A study of chemical biomarkers of nociception in saliva in healthy adults

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Abstract

In this work, we have investigated the feasibility of using the salivary biomolecules glutamate and cortisol in the detection and assessment of acute nociceptive pain. Pain was induced in 18 healthy volunteers using the cold pressor task (CPT) and saliva samples were collected before the CPT, immediately after the CPT and every 10 minutes thereafter. Statistical analysis of the biomolecules levels across all participants and time points have been done as well as calculations of change in each participant's biomolecules levels normalised to that participant's baseline. There were statistically significant differences between salivary cortisol concentration before (median 0.14 ng/μL, IQR=0.1) and 10 minutes after termination of CPT (median 0.34 ng/μL, IQR=0.4, p=0.007). At 10 minutes after the termination of CPT, normalised salivary cortisol levels increased by 134% (median, IQR=293) from baseline. Salivary glutamate levels increased immediately after the CPT from a median of 4.90 ng/μL (IQR=4.7) to a median of 5.66 ng/μL (IQR=4.6) and fluctuated thereafter but none of these changes were statistically significant except at t= +50'. Male participants exhibited greater increase in cortisol concentrations at t= +10', +20' and +30' after the CPT compared to females (p=0.01, p=0.02, and p=0.04, respectively). Further exploration of changes in salivary biomolecules related to nociception after induction of acute pain and their association with pain intensity ratings may render salivary biomarkers as a novel tool in the assessment of acute pain.

Keywords

nociception, biomarkers, induced pain, acute pain, cold pressor task, saliva, cortisol, glutamate

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1. Introduction

Pain is a universal, yet personal experience that is influenced by physical, psychological, social and genetic factors [1]. Nociception refers to the encoding and processing of noxious stimuli by the sensory nervous system and allows us to avoid potentially damaging stimuli [2]. This process relies on nociceptors, peripheral nerve endings that detect the presence of noxious stimuli [3]. The nociceptive signal may be transported to the brain and perceived as a painful stimulus or elicit a rapid nociceptive withdrawal reflex by activating the spinal reflex loop [2].

Presently, the conventional methods for assessing pain intensity in clinical practice rely on self-reporting and come in the form of either a scale (predominantly used for acute pain) or a questionnaire (predominantly used for chronic pain) [4]. Other pain assessment tools based on behavioural and physiological indicators are useful when patients are unable to self-report, for example, infants and young children, people under anaesthesia, or those with cognitive disabilities and mobility impairments [5-7]. These methods rely on the expertise of healthcare professionals, limiting their reliability [10]. Physiological parameters are not specific for pain and may be indicators of other physiological responses or pathological processes [11].

An alternative method for pain assessment could be through monitoring the biofluid levels of pain biomarkers that are related to nociceptive pathways and physiological stress. Saliva is a favourable biofluid as it can be obtained easily, painlessly and non-invasively [12]. Many biomolecules enter the saliva from the blood by transcellular or paracellular routes [13]. For example, salivary levels of cortisol - a crucial component of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis response to physiological stress (such as pain) – are an indicator of the active free cortisol level in plasma [14]. Higher salivary cortisol levels have been found in patients with acute dental pain due to pulpal or periapical inflammation compared to healthy controls [15].

Several studies have shown changes in the HPA axis activity, including an increase in salivary cortisol response after experimental pain induction [16-21]. Moreover, greater pain intensity ratings have been found to be significantly correlated with greater increases in salivary cortisol response to the cold pressure task (CPT) [22].

Glutamate is distributed throughout the human nervous system as an essential biomolecule for neurological function including in nociceptive pathways [23]. To the best of our knowledge,

there are no published studies that have investigated the changes in salivary glutamate in response to induced acute pain. However, there are several studies that investigated changes in glutamate levels in patients with chronic pain and found the levels to be elevated [24-28].

This study aimed to explore the changes in salivary glutamate and cortisol levels after the use of CPT to induce acute nociceptive pain in healthy volunteers.

