ORIGINAL

# Effect of Jersey cow defatted dry milk on salivary volume and composition in elderly persons : a pilot study

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**Abstract : The administration of Leu[57]-Leu[58]-His[59]-Lys[60] (LLHK), Leu[58]-His[59]-Lys[60] (LHK), and His[59]-Lys[60] (HK) from β-lactoglobulin C variant, which is specific to Jersey cow milk, has been shown to prevent and/or restore the age-dependent atrophy and functional decline of salivary glands by affecting gene expression in elderly rats. In this study, we investigated the effect of Jersey cow defatted milk on salivary volume and composition in elderly persons. Participants (aged 85 to 98, n = 8) were administered defatted dry milk from Jersey cows twice a day for 4 weeks. Before and after 4 weeks from the start of drinking, saliva was collected and weighed. Salivary cystatin S and amylase levels were analyzed by Western blotting. To assess the effect of Jersey cow defatted milk on taste perception, questionnaires were used. Salivary volume after oral administration of 40 g of Jersey cow defatted dry milk daily for 4 weeks was 1.8 times higher than that before administration. Salivary cystatin S and amylase levels significantly increased after administration of Jersey cow defatted dry milk. Moreover, all participants who had taste impairment reported improved taste perception after administration. The administration of Jersey cow defatted dry milk increased salivary volume and changed the composition of saliva in elderly persons. Furthermore, it improved taste perception. J. Med. Invest. 68 : 280-285, August, 2021**

***Keywords*** **:** *elderly person, salivary volume, salivary composition, Jersey cow defatted dry milk, taste perception*

## INTRODUCTION

Saliva plays many vital roles in oral and systemic health. Saliva has multiple functions, including cleaning of the oral cavity, dissolution of tastants, dilution and digestion of food, lubrication, moistening of teeth and oropharyngeal mucosa, and facilitating chewing, bolus formation, swallowing, and speech (1). Low salivary flow induces not only caries dentium and periodontal disease, but also causes difficulties in speaking, chewing, tasting, and swallowing (2, 3). Periodontal disease increases the risk of cardiovascular and atherosclerosis (4). Difficulties in chewing and swallowing lead to aspiration pneumonia (5) and impairment of nutritional status (6). Overall, low salivary flow decreases the quality of life.

Saliva is secreted from three major salivary glands (parotid, submandibular and sublingual glands) and many minor salivary glands which are innervated by autonomic nerves (7). The submandibular and sublingual glands are innervated by parasympathetic nerve derived from the superior salivatory nucleus, while parotid glands are regulated by the nerve supplied from the inferior salivatory nucleus. Minor salivary glands are innervated by the parasympathetic fibers of the buccal branch of the mandibular nerve. Therefore, cholinergic parasympathetic nerve innervates all of these salivary glands. On the other hand, adrenergic sympathetic innervation of submandibular and parotid glands is derived from the spinal cord. The sublingual and minor salivary glands supply a sparse adrenergic nerve fiber. As a result, the stimulation of cholinergic nerve induces a watery, protein-rich fluid secretion from salivary glands, whereas adrenergic stimulation leads to evoke protein-rich fluid secretion. In parasympathetic and/or sympathetic nerves, non-adrenergic and non-cholinergic neurotransmitters including substance P, vasoactive intestinal peptide, neuropeptide Y, neurokinin A, and neuronal nitric oxide synthase regulate nerve activity.

The unstimulated whole saliva and the unstimulated and stimulated saliva from submandibular and sublingual glands decrease during aging (8, 9). The unstimulated and stimulated saliva from parotid glands however does not show an age-related decrease. During aging, the parenchyma of salivary glands is gradually replaced by fat, connective tissue, and oncocytes, resulting in acinar cell atrophy in humans (10) and animals (11).

We previously reported that the administration of whey from Jersey cow milk mitigates the age-dependent atrophy and functional decline of rat salivary glands, accompanied with changes in gene expression (12). β-Lactoglobulin A and B are the most common variants, while the β-lactoglobulin C variant is observed in Jersey cattle. There is one amino acid difference between the B variant (Gln[59]) and C variant (His[59]) (13, 14). In previous experiments, we investigated the effects of Leu[57]-Leu[58]-His[59]-Lys[60] (LLHK), Leu[58]-His[59]-Lys[60] (LHK), and His[59]-Lys[60] (HK) peptides on the gene expression profile, age-dependent atrophy, and dysfunction of rat salivary glands. LLHK, LHK, and HK administration prevents and/or restores the age-dependent atrophy and functional decline of salivary glands by affecting gene expression (15). In this study, we investigated the effect

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of defatted dry milk from Jersey cows on salivary volume and composition in elderly persons.

