Increased Cerebrospinal Fluid Levels of Ubiquitin Carboxyl-Terminal Hydrolase L1 in Patients with Alzheimer’s Disease

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Key Words
Alzheimer’s disease · Biomarkers · Cerebrospinal fluid · DJ-1 · Neuron-specific enolase · Ubiquitin carboxyl-terminal hydrolase L1 · Tau phosphorylated at threonine 231

Abstract
Background: Dysfunctions of the ubiquitin proteasome system (UPS), including the highly abundant neuronal enzyme ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), and autophagy-related changes (lysosomal degradation) are implicated in several neurodegenerative disorders including Alzheimer’s disease (AD). Method: This study evaluated cerebrospinal fluid (CSF) levels of UCH-L1, protein deglycase (DJ-1), neuron-specific enolase (NSE), and tau phosphorylated at threonine 231 (P-tau231) in two independent patient and control cohorts. Cohort 1 included CSF samples from subjects having an AD biomarker profile (n = 10) or a control biomarker profile (n = 31), while cohort 2 was a monocenter clinical study including patients with AD (n = 32), mild cognitive impairment (n = 13), other dementias (n = 15), as well as cognitively healthy controls (n = 20). Results: UCH-L1 and P-tau231 were elevated in AD patients compared to controls in both cohorts. CSF levels of DJ-1 and NSE were unchanged in the AD group, whereas they were decreased in the group of other dementia compared to controls in the clinical study. Conclusion: Our main findings support that the UPS pathway may be impaired in AD, and UCH-L1 may serve as an additional CSF biomarker for AD.
Background

The ubiquitin proteasome system (UPS) selectively degrades proteins targeted for degradation by covalent conjugation to ubiquitin [1]. When these proteins have been linked to the ubiquitin chain, they are directed to degradation via the UPS [1] or in the lysosome [2]. Alzheimer’s disease (AD) is a protein-misfolding disease characterized by accumulation of amyloid β (Aβ) peptides and hyperphosphorylated tau protein into plaques and neurofibrillary tangles, respectively [3]. The ubiquitin protein is also accumulated in these structural AD changes [4–7]. This suggests that dysfunction of the quality control mechanisms regulating protein breakdown, including both the UPS and the lysosome, might be directly or indirectly involved in the pathogenesis of AD.

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1; also called neuron cytoplasmic protein 9.5 and PGP 9.5) is a highly abundant neuron-specific cytoplasmic enzyme [8–10]. It modifies the activity of the UPS by acting as a deubiquitinating hydrolase [10], ubiquitin ligase [11], and a monoubiquitin stabilizer [12]. UCH-L1 [13] and ubiquitin [4–7] are found in Aβ plaques and neurofibrillary tangles in the AD brain, supporting evidence for UPS dysfunction in AD. Furthermore, in an animal model, dysfunction of UCH-L1 affected the biological function of the tau protein as well as phosphorylation of tau [14]. Genetic studies demonstrate a link between the UCH-L1 (PARK5) gene and rare familiar forms of Parkinson’s disease (PD) [15], and most previous studies have shown a protective effect of the S18Y polymorphism (rs id 5030732) against sporadic PD [16, 17]. There are only a few reports regarding the implications of genetic variation in UCH-L1 in AD [18, 19], and there are conflicting results in terms of the role of the UCH-L1 polymorphism in AD patients [20–22]. UCH-L1 levels in the cerebrospinal fluid (CSF) of AD patients have, to our knowledge, not been reported, whereas several studies have found an increased CSF ubiquitin level in AD [23–26].

In the CSF, the fluid that surrounds the central nervous system, the AD core biomarkers total-tau (T-tau), tau phosphorylated at threonine 181 (P-tau181), and Aβ1–42 are thought to reflect neurodegeneration, neurofibrillary tangles, and aggregation of Aβ into plaques, respectively [27]. Most studies confirm a typical AD biomarker signature in AD with elevated T-tau and P-tau in addition to reduced levels of Aβ1–42. Recently, the AD core CSF biomarkers have been included in the research criteria for the diagnosis of both early and manifest AD by the International Working Group [28] and in the diagnostic guidelines from the National Institute on Aging-Alzheimer’s Association [29], respectively. However, the diagnostic performance of CSF tau phosphorylated at threonine 231 (P-tau231) compared to that of CSF P-tau181 is not well known, although in a recent study P-tau231 displayed a greater overall specificity for AD than P-tau181 [30].

