SI receives input exclusively from the VPL and SII receives inputs from the multiple nuclei in the thalamus, it seems convincing that there exists a nonlinear relationship between the SI and SII responses. In accord with our assumption, an fMRI study demonstrated that patients with a solitary infarction of the VPL showed preserved activity of SII despite the reduced activation of SI (Taskin et al., 2006).

In conclusion, the analysis of PLVs demonstrated early neuronal synchronization between SI and SII. The decrease of PLVs in MS validated the significance of increased PLVs in healthy subjects. The current study provides evidence for the gamma-band synchrony in the early-stage human somatosensory processing.

## Acknowledgment

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2010.02.001.

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# Clinical Neurophysiology

# Pre-movement gating of somatosensory-evoked potentials by self-initiated movements: The effects of ageing and its implication

Katsuya Ogata [a,*], Tsuyoshi Okamoto [b], Takao Yamasaki [a], Hiroshi Shigeto [a], Shozo Tobimatsu [a]

[a] Department of Clinical Neurophysiology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan
[b] Department of Digital Medicine Initiative, Kyushu University, Fukuoka, Japan

## ARTICLE INFO

## ABSTRACT

Objective: To study whether the gating effect of the self-initiated movements on the cortical somatosensory-evoked potentials (SEPs) is affected by ageing.
Methods: The SEPs elicited by stimulating the right median nerve were recorded in 14 young and 16 older healthy subjects, while self-initiated movements of the right fingers were performed at 5–10 s intervals. The amplitudes of the major components of the SEPs at F3 and C3' (2 cm posterior to C3) during the pre-movement period were analysed as the resting condition subserving the baseline.
Results: The amplitudes at rest were significantly greater in the elderly than in the younger subjects. The amplitudes of P27, N35 and P45 at C3' as well as N30 at F3 decreased significantly during the pre-movement period. However, the ratio of amplitudes in the pre-movement period to the resting period in the elderly was not significantly different from that in the younger subjects, except for the interaction of N30.
Conclusions: The effect of age on the gating of N30 at F3 may indicate an altered preparatory processing of self-initiated movement in the elderly. The gating effect of older subjects at C3' is almost comparable to that of young ones, which appears to be a compensatory mechanism to maintain the precise movements.
Significance: Ageing affects the SEPs differently at rest and pre-movement gating.
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## 1. Introduction

Sensorimotor integration is crucial for executing smooth voluntary movements in humans, and many studies have examined this by using evoked potentials. Sensory signals, for example, somatosensory-evoked potentials (SEPs) are modulated during active movements (Rushton et al., 1981; Cheron and Borenstein, 1987; Cohen and Starr, 1987; Tapia et al., 1987), and this modulation is called a gating phenomenon. As these studies evaluated gating during active movements, the concurrent inputs from nearby cortical areas probably interfered with the modulations by the sensory and motor areas which affect the activity of the sensory tracts.

The sensory information can also be modulated before movements by interactions with the activated motor areas. This is referred to as pre-movement gating (Shimazu et al., 1999); it is a process caused only by centrifugal processing without centripetal information. Therefore, understanding pre-movement gating is important to comprehend intra-cortical sensorimotor integration.

In our laboratory, we have used either externally triggered (ET) or self-initiated (SI) movements to study the functional connectivity amongst the motor-related cortical areas and the basal ganglia

that are related to ET and SI tasks using functional magnetic resonance imaging (fMRI) (Taniwaki et al., 2003, 2006, 2007). In the ET tasks, warning signals were used to initiate movements, while in the SI tasks, the hands were moved at the subject's pace. Our results showed different activation patterns in the motor loops for SI and ET movements. The gating of the SEPs using an ET task revealed that N30, a main component at F3, was inhibited before the movements (Cohen and Starr, 1987; Murase et al., 2000). However, the tasks could involve recognition of the external signals and other mental processes rather than sensorimotor interactions. Therefore, additional information to understand sensorimotor integration can be obtained by evaluating SEP gating under the SI paradigm. Thus far, pre-movement gating in the upper-limb using an SI task has been evaluated by magneto-encephalography (MEG) (Wasaka et al., 2003, 2005, 2007) and SEPs (Legon et al., 2008) in young subjects. The strength of the dipole at P30m, but not at N20m or P60m, decreased significantly with MEG (Wasaka et al., 2003, 2005). Legon et al. (2008) reported that frontal N30 amplitude was significantly enhanced during the movement of the hand contralateral to the median nerve stimulation. However, there was no significant effect of either the ipsilateral or contralateral hand before movement.

We have found an age-related alteration of the functional interactions within the basal ganglia and cerebellar motor loop by fMRI

* Corresponding author. Tel.: +81 92 642 5543; fax: +81 92 642 5545.
E-mail address: katuya@med.kyushu-u.ac.jp (K. Ogata).

(Taniwaki et al., 2007). However, in the studies of SEP gating, little is known about the influence of ageing. Aging effect on the gating was augmented during voluntary isometric contraction (Touge et al., 1997); in their study, the enhanced gating could be attributed to both centripetal and centrifugal gatings. Thus, if the SI task is applied to pre-movement gating, the effect of ageing on the centrifugal gating can be determined more precisely.

Therefore, the aim of this study was to evaluate the effect of ageing on the pre-movement gating of SEPs under SI tasks.

## 2. Subjects and methods

### 2.1. Subjects

Thirty subjects (17 men and 13 women) with ages ranging from 20 to 75 years were studied. The subjects were divided into two groups: younger (age range: 20–38 years; nine men and five women) and older (age range: 60–75 years; eight men and eight women). All of them were right handed and none of them were receiving neurological medications or had had neurological disorders. A written informed consent was obtained from each subject after the nature of the experiment was fully explained. The experiments were approved by The Ethics Committee at Kyushu University.

### 2.2. SEP recordings

The subjects were seated on a comfortable reclining chair and were instructed to relax during the SEP measurements. Silver–silver chloride cup electrodes were attached to the scalp by collodion and filled with conductive jelly. The recording sites were F3, C3' (2 cm posterior to C3) and P3 according to the International 10–20 system, and the potentials at F3 and C3' were referred to the linked earlobes. The impedance of the electrodes was kept below 5 kΩ. The band-pass filter on the amplifier was set between 1 and 1000 Hz and the analysis time was from −10 to 90 ms. The sampling rate was 2.5 kHz.

The median nerve at the wrist was electrically stimulated with a stimulus duration of 0.2 ms. The intensity of the stimulus was adjusted to elicit mild twitches of the right thumb. The inter-stimulus intervals were randomised between 600 and 1000 ms (mean: 800 ms). All the data were stored in a personal computer using a signal processing software (Multiscope PSTH, Medical Try System, Tokyo, Japan) for offline analyses.

### 2.3. Experimental paradigm

In the previous studies, either finger extension (Murase et al., 2000; Wasaka et al., 2003, 2005) or flexion (Cohen and Starr, 1987; Tapia et al., 1987) was used while median nerve SEPs were recorded. To study the pre-movement gating of SEPs under SI tasks, we adopted the method of finger extension (Wasaka et al., 2003, 2005). The subjects were instructed to extend their index and middle fingers as quickly as possible and then relax. They were requested to move their fingers at their own pace every 5–10 s, and also to neglect the electrical stimuli at the wrist. They practised the movement for a short time. A pair of recording surface electrodes was attached 3 cm apart on the right extensor digitorum communis muscle to detect the finger extension. Surface electromyographs (EMGs) were recorded with a band-pass filter of 20–100 Hz. The low-pass filter was set at 100 Hz to discriminate the EMGs from the SEPs; thus, the artefacts of electric stimulation were mostly eliminated. The movement was repeated 500–600 times in one movement session. To detect finger extension, electroencephalograms (EEGs) and surface EMGs were recorded for 4–5 s each time the subjects moved their fingers from 3.5 to 4.5 s

before the movement to 0.5 s after the detected EMGs. EEGs were also recorded in the resting condition for about 10 min for each subject. The EEGs under resting conditions were recorded both between and after the two movement sessions. The recordings of the first 2–3 min were used for averaging to make the number of epochs comparable to those of the movement sessions.

### 2.4. Data analyses

Trials contaminated with inappropriate movement were excluded from the analyses, and the beginning of finger extension was also adjusted by a visual inspection of the surface EMGs. The time period from −3000 to 0 ms of the finger movement was taken and divided into six segments of 500 ms for the analysis: between −3000 and −2500 ms, −2500 and −2000 ms, −2000 and −1500 ms, −2000 and −1500 ms, −1500 and −1000 ms, −1000 and −500 ms and −500 and 0 ms. For each segment, the EEGs were averaged to obtain the SEPs. The mean number of responses averaged for each bin ranged from 250 to 350. The peak-to-peak amplitudes of the SEP components were measured and compared amongst the different segments to evaluate the attenuation of the SEPs during the pre-movement period. The amplitudes of P22, N30 and P40 at F3, and N20, P27, N35 and P45 at C3' were analysed as the resting condition subserving the baseline.

The amplitude ratios were statistically analysed using two-way repeated measures analysis of variance (ANOVA). The within-subject factor was the time segment and the between-subject factor was the age group. The sphericity of the data was tested with Mauchly's test, and Greenhouse–Geisser-corrected significance values were used when sphericity was lacking. A post hoc analysis followed by Bonferroni's correction was used for multiple comparisons. The significance of the differences in the amplitudes at the resting condition between the two age groups was tested using the Student's $t$-tests. A $P < 0.05$ was considered to be significant.

## 3. Results

Several components are clearly visible in the grand-averaged SEPs at F3 and C3' as shown in Fig. 1. In general, the amplitudes of the older subjects were greater than those of the younger subjects,
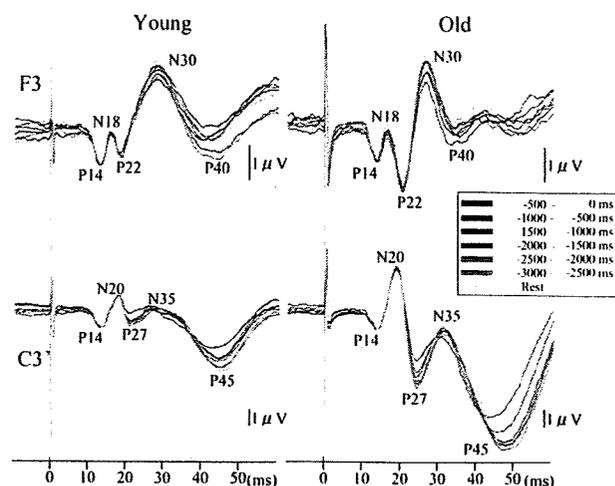


Fig. 1. Grand averaged waveforms of SEPs prior to movement and resting conditions for the younger ($n$ = 14) and older ($n$ = 16) subjects. Several components are identified at the F3 and C3' electrodes. Although the amplitudes of the older subjects are generally larger than those of younger ones except for P14, the amplitudes of the late components become smaller as the time interval moves closer to the movement for both the groups.

