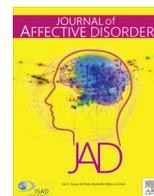




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Research paper

The CD44 ligand hyaluronic acid is elevated in the cerebrospinal fluid of suicide attempters and is associated with increased blood–brain barrier permeability

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ABSTRACT

Background: The glycosaminoglycan hyaluronic acid (HA) is an important component of the extracellular matrix (ECM) in the brain. CD44 is a cell adhesion molecule that binds to HA in the ECM and is present on astrocytes, microglia and certain neurons. Cell adhesion molecules have been reported to be involved in anxiety and mood disorders. CD44 levels are decreased in the cerebrospinal fluid (CSF) of depressed individuals, and the CD44 gene has been identified in brain GWAS studies as a possible risk gene for suicidal behavior.

Method: We measured the CSF levels of HA and the soluble CD44 (sCD44) in suicide attempters ($n=94$) and in healthy controls ($n=45$) using ELISA and electrochemiluminescence assays. We also investigated other proteins known to interact with CD44, such as osteopontin and the matrix metalloproteinases MMP1, MMP3 and MMP9.

Results: The suicide attempters had higher CSF levels of HA ($p=.003$) and MMP9 ($p=.004$). The CSF levels of HA correlated with BBB-permeability ($\rho=0.410$, $p<.001$) and MMP9 correlated with sCD44 levels ($\rho=0.260$, $p=.005$).

Limitations: Other relevant biological contributors to suicidal behavior is not addressed in parallel to the specific role of CD44-HA signaling. The gender distribution of the patients from whom CSF was analyzed was uneven.

Conclusions: Increased BBB-permeability and HA levels might be a results of increased neuroinflammation and can play a role in the pathobiology of suicidal behavior. The CD44 signaling pathway might be considered a novel target for intervention in mood disorders.

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1. Introduction

A large part of the brain volume consists of the extracellular matrix (ECM) and cell-to-ECM adhesions are important for highly coordinated brain functions such as memory and learning (Dityatev et al., 2010). Cell adhesion molecules have recently been described to be involved in anxiety and mood disorders (Sandi, 2004; Sandi and Bisaz, 2007). In addition, accumulating animal

studies indicate that adhesion molecules play a role in the response to stress (Bisaz and Sandi, 2012; Chocyk et al., 2010; Gilbert-Juan et al., 2011; Redwine et al., 2003; Sandi and Bisaz, 2007). Anxiety, mood disorders and exposure to stressful life events are all substantial risk factors contributing to suicidal behavior (Brown et al., 2000; Moscicki, 1997). Interestingly, Gene Ontology analyses in suicide victims has shown that genes involved in the expression of cell adhesion proteins, like CD44, may indeed be risk genes for suicidal behavior (Galfalvy et al., 2011; Thalmeier et al., 2008).

In the brain, the formation and structure of the ECM is dependent on the glycosaminoglycan hyaluronic acid (HA) (Bignami et al., 1993). The main receptor for HA is CD44, a cell surface adhesion molecule found on most mammalian cells (Goodison et al.,

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1999) including neurons (Glezer et al., 2009), astrocytes (Kaaijk et al., 1997) and microglia (Jones et al., 2000). CD44 has roles in regulating the inflammatory response; for example, CD44 signal transduction leads to increased inflammatory gene expression in leukocytes and in parenchymal cells (Pure and Cuff, 2001). CD44 also interacts with other ligands, such as osteopontin (OPN) and matrix metalloproteinases (MMPs). For example, during the early stage of brain damage in rats, OPN is upregulated by activated microglia and interacts with CD44 to mediate cell migration (Shin et al., 2005). CD44 is also known to act as a docking receptor to MMPs and thereby concentrates the proteolytic effect close to cell membranes (Yu and Stamenkovic, 2000). The HA and CD44 expression in brain are elevated during fetal development and during the development and migration of tumor cells (Misra et al., 2011; Naruse et al., 2013; Stylli et al., 2000), but the function of CD44-HA signaling in the intact adult brain is unclear. Interestingly, CD44 gene expression was found to be lower in dorsal lateral prefrontal cortex (Brodmann area 9) and anterior cingulate cortex (Brodmann area 24) of suicide attempters compared to controls (Galfalvy et al., 2011), and the cerebrospinal fluid (CSF) CD44 protein was found to be reduced in patients with a depressive episode compared to controls (Ditzen et al., 2012).

