TITLE PAGE

Longstanding psychological stress in relation to biomarkers of neuronal dysfunction in cerebrospinal fluid: a 25-year follow-up study in women

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ABSTRACT

Longstanding psychological stress has been associated with increased risk of neurodegenerative disorders, such as dementia and Alzheimer's disease. In a prospective population study of women (n = 81), we tested if midlife stress (mean age 49 years) was associated with late-life biomarkers of neurodegeneration in cerebrospinal fluid (CSF) (mean age 74 years) in linear regression models. It was found that women who report of stress at baseline (n = 20) had higher levels of CSF visinin-like protein-1 (VILIP-1) (age adjusted β = 0.113, p = 0.017) and CSF myelin basic protein (β = 0.060, p = 0.030) compared with women without midlife stress (n = 61). There was also a trend observed for higher CSF neurofilament light (β = 0.133, p = 0.056). In addition, longer periods of stress (i.e., stress at 2-3 midlife examinations) were associated with higher levels of CSF VILIP-1. The results suggest that longstanding stress might be associated with neurodegenerative processes in the brain, as CSF VILIP-1 is an unspecific marker for neuronal injury and CSF myelin basic protein reflects neuroaxonal demyelination.

INTRODUCTION

An increasing body of research suggests that longstanding psychological stress may trigger pathological processes in the human brain and increase the risk of neurodegenerative disorders. In a population-based study of women followed for 35 years, we found that longstanding midlife stress was associated with late-life dementia, especially Alzheimer's disease (AD) (Johansson *et al.*, 2010), as well as late-life structural brain changes, such as temporal lobe atrophy and white matter lesions (Johansson *et al.*, 2012). In addition, stressful life-events and stress-prone personality (high neuroticism) were related to increased risk of AD (Johansson *et al.*, 2013; Johansson *et al.*, 2014). Other longitudinal studies have reported associations between stress disorders, perceived stress and dementia (Low *et al.*, 2013; Greenberg *et al.*, 2014).

Little is known about the underlying biological mechanisms of those associations. Recently, we showed that longstanding stress was associated with higher levels of tau protein in cerebrospinal fluid (CSF) in a non-demented sample of women followed over 25 years (johansson 2018). Increased CSF tau concentration primarily represents AD-type neurodegeneration (ref: PMID: 29566788). However, our study found no associations between stress and levels of CSF beta-amyloid 1-42 (Aβ42) (johansson 2018), the main marker of amyloid plaques in AD (Blennow *et al.*, 2006; Jack *et al.*, 2013). These findings raised our interest to extend the knowledge about stress and neurodegenerative processes in brain. The area is not very well studied and comparable studies are few. One longitudinal observation study found that persons with stress-prone personality had more advanced neurofibrillary tangle pathology in the brain (Terracciano *et al.*, 2013). In preclinical studies, it has been found that chronic stress was associated with accumulation of tau proteins in brains of mice (Kang *et al.*, 2007; Sotiropoulos & Sousa, 2016).

Extended neurodegeneration is often a consequence of a long-lasting progression of loss of neurons and synapses in the cerebral cortex and subcortical regions, which in the end result in greater brain atrophy. Underlying trajectories to neuron loss can be due to aggregation of misfolded proteins, or other mechanisms, such as myelin and axonal injury, oxidative stress, or chronic low-grade neuroinflammation (Lupien *et al.*, 2018). Three CSF measures indicate structural and functional injury of nerve cells and can be used as prognostic and diagnostic biomarkers of brain degeneration is CSF levels of visinin-like protein 1 (VILIP-1), myelin basic protein (MBP), and neurofilament light (NF-L) (Tarawneh *et al.*, 2012; Fagan *et al.*,

2014; Tarawneh *et al.*, 2015). VILIP-1 is a calcium sensor protein that is expressed in high abundance in CNS neurons (Kiyama *et al.*, 1985; Braunewell & Klein-Szanto, 2009). The protein is excessively released because of neuronal degradation (Lee *et al.*, 2008) and has been shown to correlate well with AD progression and pathology (Tarawneh *et al.*, 2012). MBP is a main component of myelin and maintains its correct structure, interacting with the lipids in the myelin membrane (Zhan *et al.*, 2018). Increased concentrations of CSF MBP are associated with CNS demyelination, and higher amount of cytokines and oxidative stress (Zhan *et al.*, 2018). NF-L is one of the components of neurofilament, which is a main structure in the cytoskeleton, particularly in large myelinated axons (Zetterberg *et al.*, 2016). High CSF NF-L indicates structural damage of nerve cells and are associated with white matter damage and brain atrophy (Zetterberg *et al.*, 2016).