**Table 1**
Amplitudes and latencies of the major components of SEPs in the resting condition.

| | Amplitudes | | Latencies | |
|---|---|---|---|---|
| | Young | Old | Young | Old |
| *F3* | | | | |
| N18 | 1.28 ± 0.91 | 1.55 ± 0.55 | 16.40 ± 1.03 | 16.45 ± 0.84 |
| P22 | 1.08 ± 0.10 | 2.25 ± 1.09* | 18.94 ± 1.25 | 20.53 ± 0.82* |
| N30 | 3.41 ± 2.23 | 4.67 ± 2.46 | 28.26 ± 2.14 | 26.70 ± 1.04* |
| P40 | 3.01 ± 2.45 | 3.07 ± 1.87 | 39.69 ± 3.99 | 35.38 ± 3.45* |
| *C3'* | | | | |
| N20 | 2.44 ± 1.58 | 4.49 ± 1.47* | 17.97 ± 0.99 | 19.05 ± 0.73* |
| P27 | 2.59 ± 1.93 | 8.30 ± 3.91* | 21.71 ± 1.63 | 24.68 ± 1.20* |
| N35 | 1.76 ± 1.25 | 4.86 ± 2.85* | 29.69 ± 2.95 | 32.23 ± 2.86* |
| P45 | 4.14 ± 2.65 | 8.53 ± 4.27* | 45.06 ± 2.65 | 47.65 ± 2.93* |

* $P < 0.05$.

both in the resting condition and during the movements. In addition, the amplitudes of the SEPs were greatest in the resting condition and were gradually attenuated as the time interval was closer to the beginning of the finger movements in both the groups. These changes were observed at both F3 and C3'; however, the changes at C3' were more prominent. At C3', the amplitude of N20 did not change significantly, irrespective of the time segment; however, the later components were attenuated in the pre-movement period.

The amplitudes and latencies in the resting condition were compared between the two age groups using the Student's t-tests (Table 1). The amplitudes of P22 at F3 and N20, P27, N35 and P45 at C3' in the elderly were significantly greater than those of the younger age group, while the amplitudes of N18, N30 and P40 at F3 were not significantly different. The latencies of the major components at rest were also significantly different between the two groups.

The ratios of the amplitudes between each time segment and resting condition were calculated to estimate the degree of attenuation at each time segment prior to the movement in the two age groups (Tables 2 and 3 and Fig. 2). When compared between the age groups, the ratios between the younger and older age groups were not significantly different except for an interaction between time and age for N30 at F3 ($P > 0.05$; ANOVA). The mean ratios of N30 decreased gradually for the elderly, while those for the younger subjects did not show such a trend (Fig. 2). To further confirm this finding, a *post hoc* analysis was carried out. As given in Table 3, there were significant differences for the older subjects between −500 and 0 ms and −3000 and −2500 ms and also between −500 and 0 ms and −2500 and −2000 ms. However, there was no significant difference for the younger subjects in any combination of the time periods.

There was a significant main effect for the time segment for P27, N35 and P45 at C3' and for N30 at F3, which suggested that the peaks became smaller with a change in the time interval for both the younger and older age groups. A post hoc analysis also showed that P45 at C3' and N30 at F3 were significantly different only between −500 and 0 ms and the rest of the segments, while P27 and N35 at C3' had a much earlier gating. For P27, there was a significant difference

between −1500 to −1000 ms and −3000 to −2500 ms, and between −1000 to −500 ms and −3000 to −2500 ms, as well as the difference between −500 to 0 ms and the rest. For N35, the segment between −1000 to −500 ms and −2500 to −2000 ms reached a significant level. In addition, the time window between −500 and 0 ms was also significant (Table 3).

## 4. Discussion

Our results showed that the amplitudes of the SEPs in the resting condition were significantly greater in the older subjects than in the younger ones. This is in agreement with the earlier reports (Lüders, 1970; Desmedt and Cheron, 1980, 1981). However, the effect of gating before the self-paced movements was almost the same for both the age groups except for N30 at F3, where the amplitude ratios normalised by the amplitudes at rest were used as the parameters. The gating effect of SEPs during the pre-movement period is probably caused by sensorimotor interactions and we suggest that this is related to the reduction of excessive afferent information (Lidsky et al., 1985). Our results imply that the function of sensorimotor interaction for the healthy older subjects was largely maintained in younger subjects.

We also suggest that there are different mechanisms involved in the amplitude reduction from the gating during movement preparation and the amplitude enhancement at rest due to ageing. Touge et al. (1997) reported that the gating effect was enhanced in older subjects during isometric contraction. In their study, the raw amplitudes, but not amplitude ratios, were used to evaluate the gating effect. It appears that the amplitude reduction should be evaluated as the ratio, that is, normalised by the amplitude of the resting condition because of the baseline differences caused by ageing.

The interaction between the two age groups and the time segment were evident for N30 at F3, which suggested that the reduction of the ratio in the pre-movement period was greater for the older subjects than for the younger ones. The mechanism for the differential gating effect due to ageing may reflect a higher function, such as cognition or motor imagery, rather than a motor preparation alone because N30 was depressed when a contingent negative variant (CNV) paradigm was used (Murase et al., 2000). Older subjects are reported to require more activated areas such as the pre-motor (PM) areas, cerebellum and pre-supplementary motor areas for voluntary movements (Wu and Hallett, 2005), and the connectivity of the basal ganglia–thalamocortical loop is reduced (Taniwaki et al., 2007). These age-related changes could contribute to the differential gating effect. In any case, our results suggest that the age of the subjects should be matched when the gating effect of N30 is assessed.

Apart from the difference in N30 at F3 between the age groups, there was a comparable gating effect of the peaks after N20 at C3'for both the groups. In SEP studies, there have been a few papers on the effect of pre-movement gating by upper-limb stimulation using SI tasks. Legon et al. (2008) reported a trend for pre-movement

**Table 2**
ANOVA table for the pre-movement gating.

| Sites | Components | Time (main effect) | P | Time × age (interaction) | P |
|---|---|---|---|---|---|
| F3 | P22 | $F(3.663, 102.560) = 1.57$ | 0.192 | $F(3.663, 102.560) = 0.799$ | 0.519 |
| | N30 | $F(3.302, 92.450) = 3.919$ | 0.009* | $F(3.302, 92.450) = 2.792$ | 0.040* |
| | P40 | $F(3.317, 92.867) = 2.537$ | 0.056 | $F(3.317, 92.867) = 0.732$ | 0.549 |
| C3' | N20 | $F(5, 140) = 0.409$ | 0.842 | $F(5, 140) = 0.714$ | 0.614 |
| | P27 | $F(2.930, 82.045) = 22.107$ | <0.001* | $F(2.930, 82.045) = 0.971$ | 0.409 |
| | N35 | $F(3.638, 101.861) = 17.725$ | <0.001* | $F(3.638, 101.861) = 1.065$ | 0.374 |
| | P45 | $F(3.125, 87.493) = 14.848$ | <0.001* | $F(3.125, 87.493) = 1.044$ | 0.379 |

* Indicates a statistically significant difference.

**Table 3**
Multiple comparisons for main effect (time) and interaction (time × age).

| | −3000 to −2500 (ms) | −2500 to −2000 (ms) | −2000 to −1500 (ms) | −1500 to −1000 (ms) | −1000 to −500 (ms) | −500 to 0 (ms) |
|---|---|---|---|---|---|---|
| Main effect (time)[a] | | | | | | |
| P27 | | | | | | |
| −3000 to −2500 (ms) | 1.000 | 0.115 | 0.002* | 0.007* | <0.001* | |
| −2500 to −2000 (ms) | | 1.000 | 0.325 | 0.022* | <0.001* | |
| −2000 to −1500 (ms) | | | 1.000 | 0.945 | <0.001* | |
| −1500 to −1000 (ms) | | | | 1.000 | <0.001* | |
| −1000 to −500 (ms) | | | | | <0.001* | |
| −500 to 0 (ms) | | | | | | |
| N35 | | | | | | |
| −3000 to −2500 (ms) | 1.000 | 1.000 | 0.363 | 0.606 | <0.001* | |
| −2500 to −2000 (ms) | | 1.000 | 0.484 | 0.024* | <0.001* | |
| −2000 to −1500 (ms) | | | 1.000 | 1.000 | <0.001* | |
| −1500 to −1000 (ms) | | | | 1.000 | 0.001* | |
| −1000 to −500 (ms) | | | | | <0.001* | |
| −500 to 0 (ms) | | | | | | |
| P45 | | | | | | |
| −3000 to −2500 (ms) | 1.000 | 0.839 | 1.000 | 0.234 | 0.001* | |
| −2500 to −2000 (ms) | | 1.000 | 1.000 | 1.000 | <0.001* | |
| −2000 to −1500 (ms) | | | 1.000 | 1.000 | <0.001* | |
| −1500 to −1000 (ms) | | | | 0.495 | <0.001* | |
| −1000 to −500 (ms) | | | | | <0.001* | |
| −500 to 0 (ms) | | | | | | |
| N30 | | | | | | |
| −3000 to −2500 (ms) | 1.000 | 1.000 | 1.000 | 1.000 | 0.044* | |
| −2500 to −2000 (ms) | | 1.000 | 1.000 | 1.000 | 0.039* | |
| −2000 to −1500 (ms) | | | 1.000 | 1.000 | 0.045* | |
| −1500 to −1000 (ms) | | | | 1.000 | 0.008* | |
| −1000 to −500 (ms) | | | | | 0.092 | |
| −500 to 0 (ms) | | | | | | |
| Interaction (time × age)[b] | | | | | | |
| N30 | | | | | | |
| −3000 to −2500 (ms) | 1.000 | 1.000 | 1.000 | 0.004* | 0.013* | |
| −2500 to −2000 (ms) | | 1.000 | 1.000 | 1.000 | 0.013* | |
| −2000 to −1500 (ms) | | | 1.000 | 1.000 | 0.550 | |
| −1500 to −1000 (ms) | | | | 0.606 | 0.043* | |
| −1000 to −500 (ms) | | | | | 1.000 | |
| −500 to 0 (ms) | | | | | | |

Matrix chart shows P values amongst each time window.
* Indicates significant differences between each pair. Note that P45 and N30 show a significant difference only between just before movement and the rest of the segments, while earlier gating starting at the period of −1500 to −1000 is evident for P27 and −1000 to −500 is evident for N35.
[a] P values were calculated from the data of both young and old subjects.
[b] P values were calculated from the old subjects only.

gating for N30 at F3 under the SI task, but not the peaks at C3′. The differences between our results and theirs probably resulted from the methodological differences: only young subjects participated in their study and they used the peak-to-peak amplitudes, but not the ratios, as a parameter. In addition, the stimulation rate used in our study was 0.8 Hz on an average, while it was 2 Hz in their study. This is important because it has been reported that increased stimulation rates reduce the amplitudes of the SEPs (Tomberg et al., 1989; Ibanez et al., 1995). Thus, the gating effect may have been reduced in their study by the higher stimulation rate (Touge et al., 1997).