## METHODS

*Participants*

Participants (aged 85 to 98, 7 women, 1 man) (Table 1) dwelled in a care house Haiji (Tokushima, Japan). They participated in this study as volunteers.

Table 1. Characteristics of participants

| Participant | Gender | Age | Treatment |
|---|---|---|---|
| F1 | Woman | 86 | Amlodipine ($Ca^{2+}$ Channel Blocker), Reminyl (Dementia Improving Drug), Neodopaston (Parkinsonism Treatment) |
| F2 | Woman | 98 | Merislon (Anti-vertigo Medication), Plavix (Anti-platelet Medication), Mucosta (Mucosal Protection Drug), Berizyme (Digestive Enzyme Preparation), Lasix (Anti-hypertensive Drug), Predonine (Glucocorticoid Medication), Nexium (Proton Pump Inhibitor), Mucodyne (Mucolytic Medication) |
| F3 | Woman | 92 | Domperidone (Gastric Prokinetic Agent), Calfina (Active Vitamin D3), Mevalotin (Lipid-lowering Treatment), Nexium (Proton Pump Inhibitor), Viviant (Estrogen Receptor Modulator) |
| F4 | Woman | 90 | Depas (Tranquilizer), Ecabet (Anti-ulcer Agent), Ferromia (Anmtianemic Agent), Celecox (Anti-inflammatory Drug) |
| F5 | Woman | 85 | Ferromia (Anmtianemic Agent) |
| F6 | Woman | 81 | Rebamipide (Anti-ulcer Agent), Glufast (Rapid-acting Insulin Secretion-stimulating Agent), Basen (Alpha-glucosidase Inhibitor), Aricept (Acetylcholinesterase Inhibitor), Warfarin (Antic-oagulant), Bayaspirin (Anti-inflammatory Drug), Lasix (Anti-hypertensive Drug), Lanirapid (Cardiac Glycoside), Feburic (Hyperuricemia Treatment), Famotidine (Histamine $H_2$-receptor Antagonist) |
| F7 | Woman | 86 | Nicergoline (Senile Dementia Treatment), Ecabet (Anti-ulcer Agent), Cholebine (Hypercholesterolaemia Treatment), Lipidil (Dyslipidemia Treatment) |
| M1 | Man | 87 | Casodex (Anti-androgen Medication) |

*Ethical considerations*

The present study was approved by the ethics committee of Tokushima University Hospital (Approval number 2116 in 2014). Before implementation, all participants were given written and verbal information as to the purpose and method of the study, protection of personal information, and freedom of consent and withdrawal. All participants signed the informed consent forms.

*Experimental design*

Before the experiment, five samples of saliva from participants were collected at 2 p.m. using Salivette (Sarsted Inc., Numbrecht, Germany). They gently chewed a Salivette swab for 1 min and the samples were centrifuged at 1,000 g for 10 min (16, 17). The weights of supernatants were measured and counted as secreted saliva (g/min). The saliva was stored in the laboratory at -80°C until further processing. Then, the participants were administered 20 g of defatted dry milk from Jersey cows (Hiruzen Dairy Farming Farm Co-op, Okayama, Japan) twice a day for 4 weeks. Compositional characteristics of the defatted dry milk from Jersey cows are in Table 2. Each time, participants dissolved 20 g of defatted dry milk in 100 mL of hot water and drank 100 mL. After 4 weeks from the start of drinking, saliva was collected at 2 p.m. as described above and stored at -80°C until further processing.

Table 2. Compositional characteristics of the defatted dry milk from Jersey cows

| Constituents | /100g | Measurement method |
|---|---|---|
| Protein | 39.2g | Kjeldahl method |
| Fat | 0.3g | Roese-Gottlieb method |
| Carbohydrate | 49.6g | Deduction method |
| Ash | 7.9g | Direct ashing method |
| Calcium | 1400mg | Atomic absorption spectrophotometry |
| Sodium | 420mg | Atomic absorption spectrophotometry |
| Water | 3g | Normal pressure heat drying method |
| Energy | 358kcal | Conversion method |

Data were analyzed by Public Interest Incorporated Foundation Okayamakenkenkouzukurizaidan.

