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Circadian rhythms of the mRNA abundances of clock genes and glucose transporters in the jejunum of weanling-growing pigs

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Abstract

Background: Whether abundance of glucose transporter mRNAs in the small intestine of pigs shows circadian rhythms and its regulation by clock genes was still unknown.

Objectives: We examined whether the abundance of glucose transporters and clock genes mRNAs in the small intestine of pigs shows circadian rhythms.

Methods: Twenty barrows (4 weeks old) were reared under 12 h bright and 12 h dark lighting conditions. During the 3-week feeding trial, pigs were allowed free access to feed. The abundances of the mRNA of glucose transporters (*SGLT1* and *GLUT2*) and clock genes (*Bmal1*, *Per1*, *Per2*, and *Cry2*) in the intestine were measured at four time points (ZT2, ZT8, ZT14, and ZT20).

Results: In the jejunum, the abundance of *SGLT1* mRNA was higher at ZT20 and ZT2 and lower at ZT8 and ZT14 ($p < 0.05$). The abundances of *GLUT2* mRNA in the jejunum at ZTs 20 and 2 were tended to be higher than those at ZTs 8 and 14 ($p = 0.05$). In the jejunum, the abundance of *Bmal1* mRNA was higher at ZT8 and ZT14 than at ZT20 and ZT2 ($p < 0.05$). Further, the abundance of *Per1* mRNA at ZT2 was higher than those at the other sampling times ($p < 0.05$). The abundance of *Per1* mRNA at ZT8 was higher than that at ZT14 ($p < 0.05$), while that of *Per2* mRNA was higher at ZT2 than those at ZTs 20 and 14 ($p < 0.05$).

Conclusion: We speculate that these circadian rhythms of abundances of glucose transporter mRNAs are regulated by the clock genes expressed in the jejunum.

KEYWORDS
circadian rhythm, clock gene, glucose transporter, jejunum, pig

1 | INTRODUCTION

Mice and rats granted access to feed ad libitum show circadian rhythms (i.e. they actively eat during the dark period) (Asakuma et al., 2004; Fatima et al., 2009; Yamamuro et al., 2020). Circadian rhythms caused by ad libitum feed intake by pigs have also been reported; however, pigs actively eat during bright periods (Bigelow & Houpt, 1988; Ingram et al., 1980; Quiniou et al., 2000). Montgomery et al. (1978) and Gariido-Izard et al. (2020) reported more details of diurnal patterns of eating behaviours of pigs; frequencies of visits of pigs to feeders have two peaks during bright periods—one of them is found within 2 h after the beginning of bright period, while the other is found in the latter half of bright period. As for the comparison of frequencies of visits of pigs to feeders between bright and dark periods, those of during dark period

is approximately one fifth of those of bright period. These two peaks of eating behaviours, one in the morning and other in the afternoon, were also observed in commercial farms (Villagrá et al. 2007). Furthermore, pigs eat actively towards the end of bright period. Indeed, feed intake of pigs itself is high in the latter half of the bright period (de Haer & Merks 1992; Gariido-Izard et al. 2020; Katsumata & Wilkinson 2019; Montgomery et al. 1978). These previous observations suggest that pigs are diurnal animals.

In addition to eating behaviours, the activities of disaccharides in the intestine show circadian rhythms. In rats fed ad libitum, sucrase, maltase, and lactase activities were high from the dark period (active period) to the beginning of the bright period (their resting period) (George et al., 1985; Stevenson & Fierstein, 1976). However, in a previous study, the activities of sucrase, maltase, and lactase did not show circadian rhythms in pigs (Katsumata & Wilkinson, 2019). Based on the transport and absorption of glucose, a disaccharidase digestion product is transported and absorbed across intestinal epithelial cells by sodium glucose cotransporter 1 (*SGLT1*) and facilitative glucose transporter 2 (*GLUT2*) (Koepsell, 2020). The abundances of both *SGLT1* and *GLUT2* mRNA and/or protein in the intestine of rats and mice have been found to show circadian rhythms (Balakrishnan et al., 2008; Fatima et al., 2009; Iwashina et al., 2011). Furthermore, Iwashina et al. (2011) reported that the clock gene, *Bmal1*, plays a role in the circadian rhythms of the abundance of *SGLT1*. However, although several studies have revealed that nutrition or ambient temperature affects the abundance of the mRNAs of these glucose transporters in pigs (Chen et al., 2018; Li et al., 2019; Liu et al., 2016; Moran et al., 2010; Yi et al., 2020), the circadian rhythms for the mRNA abundance of these glucose transporters in pigs have yet to be reported. According to Balakrishnan et al. (2008), the circadian rhythms of glucose absorption in the intestine of rats are dependent on the abundance of *SGLT1* mRNA. Hence, although disaccharidase activity does not show circadian rhythms, it is reasonable to assume that the absorption of glucose from the intestine is dependent on the circadian rhythms of the abundance of glucose transporter mRNAs in pigs.