In an MEG study, P30m of the SEFs was depressed during the SI pre-movement period, which is consistent with our results (Wasaka et al., 2003, 2005). The MEG results probably corresponded to the mild modulation of N30 at F3 and P27 at C3′ in this study because P30m is a tangential dipole of S1 (Wasaka et al., 2003), although N30 and P27 were attenuated to different degrees. P27 and N35 of the SEPs would partly include a radial component of SI (Allison et al., 1989), which would be difficult to detect with MEG. The differential gating of N30, P27 and N35 may imply that the gating effect in the primary sensorimotor areas can be different in different areas. Several investigators have suggested that N30 at F3 originated in area 3b (Allison et al., 1989; Sonoo et al., 1991).

However, a case has been reported where N20 was impaired after a post-central lesion, while N30 was spared (Mauguière et al., 1983). Sleep has also been reported to lead to dissociated changes in the frontal and parietal SEPs (Noguchi et al., 1995). Although there is no consensus on the neural generators, our results may support the idea that N30 reflects a component generated beside S1. A recent review (Rossini et al., 2007) has suggested that physiological ageing is associated with more complex activations of the motor system to compensate the reduced motor skills. Older subjects have been shown to have enhanced activities in the motor-related areas (Wu and Hallett, 2005) and increased intra- and interhemispheric connectivity in the motor areas (Taniwaki et al., 2007). These findings are interpreted to be a compensatory increase of decreased motor performance. These compensatory activations can be partly responsible for the preserved gating effect in the elderly in our study.

Movement-related cortical potentials (MRCPs) appear before movement when SI tasks are used. The MRCPs consist mainly of 'Bereitschaftspotentials' (BPs) with a negative slope (NS). BPs are recorded from approximately −1500 ms, while NS is recorded approximately −500 ms. During the pre-movement period, the SEPs are decreased uniformly as early as −1500 to −500 ms; thus, the evolution of the MRCPs and SEP changes may be correlated.
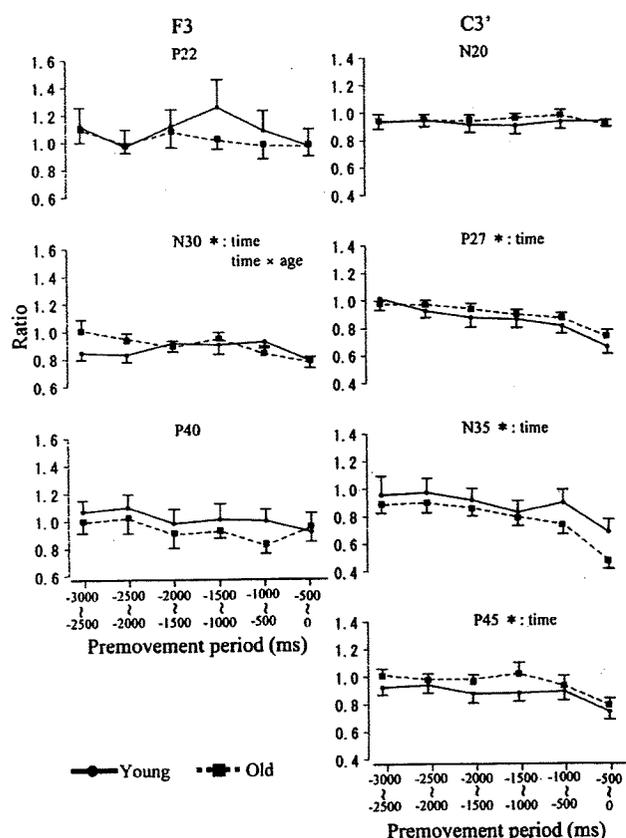
Fig. 2. Ratios of SEP amplitudes during pre-movement period compared to those during the resting condition. The ratios of P27, N35 and P45 at C3′ decrease during the pre-movement period, and N30 at F3 also decreases. There is a significant difference in the N30 ratio between the younger and older subjects at −3000 to −1500 ms. The error bars indicate standard error of the mean (SEM). $^*P < 0.05$, time: main effect of time segment, time × age: interaction of time segment and age groups.

The BPs are generated from the supplementary motor area (SMA) (Neshige et al., 1988), and an fMRI of SI tasks also showed activation of the SMA (Taniwaki et al., 2003, 2006). Thus, the SMA is a key structure for executing self-paced movements and may be related to the gating of SEPs. Matsunaga et al. (2004) reported that the SEPs at C3′ were enhanced after the sensorimotor area was activated by transcranial direct current stimulation. Similarly, inhibitory repetitive transcranial magnetic stimulation (TMS) on the primary motor area (M1) reduced the SEP amplitudes at C3′, while that of S1 and PM was unaffected (Enomoto et al., 2001). Conversely, our result showed that the activation of M1 in the pre-movement period resulted in an attenuation of the SEPs. Taken together, these findings indicate that the gating of SEPs cannot be simply explained by the direct effect of M1 activation on the SEPs.

The amplitude of N30 at F3 was selectively depressed during the pre-movement period when dual ET tasks were employed (Murase et al., 2000). The dual ET tasks refer to tasks that have two signals of warning and starting movement (Shimazu et al., 1999; Murase et al., 2000). CNVs are recorded at the central leads during these tasks, which reflect the functions of the prefrontal, PM, SMA and the primary sensorimotor areas (Gemba et al., 1990; Hamano et al., 1997). Thus, these areas may contribute to the attenuation of N30 under dual ET tasks. Gating with mental motor simulation (MMS) is reflected in N30 of the frontal leads, but not in P27 of the central leads, as that with dual ET tasks (Cheron and Borenstein, 1992). MMS requires motor images and

activates PM, SMA and M1 (Roland et al., 1980; Decety and Grezes, 2006). Therefore, it probably shares PM activation and other cognitive process with dual ET tasks, which may explain the gating of N30 by MMS and dual ET tasks. In contrast, under a single ET task that uses one signal to start the movements, the pre-movement gatings showed that P27 at the central leads was attenuated (Starr and Cohen, 1985) at approximately 100 ms before the movement, although N30 was not recorded. As the period after the signal in a single ET task would include the process of movement initiation, the SEP gating of a single ET task may share a common mechanism of gating with that of the SI task.

In summary, the amplitudes of the SEPs at rest were significantly greater in the elderly than in younger subjects. However, the degree of the gating effect during the pre-movement period was almost the same for both the younger and older groups, except for N30 at F3. The mechanism of the pre-movement gating under SI conditions is probably due to an attenuation of S1 activity, and SMA should be an important structure that underlies the pre-movement gating. The larger SEP amplitudes at rest could be related to enhanced activities in the motor-related areas with increasing intra- and inter-hemispheric connectivity in the elderly. This may result in the preservation of gating. Therefore, pre-movement gating under the SI task can be useful for the investigation of centrifugal processing of motor function. However, care should be taken when this technique is applied to the movement disorders because of the ageing effect.

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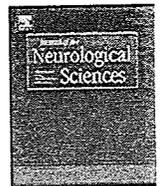
# Upregulation of water channel aquaporin-4 in experimental autoimmune encephalomyeritis

Katsuichi Miyamoto [a], Naomi Nagaosa [a], Mayumi Motoyama [a], Kazuo Kataoka [b], Susumu Kusunoki [a,*]

[a] Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
[b] Department of Neurosurgery, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan

## ARTICLE INFO

## ABSTRACT

Aquaporin-4 (AQP4) is a water channel protein that plays an important role in water movement in the central nervous system (CNS). Recently, presence of anti-AQP4 antibody has been reported in the sera from patients with neuromyelitis optica. AQP4 is therefore a possible target for inflammatory mechanisms in CNS. In the present investigation, we performed semi-quantitative analysis of AQP4-mRNA in brain and spinal cord from mice affected with experimental autoimmune encephalomyelitis (EAE) using real-time PCR. AQP4-mRNA expression was increased in EAE; reaching a peak in the spinal cord at 14 days, and in the brain at 21 days after first inoculation. Immunohistochemical analysis showed that AQP4 is expressed on astrocytes, indicating that the increase in AQP4 expression may correlate with astrocytic activation. This is the first study to demonstrate upregulation of AQP4 in EAE. The upregulation of AQP4 could be involved in the development of inflammation in the acute phase of EAE.

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## 1. Introduction

Aquaporin (AQP) is a family of water channel proteins, which are present on the plasma membrane at the boundary of various tissues, and provide a major pathway for osmotically driven water transport through cell membranes. Some members of the aquaporin family have been identified in the central nervous system (CNS) [1]. AQPs play important roles in the dynamic regulation of brain water homeostasis and in the regulation of cerebrospinal fluid production. AQP4 and AQP1 are the major species that are expressed abundantly in the brain [2,3]. AQP1 is restricted to the apical domain of choroid plexus epithelial cells [4–6]. AQP4 is abundantly present in astrocyte foot processes and cells lining the subarachnoid space and ventricles, whereas AQP9 is localized in astrocyte processes [7–9]. Astrocytes are the major components of the blood–brain barrier (BBB) and have roles as essential effectors in inflammatory reactions in the brain. The distribution of AQP4 suggests the involvement of AQP4 in the movement of water between blood and the brain and between the brain and cerebrospinal fluid (CSF).

Upregulation of AQP4 has been observed in the post-ischemic state [10], brain tumors [11], bacterial meningitis and brains with contusion [12,13]. Furthermore, AQP4 has been shown to be upregulated in experimentally-induced ischemia [14], injury or hyponatremia in rodent brain [15–17], suggesting that the regulation of AQP4 level is associated with the development of brain edema regardless of its cause.

In mice lacking AQP4, brain swelling is reduced following water intoxication and focal cerebral ischemia [18]. AQP4 deletion likely protects against cytotoxic edema in this model by slowing the entry of water into the brain parenchyma. On the other hand, remarkably increased elevations in intracranial pressure in AQP4-deficient mice have been reported in vasogenic edema models, intraparenchymal fluid infusion [19], focal cortical freeze injury [20,21], and tumor cell implantation [22,23]. Thus, in those cases, AQP4 expression may reduce vasogenic brain edema [24].

Recently, the presence of antibodies against AQP4 has been reported in patients affected with Caucasian neuromyelitis optica (NMO) and some Japanese optico-spinal type multiple sclerosis (OS-MS) patients [25–28]. NMO is an idiopathic inflammatory, necrotizing disease characterized by selective involvement of the optic nerves and spinal cord [29]. Misu et al. reported that the loss of AQP4 immunostaining was observed in dymyelinating lesions of NMO patients [30]. Therefore, AQP4 may be involved in the pathophysiological mechanism of inflammatory demyelinating disorder.

Herein, we analyzed AQP4 expression in inflammatory disease of the CNS using experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We performed semi-quantitative analysis of AQP4-mRNA in brains and spinal cord from EAE-induced mice using real-time PCR. We also performed immunohistochemical analysis.

## 2. Materials and methods

### 2.1. Mouse

Wild type C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions. All mice for experiments were aged 8–12 weeks. The

experiments involving animals were approved by the local ethics committee in Kinki University.