*Gel electrophoresis and Western blot analysis*

Saliva samples were dissolved with Laemmli buffer, and 15 μL was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 15% and 10% gels. Fifteen % and 10% gels were used for analyses of cystatin S and amylase, respectively. For immunoblot analysis, separated proteins were transferred onto a nitrocellulose membrane (Hybond ECL ; Amersham Biosciences, Little Chalfont, UK) (16, 17). The membrane was blocked overnight with 0.1% Tween 20 in Tris-buffered saline (pH 7.4). The blot was then incubated with rabbit anti-cystatin S antibody (1:500, Sino Biological Inc., Beijing, China) or rabbit anti-amylase antibody (1:1500, Calbiochem, Darmstadt, Germany) (11). The membrane was washed with Tris-buffered saline containing 0.1% Tween 20 and incubated with horseradish peroxidase-linked donkey anti-rabbit IgG, whole Ab (1:5000, GE Healthcare, Piscataway, NJ, USA) for 1 h. Immunoblots were processed using enhanced chemiluminescence (ECL) (Amersham), and the signals were visualized on a Chemi Doc apparatus (Bio-Rad, Hercules, CA, USA). The bands were quantified using Quantity One software (Bio-Rad).

*Questionnaires*

The following three questions were asked of participants. Q1. Do you feel that your regular diet tastes good? Q2. Do you choke while drinking? Q3. Does your mouth feel dry?

*Statistical analysis*

Data are expressed as the mean value ± standard error (SE). To test for statistically significant differences between two groups, a paired Student's *t* test was used for biochemical tests and Western blot analysis. The statistical significance of relative quantitative changes between two groups was evaluated by Wilcoxon signed-rank test. *p*-Values of less than 0.05 were considered statistically significant. Correlation coefficient (r) was obtained using simple regression analysis (Excel software).

## RESULTS

*Effect of Jersey cow defatted dry milk on salivary volume*

In all participants, salivary volume after oral administration of 40 g of Jersey cow defatted dry milk daily for 4 weeks was higher compared to the volume before administration (Fig. 1). It significantly increased in all participants except one (7/8). The mean ratio (salivary volume after administration/salivary volume before administration) was 1.8. Participants who had smaller initial salivary secretions had larger ratios (Fig. 2). The correlation coefficient between the ratio and the salivary volume before administration was -0.8604.

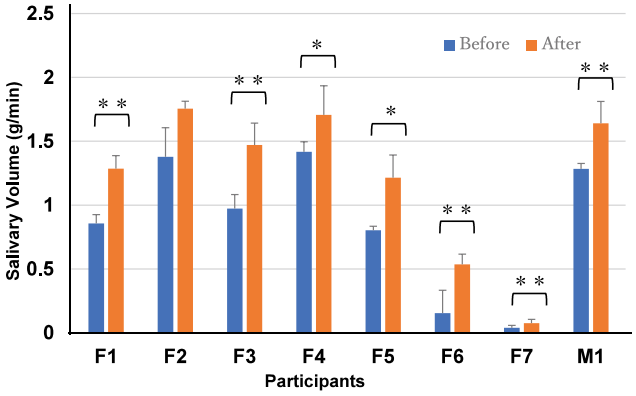


Figure 1. Salivary volume in participants before and after administration of Jersey cow defatted dry milk. Saliva was collected for 1 min from participants before and after administration of Jersey cow defatted dry milk daily for 4 weeks, and was centrifuged at 1000 g for 10 min. The resultant supernatant was used for volume measurement. Data represent means ± SE (n = 5). *$p$ < 0.05, **$p$ < 0.01 vs. salivary volume before administration.

