INTRODUCTION

Human saliva contains a variety of molecules that may be used as biomarkers for oral conditions and systemic health conditions. Recently, several neuropeptides were successfully detected and analysed in whole and glandular saliva from healthy individuals. Neuropeptides are small molecules that are released from neural cells and are commonly used by neurons to communicate with each other. The study aimed to investigate salivary levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), substance P (SP) and glutamate at five time points from morning to afternoon in a well-characterised healthy and pain-free individuals. Ten young adults were included. Unstimulated and stimulated whole saliva were collected from each participant repeatedly across the day. Blood samples were drawn in connection with the first and last saliva sample as reference standard. Levels of NGF and BDNF were determined using gel-free Western blot technology, glutamate levels were analysed using a colorimetric assay, and SP was determined using a commercially available ELISA. Salivary NGF and BDNF showed significant differences between the different collection times in both unstimulated (NGF; \( P = .006 \); BDNF; \( P = .026 \)) and stimulated whole saliva (NGF; \( P = .006 \); BDNF; \( P = .019 \)). The highest concentrations of the neuropeptides were expressed in the early morning, and they thereafter decreased across the day. In contrast, the expression of salivary glutamate and SP did not show any significant changes across the day. Plasma levels of NGF were higher in the evening sample (\( P = .028 \)); otherwise, there were no significant differences for any of the other markers between morning and evening samples. NGF and BDNF in whole saliva showed a significant variation across the day. On the contrary, no variation in the levels of SP and glutamate was detected. These findings highlight the importance of consistency in the collection time and approach in biomarker studies using saliva.
other. Several neuropeptides are co-released with other neurotransmitters. Besides their main function as neurotransmitters, neuropeptides have a variety of other significant functions.

The neuropeptide brain-derived neurotrophic factor (BDNF) promotes neurogenesis and neural plasticity during brain development and adulthood. Studies have shown an altered production and secretion of peripheral BDNF in a variety of diseases. Neurodegenerative disorders, such as Alzheimer's and Parkinson's, as well as depression appear to be associated with decreased levels of BDNF. In addition, BDNF has also been recognised as an important modulator of nociceptive pathways. A recent study found that serum BDNF is significantly elevated in patients with migraine attacks. Animal studies have indicated a diurnal endogenous cyclical change in expression of the BDNF receptor in central areas of the brain.

The first evidence of the presence of diurnal BDNF expression in human circulation emerged a decade ago. BDNF was shown to peak in the morning with a trend of constant decrease during the day. These findings have also been confirmed by later studies. Animal studies have investigated the NGF secretion across the day in stimulated and unstimulated saliva of healthy individuals. Nerve growth factor (NGF) is another neuropeptide that has been quantified in saliva. The release of NGF in the brain and periphery promotes neural growth and plasticity, in addition to regulation of endocrine and immune cell activity. It has been suggested that NGF plays an important role in hyperalgesia as the concentration of the peptide has been found to be increased in many clinical pain conditions, such as rheumatoid arthritis, chronic headache and cancer pain. Besides pain conditions, NGF has also been assumed in behavioural changes and neuropsychiatric disorders affected by the endocrine mechanism. Several studies have addressed NGF concentration in saliva and plasma but no study has investigated the NGF secretion across the day in stimulated and unstimulated saliva of healthy individuals.

Other neurotransmitters that have been discussed in pain processing are the amino-acid glutamate and the neuropeptide substance P (SP). They are present in both central and peripheral nerve terminals and released in response to noxious stimulus, and as such have been implicated in different pain conditions. Salivary glutamate and SP have mainly been analysed earlier in patients with ongoing pain. There are, however, no earlier studies on the periodicity of either glutamate or SP in healthy individuals.

Salivary glands are integrated into the neuroendocrine system through complex regulatory pathways. The accurate determination of neuropeptides and other neurotransmitters in saliva may therefore be of great interest and provide valuable information for research into pathophysiological mechanism of neurodegenerative diseases or chronic pain conditions by non-invasive sampling methods. Since there is lack of knowledge regarding the influence of sampling time on the expression of many biomarkers in saliva, the present study aimed to investigate whether the levels of NGF, BDNF, SP and glutamate in stimulated and unstimulated saliva of healthy participants differ between samples collected at different time points. Based on previous results, we expected that the salivary concentration of these neurotransmitters would show variation across the day and that this variation would differ between stimulated and unstimulated saliva.