There is a lack of studies investigating the long-term effects of psychological stress on the brain and associations to neurodegenerative biomarkers in CSF. Therefore, we examined the relationship between longstanding psychological stress in midlife and late-life levels of CSF VILIP-1, MBP, and NF-L, in a sample of 81 women followed over 25 years.

MATERIALS AND METHODS

Study population

The sample was derived from the Prospective Population Study of Women in Gothenburg, Sweden, which was initiated in 1968 (n=1462), and have had several follow-up examinations over more than four decades (Bengtsson *et al.*, 1973; Johansson *et al.*, 2010). Participants were initially sampled from the Swedish Population Register based on specific birth dates in order to yield a representative sample at the ages studied. Psychological stress was rated according to a standardized question at baseline (mean age 46 year), and in the follow-up examinations in 1974-75 (mean age=52 years) and 1980-81 (mean age=58 years). In the follow-up in 1992-94, a sub-sample of 590 women participated in an extensive neuropsychiatric examination, where 88 underwent lumbar puncture (LP) (Blennow *et al.*, 1993). Participants and non-participants in the LP examination were similar regarding baseline diastolic and systolic blood pressure, body mass index, smoking habits, cardiovascular disorders, depressive symptoms, and report of longstanding stress. There were neither no differences in Mini Mental State Examination (MMSE) score at examination in 1992-94. LP-participants were younger (p.>01), had higher education (p.04), and had a lower

5-year mortality rate (p.02), compared to non-participants. The present study consist of 81 women, born in 1908 (n=3), 1914 (n=7), 1918 (n=33), and 1922 (n=38). Five women were excluded due to missing stress-data and two were excluded due to... The Ethics Committee for Medical Research at the University of Gothenburg approved the study, and informed consent was obtained from all participants, in accordance with the provisions of the Helsinki Declaration.

Assessment of longstanding psychological stress

The question about psychological stress was asked by a physician and was as follows; "Have you experienced any period of stress, one month or longer, in relation to circumstances in everyday life, such as work, health, or family situation?" Stress was defined as feelings of irritability, tension, nervousness, fear, anxiety or sleep disturbances. The responses were categorized as experience; 0: Never a period of stress; 1: Period/s of stress more than five years ago; 2: One period of stress during the last five years; 3: Several periods of stress during the last five years; 4: Constant stress during the last year; or 5: Constant stress during the last five years. For the purpose of this study, women who acknowledged responses 3, 4 or 5 were considered to have psychological stress.

Assessment of cerebrospinal fluid biomarkers

LPs were conducted between January 1993 and March 1994. CSF samples (12 ml) were taken through the L3/L4 interspace. To eliminate cells and other insoluble material, the samples were centrifuged at 2000g for 10 minutes. The liquid was then stored at minus 80°C in 1 ml polypropylene vials until analysis. Commercially available kits were used for measuring the concentrations of VILIP-1 (Human VILIP-1 ELISA; BioVendor GmbH, Heidelberg, Germany), MBP (Active® MBP; Diagnostic Systems Laboratories Inc., Webster, Tex., USA) and NF-L (NF-light ELISA; IBL international, Hamburg, Germany) by following the manufacturers' protocols. All measurements per assay were performed in one round of experiments using one batch of reagents by board-certified laboratory technicians who were blinded to clinical data.

(Blennow *et al.*, 1993). Sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine levels of VILIP-1, MBP, and NF-L. The technical methods have previously been described in details (Blennow *et al.*, 1993; Rosengren *et al.*, 1996; Rymo *et al.*, 2017)

(Rosengren 1996 NFL, IRMA 2017 – MBP, VILIP??).The laboratory technicians were blinded to clinical data.