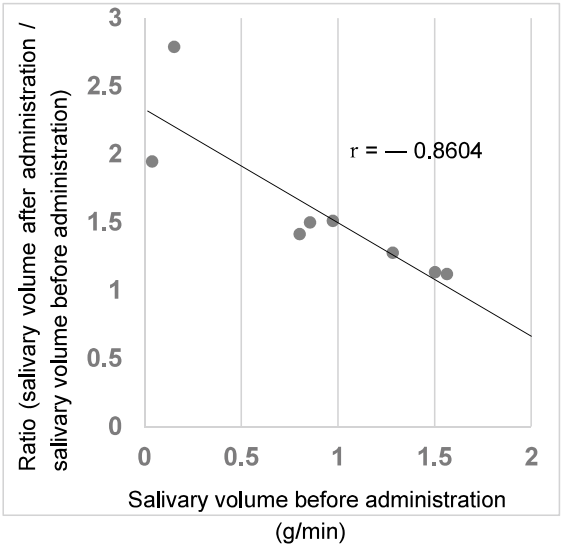


Figure 2. Correlation between the ratio and the salivary volume before administration. The vertical axis shows the ratio (the salivary volume after administration was divided by the salivary volume before administration). The horizontal axis shows the salivary volume before administration.

*Effect of Jersey cow defatted dry milk on salivary composition*

To investigate the effect of Jersey cow defatted milk on the levels of salivary cystatin S, saliva was subjected to Western blot analysis using anti-cystatin S antibody (Fig. 3, a). In all participants, the cystatin S levels in 15 µL of saliva were increased after the administration. The bands were quantified using Quantity One software, and the band density in the 1-min saliva of each participant was calculated. The density per the 1-min saliva significantly increased after the administration ($p$ = 0.0066) (Fig. 3, b). Since individual differences in the density both before and after the administration were large, relative quantification was calculated and shown in Fig. 3, c ($p$ = 0.0117). Jersey cow defatted milk increased the salivary cystatin S levels by 4.3-fold compared to before the administration.

To investigate the effect of Jersey cow defatted milk on the level of salivary amylase, saliva was subjected to Western blot analysis using anti-amylase antibody (Fig. 4, a). Amylase levels in 15 µL of saliva were increased in some participants (F1, F3, F4, F5, and F7) but decreased in others (F2, F6, and M1) after the administration. The bands were quantified using Quantity One software, and the band density in 1-min saliva of each participant was calculated. The density per the 1-min saliva significantly increased after the administration ($p$ = 0.0331) (Fig. 4, b). Relative quantification was calculated and shown in Fig. 4, c ($p$ = 0.0357). The amylase levels in whole saliva after the administration was increased by 1.9-fold compared to that before the administration.

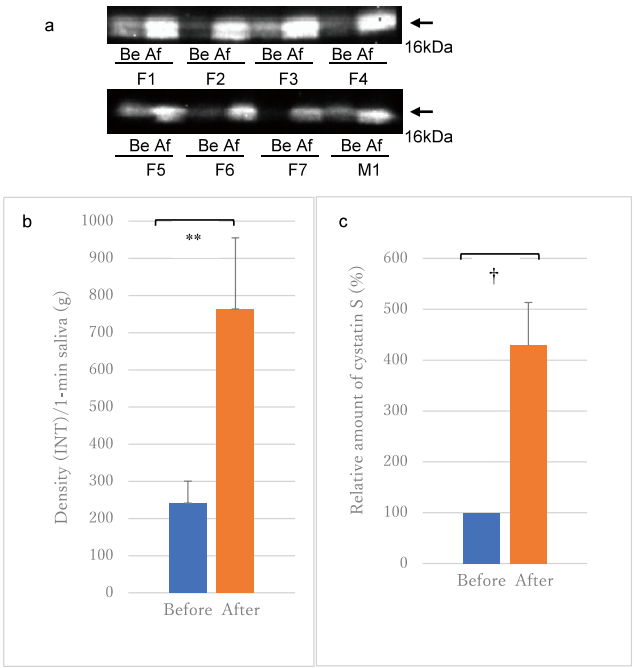


Figure 3. Salivary cystatin S levels in participants before and after administration. Saliva was dissolved with Laemmli buffer and 15 µL was subjected to SDS-PAGE. The protein was transferred to a nitrocellulose membrane and immune blotted with anti-cystatin S antibody. (a) A typical Western blot was shown in photography. (b) The bands on the blots were analyzed by Quantity One Software. The band density in 1-min saliva was calculated and expressed in the graph. Data represent means ± SE (n = 8). **$p$ < 0.01 ; Student's $t$ test. (c) Values were expressed as relative quantitative changes. The amount before administration was assigned to 100%. †$p$ < 0.05 ; Wilcoxon signed-rank test.