2 | MATERIALS AND METHODS

2.1 | Participants

Ten healthy participants, with a mean (SD) age of 26.3 ± 3.1 years, were included in the study.

Inclusion criteria were good general health, age ≥ 18 years and body mass index < 30 kg/m². Participants had also to be free of fever/or cold and maintain exceptional oral hygiene on the day of collection.

Exclusion criteria were (a) any current pain, (b) diagnosed systemic musculoskeletal or joint diseases, such as fibromyalgia and rheumatoid arthritis, (c) whiplash-associated disorder, (d) neurological or neuropsychiatric disorders, (e) pregnancy or lactation, (f) high blood pressure, (g) regular use of medications including oral contraceptives, (h) use of antidepressants or analgesics during the last week, (i) oral complaints, such as oral dryness or mucosal lesions, (j) participants with <22 teeth and extensive prosthodontic rehabilitations, (k) poor oral hygiene, hoysalivation, oral diseases, mucosal lesions or extensive dental abrasion and (l) elevated levels of psychological distress.

All participants underwent a careful clinical examination and were asked to fill in validated questionnaires around 1 week prior to the trial as described below. Participants were requested not to eat, drink or brush their teeth at least one hour prior to each saliva collection, and not to consume alcoholic beverages 24 hours prior to collection. They were also instructed to keep a detailed food log 24 hours prior to and during the day of collection. A brief interview was carried out by the examiner (HJ) at the time of collection to ensure that they had followed the instructions, which all had.

All participants received careful information regarding the objectives and procedures of the study and signed an informed written consent form before the start of the study. The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (2014/17-31/3) and followed the guidelines according to the Declaration of Helsinki.

2.2 | Questionnaires and clinical examination

Participants were evaluated by the Swedish version of the Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) axis I and II. The evidence-based protocol was used as a
screening instrument for identification of participants with TMD signs that may not be presented during the interview and general examination. Participants showing clinical signs of TMD (except for disc displacement with reduction, which was considered as normal variation) were excluded from further involvement in the study.

In addition to the DC/TMD examination, the participants underwent an oral examination. During the clinical examination, participants were checked for attrition as a sign of bruxism, decayed teeth, periodontal diseases, pericoronitis, mucosal lesions, oral hygiene and occlusal contacts.

The following brief screening instruments included in the DC/TMD axis II questionnaire were used to measure symptoms of depression, somatisation, anxiety, stress, and jaw function: the Patient Health Questionnaire (PHQ-9 and PHQ-15), the Generalized Anxiety Disorder scale (GAD-7), the Perceived Stress Scale-10 (PSS-10) and the Jaw Functional Limitation Scale (JFLS).

2.3 | Saliva collection

Unstimulated and stimulated whole saliva were collected from all participants. Prior to each saliva collection session, participants were instructed to rinse their mouth with 10 mL of distilled deionised water for 30 seconds to remove debris and moisturise the mucosa.

Unstimulated whole saliva was first collected as described earlier by Jasim et al (2016, 2018). In brief, participants were instructed to sit upright with their head slightly tilted forward and a polypropylene tube was used to collect saliva during passive drooling. Five minutes after sampling of unstimulated saliva, stimulated whole saliva was collected using paraffin gum (Orion Diagnostica). For pre-stimulation, the participants were instructed to chew the gum until it was smooth and flexible. After 60 seconds of pre-stimulation, the participants were asked to swallow the saliva present in the mouth. Subsequently, whole saliva, stimulated by the same piece of paraffin, was collected for around 3 minutes. Total collection time was recorded for both sampling methods, and salivary flow was measured (mL/min).

Saliva samples were collected at five times across the same day during the exact same circumstances. Samples were collected at 7:30, 10:30, 13:30, 16:30 and 19:30 hours. All participants were asked to come fasting to the first sample. Between each session, participants were asked to not eat/drink 1 hour prior to sample collection.