Assessment of potential confounders

Information on depression status (DSM-III criteria) (Hallstrom, 1984) and cerebrovascular disorders (documented history of stroke and/or TIA) were assessed at baseline. The mini mental state examination (MMSE) was calculated at the follow-up examination in 1992-94. Dementia was diagnosed (DSM-III-R) (1987) continuously during the study period based on information from psychiatric examinations, close informant interviews, and Hospital Discharge Registry. (Skoog *et al.*, 1993)

Statistical analyses

Due to skewed distributions, all CSF values were natural log transformed to improve symmetry. Means and standard deviations (SD) were determined for all CSF levels. Pearson correlation coefficients estimated linear relationships between age and stress, and age and CSF markers. Linear regression models analyzed the relations between stress in midlife examinations and CSF markers measured at the late-life examination. The first model adjusted for age, and in a second model adjust for age, depression, cerebrovascular disorders and MMSE score. The CSF variable was used as dependent variable, and the results were presented as β-coefficients and 95% confidence intervals (CIs).

We further examined whether number of examinations with psychological stress report influenced the association to late-life CSF levels. Stress data from the three midlife examinations were combined and the sample were divided in three groups: (1) women never reporting stress at any of the examinations, (2) women with stress at one examination, and (3) women with stress at two or three examinations. Finally, dementia disorders were taken into account and linear regression models re-analyzed the associations between midlife stress and CSF measurements, when persons with dementia were excluded. All statistical significance was defined as a *p*-value <.05, using two-tailed tests, in all analyses. The analyses were performed with IBM SPSS version 24 statistical software (Armonk, NY).

RESULTS

Table 1 presents the characteristics of the 81 participant. Twenty-five percent (n=20) of the sample reported psychological stress at baseline. In the first follow-up, six years after baseline, 22% reported stress, and in the second follow-up, twelve years after baseline, 15% reported stress. Report of stress at baseline was not related to participants' age (p=.13), but higher age was associated with higher levels of CSF VILIP-1 (p=.009), MBP (p=.018) and NF-L (p=.093).

Psychological stress at baseline was related to higher levels of late-life CSF VILIP-1 (B= XX, p=.019) and CSF MBP (B=XX, p=.039), in age-adjusted linear regression models (Table 2). There was also a trend observed for higher levels of CSF NF-L (B=, p=.056). The findings remained significant after further adjustments for depression, cerebrovascular disorders and cognitive status (Table 2). Reports of stress in the other two midlife examinations (i.e. in 1974-75 and 1980-81) were not significantly associated with late-life CSF measures (data not shown).

Among women who participated in all three midlife examinations (n=79), we tested whether a dose–response relationship could be observed, *i.e.*, if the time-period/duration when women were exposed for stress had any importance to levels of CSF markers (Table 3). Most women in the combined sample did not report stress at any of the midlife examinations (n=48, 59%), whereas there were smaller groups in which stress was reported at one examination (n=20, 25%) or two-three examinations (n=11, 14%). In a linear regression model, it was found that CSF VILIP-1 increased with the number of examinations when stress was reported (age adjusted B=XX, p= .033). There was no such dose-response relationship for CSF MBP or CSF NF-L (Table 3).

To minimize the influence of incipient dementia on the association between stress and neurodegenerative CSF biomarkers, we re-analysed the data excluding women with dementia onset before 1992 (n=2). This did not change the association between stress and CSF biomarkers (data not shown).

DISCUSSION

In a sample of women followed over 25 years, we found that longstanding midlife stress was associated with higher levels of the neurodegenerative CSF biomarkers (VILIP-1 and MBP)

in late-life. There was also a trend observed for higher levels of CSF NF-L. In addition, there was a positive correlation between levels of CSF VILIP-1 and the number of examinations when stress was reported. The findings suggest that longstanding psychological stress may injure the brain.

To our knowledge, this is the first study to show that psychological stress might influence these three CSF biomarkers. The findings shed new light on the association of stress with neurodegeneration and dementia disorders. Earlier studies have found associations between longstanding stress and accumulation of neurofibrillary tangles in post-mortem brains (Terracciano *et al.*, 2013) and higher levels of CSF tau proteins (johansson 2018). The tau pathology has a key role in synaptic dysfunction and neuronal death, due to destructed cytoskeleton, in AD and other neurodegenerative disorders (Iqbal *et al.*, 2010). Brain imaging studies have found that severe and longstanding stress may lead to structural brain changes and atrophy, in, *e.g.*, the hippocampus area (McEwen, 2000), in temporal lobe (Johansson *et al.*, 2012), and in the white matter (Johansson *et al.*, 2012). A number of studies have also found that longstanding psychological stress, stress-disorders (e.g. PTSD), numbers of stressors, and stress-prone personality increase the risk of dementia disorders (Johansson *et al.*, 2010; Johansson *et al.*, 2013; Low *et al.*, 2013; Greenberg *et al.*, 2014; Johansson *et al.*, 2014).