*Effect of Jersey cow defatted dry milk on taste perception*

To investigate the effect of Jersey cow defatted milk on taste perception, questionnaires were used (Table 3). After the administration of the milk, all participants who had previous taste impairment reported improved taste perception, saying that their meals were good or pretty good. But the administration of Jersey cow defatted milk did not influence choking when drinking or the feeling of dry mouth.

*Effect of Jersey cow defatted dry milk on side effects*

In oral cavity and systemic health, there were not the outstanding side effects caused by the administration of the milk.

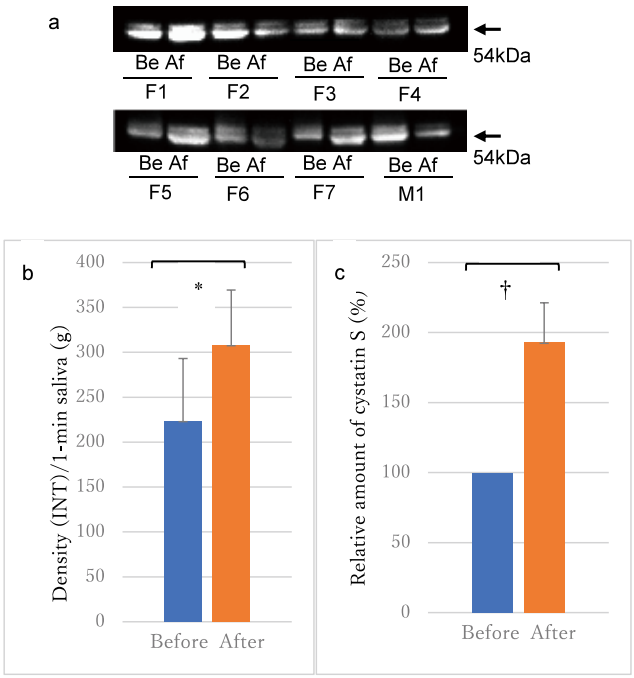


Figure 4. Salivary amylase levels in participants before and after administration. Saliva was dissolved with Laemmli buffer and 15 μL was subjected to SDS-PAGE. The protein was transferred to a nitrocellulose membrane and immune blotted with anti-amylase antibody. (a) A typical Western blot is shown in photography. (b) The bands on the blots were analyzed by Quantity One Software. The band density in 1-min saliva was calculated and expressed in the graph. Data represent means ± SE (n = 8). *$p < 0.05$ ; Student's *t* test. (c) Values were expressed as relative quantitative changes. The amount before administration was assigned to 100%. †$p < 0.05$ ; Wilcoxon signed-rank test.

## DISCUSSION

Previously, we reported that the administration of whey from Jersey cow milk mitigates age-dependent atrophy and functional decline of salivary glands accompanied with changes in gene expression in rats (12). We found that the bioactive peptides, which were LLHK, LHK, and HK derived from β-lactoglobulin C variant, prevent and/or restore the age-depending decline in structure and function of salivary gland (15). In those experiments, older rats drank the whey or the peptides. In the present study, elderly people drank commercially available Jersey cow dry milk. Participants drank 40 g of Jersey cow defatted dry milk daily, which contains about 80 mg of β-lactoglobulin C (14). Eighty mg of β-lactoglobulin C is converted to 2.5 mg of LLHK, 1.9 mg of LHK, or 1.3 mg of HK. Even though participants we exposed to small amounts of peptides, these peptides stimulated salivary secretion and improved the perceived taste of participants' diets.

The use of multiple medicines is very common in the elderly persons (18). Many medications induce salivary gland dysfunction, xerostomia, and sialorrhea (19). In all elderly participants who took multiple pharmaceuticals in this experiment, Jersey cow dry milk induced higher salivary secretion and made them feel that their diets tasted better. Jersey cow dry milk induced salivary secretion even in Participant F1, who used a $Ca^{2+}$ channel blocker that blocks signal transduction of the $M_1$ and $M_3$ muscarinic receptors or $\alpha_1$-adrenoceptor. The milk induced salivary secretion also in Participant F6, who used Aricept (an acetylcholinesterase inhibitor) and Famotidine (a histamine H2-receptor antagonist). As LLHK, LHK, and HK may activate non-adrenergic and non-cholinergic nerves (15), it seems that medications had little influence on LLHK, LHK, and HK-induced gene expression.