### 2.2. Peptides

MOG$_{35-55}$ (single letter amino acid code: MEVGWYRSPFSRVVH-LYRNGK) was synthesized by Tore Research Institute (Tokyo, Japan). The peptides were >90% pure, as determined by HPLC.

### 2.3. Induction and assessment of EAE

Mice were injected subcutaneously in the tail base bilaterally with 200 μl of inoculum containing 100 μg of MOG$_{35-55}$ and 0.5 mg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in incomplete Freund's adjuvant. Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, 200 ng) was injected intravenously on post-inoculation days 0 and 2 [31]. Immunized mice were examined daily and scored as follows: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead. Mice were examined daily for signs of EAE in a blinded fashion.

### 2.4. Pathological analysis

On days 0, 7, 14, 21, 28, and 35 after immunization for EAE, five mice per day were sacrificed under anesthesia with diethyl ether, and the brains and spinal cords were removed after perfusion with PBS. The whole brain was cut into three pieces by coronal sections, and the thoracic cord also was cut into three pieces by axial sections. The middle part of each was used for PCR analyses, and the other parts were analyzed histopathologically. For pathological analysis, thinly sliced (10 μm) frozen sections were fixed with acetone and stained with hematoxylin and eosin, and Luxol fast blue to assess inflammation and demyelination. Immunostaining with anti-AQP4 (Santa Cruz biotechnology, Santa Cruz, CA) or anti-glial fibrillary acidic protein (GFAP, BD Biosciences, San Jose, CA) antibodies was also performed.

### 2.5. Reverse transcriptase-PCR for identification of AQP4

For RT-PCR analysis, brain and spinal cords from each mouse were homogenized in RNAzol™ B (Biotecx laboratories, Friendswood, TX) for total RNA extraction. After isolation of total RNA, oligo (dT)-primed cDNA was prepared with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We performed quantitative PCR with a commercial kit (Light Cycler-DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany) using the Light-Cycler™ quantitative PCR system [32]. The PCR amplification was repeated 45 times (at 95 °C for 15 s, at 56 °C for 7 s, and at 72 °C for 15 s). The PCR primers used were as follows: AQP4-sense, TGGTGTTCAC-TATTTTTGCC and antisense, GATCAAGTCTTCCGTCTCCA.

### 2.6. Statistical analysis

The expression level of AQP4 was analyzed by Mann–Whitney-U test.

## 3. Result

### 3.1. Quantitative analysis of AQP4-mRNA

A significant increase of AQP4-mRNA was observed only in EAE-affected mice. In EAE mice, AQP4-mRNA expression in the spinal cord began to increase on post-inoculation day 7 and reached a peak on day 14. In contrast, the increase of AQP4-mRNA expression in the brain was delayed compared with that in the spinal cord; reaching a peak on post-inoculation day 21 (Fig. 1A). AQP4-mRNA expression at the peak
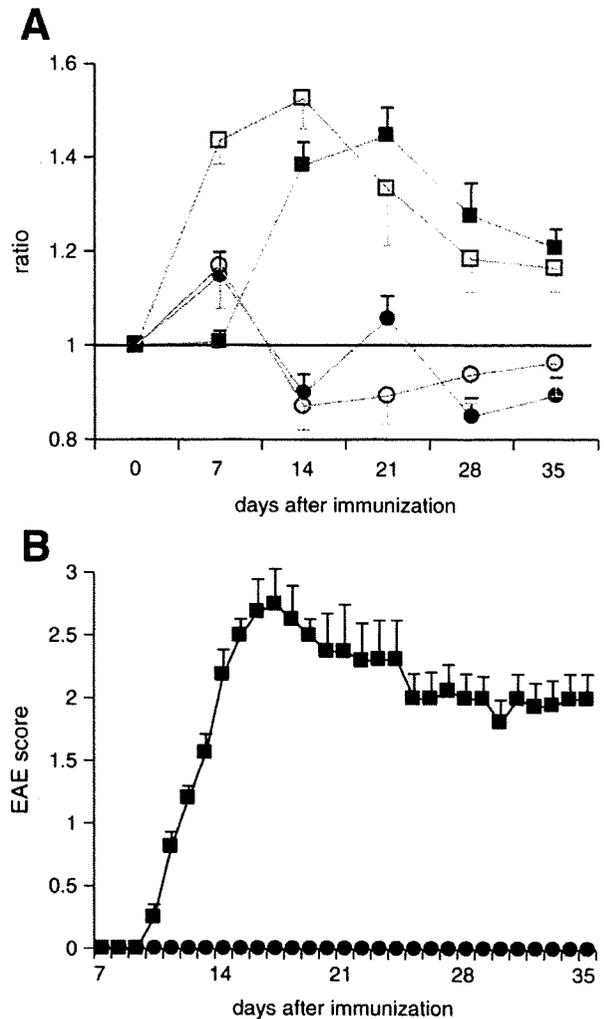


Fig. 1. Quantitative analysis of AQP4-mRNA in EAE. (A) EAE was induced in female B6 mice by immunization with MOG$_{35-55}$ in CFA, as described in the Materials and methods. Expression of AQP4-mRNA in CNS from each mouse was analyzed by LightCycler™, as described in the Materials and methods. To compare AQP4-mRNA levels among the groups, the expression level was expressed as a relative value. We determined the expression level on day 0 in each group as 1.0. In EAE mice, AQP4-mRNA expression in the spinal cord began to increase on post-inoculation day 7 and reached the peak on day 14 (□). The increase of AQP4-mRNA expression in the brain was delayed in comparison with that in spinal cord and it reached the peak on post-inoculation day 21 (■). On the other hand, no remarkable elevation of AQP4-mRNA level was observed in brain (●) and spinal cord (○) from control mice. (B) EAE scores from three independent experiments are expressed as mean±S.E.M. (■). Control mice were immunized with CFA without MOG$_{35-55}$ (●).

was 152% in the spinal cord and 145% in brain, compared with the pre-inoculation levels. The expression decreased gradually in both spinal cord and brain during subsequent weeks. The clinical score of EAE reached its peak on post-inoculation day 17 (Fig. 1B).

### 3.2. Histopathological analysis

Histopathological analysis showed that demyelination and cell infiltration were most severe on post-inoculation day 21, when the clinical manifestations were also severe. Next we performed immunohistochemical staining to analyze the protein expression of AQP4 in CNS of the animals with EAE on post-inoculation day 21. AQP4 was expressed in both spinal cord (Fig. 2A) and brain (Fig. 2B), and the staining with anti-AQP4 antibody was colocalized with anti-GFAP
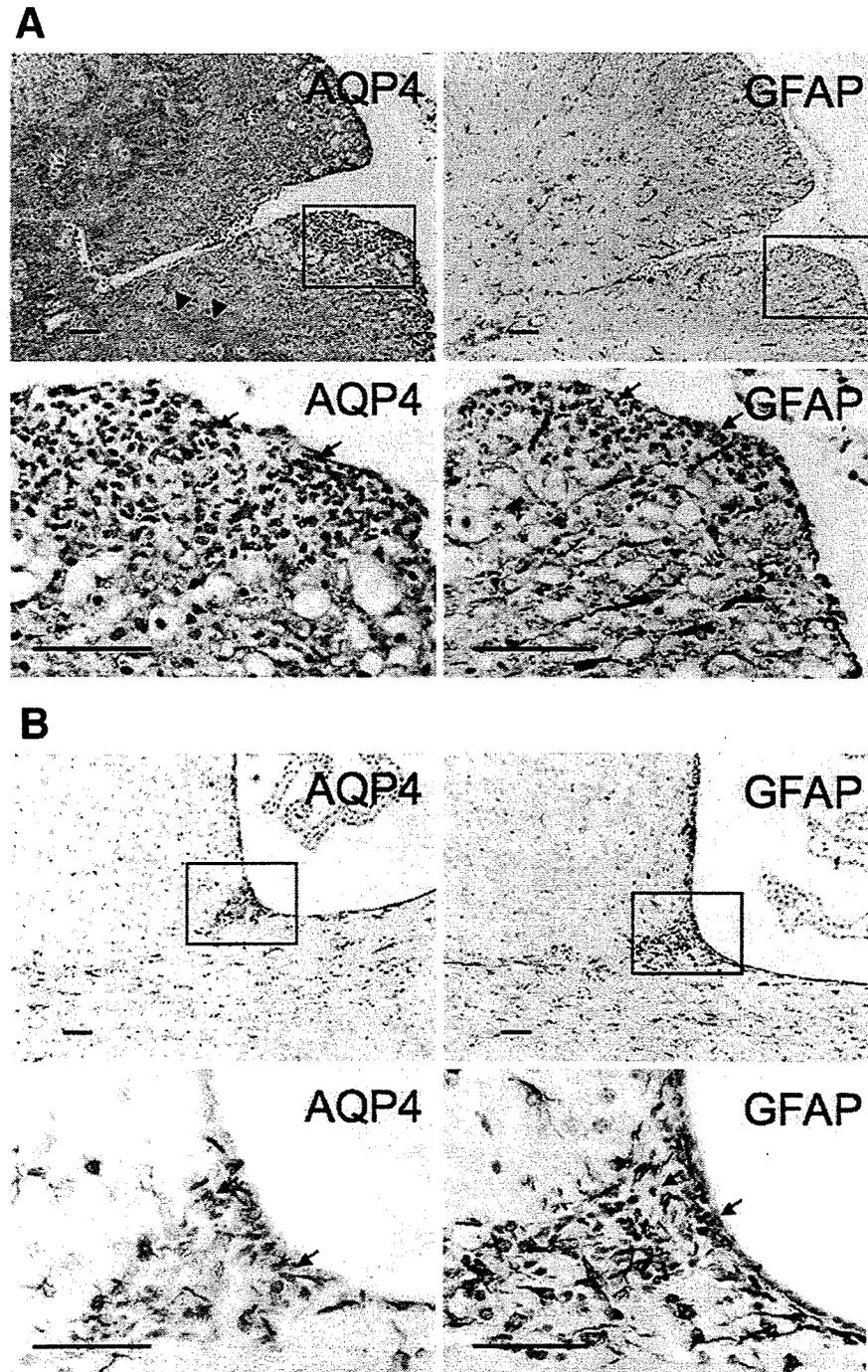
Fig. 2. Histopathological analysis. The histopathological analysis on post-inoculation day 21 is shown. Both AQP4 and GFAP were expressed (brown) in spinal cord (A) and brain (B). Each lower panel showed the enlarged square area in the upper panel. The cellular infiltration (arrows) was seen in both spinal cord (A) and brain (B). In the spinal cord, AQP4 staining in the gray matter is stronger than that in white matter (arrow head). Staining with the anti-AQP4 (green) antibody was colocalized with that of the anti-GFAP (red) antibody (C). (D) Showed the staining pattern of AQP4 and GFAP in brain from control mice immunized without MOG. Bar=100 μm.

antibody (Fig. 2C). This suggests that AQP4 expression may correlate with astrocytic activation.