2. Materials and Methods

2.1 Participants

Eighteen healthy adult individuals (nine males and nine females) were recruited to the study based on the following criteria for participation; age range 18-70 years old, overall good health (defined as requiring no regular medications and feeling well), and maintaining good oral hygiene on the day of the experiment. Exclusion criteria included pre-existing pain, diagnosed systemic muscular or joint diseases, neurological disorders, diagnosed with anxiety or depression, pregnancy or lactation, high blood pressure, tobacco use, regular use of medication (including oral contraceptives and antidepressants), and poor dental health.

The study was advertised via email within the UCL Institute of Orthopaedics and Musculoskeletal Science and the Royal National Orthopaedic Hospital, Stanmore, UK. Participants were recruited after they expressed their interest to the research team. An information sheet was given to each participant to explain the aim of the study and familiarise them with the protocol before the experimental session took place. All participants received £10 as reasonable expenses for taking part in the study.

The study was approved by the UCL Research Ethics Committee (Ref No. 15021/001) and complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects, Good Clinical Practice guidelines, and the Declaration of Helsinki. All subjects provided written informed consent prior to their participation in the study.

2.2 Experimental design and protocol

Procedures. Participants were asked to refrain from eating food and drinking anything except water for at least 3 hours before the experiment and to brush their teeth more than one hour

before the start of the experiment to remove any food residue from their mouths. There were no dietary restrictions. All experiments took place in the afternoon in a 2-hour window between 14:00 and 17:30 on separate days to ensure the participants had passed the post-lunch peak in their cortisol levels.

Pain induction. There are several methods for inflicting experimental pain in human studies [29]. We used cold stimulation in this study due to its simplicity, controllability, and effectiveness in inducing pain over a short period of time. We employed the cold pressor task experimental procedure [30-32] where the subjects submerged their (non-dominant) forearm and hand into an ice bath (at 0-5 ^{o}C) for as long as they were able to endure or up to a maximum of 5 minutes (to avoid any adverse effects) [33]. Participants had full control over when to start and terminate the immersion of the arm in the ice bath.

Pain intensity reports. Participants were asked to record the maximum pain intensity they experienced during the CPT on a 0-10 Numeric Rating Scale (NRS) after they removed their arm from the ice bath. The NRS is comprised of a line segmented into ten parts labelled with the numbers 0 to 10 in increasing order, as well as a short description above the numbers 0, 5, and 10 indicating “no pain”, “moderate pain”, and “worst pain”, respectively. The participants’ heart rate and blood pressure were monitored every 5 minutes for a full 1 hour (except during the CPT) for monitoring purposes. Additionally, we asked participants for verbal confirmation of their wellbeing throughout the experimental session.

Saliva collection and analysis. At the beginning of the session, participants were asked to rest comfortably in a chair for 10 minutes to adapt to the experimental setting. They then provided two preliminary saliva samples. The initial saliva sample was collected 30 minutes prior to the start of the CPT. The second saliva sample was collected after another 15 minutes. Additional saliva samples were collected immediately upon the termination of CPT (t= T_{CPT}) and then at 10, 20, 30, 40, 50, and 60 minutes after t= T_{CPT}.

Saliva samples of 0.5-1.0 ml volume were collected in cryovials using saliva collection aids (Salimetrics LLC, State College, PA). To collect whole saliva, we used the passive drool method as it is a gold standard in salivary proteome analysis [34]. The samples were kept on ice during the experiment to prevent the degradation of sensitive peptides. After obtaining all the saliva

samples (that is up to a maximum of 1 hour after the end of the experiment), the samples were stored at -20 ^{o}C until assayed.

The levels of biomolecules in the collected saliva samples were measured using optical techniques using a microplate reader (Infinite M200 PRO, Tecan, CH). Glutamate levels were measured using glutamate assay kits (ab83389) by Abcam (Cambridge, UK). Cortisol levels were measured using high sensitivity immunoassay kits (1-3002-SAL) by Salimetrics (State College, PA, USA). All measurements were done in duplicates as specified by the manufacturers of the kits and the average value of the two was taken and reported as data points.