The protein deglycase (DJ-1) (PARK7) gene is linked to PD [31]. Although the role of DJ-1 has not fully been evaluated, it could provide protection from oxidative stress [32]. In previous studies, CSF DJ-1 levels were unchanged in AD [33, 34]. Neuron-specific enolase (NSE) is a glycolytic enzyme present in neuronal and neuroendocrine cells and might be a marker of damage to cortical nonmyelinated neurons [27, 35]. Several previous studies have shown conflicting results with reduced [36], increased [35, 37, 38], or unchanged [39, 40] CSF levels in AD patients compared to controls.

In this study, a commercially available magnetic bead panel for neurological disorders was initially evaluated using CSF samples from subjects having an AD core biomarker profile or a control core biomarker profile, respectively. The neurological panel was then used to assess the CSF levels of UCH-L1, P-tau231, DJ-1, and NSE in a well-characterized monocenter cohort of patients with cognitive impairment and matched healthy controls [41].
Table 1. Demographic data and biomarker levels from the pilot study for the patients with AD and controls based on the biomarker profile

|                      | Control biomarker profile | AD biomarker profile |
|----------------------|---------------------------|----------------------|
| Subjects (men/women), n | 31 (16/15)                | 10 (3/7)             |
| Age, years           | 72 (70–79)                | 79 (73–85)           |
| Aβ1–42, ng/l         | 779 (636–991)             | 354 (320–414)b       |
| T-tau, ng/l          | 276 (170–332)             | 1,040 (665–1,110)b   |
| P-tau181, ng/l       | 43 (28–49)                | 101 (85–147)b        |
| UCH-L1, μg/l         | 4.5 (3.8–5.3)             | 12 (7.6–14)b         |
| P-tau231, pM         | 406 (314–495)             | 3,810 (2,870–5,070)b |
| DJ-1, μg/l           | 17 (9.3–31)               | 37 (26–52)a          |
| NSE, μg/l            | 23 (16–29)                | 42 (38–56)b          |

Data are given as median (interquartile range) unless otherwise indicated. Statistical differences were determined using nonparametric tests. a p < 0.01, b p < 0.0001 vs. control.

Materials and Methods

CSF Samples of the Pilot Study

An initial pilot study was performed using decoded human CSF samples supplied by the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal. Patients were designated as control or AD according to CSF AD core biomarker levels using in-house optimized cutoff levels of >90% specific for AD [42]: T-tau >400 ng/l, P-tau181 >80 ng/l, and Aβ1–42 <550 ng/l. The age-matched test material included 10 patients with an AD biomarker profile and 31 subjects with a control biomarker profile (table 1).

CSF Samples of the Clinical Study

All CSF samples in the clinical study were collected by lumbar puncture in the L3/L4 or L4/L5 interspace at the standardized time point 8:30 to 9:00 a.m. The first 12 ml of CSF was collected in a polypropylene tube and immediately transported to the local laboratory for centrifugation at 2,000 g at +4 °C for 10 min. The supernatant was pipetted off, gently mixed to avoid possible gradient effects, and aliquoted in polypropylene tubes that were stored at −80 °C pending biochemical analyses, without being thawed and refrozen.

The study participants as well as the AD CSF biomarker data in the clinical study have been reported previously [41, 43–46]. The study consisted of 60 patients (30 men and 30 women, all of Caucasian origin) admitted by their general practitioner for evaluation of cognitive impairment to a memory clinic in the region of Västra Götaland, Sweden. The patients were examined by a single specialized physician (P.J.) in 2000–2008. Inclusion criteria, besides being referred for evaluation of suspected dementia, were age 65–80 years, a body mass index (BMI) of 20–26, and a waist:hip ratio of 0.65–0.90 in women and 0.70–0.95 in men. Exclusion criteria were serum creatinine >175 mM, diabetes mellitus, previous myocardial infarction, malignancy including brain tumor, subdural hematoma, ongoing alcohol abuse, medication with cortisone, and previous or present medication with acetylcholine esterase inhibitors. The study also included age-matched healthy controls (10 men and 10 women) recruited contemporaneously from the same geographical area among spouses of the included patients and by advertisements in local newspapers. The control subjects had no subjective symptoms of cognitive dysfunction and had similar exclusion criteria as the patients.
All diagnoses were assessed by an independent specialized physician, as previously described [41]. The presence or absence of dementia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), criteria. Patients with dementia were classified as suffering from AD [47] or vascular dementia (VaD) according to the requirements by NINDS-AIREN [48] or the guidelines by Erkinjuntti et al. [49] for the subcortical type of VaD. Frontotemporal dementia, PD dementia, and dementia with Lewy bodies (DLB) were diagnosed as described previously [41].