Activation of the immune system has been suggested to contribute to the development of suicidal behavior (Serafini et al., 2013). We have previously shown evidence of inflammation in patients who attempted suicide recently. The suicide attempters exhibited higher CSF IL-6 levels and higher plasma IL-6 and TNF- α levels than healthy controls (Janelidze et al., 2011; Lindqvist et al., 2009). We also found signs of increased blood–brain barrier (BBB) permeability expressed as increased CSF/serum albumin ratio (Bayard-Burfield et al., 1996). Inflammatory processes are known to disrupt BBB integrity (Abbott, 2000; Michel and Curry, 1999) and recent studies in CD44 knockout (CD44KO) mice report increased BBB permeability compared to wild-type mice (Flynn et al., 2013a; Flynn et al., 2013b). We sought to investigate the relevance of CD44-HA signaling for suicidal behavior and corresponding alterations in BBB-permeability. We hypothesized that inflammation-related low expression of CD44 and altered CD44-HA signaling are linked to dysfunction of the BBB. Therefore, to evaluate the potential importance of CD44-HA signaling in the pathophysiology of suicidal behavior, we measured the CSF levels of HA and the soluble CD44 (sCD44) in suicide attempters ($n=94$) compared to healthy controls ($n=45$). We also investigated other proteins known to interact with CD44, such as OPN and MMPs.

2. Materials/subjects and methods

2.1. Study participants

Suicide attempters ($n=94$) were enrolled at admission to the Lund University Hospital after fulfillment of the criteria of Beck et al. (Resnik et al., 1973). The suicidal intent of the patients was explicit upon clinical interview. All patients gave informed consent to participate and the studies were approved by the Lund University Medical Ethics Committee. The demographic data of all participants are described in Table 1. Patients did not receive any psychotropic medication during a washout period of 14 ± 6 (mean \pm SD) days after the suicide attempt, except for occasional doses of benzodiazepines. Other ongoing medications are listed in Table 2. After the washout, CSF was drawn using a standardized protocol as previously described (Traskman-Bendz et al., 1992). Healthy controls ($n=45$), without physical or psychiatric diagnoses, were recruited at the University Hospitals in Lund and Linköping, Sweden. Psychiatric diagnoses of the suicide attempters were established by an interview conducted by a senior

Table 1

Demographic data (mean \pm SD) for suicide attempters and healthy controls. There was a significant difference in age between Suicide Attempters and Healthy Controls (Student's t -test, $p < .05$) and the distribution of gender was biased (Pearson's chi-squared test, $p < .001$).

	Suicide attempters ($n=94$)	Healthy control ($n=45$)	p
Age, Years	36.04 \pm 12.00	30.56 \pm 12.28	.015
Gender, male / female	47/47	37/8	< .001
BMI, Kg/m ²	23.55 \pm 3.70 ^a	23.79 \pm 3.23	.703

^a BMI data missing in three patients.

Table 2

Medications for suicide attempters and healthy controls. Suicide attempters on any psychotropic medication (except for occasional doses of benzodiazepines or benzodiazepine-like drugs) underwent a washout period before the lumbar punctures.