2.3 Statistical Analysis

All data analysis was performed in GraphPad Prism 8.0.1 (GraphPad Software Inc., LaJolla, CA). Descriptive statistics were generated for all concentration measures. All results are presented as the median and interquartile range (IQR) in tables. Data were checked for normality of distribution using the D' Agostino and Pearson test. The Friedman test was used followed by Dunn's multiple comparisons test to compare the levels of each biomolecule between saliva samples collected at different times points. Gender influences on the concentration levels were evaluated using unpaired t-test with Welch's correction for normally distributed data and Mann-Whitney U test for non-normally distributed data. Spearman correlation coefficients were calculated to assess the relationship between the three biomolecules and the NRS scores or CPT duration. All tests were two-tailed and a p-value of less than 0.05 was used as an indicator of statistical significance.

3. Results

3.1 Demographic characteristics

The demographic characteristics of the participants are summarised in Table 1. Samples from 9 male and 9 female subjects were analysed. The median age of all participants was 25 years, with a range of 21-40 years. In male participants, CPT duration (T_{CPT}) ranged from 1.33 to 5 minutes (the maximum duration of the test) with a median value of 5. All the female participants

left their hands and forearms in the ice bath for 5 minutes. The most frequently reported NRS pain intensity score was 6. The range of pain intensity on the NRS was 3-8 with a median value of 6.25.

Table 1. Medians and ranges (min-max values) of subjects' age, CPT duration, and NRS pain scores for both sexes. No statistically significant differences ($p>0.05$) were found between male and female participants (two-tailed Mann-Whitney test).

Parameter	Male (n=9)	Female (n=9)	*p*-value
Age (years)	23 (21-40)	24 (21-40)	0.85
T_{CPT} (min)	5 (1.33-5)	5 (-)	0.47
NRS score	6 (3-8)	6.5 (6-8)	0.49

3.2 Changes in biomolecule levels

The measured individual values of salivary glutamate and cortisol concentration are shown in Figure 1 (Graphs A and B). The medians and the IQRs of the biomolecules' concentrations are reported in Table 2. The baseline levels are calculated as the average of the values at t= -30' and t= -15' for both cortisol and glutamate as there was no statistically significant difference between the values at the two time points.

3.2.1 Glutamate. The baseline glutamate level (median= 4.90, IQR= 4.7 ng/μL) was slightly above the upper limit of the normal range of salivary glutamate reported in the literature (1.47 - 4.41 ng/μL) [24]. Immediately after the CPT, the median glutamate levels increased and then dropped below the baseline at t= +10'. The glutamate levels fluctuated thereafter, rising at t= +20' compared to baseline, and then dropping again at t= +50' to significantly lower than the baseline levels (Friedman's test with Dunn's adjusted post hoc test: p=0.014). The t= +50 time point was the only time when there was a statistically significant difference with the baseline level.

3.2.2 Cortisol. A small but significant increase from the baseline (median=0.14, IQR=0.1 μg/dL) was observed immediately after the CPT. The median salivary cortisol concentration at t= +10'

was significantly greater than the baseline level (Friedman's test with Dunn's adjusted post hoc test: p=0.007). Cortisol levels at t= +10' were also significantly higher than the levels at t=T_{CPT} and t= +60' (p=0.03 and p=0.02, respectively), but not significantly higher compared with t= +20' and t= +30', indicating that cortisol levels peaked 10-30 minutes after the CPT.

Table 2. Measured levels (medians and interquartile ranges [IQR]) of glutamate and cortisol across the different time points during the experimental session. Differences that were statistically significant between all the time points for each biomolecule are indicated with the same superscript. *: p<0.05, **: p<0.01. Differences that were statistically significant between the sexes for each time point are indicated with the same superscript. [i, ii, iii]: p<0.05. The differences between the values that are not accompanied by a superscript were not statistically significant.

Time points (min)	Glutamate Medians (IQR) (ng/ µL)			Cortisol Medians (IQR) (µg/dL)		
	Female	Male	All	Female	Male	All
Bas	4.86 (4.7)	4.50 (4.8)	4.90 (4.7)*	0.10(0.09)	0.16(0.11)	0.14 (0.1)**
T_{CPT}	5.85 (6.0)	5.37 (4.9)	5.66 (4.6)	0.13 (0.1)	0.18 (0.2)	0.16 (0.2)
+10	1.82 (2.1)	2.59 (2.7)	2.55 (1.7)	0.23 (0.2)[i]	0.56 (0.7)[i]	0.34 (0.4)**
+20	7.28 (5.5)	6.40 (6.8)	6.84 (6.7)	0.17 (0,2)[ii]	0.55 (1,0)[ii]	0.29 (0.7)
+30	1.54 (3.9)	2.60 (2.6)	2.31 (3.1)	0.18 (0.2)[iii]	0.44 (0.7)[iii]	0.28 (0.6)
+50	1.43 (4.3)	2.61 (2.9)	2.08 (3.3)*	-	-	-
+60	7.98 (8.1)	7.45 (7.3)	7.55 (7.5)	0.10 (0.3)	0.23 (0.1)	0.20 (0.2)