Although pigs are supposed to be diurnal animals which is different from nocturnal animals such as mice and rats, we hypothesized that the mRNA levels of glucose transporters in the intestine of pigs have circadian rhythms and are regulated by clock genes in the intestine. Thus, the purpose of this study was to elucidate whether the abundance of the mRNAs of glucose transporters and clock genes in the intestines of pigs fed ad libitum shows circadian rhythms.

2 | MATERIALS AND METHODS

2.1 | Animals and diets

Twenty barrows, Landrace × Large White cross-breeds, from five litters (four barrows from each litter; age, 3 weeks old) were purchased from a commercial breeder company (Cimco Corp., Tokyo, Japan). Upon arrival, the pigs were housed in pens (1.62 m × 0.90 m). For the first 4 days, two pigs shared the same pen; thereafter, the pigs were individually housed until the end of the experiment. The acclimation period was the first week, while the experimental period was the subsequent 3 weeks. During the acclimation period, the pigs were allowed free access to a commercial pig starter for weaning pigs (diet A, crude protein content 23.0%, Nosan Corporation, Yokohama, Japan). Thereafter, the diet was gradually replaced with another commercial pig starter for weanling-growing pigs (diet B, crude protein content was 18.5%, Nosan Corporation). The replacement was carried out as follows: the pigs were allowed free access to a mixture of pig starters: a mixture of 75% of diet A and 25% of diet B for 1–2 days, a mixture of 50% of diet A and 50% of diet B for the next 1–2 days, and finally a mixture of 25% diet A and 75% diet B for another 1–2 days. Thereafter, the pigs were allowed free access to diet B until the end of the experimental period. Drinking water was always available throughout the acclimation and experimental periods.

2.2 | Outline of the feeding trial and sample collection

The pens were bright from 7:00 to 19:00 h (12 h bright: 12 h dark). Of note, 7:00 refers to ZT0 in this study. To remove the effects of natural light, the windows were shielded. The pens were kept warm with floor heating. In addition, an electric infrared heater (Panasonic, Kadoma, Japan) was used to maintain the temperature of the pen. The electric infrared heaters were controlled from 7:00 (ZT0) to 19:00 (ZT12) h to ensure the pens were kept completely dark during the dark period [from 19:00 (ZT12) to 7:00 (ZT0) h]. At the end of the experimental period, samples of the jejunum and ileum were collected at 3:00 (ZT20), 9:00 (ZT2), 15:00 (ZT8), and 21:00 (ZT14) h. The proximal region of the jejunum was 100 cm from the stomach, whereas the distal region of the ileum was 100 cm from the caecum. According to Mekbungwan et al. (2002), the length of the duodenum of 30 kg pig is approximately 50 cm. Before sample collection, the pigs were sedated with an intramuscular injection of a mixture of medetomidine hydrochloride [0.06 mg/kg body weight (BW)], midazolam (0.2 mg/kg BW), and butorphanol tartrate (0.2 mg/kg BW). Thereafter, the pigs were deeply anaesthetized by inhalation of isoflurane and killed by exsanguination. One littermate was killed at each sampling time point. Tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis.