Mild cognitive impairment (MCI) was diagnosed in the clinical setting in patients with cognitive impairment who did not fulfill the criteria for dementia [50]. Patients with MCI were followed at least annually for a median of 3 (range 1–7) years to evaluate whether they later developed dementia. During the follow-up visits, 13 MCI patients remained in stable cognitive function (sMCI). Others progressed to dementia during the follow-up period and were diagnosed with AD (n = 7), VaD (n = 3), or frontotemporal lobe dementia (n = 1). MCI patients diagnosed with AD on follow-up visits did not differ in CSF levels of Aβ_{1–42}, T-tau, or P-tau from patients with established AD at baseline. In total, the study population consisted of patients with AD dementia or with MCI diagnosed with AD dementia upon follow-up (n = 32), patients with sMCI (n = 13), patients with other dementias (n = 15), and healthy controls (n = 20). The distribution of diagnoses in the other dementia group were VaD or MCI diagnosed with VaD upon follow-up (n = 10), DLB (n = 4), and MCI that later converted to frontotemporal lobe dementia (n = 1). Before the test day, a mini-mental state examination (MMSE) [51] was performed.

**CSF Analyses**

Measurements of the core AD biomarkers (Aβ_{1–42}, T-tau, and P-tau_{181}) were performed using commercially available assays from Fujirebio, Ghent, Belgium [INNOTEST® β-AMYLOID(1–42), INNOTEST® hTAU Ag, and INNOTEST® PHOSPHO-TAU(181P)]. For the clinical study, the core AD biomarkers were analyzed on one occasion using the same batch of reagents, which has previously been reported [41, 43–46]. Furthermore, for the clinical study, CSF hemoglobin concentrations were measured using a human hemoglobin ELISA kit (Bethyl Laboratories, Inc.) according to the manufacturer’s protocol. For the clinical study, red blood cells (RBCs) were counted in most of the samples. Hemoglobin levels above 1,000 ng/l [52] and/or more than 500 erythrocytes per μl were indicative of significant blood contamination. Only two of the 80 CSF samples in the clinical study fulfilled these criteria. However, these two samples were not excluded from the study since statistical analyses showed that our results were not affected by these two samples (data not shown).

MILLPLEX MAP Human Neurological Disorders Magnetic Bead Panel 1 HND1MAG-39K (Merck Millipore) was used for quantification of UCH-L1 (PARK5), DJ-1 (PARK7), NSE, P-tau_{231}, NGF-β, and α-synuclein in accordance with the protocol provided by the manufacturer, and 25 μl neat CSF was analyzed. DJ-1 levels <4.8 μg/l and levels of NSE >60 μg/l were set to 4.8 and 60 μg/l, respectively. Samples were analyzed on a MAGPIX® system (Merck Millipore). Quality control (QC) samples (QC1 and QC2) analyzed in duplicate supplied by the manufacturer fulfilled the specified concentration levels. Coefficients of variation were <8% for all analytes. The levels of NGF-β and α-synuclein are not reported since they were below the detection limit in all CSF samples of both the pilot study and the clinical study.