3.3 Normalised changes in biomolecule levels

The following equation was used to calculate normalised substance levels ($[sub]_{N,t=x}$):

$$[sub]_{N,t=x}\,\% = \frac{[sub]_{t=x} - [sub]_{t=bas}}{[sub]_{t=x}} * 100$$

Here, the changes in the measured biomolecule levels for each participant were normalised to the baseline level of the same participant. The medians of the normalised (%) changes in the levels of the two biomolecules at each time point are presented in Figure 2 (graphs C and D).

3.3.1 Glutamate - The normalised glutamate levels increased from the baseline by 38% (median) immediately after the CPT. The median values were higher than the baseline levels also at t= +20' and t= +60' but not at t= +10', t= +30' and t= +50', (see Fig. 2; graph C).

3.3.2 Cortisol - The median of the normalised cortisol levels was higher than the baseline at all time points. In particular, the levels at t= +10' were 134% higher than the baseline. Cortisol levels in 15 out (of 18) participants increased from the baseline at t= +10'. The normalised levels gradually reduced to within 23% of their baseline by the end of the experiment (see Fig. 2; graph D).

3.3.3 Correlation analysis with NRS scores and CPT duration

We found a moderate positive correlation between the NRS scores and the normalised glutamate levels at t= T_{CPT} (Spearman correlation coefficient (r=0.51, p=0.02). For cortisol, moderate positive correlation between the NRS scores and the normalised levels at t= +20' and t= +30' was found (Spearman correlation coefficient (r=0.52, p=0.02, and r=0.54, p=0.01, respectively).

There was no correlation between the duration of the CPT and the normalised levels of glutamate or cortisol at any time point (Spearman correlation coefficient, p>0.05).

3.4 Gender differences

The changes in the levels of biomolecules in male and female participants are depicted in Figure 3 (Graphs E and F).

3.4.1 Glutamate - Wide biological variability was observed for salivary glutamate concentrations both in male and female participants. Male and female subjects exhibited a similar glutamate pattern of change. No statistically significant differences were found between the sexes (t-test with Welch's correction and Mann-Whitney U test performed, p>0.05) at any of the time points.

3.4.2 Cortisol - Male participants exhibited a greater increase in cortisol concentrations at t= +10', +20' and +30' compared to females and these differences were found to be statistically significant (Mann-Whitney U test, p=0.01, p=0.02, and p=0.04, respectively).

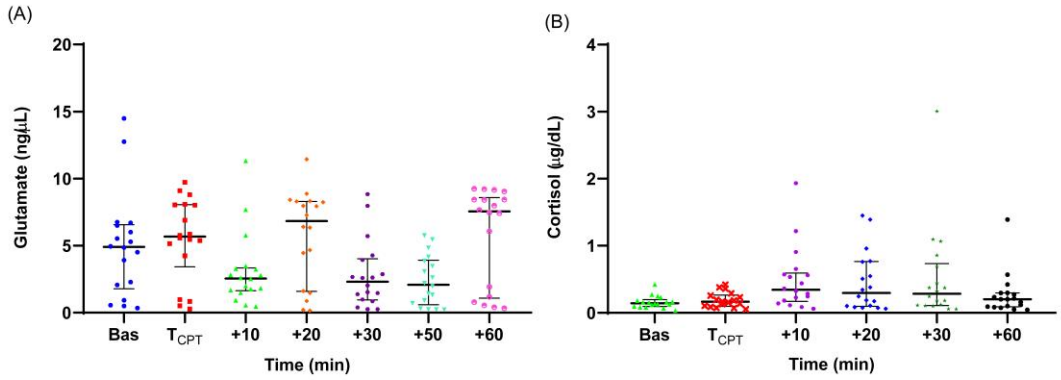

Figure 1. Individual salivary glutamate (A) and cortisol (B) concentration values during the experiment. Medians are shown with horizontal lines. The interquartile ranges (IQR) are presented with vertical lines.