## 4. Discussion

We demonstrated that AQP4-mRNA expression was elevated in the CNS from mice affected with EAE in the acute phase. Immunohisto-chemical analysis showed that AQP4 was expressed in both spinal cord and brain in EAE mice. The distribution of the staining with anti-AQP4 antibody was similar to that of anti-GFAP antibody. AQP4 therefore may be expressed in activated astrocytes. This is the first study to investigate the regulation of AQP-4 in EAE.

The peak expression of AQP4 mRNA was detected on day 14 in the spinal cord, and on day 21 in the brain after immunization for EAE. It is unclear why the expression in the spinal cord reached its peak earlier than that in the brain. We consider that the time course of AQP4-mRNA
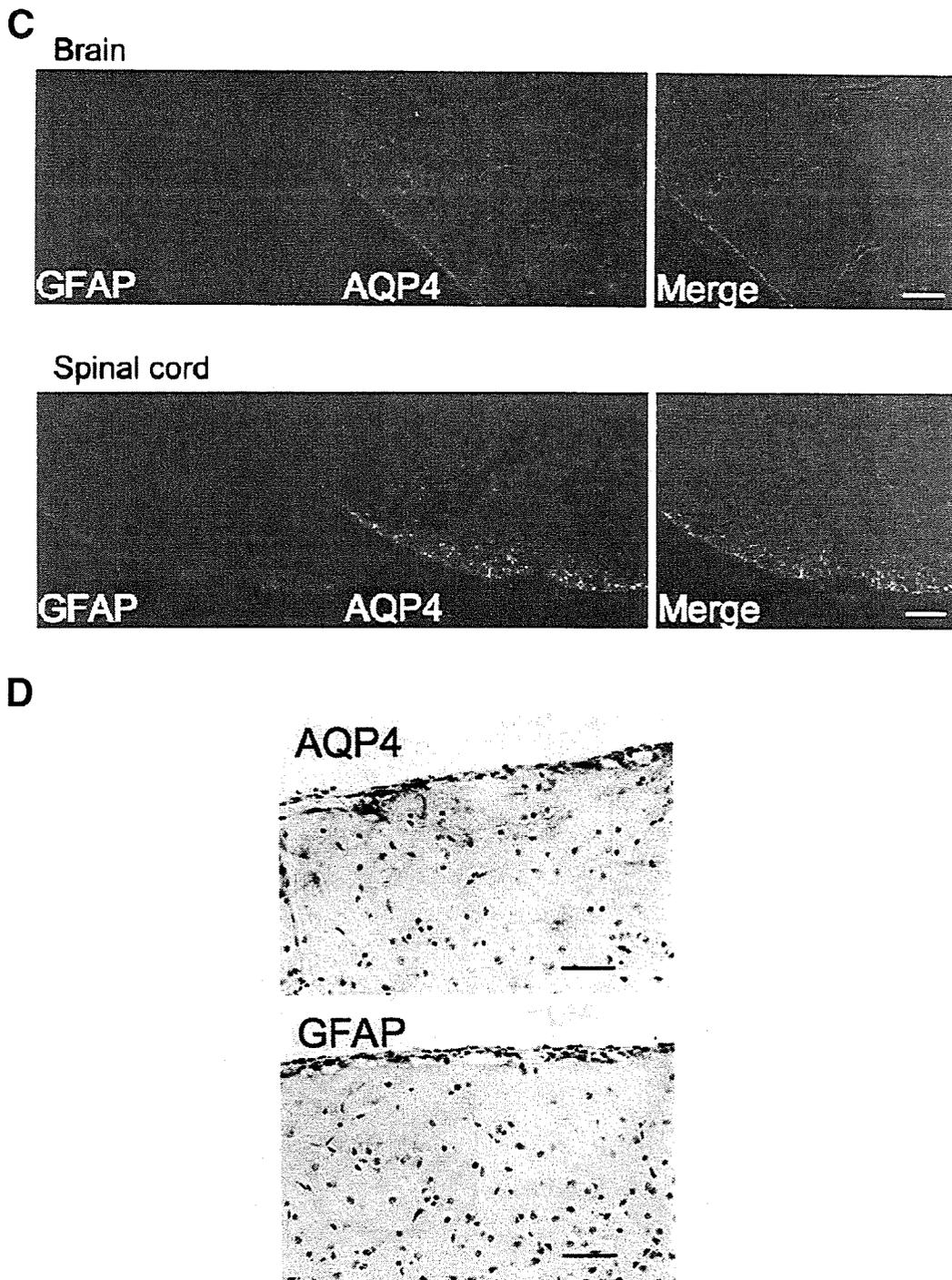
**C**

**Brain**



**Spinal cord**



**D**



Fig. 2 (continued).

expression depends on the clinical course of EAE. The clinical signs of EAE induced by active-immunization with MOG$_{35-55}$ in the present study usually appear first in tail, followed subsequently by the hind limbs and ore limbs are affected. The pathological changes also appear first in the lumbar spinal cord. We generally inoculate the mice at the lower part of the body, tail base or hind limbs. Thus, the inflammation begins at the lower part of the spinal cord, and then ascends gradually up to the brain. Astrocytic activation also may occur first in the spinal cord, followed by the brain. The astrocytic activation could also be related to AQP4 expression, as described above.

Previous studies in AQP4-deficient mice have shown reduced cellular brain edema following water intoxication and ischemic stroke [18]. On the other hand, higher intracranial pressure and brain edema have been reported in AQP4-deficient mice after continuous intraparenchymal fluid infusion [24]. Thus, the effect of AQP4 deletion is difficult to predict for each situation. Future investigations should determine whether the increased expression of AQP4 in EAE aggravates or ameliorates the inflammatory edema.

In human MS, AQP4 immunoreactivity was well preserved and stained strongly in the demyelinating plaques [33]. AQP4-positive

astrocytes were more abundant at the periphery of plaques than in their center, as seen in ischemic foci. AQP4 stained from the very acute phase of necrosis to the chronic stage of astrogliosis. In contrast, the immunoreactivities of AQP4 and GFAP in NMO were consistently lost from the early stage in NMO lesions. These distinct features suggest that the different mechanisms of initiation and progression between MS and NMO. Thus, astrocytic impairment associated with the loss of AQP4 and humoral immunity may be important in the pathogenesis of NMO lesions [30]. Our results show that the regulation of AQP-4 levels in EAE is similar to that of MS but not of NMO.

Our results showed that the upregulation of AQP4 could be involved in the development of inflammation in the acute phase of EAE. Future studies using EAE to examine whether the anti-AQP-4 antibody has any effect on astrocytes with enhanced AQP-4 expression may be warranted. Such investigations may provide us with an important clue to solve the pathogenesis of NMO.

## Acknowledgements

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# Atherosclerosis

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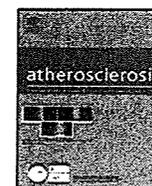
# Predictive value of plasma B-type natriuretic peptide for ischemic stroke: A community-based longitudinal study

Tomohiro Takahashi[a,*], Motoyuki Nakamura[a], Toshiyuki Onoda[b], Masaki Ohsawa[b], Kozo Tanno[b], Kazuyoshi Itai[b], Kiyomi Sakata[b], Masafumi Sakuma[a], Fumitaka Tanaka[a], Shinji Makita[a], Yuki Yoshida[c], Akira Ogawa[c], Kazuko Kawamura[d], Akira Okayama[e]

[a] Division of Cardiology, Department of Internal Medicine, Iwate Medical University, Morioka, Japan
[b] Department of Hygiene and Preventive Medicine, Iwate Medical University, Morioka, Japan
[c] Department of Neurosurgery, Iwate Medical University, Morioka, Japan
[d] Iwate Health Service Association, Morioka, Japan
[e] Japan Anti-Tuberculosis Association, Tokyo, Japan

## ARTICLE INFO

## ABSTRACT

*Objective:* Structural heart diseases including atrial fibrillation are precursors for ischemic stroke. Plasma B-type natriuretic peptide (BNP) has been reported to be increased in patients with several types of structural heart diseases. However, the predictive value of plasma BNP for ischemic stroke remains unknown. We have studied the predictive ability of plasma BNP for future development of stroke in community dwelling adults.
*Methods:* Subjects of this community-based study were recruited from the general population ($n = 13,466$). Plasma BNP levels and cardiovascular risk factors were determined at baseline. The incidence of ischemic stroke in the cohort was identified from regional stroke registry data. A multivariate Cox regression analysis was performed to analyze the relationship between plasma BNP levels and the risk of stroke.
*Results:* During a mean follow-up period of 2.8 years, 102 participants (65 males, 37 females) experienced a first ischemic stroke. In men, after adjustment for classical cardiovascular risk factors and atrial fibrillation, the hazard ratio (HR) for ischemic stroke was significantly elevated in the highest plasma BNP quartile (HR = 2.38; 95% CI = 1.07–5.29). In women, the relationship between plasma BNP levels and risk of ischemic stroke was of marginal significance after adjusting for the presence or absence of atrial fibrillation (HR = 3.03; 95% CI = 0.84–10.92, $P = 0.09$).
*Conclusion:* Elevated plasma BNP levels predict the risk of ischemic stroke within men from the general population.

## 1. Introduction

B-type natriuretic peptide (BNP) is a cardiac hormone secreted from the myocardium in response to changes in intracardiac volume and pressure [1,2]. Plasma BNP levels are known to be elevated in patients with symptomatic left ventricular systolic dysfunction [3,4] and correlate to New York Heart Association (NYHA) class as well as prognosis [5,6]. In addition, irrespective of the degree of left ventricular dysfunction, plasma BNP levels have been shown to be elevated in patients with various structural heart diseases including previous myocardial infarction, cardiomyopathy, valvu-

lar heart disease, hypertensive heart disease, and atrial fibrillation [3,7–13].

These structural heart diseases are precursors not only for heart failure, but also for ischemic stroke, and especially cardioembolic stroke [14]. However, there have been very few reports on the association between plasma BNP levels and the risk of stroke. The Framingham Heart Study [15] has described a 4.9-fold increase in the crude incidence of stroke or transient ischemic attack in the highest tertile of BNP levels compared to the lowest tertile. Kistorp et al. [16] reported that plasma levels of N-amino terminal fragment of the prohormone BNP (NT-proBNP) predicted the risk of stroke or transient ischemic attack, with a 3.6-fold increase in risk of stroke for participants with values above the 80th percentile vs those with values equal to or below the 80th percentile in the general population. However, the association between plasma BNP levels and risk of stroke subtypes remains unclear. The predictive value of plasma BNP measurement for ischemic stroke remains unknown.

* Corresponding author at: Division of Cardiology, Department of Internal Medicine, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka 0208505, Japan. Tel.: +81 19 651 5111x2324; fax: +81 19 651 0401.
*E-mail address:* ttomo@ruby.ocn.ne.jp (T. Takahashi).

We have studied the predictive ability of plasma BNP for future development of ischemic stroke in community dwelling adults.