**Statistical Analysis**

Because the distribution of most analytes was skewed (Shapiro-Wilk test, p < 0.05), nonparametric statistics were used for the statistical analysis using SPSS version 20.0 statistical software (SPSS Inc., Chicago, Ill., USA). Data are given, if not otherwise stated, as the median (interquartile range). Differences between more than two groups were assessed with
the Kruskal-Wallis test. If statistically significant (p < 0.05), the Mann-Whitney U test was then used for pairwise comparisons. The diagnostic value of each biomarker was assessed using receiver operating characteristic (ROC) curves with values of the area under the curve (AUC) and a 95% confidence interval (CI) calculated using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, Calif., USA). The correlation coefficients (ρ) were calculated using the Spearman two-tailed correlation test. Significance was obtained if the two-tailed p value was <0.05.

Statement of Ethics
The study was approved by the ethical committee of the University of Gothenburg, and informed consent was obtained from all participants. The study was conducted according to the Declaration of Helsinki.

Results

Pilot Study
In the pilot study, CSF levels of all the analytes (UCH-L1, DJ-1, NSE, and P-tau231) were significantly higher in the group with an AD biomarker profile (n = 10) than in the group with a control biomarker profile (n = 31) (table 1).

Clinical Samples
Demographic Results and CSF AD Biomarkers
Table 2 shows the demographic characteristics of the groups. In the clinical cohort, patients and controls were comparable in terms of age, gender, BMI, and waist:hip ratio. Patients with AD and other dementia had both significantly lower MMSE scores compared to controls. The core AD biomarkers for the clinical study have previously been reported [41, 43–45]. The AD group showed significantly higher CSF levels of T-tau and P-tau181 than the control group, while the Aβ1–42 concentration was significantly decreased (table 2). CSF levels of P-tau181 and Aβ1–42 were also significantly altered in the other dementia group (table 2).

Table 2. Demographic data and biomarker levels for the clinical study

|                      | Controls | sMCI | AD            | Other dementia |
|----------------------|----------|------|---------------|----------------|
| Subjects (men/women), n | 20 (10/10) | 13 (5/8) | 32 (15/17) | 15 (10/5) |
| Age, years           | 75 (70–78) | 72 (69–74) | 75 (71–77) | 74 (72–77) |
| MMSE                 | 29 (27–29) | 29 (27–29) | 23 (19–25) | 24 (20–26) |
| Aβ1–42, ng/l         | 992 (786–1,038) | 671 (544–833) | 420 (336–493) | 404 (354–816) |
| T-tau, ng/l          | 327 (223–398) | 270 (230–390) | 584 (434–747) | 311 (260–380) |
| P-tau181, ng/l       | 65 (50–79) | 60 (38–74) | 98 (78–113) | 47 (36–63) |
| UCH-L1, μg/l         | 7.2 (6.2–8.9) | 7.4 (5.1–8.9) | 11 (8.7–13) | 7.2 (5.8–9) |
| P-tau231, pm         | 1,000 (900–1,200) | 1,200 (700–1,700) | 3,400 (2,700–3,850) | 1,500 (1,100–1,800) |
| DJ-1, μg/l           | 19 (16–20) | 14 (12–16) | 17 (13–19) | 7.1 (6.3–18) |
| NSE, μg/l            | 40 (33–60) | 33 (31–60) | 50 (34–60) | 30 (26–34) |

Data are given as median (interquartile range) unless otherwise indicated. Statistical differences were determined using nonparametric tests. The demographic data and the core AD biomarkers have previously been reported [41, 43–46]. a p ≤ 0.05, b p ≤ 0.001, c p ≤ 0.0001 vs. control; d p ≤0.05, e p ≤ 0.001, f p ≤ 0.0001 vs. AD.
The CSF levels of UCH-L1 and P-tau\textsubscript{231} were significantly increased in patients with AD compared to controls, while these biomarkers were unaltered in other dementia and sMCI (fig. 1a, b; table 2). The CSF levels of DJ-1 and NSE were unchanged in the AD group, whereas they were significantly decreased in the group of other dementia compared to controls (fig. 1c, d; table 2).