Medication	Suicide attempters	Healthy controls
Analgesic	2	1
Antihistamine	0	1
Benzodiazepine-like drug	4	0
Benzodiazepine	31	0
Contraception	3	5
Glucocorticoid	0	1
Naturopathic drug	1	8
Non-Steroidal Anti Inflammatory drug	2	1

psychiatrist according to the DSM-III-R and DSM-IV criteria. The severity of the symptoms was assessed by the Comprehensive Psychopathological Rating Scale (CPRS) (Asberg et al., 1978), the Suicide Assessment Scale (SUAS) (Stanley et al., 1986), as well as the Montgomery–Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) and Brief Scale of Anxiety (BSA) (Tyrer et al., 1984) as derived from the CPRS. All participants underwent a physical examination in concurrence with the lumbar puncture. Psychiatric diagnoses (Axis I in DSM-IV) and somatic conditions of suicide attempters are listed in Table 3.

2.2. Analyte measurements

OPN, MMP9, MMP3, and MMP1 were assessed by an electrochemiluminescence-based immunoassay (MSD, Rockville, MD, US). HA and sCD44 levels were measured by an ELISA (R&D

Table 3

Psychiatric and somatic diagnoses of the suicide attempters. The controls were thoroughly checked for psychiatric comorbidity by an evaluating senior psychiatrist and with the structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Axis I diagnoses of the suicide attempters were established at an interview by a senior psychiatrist according to the DSM-III-R and DSM-IV criteria (codes in brackets).

Psychiatric Diagnosis (Axis I)	Suicide attempters	Somatic diagnosis	Suicide attempters
Adjustment disorder (309)	17	Anemia	1
Anxiety disorder (300)	3	Chronic headache	1
Depression NOS (312)	12	Diabetes	1
Dysthymia (300.4)	9	Gastritis	3
Major Depression disorder (296)	27	Irritable bowel syndrome	1
Psychotic disorder (295)	5	Migraine	2
Substance dependence/abuse (303–305)	6	Sciatica	1
Other	5		
No Diagnosis	10		

Table 4

The characteristics of the analyte measurements.

	Sample dilution	Detection limit (pg/mL)	Mean intra-assay CV%-values (Between duplicates) (%)
HA	10	370	2.36
sCD44	10	20	5.72
Osteopontin	10	17.8	4.27
MMP9	1	12	10.78
MMP3	1	9	7.10
MMP1	1	1	11.34

Systems Europe, Abingdon, England and eBioscience, San Diego, CA, US, respectively). All assays were conducted according to manufacturer's protocol. All samples were assayed in the same run to avoid inter-assay variability. The levels of all analytes were above the detection limits. CSF/serum albumin ratio (Chen, 2011) was reported in our previous study (Bayard-Burfield et al., 1996). Sample dilutions and intra-assay CV% values are depicted in Table 4.

2.3. Statistical analysis

The statistical analysis was conducted using SPSS Statistics 21 (IBM, Armonk, New York, US). Analysis of co-variance (ANCOVA) was used to compare means between healthy controls and suicide attempters controlling for age and gender. The variables were checked for the assumptions of normality, homogeneity of variance and homogeneity of regression slopes. Linear Regression with HA levels as outcome variable was used. Non-parametric tests were used (Mann–Whitney *U* test) to compare means of MMP9 due to non-parametrical distribution. Correlations were analyzed with Spearman's rho. For the correlation between CSF/serum albumin ratio and HA additional Pearson product-moment correlation coefficient was calculated as well.

3. Results

3.1. HA, sCD44, MMP1, MMP3, MMP9 and OPN levels in CSF of suicide attempters

There was a significant correlation between age and sCD44 ($r=.314$), HA ($r=.425$) and OPN ($r=.346$), $p < .001$ for all. There was a trend towards gender effects of HA ($p=0.086$); such gender

effects have been reported previously for HA (Nielsen et al., 2012). There were no relationships between the analytes and BMI values.

The levels of HA were significantly higher (+49.6%, adjusted for the effect of age and gender) in suicide attempters ($n=94$) than in healthy controls ($n=45$), Fig. 1A (ANCOVA, $p=.003$). Suicide attempters also had significantly higher levels (mean \pm SD) of sCD44, 39.69 ± 19.72 pg/mL compared to 33.49 ± 11.94 pg/mL, but this significance was lost after adjusting for age. There was a positive correlation between sCD44 and HA levels in both suicide attempters and healthy controls, (Spearman's rho=.323, $p < .001$, $n=139$, Fig. 2B). A linear model of predictors of HA levels is depicted in Table 5.