2.3 | Determination of the abundances of the mRNAs of clock genes and glucose transporters

Total RNA was extracted from jejunum and ileum samples using a commercial kit (RNeasy Mini Kit; Qiagen, Tokyo, Japan). Thereafter, first-strand complementary DNA was synthesized using a commercial kit (PrimeScript II 1st strand cDNA synthesis kit; Takara Bio Inc. Shiga, Japan) and random hexamer primers. The abundance of the mRNA of clock genes, brain and muscle Arnt-like potein-1 (*Bmal1*), *Clock*, Period 1 (*PER1*), Period 2 (*PER2*), Cryptochrome 2 (*CRY2*), and the glucose transporters (*SGLT1* and *GLUT2*) were determined by

TABLE 1 Primer sequences used for real-time reverse transcription polymerase chain reaction (RT-PCR)

Genes	Forward and reverse	(5′) Sequences (3′)	PCR products (bp)
Bmal1	Forward	AGAGGGTCATCGCCTTCC	63
	Reverse	CAGGGGGAGGTGTACTTGTG	
Clock	Forward	CTGCCTCAACACCAACAAAG	71
	Reverse	TCTCATGGGCTGGCAGAT	
Per1	Forward	ATCCACAGGTGACCTTCCAG	140
	Reverse	AGTCTTGGCCTTGAASTGTGC	
Per2	Forward	GTCTCCCTGCCACCATTACT	102
	Reverse	TTCCAAGATGAGTCCACCCC	
Cry2	Forward	TGACGTGTTCCCAAGGCTAT	148
	Reverse	ATGCGAGTTCTCAGTCACCA	
SGLT1	Forward	TGTATTTGAGGCCAGTGTCA	198
	Reverse	GGGCGACCACAACTCTTAAA	
GLUT2	Forward	TGGAATCAGCCAACCTGTTT	165
	Reverse	ACAAGTCCCACCGACATGA	
RPL4	Forward	AGGAGGCTGTTCTGCTTCTG	185
	Reverse	TCCAGGGATGTTTCTGAAGG	
ACTB	Forward	TCCAGCCCTCCTTCCTGGGC	112
	Reverse	AGCACCGTGTTGGCGTAGAG	

real-time reverse transcription polymerase chain reaction (RT-PCR) with SYBR Premix Ex Taq™ II (Takara Bio Inc.) and a Light Cycler96 (Roche Diagnostics K.K., Tokyo, Japan). The abundances of the target mRNAs were normalized to the abundance of the mRNA of internal standard genes. The relative abundance of mRNA of the target genes was calculated using the ⊿⊿Cq method. The internal standard genes were 18S ribosomal RNA (*18SRNA*), ribosome protein L4 (*RPL4*), hyposanthine phosphoribosyltransferase 1 (*HPRT1*), beta-actin (*ACTB*), hydroxymethylbilane synthase (*HMBS*), and TATA box binding protein (*TBP*). The stability of the abundances of these candidate genes was verified using the NormFinder software (http://www.mdl.dk/publicationsnormfinder.htm), and *RPL4* and *ACTB* were selected as the internal standards. The PCR conditions were as follows: predenaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 10 s. To assess whether the PCR products were single and had specific amplicons, we checked the melt curves of the amplicons produced by each primer set and confirmed that all the curves had single peaks. The primer sets for *Bmal1* and *Clock* were designed based on the sequences of porcine *Bmal1* and *Clock* (accession numbers EF216896.1 and XM_021101335, respectively) with Roche/UPL-Probe Finder web-based software (https://qpcr.probefinder.com/organism.jsp). We referred to the sequences of the primer sets of *PER1*, *PER2*, and *CRY2* published by Cardoso et al. (2018); *SGLT1* and *GLUT2* published by Su et al. (2020); and *18SRNA*, *RPL4*, *HPRT1*, *ACTB*, *HMBS*, and *TBP* published by Ishida et al. (2017). The sequences of the primer sets used are listed in Table 1.

2.4 | Statistical analysis

Statistical significances of the effects of sampling times on the abundance of mRNAs of clock genes and glucose transporters were assessed by one-way analysis of variance based on a randomized block design, where litters were blocked and sampling times were the main effect using the GLM procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). When the effects of sampling times were significant, the significance of the differences between means was assessed using the Tukey statement of the GLM procedure of SAS. All statistical analyses were performed using the supercomputer of Academic Center for Computing and Media Studies, Kyoto University.