To prevent degradation of sensitive peptides, all samples were collected on ice in pre-cooled polypropylene tubes. Immediately after collection a Protease Inhibitor Cocktail (Sigma Aldrich v/v 1:500) was added. All samples were then centrifuged at 1500 × g for 15 minutes at 4°C to remove debris. The supernatant (upper 2/3) of each sample was fractionated into 100 µL aliquots and frozen at −70°C until analyses.

2.4 | Blood collection

After the first and the last saliva sample, venous blood samples were collected in 8.5 mL EDTA PPT tubes (BD Vacutainer® PPT™, BD) from all subjects. The sample was mixed gently for 1 minute and then immediately placed on ice for maximum 30 minutes. The samples were then centrifuged at 1000 × g for 15 minutes at 4°C, and the upper 2/3 of the plasma was stored as aliquots at −70°C until analysis.

2.5 | Glutamate quantification

The concentration of glutamate was determined essentially as described previously. Briefly, 20 µL saliva or plasma was centrifuged at 4°C for 5 minutes at 12 000 × g. The supernatant was collected and transferred to a new tube, and 5 µL of it was immediately analysed using an ISCUS Analyser (CMA Microdialysis). The detection limit was 1.0-150 µmol/L.

2.6 | Enzyme-linked immunosorbent assay

SP quantitation was performed by using an enzyme-linked immunosorbent assay kit from Enzo Life Sciences (ADI-900-018). Saliva and plasma samples were prepared and analysed according to the manufacturer’s recommendations. The detection limit was 8.04 pg/mL.

2.7 | Capillary isoelectric focusing immunoassay

NGF and BDNF were analysed with a Capillary Isoelectric Focusing (CIEF) immunoassay. Saliva samples were centrifuged to remove debris, and the supernatants were extracted to a new tube. The samples were then diluted/buffer-exchanged with Bicine/Chaps and concentrated and desalted using Amicon® Ultra centrifugal filters (Merck Millipore). Total protein of saliva was measured with 2D-Quant kit according to the user manual (GE Healthcare). The treated saliva samples were analysed using a size assay molecular weight 1-40 kDa using CIEF with Peggy system (ProteinSimple) according to the user manual. A protein concentration of 0.5 mg/mL was used for analysis of both BDNF and NGF. Antibodies for BDNF (Mouse monoclonal, ab10505) and NGF (Rabbit polyclonal, ab6199) were diluted with antibody diluent 1:50 and 1:12.5, respectively (Abcam). All including secondary antibodies were loaded onto a 384-well plate. Proteins were separated in the capillary by applying 250 volts over 42 minutes, UV immobilisation and incubation with primary antibody were 5 and 30 minutes, respectively. Signal was detected with Luminol and Peroxide and scanned with a CCD camera. A high chemiluminescence equals high expression. Negative controls for BDNF and NGF were included in every run. Data generated were analysed in compass software version 3.1.7 (ProteinSimple).
2.8 | Statistics

The normal distribution of the data was tested with Shapiro-Wilk test. Only substances that were detected in more than half of the samples were included in the statistical analysis. Differences in background variables between males and females in the study were tested with the Mann-Whitney U test since most variables did not show normal distribution. Salivary flow showed a normal distribution, why repeated measures analysis of variance (ANOVA) was applied. Bonferroni was used as post hoc test. To analyse the concentrations of neuropeptides in saliva over time, Friedman’s ANOVA was used for each substance separately, since these did not show a normal distribution. When significant, post hoc analysis with Wilcoxon matched pair test was applied with Bonferroni correction for multiple comparison between time points as well as between unstimulated and stimulated saliva levels at the different time points. Wilcoxon test was also used when analysing differences in plasma concentration between morning and afternoon samples. Correlations between saliva and plasma were tested for statistical significance with Spearman correlation test. Descriptive data are presented as mean ± SD or median and interquartile range (IQR). For all analyses, the significance level was set at $P < .05$. The statistical analyses were performed using Statistica version 13 (StatSoft).

3 | RESULTS

3.1 | Descriptive data

Descriptive data for the men and women in the study are presented separately in Table 1. Participants included in the study reported no perceived signs of psychological distress. There were no significant differences between sexes in any background variables ($P > .05$).