The suicide attempters ($n=86$) had significantly higher CSF levels of MMP9 (mean \pm SD) compared to the healthy controls ($n=30$), Figure 1C. (135.87 ± 107.47 pg/mL vs. 94.43 ± 74.76 , Mann–Whitney *U* test $p=.004$). The CSF levels of MMP9 correlated with the levels of sCD44, Spearman's rho=.0260, $p=.005$, $n=116$, Fig. 2B. There were no differences in the CSF MMP3, MMP1 or OPN levels between suicide attempters and controls. Comparisons of a subgroup suicide attempters, MDD patients ($n=27$) and non-MDD patients ($n=67$), to healthy controls, did not reveal any significant differences in mean values of any of the analytes (data not shown).

3.2. Associations between analytes and blood–brain permeability

To further investigate the source of CD44-ligands in the CSF, the relationship between the analytes and the blood–brain barrier (BBB) permeability, measured as CSF/serum albumin ratio, was performed. There was a correlation between CSF HA levels (rho=.0410, $p < .001$) and the BBB permeability (Fig. 2A) as well as CSF sCD44 and the BBB permeability (Spearman's rho=.0241, $p=.033$), Fig. 2B.

3.3. Associations between sCD44, HA and psychiatric rating scales

There was no significant correlation between any of the analytes and total scores of Suicide Assessment Scale (SUAS), Montgomery–Asberg Depression Rating Scale (MADRS) and the Brief Scale of Anxiety (BSA) without gender or age stratification.

After gender stratification, there was a positive correlation between HA and total scores of SUAS in women (Spearman's rho=.327, $p=.037$, $n=41$).

HA levels correlated positively with age, therefore two subgroups were stratified based on the median age of the suicide attempters. The younger population of suicide attempters (< 37