3 | RESULTS

Figure 1 shows the abundance of the mRNA of clock genes and glucose transporters in the jejunum. The abundance of *Bmal1* mRNA was higher at ZT8 and ZT14 than at ZT20 and ZT2 ($p < 0.05$). However, the abundance of *Per1* mRNA was the highest at ZT2; differences were detected between the abundances at ZT2 and those at the other tissue sampling times ($p < 0.05$). Furthermore, the abundance of *Per1* mRNA at ZT8 was higher than that at ZT14 ($p < 0.05$), while that of *Per2* mRNA was the highest at ZT2; differences in the abundances between ZT2 and ZT20 and at ZT14 were significant for *Per2* ($p < 0.05$). The abundance of the *Clock* and *Cry2* mRNAs did not show circadian rhythms. Although multiple comparisons using the Tukey–Kramer test did not reveal

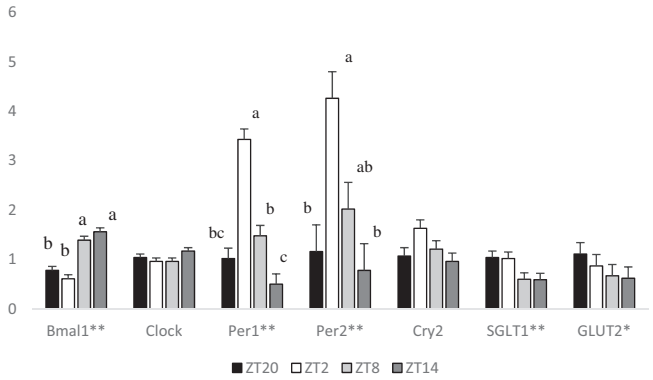

FIGURE 1 Abundances of the mRNAs of clock genes and glucose transporters in the jejunum. Bars represent means and error bars represent their pooled standard errors, $n = 5$. The abundances are expressed as relative ratios of the target mRNAs to the internal standard mRNAs (*RPL4* and *ACTB*). * and ** with the target mRNAs indicate that the effects of sampling times are significant, $p < 0.05$ and $p < 0.01$, respectively. a, b, and c: the differences between the means ($p < 0.05$)

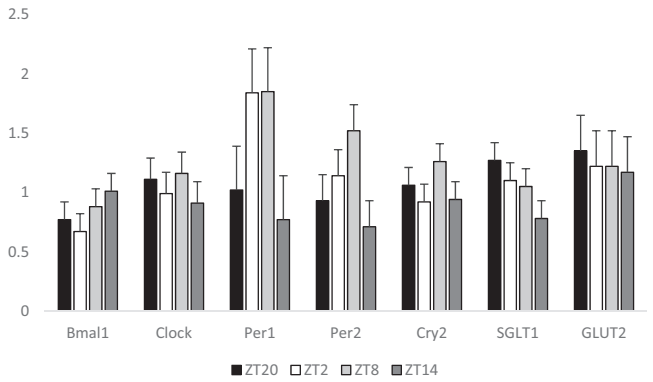

FIGURE 2 Abundances of the mRNAs of clock genes and glucose transporters in the ileum. Bars represent means and error bars represent their pooled standard errors, $n = 5$. The abundances are expressed as relative ratios of the target mRNAs to the internal standard mRNAs (*RPL4* and *ACTB*). The sampling times did not affect the abundances of the target mRNAs

differences between the sampling times, we assessed the effects of the sampling times on the abundance of *SGLT1* mRNA ($p < 0.05$). The abundance of *SGLT1* mRNA was found to be higher at ZT20 and ZT 2 and lower at ZT8 and ZT14. Further, the abundances of *GLUT2* mRNA in the jejunum between those at ZTs 20 and 2 and those at ZTs 8 and 14 (i.e. between the morning and afternoon) were compared by analysis of variance. Based on the result, the abundances at ZTs 20 and 2 tended to be higher than those at ZTs 8 and 14 ($p = 0.05$).

Figure 2 shows the abundance of the mRNA of clock genes and glucose transporters in the ileum. Neither clock genes nor glucose transporters were found to exhibit circadian rhythms.