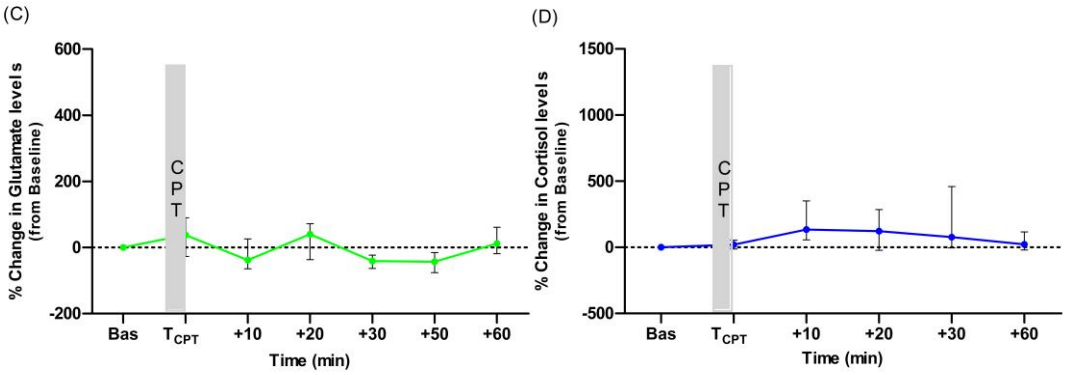

Figure 2. Medians of the normalised (to the baseline levels) percentile changes of glutamate (C) and cortisol (D) levels. The interquartile ranges (IQR) are presented with vertical lines.

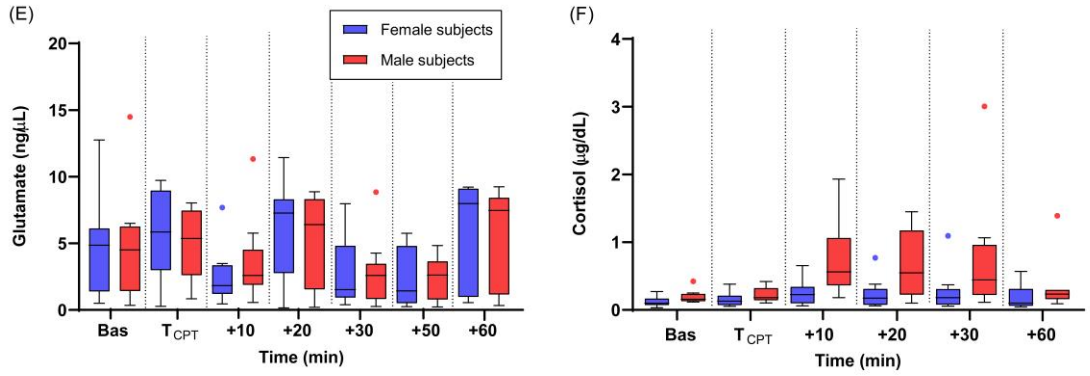

Figure 3. Medians of salivary glutamate (E) and cortisol (F) concentrations in the male (red color line) and female (blue color line) groups. The interquartile ranges (IQR) are presented with vertical lines.

4. Discussion

This study investigated the salivary concentrations of two nociception-related biomolecules (glutamate and cortisol) after experimentally induced acute pain in healthy volunteers.

4.1 Glutamate variations with pain

After the CPT, glutamate levels fluctuated. These fluctuations were not statistically significant except at t= +50' when there was a drop below baseline. The fluctuation in glutamate levels after the initial rise is difficult to explain. There is evidence that salivary glutamate levels are elevated in patients with migraines [24-26] and chronic musculoskeletal pain [27,28], however, the literature is limited on acute pain-induced changes in salivary glutamate. We have found no other studies that have reported on glutamate levels after experimental induction of acute pain.

4.2 Cortisol variations with pain

Salivary cortisol levels were significantly elevated compared to baseline 10 minutes after the CPT. One hour after the termination of the painful task, cortisol levels returned close to the baseline. Although a similar pattern was observed in both sexes, cortisol levels in males were significantly higher than the levels in females.