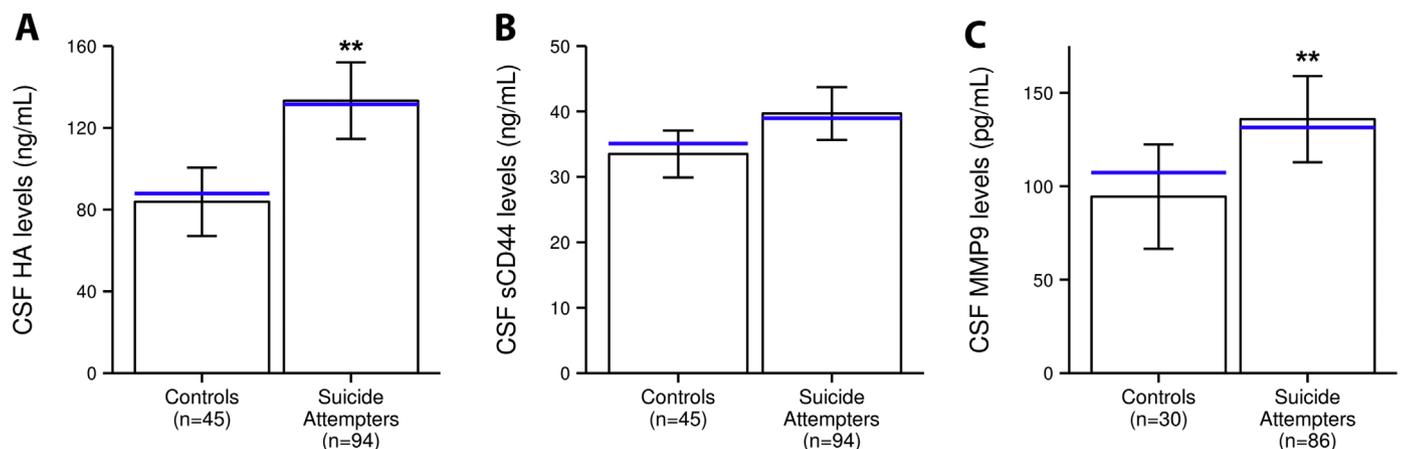


Fig. 1. (A–C) Mean values of CSF hyaluronic acid (HA), sCD44 and matrix metalloproteinase-9 (MMP9) levels of suicide attempters and healthy controls. There were significantly higher levels of HA in CSF of suicide attempters compared to healthy controls after adjusting for age and gender (ANCOVA, $F_{(1, 135)}=8.94$, $p=.003$). The levels of MMP9 were significantly higher in suicide attempters compared to healthy controls, Mann–Whitney *U* test, $p=.004$. The blue lines represent the adjusted mean (ANCOVA) levels (age and gender when appropriate). Error bars: 95% CI (raw uncorrected data), ** $p < .01$.

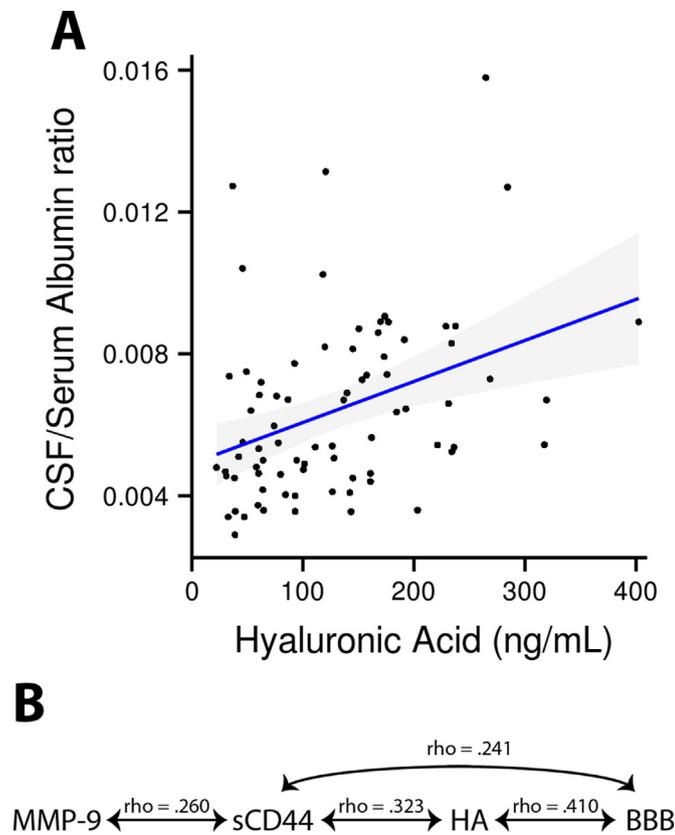


Fig. 2. (A) Association between CSF hyaluronic acid levels and the CSF/serum albumin ratio as a measure of BBB integrity. CSF/serum albumin ratio correlated with the CSF hyaluronic acid levels (Pearson's $r=0.376$, $p=.001$). The shaded area around the regression line is the 95% CI around the line. (B) Significant ($p < .05$) associations between the analytes measured in the CSF of suicide attempters and controls (Spearman's rho). BBB=Blood-Brain Barrier permeability as measured by CSF/serum albumin ratio.

Table 5
Linear model (blockwise entry) of predictors of HA levels in CSF.

	B	SE B	Beta	P
Step 1				
Constant	133.30	8.43		<.001
Group (Control or Suicide Attempter)	-49.47	14.81	-.27	.001
Step 2				
Constant	38.14	20.74		.068
Group (Control or Suicide Attempter)	-34.99	14.00	-.19	.014
Age	2.64	.53	.38	<.001
Step 3				
Constant	52.39	21.86		.018
Group (Control or Suicide Attempter)	-43.56	14.57	-.24	.003
Age	2.61	.53	.38	<.001
Gender (male/female)	-26.02	13.64	-.15	.059
Step 4				
Constant	19.00	26.37		.47
Group (Control or Suicide Attempter)	-40.29	14.27	-.22	.005
Age	2.00	.55	.29	<.001
Gender (male/female)	-30.19	13.15	-.18	.02
sCD44	1.31	.39	.28	.001
OPN	.08	.40	.08	.833