It is well known that severe psychological stress can be seen as increased levels of, *e.g.*, corticosteroids, catecholamines and inflammatory proteins (Lupien *et al.*, 2018). For example, internal and external stress triggers the activation of the hypothalamic-pituitary-adrenal (HPA) axis and elevates levels of glucocorticoid. High levels of glucocorticoids over an extended period of time can cause neuronal damage in hippocampus (McEwen, 2000), a brain structure that is particularly vulnerable to AD pathology (Henneman *et al.*, 2009). The long-term biological mechanisms of stress, glucocorticoids and neurodegeneration are complex and not much studied. Neurodegeneration is a progressive loss of neurons in the cerebral cortex and subcortical regions, which results in loss of brain tissue and brain atrophy (Zetterberg *et al.*, 2016).

To gain new insights into the molecular pathology of stress, several pathophysiological pathways can be explored. VILIP-1 is a neuronal calcium sensor protein that is abundantly expressed in the brain, and plays a functional role in integrating the cytosolic calcium

concentration and the oxidative status of the cell (Liebl *et al.*, 2014). The protein has been demonstrated as a marker of neuronal injury and can be excessively released because of neuronal degradation (Lee *et al.*, 2008). High CSF VILIP-1 correlates with both regional and whole-brain atrophy (Tarawneh *et al.*, 2015), ischemic stroke (Laterza *et al.*, 2006), and AD pathology (Tarawneh *et al.*, 2012; Fagan *et al.*, 2014). Different pathological features, where VILIP-1 may reflect the end result of the disease, *i.e.*, neuronal cell death (Lee *et al.*, 2008). In this study, analyses indicate that stress had a dose-response relationship to increased levels of CSF VILIP-1. According to our knowledge, no other study have found similar association.

Psychological stress in midlife was also associated with higher levels of CSF MBP, a sign of myelin disintegration that may lead to neuronal damage (Bjerke *et al.*, 2011). MBP is a main component of the myelin membrane and important to the process of myelination of neurons (Zhan *et al.*, 2018). The protein is highly expressed in some specific brain areas, *e.g.*, in the hippocampus area (ref). During chronic demyelinating conditions, MBP precipitate and aggregate, and outside the membrane have the aggregated protein damaging effect on the neighboring neurons (Zhan *et al.*, 2018). Increased concentrations of CSF MBP are seen in, *e.g.*, demyelinating diseases (Romme Christensen *et al.*, 2013), white matter degeneration (Omlin *et al.*, 1980), and after subcortical infarcts (Brouns *et al.*, 2010). Destructed myelin has been suggested as an important component of the amyloid plaque in AD (Zhan *et al.*, 2018).

CSF NF-L was not significantly higher in persons with midlife stress, but there was a trend in that direction (p .056). Neurofilaments are major structural proteins in the neuronal cytoskeleton and play a central role in controlling axonal caliber, maturation of regenerating myelinated axons, and growth of dendrites (Zetterberg *et al.*, 2016). Increased CSF NF-L is a marker of damage to large-caliber myelinated axons and can be used to monitor general neurodegeneration (Zetterberg *et al.*, 2016). Elevated CSF NF-L has been seen in cerebral disorders such as white matter lesions (Sjogren *et al.*, 2001) and AD (Zetterberg *et al.*, 2016), but do not correlate very well with levels of Aβ42, indicating that the changes are not primarily driven by amyloid pathology (Zetterberg *et al.*, 2016).

Some limitations and methodological issues need to be considered, according to this study. First, psychological stress was based on a subjective personal response to a single question. Our question on stress has however been used in several previous studies and reflects the

number of life-stressors (Johansson *et al.*, 2013) and high neuroticism (Johansson *et al.*, 2014). Second, all analyses were conducted in a relatively small sample, resulting in low statistical power. The results may therefore be underpowered to detect small differences between groups. Further, the ability to adjust for possible confounders was limited. Third, cumulative attrition is a problem in long follow-up studies, and the participation in the LP study was only 10% of the eligible sample. However, participants and non-participants were similar in a number of variables. Fourth, prolonged stress may predispose individuals to a number of physiological and psychiatric disorders. Due to the small sample size, there were limited opportunities for controlling for possible confounders. Finally, as CSF biomarkers were measured in late-life, we cannot exclude the possibility of survival effects.