The peak in participants' salivary cortisol levels occurred 10-30 minutes after the completion of the CPT in this study. This finding is consistent with the results of previous studies, which have examined pain-related changes in cortisol levels in response to the CPT. A peak in cortisol concentration 10-20 minutes after the CPT has consistently been reported in the literature on this topic [22,35-42]. Similarly to our results, Goodin et al. found pain intensity reports were significantly and positively correlated with elevations in cortisol response [22].

We found gender differences in the cortisol response: male participants exhibited higher cortisol responses than female participants and the differences between them were statistically significant at multiple time points. This is consistent with previous studies of experimentally induced acute pain [38,39], in which men had a significantly greater cortisol response to the CPT, even after adjustment for sex differences in the CPT tolerance time and despite a lack of sex differences in baseline cortisol levels. A possible explanation of our findings may be the gender

differences in stress reactivity due to different HPA response patterns to stress between the sexes [43,44]. In contrast to our results, other studies have not identified similar differences between males and females [36,37,40,41].

4.3 Limitations & future studies

In this work, we studied only one type of painful stimulus and studied two biomolecules, cortisol and glutamate. One of these, cortisol, has been studied extensively in the past. Encouragingly there is consistency in the pattern and timing of change in cortisol levels in data reported by various investigators which implies that salivary cortisol may be a suitable biomarker for the detection and assessment of acute pain in individuals. Cortisol levels rise slowly after induction of acute pain and reach a peak after 10-30 minutes. This is a significant limitation to using cortisol alone as a marker for acute nociception. Moreover, the correlation between the changes in the concentration of salivary cortisol and pain intensity remains debatable. Other factors to consider are that cortisol production follows a circadian rhythm [46], which can impact the salivary cortisol response to pain, and that cortisol levels can change as a result of certain diseases (e.g. Cushing's syndrome) as well as physiological stress [45].

Studies of other pain-related biomarkers are therefore warranted, alongside cortisol, to build a clear picture of how these molecules could reliably be used to detect nociception and measure its intensity. In this study, we also chose to look at salivary glutamate for the first time. We found no rise in salivary glutamate levels but it is possible that we missed a peak in glutamate concentration in the first 10 minutes after the CPT due to the saliva sampling time points in our experimental design.

Another biomarker candidate would be substance P (SP), a neuropeptide in the tachykinin family that is a key neurotransmitter in afferent nociceptive pathways, often alongside glutamate [47,48]. SP is also easily measurable in the saliva [49]. Elevated levels of SP were measured in the saliva of patients with dental pain [50]. Salivary SP levels were also higher in chronic migraine patients compared to healthy subjects [51]. The feasibility of using salivary SP as a pain-associated biomarker remains open to investigation. We would propose that in future experiments glutamate and substance P should be studied alongside cortisol whose pattern of

rising after acute nociception is now well described and that measurements are done closer to the time of pain induction.

An important factor to consider when investigating gender differences in cortisol levels is the menstrual cycle of female subjects, which has been reported to influence the HPA responsiveness to psychosocial stress in healthy subjects [52]. Therefore, the stage of the menstrual cycle in female subjects should be recorded and considered in future pain biomarker studies. Despite the potentials of salivary biomarkers, the literature regarding pain-associated molecules in human saliva remains limited, and the feasibility of using them as salivary biomarkers of acute pain remains debatable and needs to be further explored.

4.4 Conclusion

In this study, we have confirmed the previous finding that a short period of induced acute pain elicits a rise in the concentration of cortisol in the saliva of healthy volunteers and that these changes show sex-dependent differences. We report the first study of salivary glutamate as a nociception-associated biomolecule. Future work is needed to examine changes in salivary biomolecules after other painful stimuli and determine whether they can be used to detect nociceptive intensity and guide treatment in situations where self-assessment is impossible or unreliable.

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Author Contributions

AA, RZ and SG developed the concept, the research question, and designed the study. AA and AV conducted the experiments. All authors contributed to the analysis and interpretation of the data. AV, RZ and SG also contributed to revising the manuscript. All authors approved the final version of the manuscript.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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