years) with high HA levels (> 118 ng/ml, median value) ($n=12$) revealed significantly higher SUAS scores (33.8 ± 11.0 vs. 23.6 ± 15.0 points [mean \pm SD], $p=.040$) and MADRS scores (24.3 ± 8.4 vs. 16.4 ± 9.7 points, $p=.017$) compared to the other young suicide attempters (low age and low HA levels, $n=32$).

4. Discussion

In the current study, we found that suicide attempters had higher CSF levels of HA and MMP9 than healthy controls. Suicide attempters also had higher levels of sCD44, but this significance was lost after adjusting for age. Since a previous GWAS study, conducted with post-mortem brains of suicide victims, identified CD44 as a candidate gene (Galfalvy et al., 2011), our study supports the notion that CD44-HA signaling might play a role in the pathobiology of suicidal behavior.

Increased HA levels in CSF have previously been reported in patients with CNS tumors (Laurent et al., 1996), meningitis (Laurent et al., 1996), spinal disorders (Sakayama et al., 2006), cerebral ischemia (Wang et al., 2002) and dementia (Nagga et al., 2014; Nielsen et al., 2012). In the studies of dementia patients, the levels of HA strongly correlated with the CSF/serum albumin ratio, a measure of blood-brain barrier (BBB) permeability (Ganrot and Laurell, 1974). The association between CSF HA levels and BBB permeability was replicated in our study. Increased levels of CSF HA might thus be a result of a general increase of proteins in the CSF, due to leakage from the blood to the CSF. However, HA is a large molecule, ranging from 5 to 20,000 kDa, and the normal CSF HA levels are usually many times higher than the plasma levels (Engstrom-Laurent et al., 1985; Laurent et al., 1996; Nielsen et al., 2012). Therefore, it is unlikely that the increased CSF HA levels are a result of leakage of HA from the blood. This is supported by the fact that no correlation between plasma and CSF HA levels were found in a study in neurodegenerative dementia (Nielsen et al., 2012). Instead, increased CSF HA levels and increased BBB permeability might be the result of the same underlying mechanism.

Dysfunction of the BBB has been suggested as a contributor to the development of psychiatric disorders (Shalev et al., 2009) and the HA receptor CD44 might have a direct role in maintaining the BBB integrity. A recent study in CD44 knockout mice reported increased BBB permeability in an experimental autoimmune encephalomyelitis model (Flynn et al., 2013b). Another study reported increased histamine-induced BBB permeability in CD44KO compared to wild-type mice (Flynn et al., 2013a). We could not find statistically significant differences in mean values of sCD44 levels, possibly due to insufficient sample size; still it is not known whether sCD44 levels are correlated with the amount of membrane-bound CD44 receptors or CD44 gene expression. Future studies are warranted to investigate the expression of membrane-bound CD44 receptors in the brain of suicide victims. However, mice lacking CD44 do not compensate by upregulating other HA receptors such as RHAMM (Receptor for Hyaluronan Mediated Motility), but rather have increased levels of HA in blood and tissues (Inoue et al., 2009); unfortunately, it is unknown whether a reduction in CD44 is associated with a compensatory increase in the levels of HA in CSF. Nevertheless, acute stress also increases HA levels in the tissues and blood of mice (Inoue et al., 2009). Therefore, we suggest that the increased levels of HA and increased BBB permeability in suicide attempters might be a compensatory response to altered number of membrane-bound CD44 molecules. This is supported by a pilot post-mortem study, showing lower expression of CD44 in the brain of suicide victims (Galfalvy et al., 2011).