## 2. Methods

### 2.1. Study population

The Iwate-Kenpoku Cohort (Iwate-KENCO) study was designed to prospectively investigate the risk of cardiovascular diseases including stroke and malignant tumor in the general Japanese adult population as described previously [17,18]. Subjects consisted of residents of the Ninohe, Kuji and Miyako districts in the northern Iwate prefecture, Japan. Between April 2002 and January 2005, 26,469 of these residents (men = 9161, women = 17,308) who were participating voluntarily in a multiphasic health checkup agreed to join the study (original cohort). The baseline survey included routine anthropometrical measurement, blood pressure measurement, ECG, routine laboratory assessment, a self-administered lifestyle questionnaire, and a food-frequency questionnaire. This study protocol was approved by our institutional ethics committee. All participants gave written informed consent.

Of the original cohort living in the Ninohe and Kuji districts ($n$ = 15,927), 15,394 subjects (men = 5288, women = 10,106) underwent BNP measurement (BNP cohort). Subjects were excluded from this cohort on the basis of the following characteristics: age under 40 years ($n$ = 575), history of cardiovascular or cerebrovascular events ($n$ = 507), non-measurement of adjustment factors ($n$ = 846). The final statistical analysis was therefore performed in 13,466 subjects (men = 4527, women = 8939, mean age = 62.7 years).

### 2.2. Outcome

In this cohort study, the primary endpoint was all-cause death, in addition to any nonfatal cardiovascular events such as myocardial infarction, cerebral infarction, or other strokes. Information about death and emigration was obtained from local government records. Stroke events were identified by accessing the Iwate prefecture stroke registration programme, which has been conducted since 1991 by the Iwate Medical Association with the support of the government of the Iwate prefecture [19]. Registration forms were submitted to the registration office of the Iwate Medical Association by mail when a patient with stroke was discharged from a medical facility. Diagnostic criteria for stroke used by the registry correspond with those published by the World Health Organization, based on a definition of sudden onset of neurological symptoms [20]. For diagnosis of stroke subtypes, computed tomography and/or magnetic resonance imaging were performed within each hospital. In order to improve accuracy of registration, trained research nurses checked medical charts in all hospitals located within these districts. Follow-up was conducted until August 2007.

### 2.3. Measurement

At the time of baseline survey, participants underwent anthropometrical measurement, ECG, blood pressure measurement, and routine laboratory assessment. In addition, a self-administered questionnaire was used to ascertain family history, symptoms, and lifestyle factors such as smoking habits, alcohol consumption, and exercise habits. A medical history including the status of drugs prescribed for hypertension, hyperlipidemia, diabetes, angina, myocardial infarction, congestive heart failure, and stroke was recorded by trained research staff. Using a 3-channel device, a standard 12-lead ECG was recorded in a supine position. Atrial fibrillation was defined by this 12-lead ECG at the time of baseline survey. Systolic and diastolic blood pressures were determined with an automatic device placed on the right arm of seated sub-

jects who had rested in a sitting position for at least 5 min before measurement. Measurement was performed twice, with the mean value used for statistical analysis. Hypertension was defined as systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg, and/or current anti-hypertensive therapy. Hyperlipidemia was defined as total cholesterol level $\geq$240 mg/dL, and/or current lipid lowering therapy. Diabetes was defined as non-fasting glucose concentration $\geq$200 mg/dL, and/or glycosylated hemoglobin (HbA1c) value $\geq$6.5%, and/or current anti-diabetic therapy. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m$^2$). Smoking was defined as current smoker. Regular alcohol consumption was defined as drinking alcohol 5 days or more per week. Regular exercise was defined as exercising (at least 60 min) 8 days or more per month.

Venous blood samples for plasma BNP measurement were drawn from the antecubital vein of seated participants with minimal tourniquet use. Samples were collected into vacuum tubes containing ethylenediaminetetraacetic acid sodium. Tubes were stored in an icebox immediately after sampling and were transported to our laboratory within 8 h of collection. These were then centrifuged at 1500 × $g$ for 10 min. After separation, plasma samples were stored frozen at −20 °C until the time of assay. Plasma BNP levels were measured by direct radioimmunoassay using monoclonal antibodies specific for human BNP (ShionoRIA BNP, Shionogi, Japan) within 4 months of separation. The intraassay and interassay coefficients of variation were 5% and 6%, respectively. The lower detection limit of the assay was 0.05 pg/mL. Enzymatic methods were used to measure serum total cholesterol levels, serum creatinine, and blood glucose. HbA1c was measured quantitatively with an HPLC method.

### 2.4. Statistical analysis

Participants were divided into quartiles according to their baseline plasma BNP levels. Continuous variables were expressed as mean ± SD. Group comparisons were based on the unpaired $t$-test and multiple group comparisons across BNP quartiles were based on the one-way analysis of variance. Because BNP values were not normally distributed, these were expressed as median and the Mann–Whitney $U$-test was used for comparison. Categorical parameters were expressed as proportions (percentage) and group comparisons were based on the chi-square test.

The ischemic stroke event free rates according BNP quartiles were estimated using the Kaplan–Meier method, followed by Log-rank test. A multivariate Cox regression analysis was performed to analyze the relationship between plasma BNP levels and risk of stroke. For all models, the hazard ratios were adjusted for age, BMI, blood hemoglobin levels, serum creatinine levels, presence or absence of hypertension, hyperlipidemia, diabetes, smoking, regular alcohol consumption, and regular exercise. The analysis was not adjusted for presence or absence of atrial fibrillation in Model 1 and was adjusted in Model 2. Additional multivariate Cox regression analysis using covariates in Model 1 was performed using 1 SD increments in natural logarithm-transformed BNP values. For the analysis of stroke incidence, person-years were censored at the date of stroke diagnosis, the date of emigration from the study area, the date of death, or the end of the follow-up period, whichever came first. All statistical analysis was performed using SPSS software, version 11.0. A significant difference was defined as $P<0.05$.

## 3. Results

Baseline characteristics of participants by sex are shown in Table 1. The mean age of men was higher than that of women. The percentages of hypertension, diabetes, atrial fibrillation, smoking, regular alcohol consumption, regular exercise, and mean values for

**Table 1**

Comparison of baseline characteristics between men and women.

| Characteristic | Men ($N=4527$) | Women ($N=8939$) | $P$-value |
|---|---|---|---|
| Age (years) | 64.1 ± 10.3 | 62.0 ± 10.0 | <0.001 |
| Hypertension (%) | 44.4 | 38.8 | <0.001 |
| Hyperlipidemia (%) | 10.3 | 20.3 | <0.001 |
| Diabetes (%) | 8.0 | 4.3 | <0.001 |
| Body mass index (kg/m$^2$) | 23.9 ± 2.9 | 24.2 ± 3.4 | <0.001 |
| Smoking (%) | 33.4 | 2.5 | <0.001 |
| Regular alcohol consumption (%) | 47.4 | 4.2 | <0.001 |
| Regular exercise (%) | 17.0 | 10.5 | <0.001 |
| Atrial fibrillation (%) | 3.0 | 0.6 | <0.001 |
| Hemoglobin (g/dL) | 14.6 ± 1.3 | 13.0 ± 1.1 | <0.001 |
| Creatinine (mg/dL) | 0.82 ± 0.19 | 0.63 ± 0.12 | <0.001 |
| BNP (median) (pg/mL) | 14.8 | 17.1 | <0.001 |

Continuous variables are expressed as mean ± SD.

Comparison of BNP data are performed using a Mann-Whitney U test.

hemoglobin and serum creatinine were significantly higher in men. The percentage of hyperlipidemia and mean BMI were significantly higher in women. The median value for plasma BNP was higher in women.

Table 2 shows baseline characteristics among the BNP quartiles. In men, mean age and BMI and mean levels of hemoglobin, and serum creatinine were different among the BNP quartiles ($P < 0.001$). Although the percentages of hypertension, hyperlipidemia, current smoking, and regular exercise were different ($P < 0.001$), the percentages of diabetes and regular alcohol consumption did not differ among the BNP quartiles. In women, although mean age and mean levels of hemoglobin, and serum creatinine were different among the BNP quartiles ($P < 0.001$), the mean BMI did not differ among the BNP quartiles. Although the percentages of hypertension, hyperlipidemia, diabetes, current smoking, and regular alcohol consumption were different ($P < 0.05$), the percentage undertaking regular exercise did not differ among the BNP quartiles. Subjects with atrial fibrillation were concentrated in the highest BNP quartile in both men and women.

During a mean follow-up period of 2.8 years, 102 participants (65 males, 37 females) had a first ischemic stroke event. Ranges of BNP levels in men and women are shown in Table 2. The crude incidences of ischemic stroke (per 1000 person-years) among BNP quartiles in men and women are shown in Tables 3 and 4. The crude incidence of ischemic stroke in men was 2.76 per 1000 person-years in Q1 (the lowest quartile) and 12.51 per 1000 person-years in Q4 (the highest quartile). The crude incidence of ischemic stroke in women was 0.44 per 1000 person-years in Q1 and 2.95 per 1000 person-years in Q4. The crude incidence of ischemic stroke elevated in the highest quartile in both men and women.

The Kaplan–Meier curves for ischemic stroke event free rates according to BNP quartiles in men and women are shown in Fig. 1. The ischemic stroke event free rates differed significantly among the BNP quartiles in both men and women (men: $P < 0.001$; women: $P < 0.001$ by log-rank test).