**ROC Curve Analysis**

UCH-L1 and P-tau\textsubscript{231} could differentiate AD from controls with an AUC of 0.854 (95% CI 0.746–0.963; \(p < 0.0001\)) and 0.915 (95% CI 0.813–1.018; \(p < 0.0001\)), respectively (fig. 2). The AUC for the core AD biomarkers A\beta\textsubscript{1–42}, T-tau, and P-tau\textsubscript{181} were 0.938 (95% CI 0.865–1.010; \(p < 0.0001\)), 0.909 (95% CI 0.833–0.986; \(p < 0.0001\)), and 0.844 (95% CI 0.736–0.954; \(p < 0.0001\)), respectively (fig. 2). DJ-1 and NSE could differentiate other dementia from controls with an AUC of 0.835 (95% CI 0.679–0.990; \(p = 0.001\)) and 0.744 (95% CI 0.562–0.927; \(p = 0.02\)), respectively.

**Correlation Analysis**

None of the investigated CSF biomarkers correlated with age, MMSE score, or hemoglobin concentration in either the control group or in patients with AD (table 3). The levels of UCH-L1 correlated positively with the levels of P-tau\textsubscript{181}, P-tau\textsubscript{231}, DJ-1, and NSE in both the
control group and in AD patients (table 3). Moreover, UCH-L1 levels correlated positively with T-tau in the AD group but not in the control group (table 3). The levels of P-tau\textsubscript{231} correlated positively with the CSF levels DJ-1 and NSE in AD patients but not in controls (table 3). Finally, the CSF levels of P-tau\textsubscript{231} correlated positively with P-tau\textsubscript{181} and T-tau both in the control group and in patients with AD (table 3).
Discussion

In both investigated CSF materials, we found that the levels of UCH-L1 and P-tau<sub>231</sub> were significantly increased in AD patients compared to controls, while these biomarkers were unaltered in other dementias and sMCI. Moreover, the separation of AD from controls was of similar magnitude when using these markers as when using the CSF core AD biomarkers (Aβ<sub>1-42</sub>, T-tau, and P-tau<sub>181</sub>). In the clinical study, CSF levels of DJ-1 and NSE were unchanged in the AD group, whereas they were decreased in the group of other dementias compared to the controls.

To our knowledge, this is the first study to assess the potential of UCH-L1 as a CSF biomarker for AD. UCH-L1, a neuronal-specific enzyme that is highly abundant in the brain [8–10], is one of the enzymes involved in the regulation of proteosomal degradation. During this process, normal proteins with short half-life or misfolded proteins are conjugated with ubiquitin, which destines them for proteosomal [1, 10–12] and/or lysosomal degradation [2]. The UPS has previously been implicated in the pathogenesis of AD, and UCH-L1 is present in neurofibrillary tangles in the AD brain [13, 53]. Several previous studies have reported increased CSF ubiquitin levels in AD patients [23–26], but little has previously been known of the CSF UCH-L1 level in AD. Our results show that the CSF UCH-L1 level is increased in AD and that CSF UCH-L1 can separate AD patients from controls with high diagnostic accuracy in a ROC curve analysis.

The CSF UCH-L1 level did not only correlate with CSF levels of T-tau and P-tau, but there was also a positive correlation between CSF levels of UCH-L1 and NSE both in the AD group and in the controls. Both T-tau and NSE have previously been suggested to be general markers of damage to cortical nonmyelinated neurons [27, 35], which might indicate that our finding of an elevated CSF UCH-L1 level in AD to some extent reflects neurodegeneration. Recent reports suggest that UCH-L1 is released after brain damage caused by acute neurological insults such as traumatic brain injury and subarachnoid hemorrhage [54, 55]. Therefore, the diagnostic accuracy of CSF UCH-L1 to separate AD from brain damage of other causes needs to be investigated in further studies. In contrast, P-tau might be a more specific marker for AD [27], since high CSF levels of P-tau have been found to correlate with the accumulation of cortical neurofibrillary tangles [56, 57]. Thus, the positive correlation between CSF UCH-L1 and CSF P-tau found in our study combined with previous findings that neurofibrillary tangles in AD are ubiquitinated [4–7], and that the numbers of neurofibrillary tangles in AD brains relate negatively to the level of soluble UCH-L1 [18], support the hypothesis that the elevated UCH-L1 levels in AD could reflect a higher expression of UPS enzymes to compensate for a higher load of misfolded proteins. Furthermore, dysfunction of UCH-L1 in an animal model affected the biological function of tau as well as the phosphorylation of tau [14].