4 | DISCUSSION

Importantly, the abundance of the mRNA of clock genes (*Bmal1*, *Per1*, and *Per2*) in the jejunum was found to display circadian rhythms in the present study. The abundance of *Bmal1* mRNA was high at ZT8 and ZT14 (from the end of the bright period to the beginning of the dark period) and low at ZT20 and ZT2 (from the end of the dark period to the beginning of the bright period) (Figure 1). The complex of Bmal1 and Clock proteins promotes the transcription of *Per1* and *Per2* via a clock gene control sequence E-box. This mode of action suggests that increases in the abundance of the *Pers* mRNAs follow that of *Bmal1*. Indeed, the abundance of *Pers* mRNA was high at ZT2. Subsequently, the transcription of *Bmal1* and *Clock* was downregulated; the complex of the *Pers* and *Crys* proteins downregulates the transcription of *Bmal1*. Thus, there is a feedback loop in the circadian rhythm for the expression of clock genes. In fact, the abundance of *Bmal1* mRNA was low at ZT2. Therefore, we infer that the jejunum of pigs fed ad libitum has a mechanism that regulates the circadian rhythm of the expression of clock genes, *Bmal1* and *Pers* mRNAs, via E-box. However, the abundance of *Clock* mRNA did not show a circadian rhythm. Pan and Hussain (2009) reported that the mRNA expression of *Clock* did not show a clear circadian rhythm in the jejunum of mice fed ad libitum, while that of *Bmal1* and *Pers* showed circadian rhythms. Based on the observations of previous and present studies, we infer that the activity of E-box in the jejunum of pigs fed ad libitum is mainly regulated by circadian rhythms of *Bmal1* abundance.

The abundance of *SGLT1* mRNA in the jejunum was high at ZT20 and ZT2 and low at ZT8 and ZT14; their abundances were high from the end of the dark period to the beginning of the bright period, and low from the end of the bright period to the beginning of the dark period. Such a finding indicates that the abundance of *SGLT1* mRNA in the jejunum of pigs fed ad libitum was high from the end of their resting period to the beginning of their active period. Similar findings were also obtained in mice; the abundance of *SGLT1* mRNA in the jejunum of mice fed ad libitum was high from the end of their resting period to the beginning of their active period (Balakrishnan et al., 2008; Fatima et al., 2009; Iwashina et al., 2011). Thus, patterns of circadian rhythms in the abundance of *SGLT1* mRNA in the jejunum of pigs and mice were similar when both were fed ad libitum. According to Iwashina et al. (2011), the abundance of *Bmal1* mRNA in mice fed ad libitum was high at ZT4 (their resting period), while that of *SGLT1* was low at ZT20 and ZT0 (end of their active period); thereafter, those of *SGLT1* gradually increased and reached their peak level at ZT12. Such a finding indicates that the abundance of *Bmal1* mRNA increased prior to an increase in that of *SGLT1* mRNA. *Bmal1* binds to the promoter region of the *SGLT1* gene (Iwashina et al., 2011), suggesting that *Bmal1* promotes the transcription of *SGLT1*. We also observed that the abundance of *Bmal1* increased prior to the increase in the levels of *SGLT1* in the jejunum of pigs (Figure 1). Thus, we speculate that *Bmal1* promotes the transcription of *SGLT1* in the jejunum of pigs fed ad libitum.

The downregulation of *Per1* transcription has been reported to downregulate the transcription of *SGLT1* in cultured Caco-2 cells

(Balakrishnan et al., 2012). In the jejunum of mice fed ad libitum, the downregulation of *SGLT1* followed the upregulation of *Per2* (Iwashina et al., 2011). In the present study, the mRNA levels of both *Pers* and *SGLT1* in the jejunum were high at ZT2. Thereafter, the abundance of *SGLT1* mRNA was low at ZT8. Such a finding suggests that the elevated transcription of *SGLT1* promoted by *Bmal1* is downregulated by *Pers*. This mode of action may contribute to the formation of circadian rhythms in the transcription of *SGLT1* in the jejunum of pigs fed ad libitum. However, the overexpression of *Bmal1* in Caco-2 cells downregulated both glucose uptake and *SGLT1* protein expression, whereas the knockout of *Bmal1* resulted in their upregulation (Sussman et al., 2019). These observations by Sussman et al. (2019) opposed those of Iwashina et al. (2011), Balakrishnan et al. (2012), and the present study. The effects of extreme treatments, such as overexpression and/or knockout, may differ from those of treatments with physiological levels. Nevertheless, *Bmal1* might regulate glucose uptake via *SGLT1* expression.