Several studies have demonstrated that blood hemoglobin levels [21], renal function [22] and BMI [23] influence plasma BNP levels. For that reason, after adjustment for classical cardiovascular risk

**Table 2**

Comparisons of baseline characteristics among BNP quartiles.

| BNP quartiles | Q1 | Q2 | Q3 | Q4 | $P$-value |
|---|---|---|---|---|---|
| *Men* | | | | | |
| Number of subjects | 1131 | 1134 | 1129 | 1133 | |
| Range of BNP levels (pg/mL) | <6.5 | 6.5–14.8 | 14.9–29.9 | 30.0< | |
| Age (years) | 57.4 ± 10.1 | 61.9 ± 9.8 | 66.7 ± 8.3 | 70.3 ± 7.8 | <0.001 |
| Hypertension (%) | 33.3 | 40.0 | 47.5 | 56.8 | <0.001 |
| Hyperlipidemia (%) | 16.5 | 9.8 | 7.9 | 7.1 | <0.001 |
| Diabetes (%) | 7.6 | 8.2 | 8.4 | 7.6 | 0.846 |
| Body mass index (kg/m$^2$) | 24.1 ± 2.9 | 24.1 ± 2.9 | 23.8 ± 2.9 | 23.6 ± 3.0 | <0.001 |
| Smoking (%) | 40.4 | 34.9 | 31.6 | 26.6 | <0.001 |
| Regular alcohol consumption (%) | 48.2 | 45.7 | 47.3 | 48.3 | 0.576 |
| Regular exercise (%) | 11.6 | 17.1 | 20.0 | 19.3 | <0.001 |
| Atrial fibrillation (%) | 0.53 | 0.00 | 0.71 | 10.59 | <0.001 |
| Hemoglobin (g/dL) | 15.0 ± 1.1 | 14.8 ± 1.1 | 14.5 ± 1.2 | 14.2 ± 1.4 | <0.001 |
| Creatinine (mg/dL) | 0.80 ± 0.14 | 0.81 ± 0.16 | 0.83 ± 0.24 | 0.84 ± 0.19 | <0.001 |
| | | | | | |
| *Women* | | | | | |
| Number of subjects | 2235 | 2228 | 2242 | 2234 | |
| Range of BNP levels (pg/mL) | <8.9 | 8.9–17.0 | 17.1–30.4 | 30.5< | |
| Age (years) | 57.6 ± 9.5 | 60.0 ± 9.8 | 62.8 ± 9.4 | 67.4 ± 8.7 | <0.001 |
| Hypertension (%) | 28.6 | 33.4 | 40.7 | 52.4 | <0.001 |
| Hyperlipidemia (%) | 24.3 | 20.4 | 20.2 | 16.3 | <0.001 |
| Diabetes (%) | 5.0 | 3.1 | 4.1 | 4.9 | 0.006 |
| Body mass index (kg/m$^2$) | 24.3 ± 3.4 | 24.2 ± 3.3 | 24.0 ± 3.3 | 24.1 ± 3.5 | 0.206 |
| Smoking (%) | 3.9 | 2.4 | 2.2 | 1.5 | <0.001 |
| Regular alcohol consumption (%) | 5.0 | 4.6 | 3.8 | 3.4 | 0.027 |
| Regular exercise (%) | 10.2 | 10.2 | 10.9 | 10.9 | 0.756 |
| Atrial fibrillation (%) | 0.09 | 0.05 | 0.04 | 2.24 | <0.001 |
| Hemoglobin (g/dL) | 13.2 ± 1.1 | 13.1 ± 1.1 | 13.0 ± 1.1 | 12.8 ± 1.1 | <0.001 |
| Creatinine (mg/dL) | 0.61 ± 0.10 | 0.63 ± 0.11 | 0.63 ± 0.10 | 0.66 ± 0.15 | <0.001 |

Continuous variables are expressed as mean ± SD.

**Table 3**

The crude incidence and multivariate hazard ratio of ischemic stroke among BNP quartiles in men.

| BNP quartiles | Q1 | Q2 | Q3 | Q4 | P for trend |
|---|---|---|---|---|---|
| Observed person-years | 3619 | 3198 | 3116 | 3036 | |
| *Ischemic stroke* | | | | | |
| Crude incidence (/1000 person-years) | 2.76 | 2.19 | 3.21 | 12.51 | |
| *Multivariate HR (95%CI)* | | | | | |
| Model 1 | 1.0 (ref.) | 0.71 (0.27–1.89) | 0.85 (0.34–2.12) | 2.83 (1.29–6.20) | <0.001 |
| Model 2 | 1.0 (ref.) | 0.71 (0.27–1.88) | 0.81 (0.33–2.03) | 2.38 (1.07–5.29) | <0.005 |

For all models, the hazard ratios were adjusted for age, presence or absence of hypertension, hyperlipidemia, diabetes, smoking, regular alcohol consumption, and regular exercise. BMI, blood hemoglobin levels, and serum creatinine levels.
Model 1: The analysis was not adjusted for presence or absence of atrial fibrillation.
Model 2: The analysis was adjusted for presence or absence of atrial fibrillation.
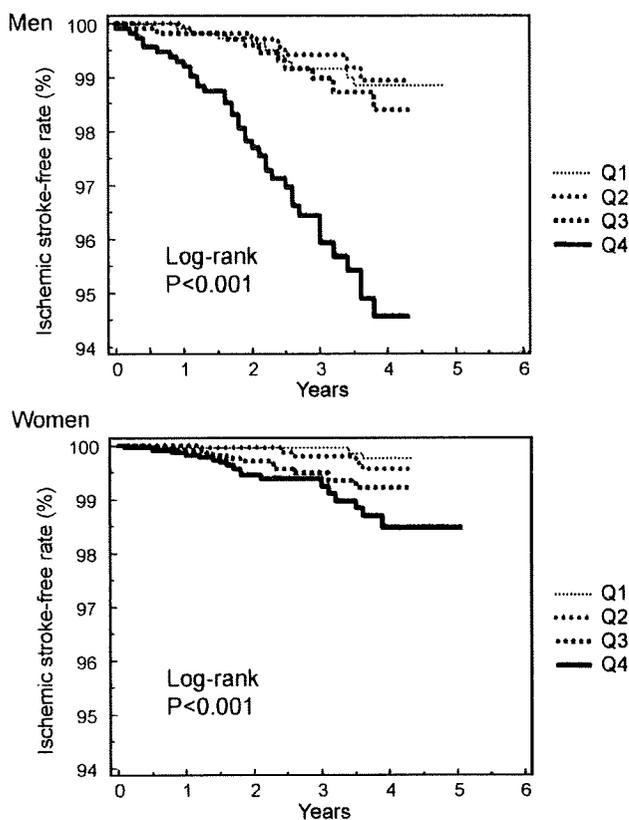


Fig. 1. Kaplan–Meier curves for ischemic stroke event free rate according to BNP quartiles by sex.

factors, blood hemoglobin levels, serum creatinine levels, and BMI, a multivariate Cox regression analysis was performed to analyze the relationship between plasma BNP levels and the risk of stroke. In men, the hazard ratio (HR) obtained from a Cox proportional model for ischemic stroke in the highest BNP quartile was significantly elevated in Model 1 (HR = 2.83; 95% CI = 1.29–6.20; Table 3). After also adjusting for the presence or absence of atrial fibrillation (Model 2), HR in the highest BNP quartile was still significantly elevated (HR = 2.38; 95% CI = 1.07–5.29; Table 3). The risk of incidence of ischemic stroke increased in association with BNP levels ($P < 0.01$). In women, HR for ischemic stroke in the highest BNP quartile was significantly elevated in Model 1 (HR = 3.61; 95% CI = 1.01–12.93; Table 4). After adjusting for the presence or absence of atrial fibrillation (Model 2), the relationship between plasma BNP levels and the risk of ischemic stroke was of marginal significance (HR = 3.03; 95% CI = 0.84–10.92, $P = 0.09$; Table 4).

An additional multivariate Cox regression analysis was performed using 1 SD increments in natural logarithm-transformed BNP values. Elevated plasma BNP levels were associated with an elevated risk of ischemic stroke in both men and women (HR = 1.70; 95% CI = 1.17–2.45 in men; HR = 1.69; 95% CI = 1.04–2.75 in women).

## 4. Discussion

There have been very few reports on the association between plasma BNP levels and the risk of stroke, [15,16] and the relationship with risk of stroke subtypes therefore remains unclear. The present study suggests that high plasma BNP levels predict the risk of ischemic stroke within the general Japanese population. Ischemic stroke is classified into atherothrombotic infarction, cardiogenic embolic infarction, and lacunar infarction. Several types of structural heart diseases including atrial fibrillation, which are associated with elevated plasma BNP levels, may be an important cause of ischemic stroke, especially cardioembolic stroke. In view of this, elevated plasma BNP levels may be a biomarker for high risk of ischemic stroke.

Cardiac disorders linked with ischemic stroke, especially cardioembolic stroke, are nonvalvular atrial fibrillation, acute

**Table 4**

The crude incidence and multivariate hazard ratio of ischemic stroke among BNP quartiles in women.

| BNP quartiles | Q1 | Q2 | Q3 | Q4 | P for trend |
|---|---|---|---|---|---|
| Observed person-years | 6794 | 6283 | 6188 | 6099 | |
| *Ischemic stroke* | | | | | |
| Crude incidence (/1000 person-years) | 0.44 | 0.80 | 1.78 | 2.95 | |
| *Multivariate HR (95%CI)* | | | | | |
| Model 1 | 1.0 (ref.) | 1.72 (0.41–7.25) | 3.07 (0.84–11.16) | 3.61 (1.01–12.93) | 0.168 |
| Model 2 | 1.0 (ref.) | 1.79 (0.43–7.55) | 3.15 (0.87–11.44) | 3.03 (0.84–10.92) | 0.269 |

For all models, the hazard ratios were adjusted for age, presence or absence of hypertension, hyperlipidemia, diabetes, smoking, regular alcohol consumption, and regular exercise. BMI, blood hemoglobin levels, and serum creatinine levels.
Model 1: The analysis was not adjusted for presence or absence of atrial fibrillation.
Model 2:The analysis was adjusted for presence or absence of atrial fibrillation.

myocardial infarction, ventricular aneurysm, and valvular heart disease. According to the Cerebral Embolism Task Force [14], non-valvular atrial fibrillation is the most common cardiac disorder associated with embolic stroke, accounting for 45% of embolic strokes. Several previous studies have suggested that plasma BNP levels were significantly higher in patients with atrial fibrillation than in those without atrial fibrillation [11,13]. The Framingham Heart Study [15] has indicated that higher plasma BNP levels predict risk of atrial fibrillation. It is therefore possible that atrial fibrillation-related high plasma BNP levels are associated with increased risk of ischemic stroke. We therefore analyzed the relationship between plasma BNP levels and risk of ischemic stroke after adjusting for the presence or absence of atrial fibrillation. Even after this adjustment, HR was still significant in men. This suggests that there may be factors other than atrial fibrillation underlying the apparent relationship between plasma BNP levels and risk of ischemic stroke. As the present study did not perform echocardiography as a baseline examination, some subjects may have had asymptomatic structural heart disease (i.e. left ventricular dysfunction, valvular heart disease, or left ventricular hypertrophy) characterized by elevated plasma BNP [12] which would account for the significant relationship between plasma BNP levels and risk of ischemic stroke. This study was therefore unable to show a correlation between plasma BNP levels and risk of stroke independent of the presence of heart disease. However, it is difficult to perform echocardiography routinely for participants in a community-based multiphasic health checkup. A simple blood test for BNP is an ideal approach for selecting males at high risk for ischemic stroke within the general population. In addition, a previous study examining the relationship between traditional and nontraditional risk factors and the incidence of ischemic stroke subtypes has reported that left ventricular hypertrophy increases the risk not only of cardioembolic stroke but also of atherothrombotic stroke [24]. It follows that high plasma BNP levels may be associated with both cardioembolic and atherothrombotic stroke.

The present study has shown a median plasma BNP level of 14.8 pg/mL and the threshold plasma BNP levels associated with elevated risk of ischemic stroke of 30.0 pg/mL in men. The Framingham Heart Study [15] found a median plasma BNP level of 6.2 pg/mL and the threshold plasma BNP levels associated with elevated risk of stroke or transient ischemic attack of 20.0 pg/mL in a community-based male sample. Both studies have shown that excess risk is apparent at plasma BNP levels well below the thresholds currently used to diagnose heart failure [25].