**TABLE 1** Background data of age, anthropometric data, psychological distress and jaw functional limitations of the healthy participants in the study ($n = 10$)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Males (n = 5)</th>
<th>Females (n = 5)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>26.1 ± 3.2</td>
<td>26.4 ± 3.4</td>
<td>1.000</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>21.5 ± 2.8</td>
<td>21.6 ± 3.6</td>
<td>1.000</td>
</tr>
<tr>
<td>Number of teeth</td>
<td>30 (1)</td>
<td>32 (0)</td>
<td>.256</td>
</tr>
<tr>
<td>PHQ-9 Score</td>
<td>0 (5)</td>
<td>2 (2)</td>
<td>.671</td>
</tr>
<tr>
<td>PHQ-15 Score</td>
<td>1 (4)</td>
<td>4 (2)</td>
<td>.083</td>
</tr>
<tr>
<td>GAD-7 Score</td>
<td>0 (2)</td>
<td>1 (3)</td>
<td>.449</td>
</tr>
<tr>
<td>PSS-10 Score</td>
<td>8 (3)</td>
<td>7 (6)</td>
<td>.917</td>
</tr>
<tr>
<td>JFLS Score</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>.424</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean (SD) or median (IQR).

Abbreviations: BMI, Body Mass Index; GAD, Generalized Anxiety Disorder; JFLS, Jaw Functional Limitation Scale; n, number of subjects in each group; PHQ, Patient Health Questionnaire; PSS, Perceived Stress Scale.

3.2 | Salivary flow rate

The secretion rate between collection points differed significantly for both stimulated ($F = 6.50; P < .001$) and unstimulated saliva ($F = 6.84; P < .001$) and showed an increasing rate during the day (Table 2). Unstimulated saliva showed higher secretion rate at 19:30 hours compared to the fasting sample at 07:30 hours, while stimulated saliva showed significantly higher flow rate at 16:30 hours compared to 7:30 hours after adjustment for multiple comparison. Salivary flow differed between simulated and unstimulated saliva but was not affected by sex ($F = 0.94; P > .6$). As there were no sex differences in either background variable or salivary flow rate and the sample size was small, data are further presented for the group as a whole.

3.3 | Nerve growth factor

NGF was detected as two different isoforms in saliva based on size, described as NGF-1 and NGF-2 (Figure 1). The former had a molecule weight of 33 kDa, while NGF-2 displayed a molecular weight of 60 kDa. The different isoforms were quantified separately.

NGF-1 showed significant differences between the different collection points in both unstimulated and stimulated whole saliva. The post hoc test showed that NGF-1 in stimulated saliva was significantly higher in the fasting sample compared to the sample taken at 13:30 hours, but there were no significant differences between collection times in unstimulated saliva after adjustment for multiple comparisons. Neither were there any significant differences between stimulated and unstimulated saliva samples at any time point.

For NGF-2, there were no significant differences over time, either for unstimulated or stimulated whole saliva, but the levels in stimulated saliva were significantly higher than in unstimulated saliva at all time points ($P < .05$).

The plasma NGF expression showed a similar tendency to NGF-2 in saliva and was significantly higher expressed in the evening.

**TABLE 2** Mean (±SD) salivary flow during collection of unstimulated and stimulated whole saliva at different time points among 10 healthy individuals

<table>
<thead>
<tr>
<th>Collection time</th>
<th>Unstimulated whole saliva mL/min</th>
<th>Stimulated whole saliva mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30 AM</td>
<td>0.217 ± 0.149</td>
<td>1.810 ± 0.905</td>
</tr>
<tr>
<td>10:30 AM</td>
<td>0.294 ± 0.179</td>
<td>2.106 ± 0.984</td>
</tr>
<tr>
<td>1:30 PM</td>
<td>0.338 ± 0.178</td>
<td>2.283 ± 1.080</td>
</tr>
<tr>
<td>4:30 PM</td>
<td>0.357 ± 0.211</td>
<td>2.581 ± 1.449b</td>
</tr>
<tr>
<td>7:30 PM</td>
<td>0.536 ± 0.321$^a$</td>
<td>2.566 ± 1.450</td>
</tr>
</tbody>
</table>

Note: For both unstimulated and stimulated saliva, the flow rate varied significantly throughout the day (ANOVA, $P < .001$).