The CSF used in this study was collected from a cohort of suicide attempters who have previously been reported to have increased inflammation markers compared to healthy controls, i.e. higher levels of the proinflammatory cytokine IL-6 (Lindqvist et al., 2009). Previous studies have suggested that inflammation might be the cause of increased levels of HA in CSF (Sakayama et al., 2006). In blood vessels, the endothelial glycocalyx contribute to the BBB integrity and HA is essential for the function of the glycocalyx (Gao and Lipowsky, 2010). Inflammatory processes open

up the BBB to support the migration of reactive cells into the brain (Abbott, 2000; Michel and Curry, 1999) and it has been suggested that HA levels in CSF increase when the BBB glycocalyx is degraded (Nagga et al., 2014). In this study, CSF HA levels correlated positively with an indicator of increased BBB permeability, and a previous study on the same patient cohort, showed amplified BBB permeability in suicide attempters compared to controls (Bayard-Burfield et al., 1996). Augmented inflammation also increases the levels of MMPs (Rosenberg, 2002) which are associated with BBB opening. For example, MMP9 contribute to BBB disruption and subsequent brain edema following traumatic brain injury (Shigemori et al., 2006). MMPs are also involved in both shedding of membrane-bound CD44 molecule and in modulation of the glycocalyx (Kajita et al., 2001; Lipowsky, 2011; Okamoto et al., 1999). MMP-9 is concentrated adjacent to cell membranes through its binding of adhesion molecules, including CD44 (Bourguignon et al., 2007). Our data indicate that CSF MMP9 levels correlate with sCD44 levels, which is consistent with the capacity of MMP9 to cleave CD44 from cell membrane (Chetty et al., 2012). Furthermore, the CSF sCD44 levels correlated significantly with the levels of CSF HA. CD44-HA signaling acts as a regulator of immune response (Jiang et al., 2011), thus the elevated levels of CSF HA and MMP9 in the suicide attempters might reflect increased activity of inflammatory processes in the brain, resulting in increased BBB permeability and presence of abnormal number of membrane-bound CD44 molecules.

In studies of inflammation in the central nervous system, pro-inflammatory cytokines have usually been measured. However, the use of cytokines as a biomarker has some disadvantages. Many cytokines are pleiotropic and due to their local mode of action, involving paracrine or autocrine processes, cytokines circulate at very low levels, often below or near the detection limit of the majority of assays (Bienvenu et al., 2000). There are also many other confounders, which could affect the levels of cytokines. For example, correlations with cytokines and BMI, age, smoking, sex, and physical activity have been reported (Haack et al., 1999; Himmerich et al., 2009). In this study, we hypothesized that increased levels of hyaluronic acid (HA) is an effect of increased BBB permeability and low-grade inflammation. In patients where increased inflammation might be a factor contributing to suicidal behavior, an add-on treatment with anti-inflammatory drugs to conventional anti-depressive treatments might be beneficial. Even though the HA levels correlated with age and possibly differs between men and women. The fact the levels are in thousand-fold higher concentration than cytokines, very little CSF is needed to get a robust quantification. Since specifically low molecular weight HA is associated with inflammation (Jiang et al., 2011), future studies investigating the potential of low molecular weight HA as a biomarker of low grade inflammation and of finding candidates for add-on treatment with anti-inflammatory drugs is warranted.

There are some limitations to our study. The gender distribution of the patients from whom CSF was analyzed was uneven, although we did not find any gender-dependent differences in the relevant biological measures. Another limitation is the use of an exploratory approach and the examination of the specific role of CD44-HA signaling in the development of suicidal behavior without addressing the putative role of other relevant contributors, like CSF markers of inflammation and monoamines.

In summary, the current study demonstrates that increased CSF HA and MMP9 levels and increased BBB-permeability may be implicated in the pathobiological mechanisms underpinning suicidal behavior. Future studies should focus on CD44-HA signaling components as putative biomarkers for mental distress and suicidality, as previous studies have shown that levels of certain immune mediators may discriminate between suicide attempters and controls (Janelidze et al., 2011; Lindqvist et al., 2011). In

addition, the CD44 signaling pathway may be considered a novel target for intervention in stress-related mood disorders.

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