A possible reason for the marginal significance of the relationship between plasma BNP levels and risk of ischemic stroke in women after adjusting for the presence or absence of atrial fibrillation may be the low incidence of stroke in the female cohort. The crude incidences of stroke in women were clearly lower than those in men, and thus, the statistical power to show any relationship between risk and incidence of stroke might be limited in women. As the statistical results concerning the relationship between plasma BNP levels and risk of stroke in women were not so robust, more events should be gathered to investigate the predictive power of plasma BNP with regard to stroke in women.

Although our study was a large, prospective community-based longitudinal study, several limitations must be considered when interpreting the results. Since ECG testing was performed only at the time of baseline survey, paroxysmal atrial fibrillation had not been detected and new incidence of atrial fibrillation was not captured after the baseline survey. Hence the impact of atrial fibrillation on the association between plasma BNP levels and risk of ischemic stroke may not have been accurately estimated. In addition, since the attending physicians participating in the registration survey were not all neurological specialists, the diagnosis of stroke subtypes was occasionally carried out by general physicians. How-ever, since most of the patients registered were diagnosed using computed tomography or magnetic resonance imaging, the differential diagnosis between ischemic stroke and hemorrhagic stroke was made correctly.

In conclusion, this community-based study has shown that elevated plasma BNP levels predict the risk of ischemic stroke within Japanese men from the general population. This suggests that a simple blood test for BNP is an ideal approach for selecting men at high risk for ischemic stroke within the general population.

## Acknowledgements

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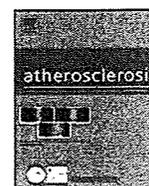
# Serum C-reactive protein levels can be used to predict future ischemic stroke and mortality in Japanese men from the general population

Shinji Makita [a,*], Motoyuki Nakamura [a], Kenyu Satoh [a], Fumitaka Tanaka [a], Toshiyuki Onoda [b], Kazuko Kawamura [e], Masaki Ohsawa [b], Kozo Tanno [b], Kazuyoshi Itai [b], Kiyomi Sakata [b], Akira Okayama [f], Yasuo Terayama [c], Yuki Yoshida [d], Akira Ogawa [d]

[a] Department of Internal Medicine II and Memorial Heart Center, Iwate Medical University, 19-1 Uchimaru, Morioka 0208505, Japan
[b] Department of Hygiene and Preventive Medicine, Iwate Medical University, Morioka, Japan
[c] Department of Neurology, Iwate Medical University, Morioka, Japan
[d] Department of Neurosurgery, Iwate Medical University, Morioka, Japan
[e] Iwate Health Service Association, Morioka, Japan
[f] The First Institute of Health Service, Japan Anti-Tuberculosis Association, Osaka, Japan

## ARTICLE INFO

## ABSTRACT

Background: High C-reactive protein (CRP) levels have been reported to be associated with an increased risk of atherosclerotic cardiovascular events. The relationship of CRP levels to the risk of cerebrovascular events in the Japanese population, which has a lower prevalence of coronary artery disease and a lower CRP level than Western populations, has not been fully clarified. The present study examined the predictive value of serum high sensitivity CRP (hs-CRP) levels for future cerebrovascular events and mortality in the general Japanese population.
Methods: The subjects for this community-based, prospective cohort study were recruited from the general population ($n = 7901$, male only, mean age = 64.0 years). Serum hs-CRP levels and cardiovascular risk factors were determined at baseline. The mean follow-up period was 2.7 years. After excluding subjects with a cardiovascular history, the relationships between hs-CRP levels and cerebrovascular events and mortality were assessed.
Results: During follow-up, 130 participants had a first stroke (95 ischemic strokes), and 161 participants died. The hs-CRP tertile level was a significant predictor for a first ischemic stroke (3rd tertile, HR = 1.77: 95% CI, 1.04–3.03, compared with the 1st tertile), after adjustment for age and classical cardiovascular risk factors. Similar trends were observed for the prediction of all-cause mortality (3rd tertile, HR = 2.26: 95% CI, 1.49–3.42, compared with the 1st tertile).
Conclusion: CRP levels can be used to predict future ischemic stroke and mortality in Japanese men from the general population, independently from traditional cardiovascular risk factors.

## 1. Introduction

The degree of systemic inflammation that is represented by elevated high sensitivity C-reactive protein (hs-CRP) levels has been associated with an increased risk of cardiovascular events in studies conducted in the United States and Europe [1–3]. In the prospective Physicians' Health Study (PHS), elevated hs-CRP levels were associated with an approximately twofold increase in the risk of stroke [1].

We previously reported that, in apparently healthy males living in Japan, hs-CRP levels were closely associated with atherosclerotic changes as measured by carotid plaque formation [4]. Thus, the extent of inflammation may reflect the propensity of atherosclerotic lesions to precipitate clinical vascular events. However, the serum hs-CRP levels of the general Japanese population have been reported to be lower than those of other ethnic groups [5,6]. One must clarify whether associations between a future risk of cerebrovascular diseases and elevated hs-CRP levels also exist in a population that has a relatively lower hs-CRP level. Only one study has reported the association between hs-CRP and ischemic stroke in a rural area of Japan [7]. Therefore, we evaluated the ability of hs-CRP levels to predict future cerebrovascular events and mortality in a larger cohort of the general Japanese population.

* Corresponding author. Tel.: +81 19 651 5111x2324; fax: +81 19 651 7072.
E-mail address: makitas@seagreen.ocn.ne.jp (S. Makita).

## 2. Methods

### 2.1. Study subjects

The study subjects were recruited from the community-dwelling population living in the Ninohe, Kuji, and Miyako districts of Iwate in northern Japan (the Iwate-Kenpoku Cohort study). This study was conducted as part of a government-sponsored, multiphasic health checkup program aimed at the general population. Between April 2002 and January 2005, invitations to participate in this health checkup program were issued by government offices in 17 rural municipalities located in these districts; 26,469 individuals (9161 males) took part in the program and agreed to join the present study. Of these, 25,925 subjects (8957 males) had hs-CRP measurements. Subjects aged over 80 years (280 males) and those under 40 years (300 males), as well as those with a history of cardiovascular disease or stroke (527 males), were excluded. Thus, the data of 7901 males (mean age, $64.0 \pm 9.7$ years) were analyzed. Baseline clinical examinations included a standard 12-lead electrocardiogram, and a self-reported questionnaire was administered to document subjects' medical history and lifestyle. Hospital inpatients, persons who could not walk independently, and persons with recent inflammatory conditions, such as major trauma, surgery, or obvious acute infectious disease, were not included in the present study.

The study was approved by our institutional ethics committee, and all of the participants provided their written informed consent.

### 2.2. Risk factor definitions

The presence of baseline cardiovascular risk factors, including hypertension, diabetes mellitus, hypercholesterolemia, obesity, and smoking, was determined. Hypertension was defined as at least one of: systolic blood pressure $\geq 140$ mmHg; a diastolic blood pressure $\geq 90$ mmHg; or current antihypertensive therapy. Diabetes mellitus was defined as a history of a random blood glucose level $\geq 200$ mg/dL or an HbA1c level $\geq 6.5\%$ or current anti-diabetic therapy. Dyslipidemia was defined as a total cholesterol level $\geq 240$ mg/dL or high density lipoprotein cholesterol level $<40$ mg/dL or current cholesterol-lowering therapy. Obesity was defined as a body mass index $\geq 25.0$ kg/m$^2$. The estimated glomerular filtration rate (eGFR) was calculated using the modified equation of the Modification of Diet in Renal Disease (MDRD) study [8].

An electrocardiogram was not done in 225 males (2.8%). Body height or body weight was missing in 10 males, and blood pressure data were missing in 2 males. These participants were considered to have no risk factors such as atrial fibrillation, obesity, or hypertension if they had no history of atrial fibrillation or hypertension.

### 2.3. Blood samples and hs-CRP measurement

Blood samples were collected from an antecubital vein. The samples were collected into vacuum tubes containing EDTA or a serum separator gel (CRP, lipids). After sampling, tubes were stored immediately in an icebox and centrifuged at $1500 \times g$ for 10 min within 8 h of collection. Aliquots of serum were stored at $-20\,°C$, and routine hematology and biochemistry tests, including hs-CRP, were done within a few days after blood sampling. hs-CRP levels were determined using a highly sensitive immunonephelometric method with a coefficient of variation $<5\%$ (N Latex CRP, Dade Behring). The detection limit of CRP assay is 0.1 mg/L, and cases with levels below the limit of detection were considered as 0.1 mg/L.

### 2.4. Outcome measures

In this cohort study, the primary endpoint was all-cause death, as well as any non-fatal cardiovascular events, such as myocardial infarction, cerebral infarction, or other strokes. The dates of death and move-out were confirmed by the investigators reviewing population-register sheets in each local government. Persons who were known to be alive at the end of follow-up and those who had moved away from the study area were treated as censored cases.

Stroke events were identified by accessing the Iwate prefecture stroke registration program, which included the entire area where the subjects lived; details of this registry have been described previously [9]. Since 1991, the stroke registration program has been coordinated by the Iwate prefecture government and the Iwate Medical Association; the medical records of all medical facilities within the survey area are verified to ensure complete capture of all data. Incidents of acute myocardial infarction were identified by accessing data from the Northern Iwate Heart Disease Registry Consortium, which has been collecting data since 2002. The registration of acute myocardial infarction and sudden death was based on the criteria of the MONICA study [10]. To verify the accuracy of the data, a physician or trained research nurse visited and checked the medical records of the referral hospitals.

Females were excluded from the analysis due to a low incidence of ischemic stroke events (59 events in 15,457 females; 0.4%). For the same reason, coronary heart disease events (non-fatal myocardial infarction, 34 events in 7901 males; 0.4%) were also not analyzed.
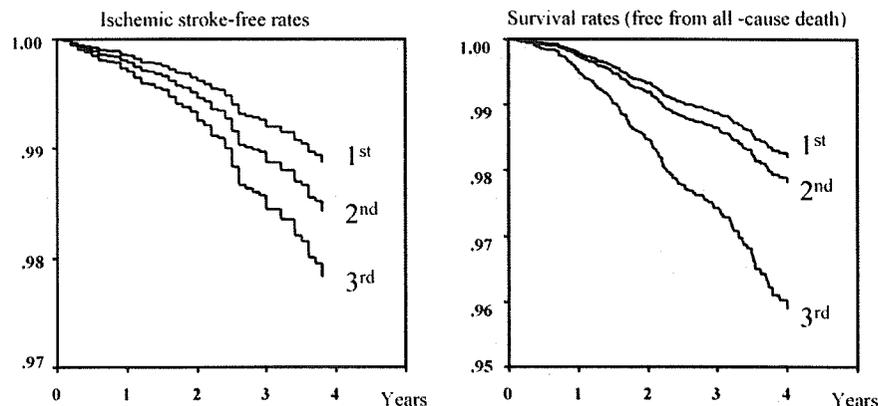


Fig. 1. Cumulative ischemic stroke-free rates and survival rates by age-adjusted Cox regression model for hs-CRP tertiles.