Conclusion

Stress has been suggested to be a risk factor for neurodegenerative disorders, such as AD. There is a lack of knowledge about the underlying molecular mechanisms. In this study, we found that longstanding midlife stress was associated with late-life neurodegenerative biomarkers in CSF. The findings suggest that stress might stimulate neural damaging processes in brain, which reflect neuronal death rather than specific markers of disease pathogenesis.

Conflict of interests

HZ and KB are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics and Wave, and has received travel support from Teva.

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Table 1 Characteristics of the study participants (n=81)

Total sample	Mean±SD or n (% of total sample)
Age at baseline, years	48.8±3.4
Psychological stress	
Baseline	20 (24.7)
1st follow-up, 6 years after baseline	18 (22.2)
2nd follow-up, 12 years after baseline [a]	12 (14.8)
CSF biomarkers [b]	
VILIP-1, xx	13.4±5.4
MBP, xx	0.99±0.28
NF-L, ng/l	324.7±243.8
Depression [c]	10 (12.2)[d]
Cerebrovascular disorder [d]	7 (8.5)[f]
MMSE [b]	28.4±1.4[e]

CSF= cerebrospinal fluid; VILIP-1= visinin-like protein-1; MBP, myelin basic protein; NF-L=neurofilament light; MMSE=mini mental state examination
a Two persons with missing data, n=79
b Measured in 1992-94 examination
c Baseline depression, measured in a sub-sample with psychiatric data (n=53)
d Stroke and/or TIA between 68-94

Table 2 Associations between midlife stress (BASELINE) and late-life CSF biomarkers, in a population sample of women, followed over 25 years (n=81)

	No stress	Psychological stress						
	N=61 (75.3%)	N=20 (24.7%)	**Model 1**			**Model 2**		
	mean±SD	mean±SD	β-Coefficient	95% CI	*p*-value	β-Coefficient	95% CI	*p*-value
VILIP-1 (pg/mL)	12.3 ± 4.7	16.5±6.3	0.59	0.10, 1.07	**0.019**			
MBP ()	0.95±0.25	1.12±0.32	0.06	0.003, 0.116	**0.039**			
NFL (pg/mL)	291.8±213.4	425.1±304.2	0.49	-0.01, 0.99	0.056			

CSF= cerebrospinal fluid; VILIP-1= visinin-like protein-1; MBP, myelin basic protein; NF-L=neurofilament light
Psychological stress measured in 1968-69, CSF biomarkers measured in 1993-94, Model 1=Adjusted for age, Model 2= Adjusted for age, depression, cerebrovascular disorder, and MMSE

Table 3 Associations between midlife stress (BASELINE) and late-life CSF biomarkers, in a population sample of women, followed over 25 years **(n=79)**

	Never stress,	Stress in 1 examination	Stress in 2-3 examinations						
	n=48 (59.3%)	n=20 (24.7%)	n=11 (13.6%)	**Model 1**			**Model 2**		
	mean±SD	mean±SD	mean±SD	β-Coefficient	95% CI	*p*-value	β-Coefficient	95% CI	*p*-value
VILIP-1 (pg/mL)	12.2±4.9	12.6±5.4	15.5±6.8	0.061	0.005-0.118	**0.033**			
MBP ()	0.97±0.26	1.03±0.35	0.99±0.23	0.008	-0.027-0.043	0.643			
NFL (pg/mL)	312.0±229.8	349.8±285.2	337.1±253.1	0.016	-0.067-0.099	0.704			

CSF= cerebrospinal fluid; VILIP-1= visinin-like protein-1; MBP, myelin basic protein; NF-L=neurofilament light
Psychological stress measured in 1968-69, CSF biomarkers measured in 1993-94
Model 1=Adjusted for age, Model 2= Adjusted for age, depression, cerebrovascular disorder, and MMSE

STATISTIK

NEVER STRESS – STRESS one/two/three

Figure 1 Box plot of xxx levels in the non-stressed and stressed group (n=81)

Figure 2 Box plot