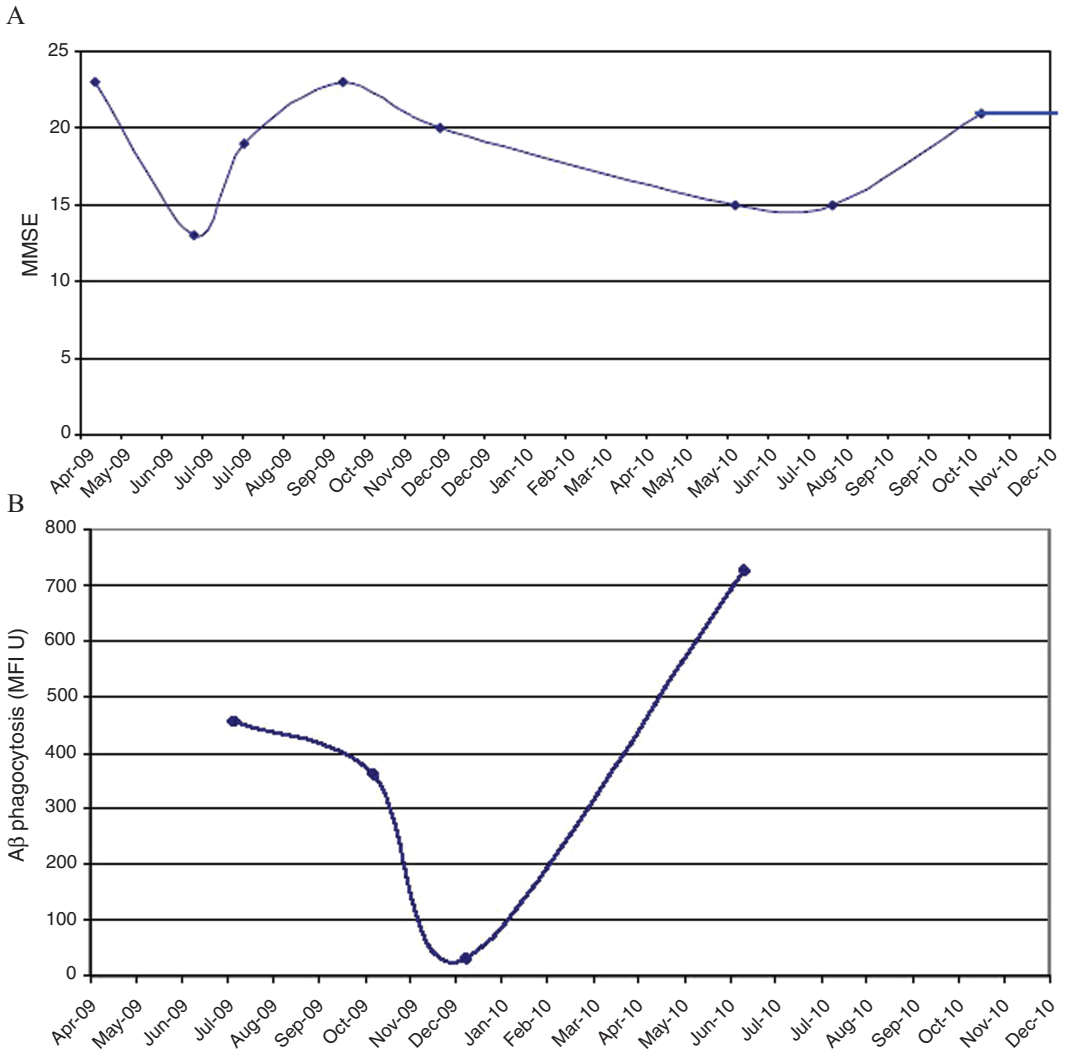

Fig. 4. Relation between the cognitive function (A) and Aβ phagocytosis (B) in patient #2 (see MMSE course from June 2007 to October 2010 of patient #2 in Fig. 3). MMSE (from April 2009 to October 2010) showed nadir in July 2009 following surgery and rebound followed by decline and another rebound. Aβ phagocytosis showed improved phagocytosis in June 2010 preceding improved MMSE score in October 2010 and (not shown) identical score in January 2010.

independently). At the end of the study, five of 8 Type I and II patients remained living independently versus none of 6 Type 0 patients ($p = 0.03$) (Table 2).

Cognitive decline in one patient in each group is shown in Fig. 3. Patient #1 (Type I patient) maintained her MMSE score between 27 and 28 during the fourth to sixth year of her illness when she had defective delayed episodic memory but has functioned independently. Patient #2 (Type II patient) had a precipitous cognitive decline after hip surgery with prolonged anesthesia but recovered all but two points of her preoperative score in the eighth to tenth year of her illness when she was taking high dose vitamin D3 supplementation and maintained independent living (Figs 3 and 4). Patient #3 (Type 0 patient) lost > 12 points in the two-year study during the fourth to sixth year of illness and was placed in full time nursing care.