In general, low-fat milk products have many health benefits, including the prevention of age-related cognitive decline and dementia (20), a decrease in blood pressure (21), a decreased risk of diabetes (22), and a decreased risk of stroke and heart disease (23). Furthermore, these products possess antimicrobial,

Table 3. Participants' perception before and after the administration of Jersey cow defatted dry milk

| | Grade | F1 Be → Af | F2 Be → Af | F4 Be → Af | F5 Be → Af | F6 Be → Af | F7 Be → Af | M1 Be → Af |
|---|---|---|---|---|---|---|---|---|
| Q1 : Do you feel that your regular diet tastes good? | 1 Bad<br>2 Somewhat bad<br>3 Pretty good<br>4 Good | 4 → 4 | 2 → 3 | 1 → 4 | 4 → 4 | 4 → 4 | 1 → 4 | 2 → 3 |
| Q2 : Do you choke while drinking? | 1 Often<br>2 Sometimes<br>3 Rarely<br>4 Never | 2 → 3 | 4 → 4 | 4 → 3 | 4 → 4 | 4 → 4 | 4 → 3 | 4 → 4 |
| Q3 : Does your mouth feel dry? | 1 Yes<br>2 No | 1 → 1 | 1 → 1 | 2 → 2 | 2 → 2 | 2 → 2 | 1 → 1 | 1 → 1 |

Be : before administration, Af : after administration

anticancer, immunomodulatory, and opioid activities (24-26). Milk proteins consist of many peptides and amino acids that are often inactive within the sequence of the parent protein, but are released during food processing and digestion, changing into biologically active compounds. Branched-chain amino acids (22, 27) and peptides such as LKPTPEGDL and LKPTPEGDLEIL derived from β-lactoglobulin (24) inhibit dipeptidyl peptidase IV activity, leading to an insulinotrophic effect. ALPMHIR and WLAHK peptides derived from β-lactoglobulin and α-lactoalbumin respectively inhibit angiotensin-I-converting enzyme activity, leading to decreased hypertension (25, 26). IIAQK, GLDKQK, ALPMH, and TPEGDLEILLQK peptides derived from β-lactoglobulin inhibit cholesterol absorption, resulting in decreasing serum cholesterol levels (25, 28). Peptides such as YLLF and YGLF, derived from β-lactoglobulin and α-lactoalbumin respectively, behave as μ-opioid receptor agonists, inducing ileum-contraction and endothelium-independent relaxation (25, 28, 29). The peptide CRRWQWRMKKLGAPSICV, derived from lactoferrin, possesses antimicrobial activity against oral pathogens (25, 30). Glycomacropeptide, which is derived from κ-casein, prevents the adhesion of cariogenic bacteria to tooth surfaces, inhibiting dental plaques and caries (25, 31).

As previously mentioned, oral administration of LHK upregulates proline-rich protein genes (*Prpmp5, Prb3, Prp2, Prb1, Prp15*), cystatin genes (*Cst5, Cyss, Vegp2*), amylase genes (*Amy1a, Amy2a3*), and lysozyme gene (*Lyzl1*), and AP-2 transcription factor gene (*Tcfap2b*) (15). The administration of LLHK, LHK, and HTK prevents and/or restores the age-dependent atrophy and functional decline of salivary glands. These peptides, which derived from β-lactoglobulin C variant, increase salivary volume and change the salivary compositions in rats, suggesting that these peptides are effective in improvement at the quality of human life. This study showed that the milk including β-lactoglobulin C variant increased salivary volume, changed the composition of saliva, and improved taste perception in elderly persons.

## CONCLUSIONS

The administration of Jersey cow defatted dry milk increased salivary volume, changed the composition of saliva, and improved taste perception in elderly persons.

## DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no competing interests.

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## FUNDING

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## ETHICAL CONSIDERATION AND CONSENT TO PARTICIPATE

All protocols of this study were approved by the Medical Ethics Committee of Tokushima University Hospital (Approval number 2116, date of approval : October 27th, 2014) and was conducted in accordance with the Declaration of Helsinki ethical principles. Written informed consent from all participants were obtained prior to start of the study.

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