$^a$Significantly higher than at 7:30 AM (Bonferroni test, $P < .001$).

$^b$Significantly higher than at 7:30 AM (Bonferroni test, $P < .01$).
3.4 | Brain-derived neurotrophic factor

Salivary BDNF showed significant differences between the different collection points in both unstimulated and stimulated whole saliva (Figure 1). The highest concentration of the neuropeptide was expressed in stimulated whole saliva during the early morning, and the concentration thereafter significantly decreased throughout the day. Also, unstimulated whole saliva showed higher concentration at 07:30 hours compared to 10:30 hours ($P = .007$) and 19:30 hours ($P = .011$). The expression of BDNF in unstimulated whole saliva was significantly higher than those in stimulated whole saliva at 10:30, 13:30 and 19:30 hours ($P < .05$).

The expression of BDNF in plasma did not express any significant differences between early morning and evening and did not correlate to saliva. There were further no correlations between stimulated and unstimulated saliva in BDNF expression.

3.5 | Glutamate

The expression of salivary glutamate was relatively constant during the day (Figure 1). Consequently, neither unstimulated nor stimulated whole saliva expressed any significant differences between the collection points. However, glutamate levels in unstimulated and stimulated whole saliva showed a moderate correlation at 13:30 hours ($r_s = .720; P < .05$), 16:30 hours ($r_s = .733; P < .05$) and 19:30 hours ($r_s = .720; P < .05$).

Plasma glutamate expression was lower in the morning ($44.2 \pm 9.9 \mu mol/L$) compared to in the evening ($51.4 \pm 5.3 \mu mol/L$), with a trend towards significance. There was no significant correlation between plasma and saliva levels of glutamate.

3.6 | Substance P

SP expression in unstimulated whole saliva and plasma did not change significantly over time (Figure 1). In stimulated whole saliva, the expression could not be studied accurately due to failure to detect the peptide in all samples. There was no significant correlation in SP levels between plasma and unstimulated whole saliva.
The present study aimed to investigate the variation of salivary NGF, BDNF, SP and glutamate in healthy young adults during awakening hours. The main findings were that salivary NGF and BDNF levels, but not glutamate or SP levels, differed across the day and that the levels of NGF-2 and BDNF differed between unstimulated and stimulated saliva. To our knowledge, this is the first study designed to study daytime periodicity of these peptides in both unstimulated and stimulated saliva. Most earlier studies on neuropeptide and other neurotransmitter levels focus mainly on the plasma concentration. Nevertheless, saliva has the advantages such as an easy and non-invasive collection method, and the close relationship between saliva and plasma levels of various substances makes saliva a suitable diagnostic and prognostic fluid.

In our study, NGF was detected using gel-free Western blot based on capillary electrophoresis technology based on size, and the predicted band sizes were at about 27 and 53 kDa for the dimer of NFG. However, in saliva two notable bands were detected at around 33 and 60 kDa, which indicates expression of both NGF and the dimer of NGF in human saliva. Since NGF goes through several transitions before becoming fully matured, multiple bands for salivary NGF were somehow expected. Indeed, in a previous study from our group five different isoforms of NGF were detected in both unstimulated and stimulated saliva. Furthermore, as described earlier, stimulated whole saliva showed higher concentration of NGF compared to unstimulated whole saliva. Though there are some previous studies on the salivary and plasma concentration of NGF, there is only one study that have studied the daily variation of the neuropeptide in human plasma samples. Nevertheless, there are no studies on salivary regulation. Bersani et al reported a rhythmic NGF regulation in plasma with higher levels in the morning which decreased in the evening. Similar findings were observed for salivary NGF-1 in the current study. In particular, the morning NGF-1 chemiluminescence in stimulated whole saliva was higher than those measured around noon. The elevated NGF levels in the morning suggest an increased NGF production during the night, similar to what is observed fornoon.

The plasma NGF, with similar molecular mass as NGF-2, was higher expressed in the evening compared to in the morning. Since plasma samples were only collected in the morning and evening in connection with the first and last saliva sample, it is difficult to conclude if salivary NGF completely mirrors the plasma NGF. Further research dealing with simultaneous daytime periodicity of plasma and salivary NGF is needed to reveal such interface.