All 14 professors who were controls in the 2008 study [8] are still academically active.

Post-operative cognitive dysfunction and Aβ phagocytosis

Patient 2 (Type II) was diagnosed with AD at the age 69 years. She started taking 5,000 U vitamin D3 daily in April 2009 and has continued until present (October

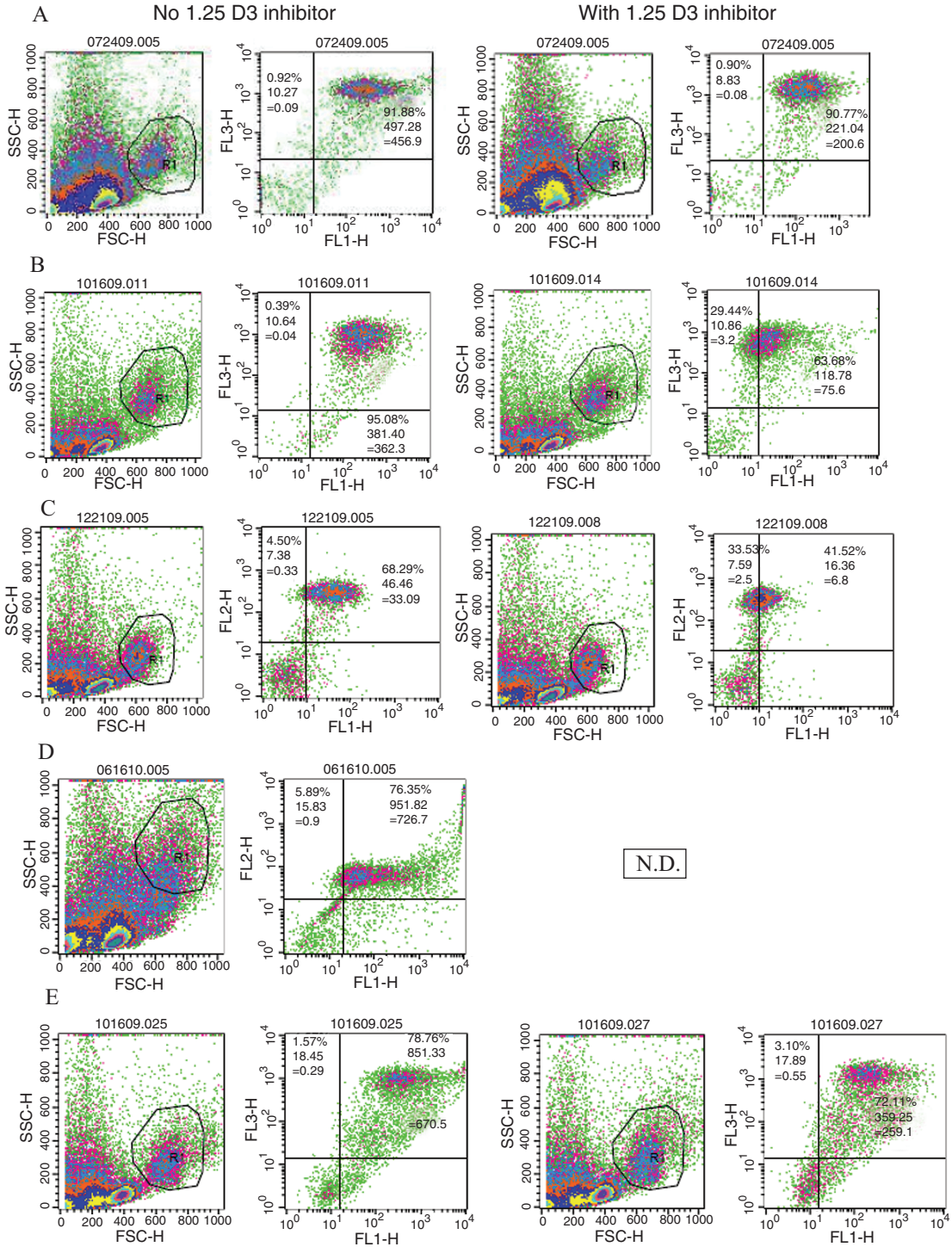

Fig. 5. Course of Aβ phagocytosis and effect of the VDR inhibitor on Aβ phagocytosis. Phagocytosis of FITC-Aβ by mononuclear cells was measured by a flow cytometric test without (left two columns) and with (right two columns) *in vitro* treatment by a specific VDR antagonist "MK". The gating on monocytes R1 is showed on the left and phagocytosis of FITC-Aβ (FL1) by PE-CD14 (FL2 or FL3) monocytes on the right of each section. Phagocytosis of Aβ = Mean fluorescence intensity (MFI) of FITC-Aβ in upper right corner times % cells upper right corner. Patient #2 (type II) A. 1 month after surgery a. not treated, b. treated with "MK"; B. 4 months after surgery a. not treated, b. treated with "MK"; C. 6 months after surgery a. not treated, b. treated with "MK"; D. 12 months after surgery. Patient #1 (Type I) E. a. not treated, b. treated with "MK".