The elevated P-tau<sub>231</sub> level in the AD group is in concordance with previous reports that both P-tau<sub>231</sub> and P-tau<sub>181</sub> are increased in AD [58–60]. We also confirm that CSF levels of P-tau<sub>231</sub> and P-tau<sub>181</sub> correlate tightly [58], suggesting that these P-tau epitopes reflect the same pathogenic process and may be used interchangeably to measure brain neurofibrillary tangle load [56, 57]. The results of a recent study suggested that P-tau<sub>231</sub> has a greater overall specificity for AD compared to P-tau<sub>181</sub> [30]. However, another study found a similar performance for P-tau<sub>231</sub> and P-tau<sub>181</sub> in differentiating AD patients from controls, while P-tau<sub>181</sub> performed better in differentiating AD from Lewy body dementia and P-tau<sub>231</sub> performed better in differentiating AD from frontotemporal dementia [58]. Further studies are warranted to settle whether there is a difference in the diagnostic performance of P-tau<sub>231</sub> and P-tau<sub>181</sub> to identify AD and other neurodegenerative disorders.

DJ-1 (PARK7) is genetically linked to PD [31]. Even though the physiological function of DJ-1 has not fully been evaluated, it is thought to play a protective role during oxidative stress
In accordance with previous reports, we found that the DJ-1 level was not changed in AD compared to controls [33, 34]. NSE is a glycolytic enzyme present in neuronal and neuroendocrine cells and is associated with neurodegeneration [27, 35]. Previous studies have shown conflicting results with reduced [36], increased [35, 37, 38], or unchanged [39, 40] CSF NSE levels in AD compared to controls. In the present study, CSF levels of NSE were unaltered in AD, whereas in patients with other dementias CSF levels of NSE as well as of DJ-1 were reduced. However, the other dementia group was heterogeneous with relatively few cases of each specific diagnosis such as VaD and DLB. Therefore, further studies are needed to explore the roles of NSE and DJ-1 in dementias other than AD.

The present study represents the monocenter design of the clinical study with strictly defined procedures regarding lumbar puncture and laboratory assays. Patients and controls were matched in terms of age, gender, BMI, and waist:hip ratio, and none of the participants had diabetes mellitus or received treatment with acetylcholine esterase inhibitors or glucocorticoids. One limitation of the clinical study is the cross-sectional design, and changes over time could therefore not be studied. Furthermore, the lack of separation between AD patients and controls in terms of CSF NSE levels might be explained by NSE levels exceeding the highest allowed level being set to the highest standard concentration. Moreover, since both DJ-1 and NSE are abundant in RBCs, their CSF levels could be falsely elevated due to blood contamination [33, 61]. We investigated RBC contamination by assessment of the hemoglobin levels and RBC counting and found that only two samples were confounded by RBC contamination. These two samples were not excluded from the study since additional statistical analyses showed that these samples did not affect the final CSF result of DJ-1 and NSE. In addition, neither DJ-1 nor NSE correlated with CSF hemoglobin. Finally, the evaluated magnetic bead panel was not able to measure NGF-β or α-synuclein in any of the analyzed CSF samples. The QC samples provided by the manufacturer fulfilled the specified concentration levels, suggesting that the multiassay was not sufficiently sensitive to measure NGF-β and α-synuclein in our CSF samples.

Conclusions

In this study, we evaluated a magnetic bead panel for neurological disorders. CSF UCH-L1 and P-tau231 levels were elevated in patients with AD compared to healthy controls. The elevated CSF levels of UCH-L1 might indirectly reflect disturbed proteosomal degradation or that it is released in response to general neurodegeneration in AD. In addition, the clinical study suggests UCH-L1 to be an additional CSF biomarker for AD and that CSF P-tau231 has a high diagnostic accuracy for AD, suggesting that CSF P-tau231 is a valid alternative to CSF P-tau181. CSF levels of DJ-1 and NSE were decreased in the other dementia group, but this group was relatively small, and further studies are needed to clarify the role of DJ-1 and NSE in dementing disorders other than AD.

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Disclosure Statement

K.B. has served as a consultant for Eli Lilly and Roche Diagnostics and at Advisory Boards for Amgen and IBL International. K.B. and H.Z. are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU venture-based platform company at the University of Gothenburg. The other authors have nothing to disclose.

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