Several studies have shown that BDNF modulates several neuronal activates and influence cell survival, proliferation and maturation. BDNF has also been suggested to play an important role modulating synaptic plasticity, and altered secretion of BDNF has been proposed in several diseases. Experimental animal studies have shown that the light-darkness cycles may influence BDNF expression by modulating the cerebral circadian pacemaker localised in the suprachiasmatic nucleus of the hypothalamus gland. Both BDNF mRNA and protein levels in these regions as well as its receptors have been shown to fluctuate rhythmically in the rat central nervous system with peak levels during the dark hours and the lowest levels in the light hours. Several human studies have also confirmed a systemic circadian variation of BDNF secretion in plasma, with significant decrease throughout the day. These fluctuations resemble the cortisol circadian rhythm that allows for speculations that BDND and cortisol may be physiologically co-regulated in order to maintain homeostasis. In women, the amplitude of the fluctuation has been shown to vary across the menstrual cycle in fertile women and to be reduced in post-menopausal women. However, these studies have been conducted on blood samples. Our study showed that salivary BDNF presents a characteristic trend during the day; the highest BDNF expression was detected in the early morning, where after it decreased across the day and had the lowest expression in the evening. These results are in accordance with a previous study measuring BDNF in unstimulated whole saliva. Plasma expression of BDNF was also in accordance with earlier studies with peak expression in the morning (906 ± 431) and decrease towards the evening (809 ± 376). The results emphasise a positive interaction between salivary and plasma BDNF.

Salivary glutamate and SP, on the other hand, showed no clear fluctuations across the day. In our previous study, we found that plasma glutamate and SP had higher concentration in plasma compared to saliva and that SP exhibit a higher concentration in unstimulated saliva compared to stimulated saliva. A similar tendency was also observed in the current study population, with higher concentrations of glutamate and SP in plasma compared to saliva. When comparing the variability between saliva types, it clearly appears that stimulated whole saliva is less variable than unstimulated whole saliva. The latter is far more exposed to external stimuli and more difficult to control.

A strength in our study design was that stimulated and unstimulated whole saliva were structurally collected each third hour from ten individuals during the exactly same time and surrounding conditions. It was ensured via the anamnesis and the careful oral and dental examination that the participants were healthy. The inclusion criteria were very strict to reduce the influence of external factors on salivary flow and secretion. The participants were also closely related in age and in order to minimise the influence of this factor on the flow rate and peptide expression.

Nevertheless, our findings should be interpreted within the context of certain limitations. For instance, the study was performed in healthy young adults and included a small number of participants, different age groups were not taken into consideration because of the possibility of age-variability and the chemiluminescence and not absolute concentration was measured for NGF and BDNF. Moreover, the female subjects were not screened for menstrual phase cycle, although one earlier study has shown possible influence of sex hormones on BDNF secretion. The absence of samples during the night may have prohibited observations of some important fluctuations affected by the sleep-wake phase. On the other
hand, the current study was not undertaken to investigate diurnal variation throughout 24 hours. Instead, the intention was to explore if samples collected at different time points differ during a day, that is if sampling time is important to consider in studies exploring salivary levels of these biomarkers.

In conclusion, the present study indicated that NGF and BDNF show a dynamic daily variation in saliva, which somehow resemble the fluctuations observed in plasma. It further demonstrated that daily variation may not exist for SP and glutamate. These findings are the first to show salivary fluctuations in secretion of these neurotransmitters in healthy individuals. It also emphasise the great significance in clinical and research settings of consistency in the collection time and approach.

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CONFLICT OF INTEREST
The authors declare no conflict of interest. No financial or personal relationships have inappropriately influenced this work.

AUTHOR CONTRIBUTION
AC, BG, BM, HJ and ME designed the experiment. HJ examined and collected the samples. AC and BG performed the analysis. HJ performed the statistics. HJ wrote the first draft of the manuscript and prepared figures and tables. All authors wrote and revised the manuscript and approved the final version of the manuscript.

ORCID
Hajar Jasim https://orcid.org/0000-0003-2352-8070
Malin Ernberg https://orcid.org/0000-0003-4152-5439

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