2010). She had a total arthroplasty of right hip (with a 2-hour general anesthesia) in June 2009. Her MMSE score was fairly stable with the score of 22 before the surgery but declined precipitously after the surgery to MMSE score of 12. She had fluctuating improvement with steady improvement at 17 months to MMSE score of 21, which was preceded by improvement of Aβ phagocytosis at 12 months (Fig. 4).

Role of 1,25D3 in Aβ phagocytosis

To determine the effect of endogenous 1,25D3 level in Aβ phagocytosis, it was of interest to test Aβ phagocytosis by PBMCs treated or not treated *in vitro* by the specific inhibitor of VDR "MK". In patient 2 (Type II), the inhibitor reduced mean phagocytosis of Aβ by 56% at 1 month post-operatively, by 79% at 4 months and by 78% at 6 months. In patient 3 (Type I), the inhibitor reduced mean phagocytosis by 69% (Fig. 5).

DISCUSSION

The validity of this pilot study is limited by its small size. The results suggests that the frequency of Type 0 macrophages is greater among patients than controls, and in a 2-year study Type 0 patients have faster progression to full time nursing care than Type I and II patients (Table 2 and Fig. 3). The definition of *MGAT3* Type is based upon up or down regulation of *MGAT3*. The regulatory mechanisms of *MGAT3* may include transcriptional regulation by nuclear transcription factors, such as RXR-VDR. Post-transcriptional regulation of *MGAT3* expression by miRNAs might be also important. The expression level of some miRNAs may vary by 3 to 5 logs resulting in large heterogeneity in expression. Whereas pre-clinical Aβ plasma levels have been found altered either up or down in recent studies [12–15], the *MGAT3* up regulation by Aβ and down regulation by Aβ/BDC has been stable on repeat testing of a control type II subject over one year and, therefore, might be a personal biomarker preceding the clinical onset. However, the stability of the Type I or II in AD patients is not known and over time some patients might veer to Type 0. Control subjects showed divergent results in phagocytosis: good to excellent in professors and low in caregivers. The stress of caring for patients may be adversely affecting caregivers' immunity, including Aβ phagocytosis [30].

Nutritional supplementation might be important for clearance of Aβ because both 1,25D3 and BDC bind to VDR and improve Aβ phagocytosis *in vitro* [25]. Although this study was not a control study of vitamin D3 supplementation, direct evidence of the effect of vitamin D3 on Aβ phagocytosis was gleaned by use of the specific 1,25D3-VDR inhibitor "MK" [25, 28]. Patient #2 showed profound post-operative cognitive decline with slow but ultimately good recovery. During her recovery, this patient was receiving high dose of vitamin D3 and her phagocytic function of Aβ was dependent on 1,25D3, as it was strongly inhibited by the "MK" inhibitor of the 1,25D3-VDR-activated nuclear receptor transcription factor complex formation and subsequent trans regulation of genes that contain a vitamin D response element [28] (Fig. 5). Her steady cognitive recovery was preceded by recovery of Aβ phagocytosis while taking high dose of vitamin D3 (Fig. 4).

The incidence of post-operative cognitive dysfunction (POCD) is a well-documented occurrence reported in the scientific literature for over a century, particularly after surgeries requiring the use of general anesthesia. A number of observational studies and case reports have noted a decrease in MMSE score post-operatively, followed by an improvement back to pre-operative level within several months [31]. Although most patients will see rapid recovery of their cognitive abilities within a few months after surgery, a small proportion will have long-term sequelae. Although the etiology of the observed POCD remains controversial, both general anesthesia and the actual surgical procedure are believed to contribute by deregulation of neuronal calcium, increased production of Aβ, and anti-cholinergic effects of medications. In our patient #2, the phagocytic function declined and then improved before cognitive amelioration. Immune suppression might therefore be an important cause of POCD. Vitamin D3 supplementation may have benefits for cognitive health and recovery from POCD given its positive effects on Aβ phagocytosis.

MGAT3 type may have importance not only for prognosis but also for therapies with natural products, such as vitamin D. *In vitro*, 1,25D3 benefits the immunity of Type I and II patients, whereas curcuminoids benefit only Type I patients and may even decrease the immunity of Type II patients. The results of this pilot investigation point to a new direction but must be confirmed in a large prospective trial before their validity is accepted.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=753).

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