We found that the abundance of *GLUT2* mRNA in the jejunum at ZTs 20 and 2 tended to be higher than that at ZTs 8 and 14 ($p = 0.05$). This finding suggests that, although not as clear as those of *SGLT1*, there were circadian rhythms of *GLUT2* transcription in the jejunum of pigs fed ad libitum. In particular, the transcription of *GLUT2* in the jejunum was high from the end of the resting period to the beginning of the active period. Fatima et al. (2009) also observed that the transcription of *GLUT2* in the jejunum of mice fed ad libitum was high at the end of their resting period and low from the end of their active period to the beginning of their resting period.

In contrast to the jejunum, we failed to observe circadian rhythms for the abundance of clock genes and glucose transporters in the ileum. In rodents, the mRNA levels of both *SGLT1* and *GLUT2* displayed circadian rhythms in the ileum of rats and mice fed ad libitum. However, the magnitudes of the rhythms were as small as one-quarter to one-half of those of the jejunum (Balakrishnan et al., 2008; Fatima et al., 2009). We collected samples of the jejunum from the proximal position and those of the ileum from the distal position. Feeding behaviour may have a stronger influence on the arrival time of the feed bolus to the proximal jejunum, while the influence of feeding behaviour on the arrival time of the feed bolus to the distal ileum may be smaller. Furthermore, the activities of disaccharidases (maltase, sucrase, and lactase) and amino peptidase N in the proximal jejunum were two- to threefold higher than those in the distal ileum (data not shown). This finding suggests that the significance of the proximal jejunum as a digestion site is larger than that of the distal ileum. This difference in significance regarding digestion sites may be related to the magnitudes of the circadian rhythms for the transcription of glucose transporters.

We measured abundances of mRNA of both glucose transporters and clock genes, but we did not measure abundances of those proteins in this study. This is a major limitation of this study because mRNAs themselves do not regulate the expression of other mRNAs. Although we recognize that there is a such limitation in this study, we summarize the results of this study as follows.

The abundance of the glucose transporter mRNAs (*SGLT1* and *GLUT2*) in the jejunum of pigs fed ad libitum showed circadian rhythms; their abundances were high from the end of their resting (dark) period to the beginning of their active (bright) period. We speculate that these circadian rhythms are regulated by clock genes. Specifically, the circadian rhythms of glucose transporter abundance may be dependent on those of clock genes. *Bmal1* may promote the transcription of glucose transporters, whereas *Pers* may inhibit their transcription. Pigs were granted access to feed ad libitum in this study, indicating that they actively ate during the bright period. Thus, we could not separate the effects of lighting conditions and feed intake in this study. However, these effects can be separated by employing restriction feeding programmes, such as ad libitum feeding only during bright or dark periods, to clarify the effect of stronger lighting conditions or feed intake.

We planned this study to establish time-restricted feeding procedures which allow feed bolus reaching intestine during time zone when digestive enzymes and nutrient transporters are active—such procedures may be called time-restricted feeding procedure based on chrono-nutrition. We expect that such procedures improve productivities of the pig industry. However, if we view from a different angle, shifting feeding procedures from ad libitum to time-restricted may affect the welfare of pigs through changes in their behaviours. Thus, to study the effects of time-restricted feeding procedures based on chrono-nutrition on the welfare of pigs will be a future research subject. On the other hand, time-restricted feeding procedures may contribute to reduce the magnitude of metabolic stress of pigs because they allow the digestive tract of pigs to rest for certain period during the day.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

All animal experimentation procedures in this study were conducted in accordance with the Regulation for Animal Experiment at Azabu University and were approved by the Azabu University Animal Experimentation Committee (certification number, 170217).

AUTHOR CONTRIBUTIONS

Formal analysis and investigation: Yuki Kinoshita and Hayata Takahashi. *Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, and writing—original draft*: Masaya Katsumata.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [M.K.], upon reasonable request.

PEER REVIEW

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