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Evaluation of the One-Hour ^{13}C-Propionate Breath Test in 49 Patients from a Single Center in Japan to Detect Vitamin B_{12} Deficiency

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG Yosuke Sasaki
BDF Takahiro Sato
BDFG Tadashi Maeda
BCD Fumiya Komatsu
BC Naoyuki Kawagoe
ACDF Tsunehiko Imai
BF Tomoyuki Shigeta
B Naoyasu Kashima
ABDFG Yoshihisa Urita

Department of General Medicine and Emergency Care, Toho University School of Medicine, Tokyo, Japan

Corresponding Author: Yosuke Sasaki, e-mail: yousuke.sasaki@med.toho-u.ac.jp
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Background: Given the unavailability of reliable biomarkers for vitamin B_{12} (VB_{12}) deficiency in clinical settings, the usefulness of the ^{13}C-propionate breath test (PBT), utilizing VB_{12} as a coenzyme of methylmalonyl-CoA in propionate metabolism, as a diagnostic modality for VB_{12} deficiency has been studied. However, a collection time of 2 h reduces its convenience. Hence, we evaluated the effectiveness of 1-h PBT for detecting VB_{12} deficiency in 49 patients with suspected VB_{12} deficiency.

Material/Methods: We collected 100-200 mL breath gas every 10 min until 1 h after the administration of 1 g of ^{13}C-propionate from 49 patients (31 men, 18 women; median age, 70 years) with clinically suspected VB_{12} deficiency and calculated the $^{13}CO_2$ recovered in the breath per hour as the recovery rate (RR [%dose/h]) from $^{13}CO_2/^{12}CO_2$ using infrared isotope spectrometry. We compared the RRs between groups: (1) with serum VB_{12} levels ≥145 pg/mL and <145 pg/mL, (2) with mean corpuscular volume ≤100 fL and >100 fL, and 3) pre- and post-VB_{12} supplementation.

Results: The RRs peaked within 30 min. The RRs at 20 min (RR_{20}) and 30 min (RR_{30}) were significantly lower in macrocytotic patients (41.28 vs 50.07, p=0.026 and 37.82 vs 43.93, *P*=0.003). The RR_{30} was higher in the supplemented patients (41.93 vs 32.84, *P*=0.024). There was no significant difference in RRs between the patients with normal and low serum VB_{12} levels.

Conclusions: The 1-h PBT can be a diagnostic modality for VB_{12} deficiency because 1 h is a sufficient collection time.

Keywords: Anemia • Anemia, Megaloblastic • Breath Tests • Propionates • Vitamin B_{12}

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Background

Vitamin B_{12} (VB_{12}) deficiency is known to cause various disorders, including megaloblastic anemia, cognitive defects, myelopathy, peripheral neuropathy, and loss of taste [1]. Furthermore, VB_{12} deficiency in older adults due to various causes, including adverse drug events or social isolation, is increasingly problematic because of the aging society [2,3]. As VB_{12} deficiency can be treated with VB_{12} supplementation, early diagnosis is crucial. However, there are challenges in the development of diagnostic methods for VB_{12} deficiency in Japan, such as the low reliability and unavailability of many biomarkers. Although many researchers have evaluated the association between serum VB_{12} levels and clinical symptoms, particularly cognitive function [4,5], many other studies have shown a poor association between VB_{12} and clinical symptoms [6,7]. Therefore, the measurement of other VB_{12} deficiency markers, such as serum homocysteine (HC) or methylmalonic acid (MMA), is required for the diagnosis of VB_{12} deficiency [8,9]. A systematic review of randomized controlled trials concluded that serum VB_{12} level is an effective biomarker for detecting change in VB_{12} intake. However, a systematic review pointed out the heterogeneity of serum VB_{12} levels among different studies [10]. The current guidelines recommend suspecting VB_{12} deficiency if patients have serum VB_{12} level <200 pg/mL and measuring plasma total HC or MMA levels to confirm the diagnosis [11]. Serum holotranscobalamin is also a promising candidate biomarker of VB_{12} deficiency; however, the unavailability and high costs of measuring these biomarkers hinder their use in clinical practice [11]. In addition, there are limited commercial methods for measuring serum MMA levels in Japan. Furthermore, the guidelines mention that there is no "gold-standard" test for the diagnosis of VB_{12} deficiency because there are no definitive cut-off values for HC or MMA levels, since a variety of methodologies are used to measure them [11]. Therefore, accessible noninvasive diagnostic modalities including screening tests are needed to better address VB_{12} deficiency in various clinical settings.

Utilizing the role of VB_{12} as a coenzyme of methylmalonyl-CoA in propionate metabolism to CO_2 (**Figure 1**), the use of ^{13}C-propionate breath test (PBT) as a noninvasive diagnostic modality for VB_{12} deficiency has been studied previously [12]. In the conventional PBT, the examiner collects 100-200 mL of breath gas every 10 min for 120 min after the administration of ^{13}C-propionate. We believe that the collection time of 2 h for the convention PBT is too long and reduces the convenience for the participants; a collection period of 60 min appears to be sufficient to evaluate the propionate metabolism in patients with VB_{12} deficiency. Therefore, this study aimed to evaluate the usefulness of a 1-h PBT for detecting VB_{12} deficiency in patients with clinically suspected VB_{12} deficiency.

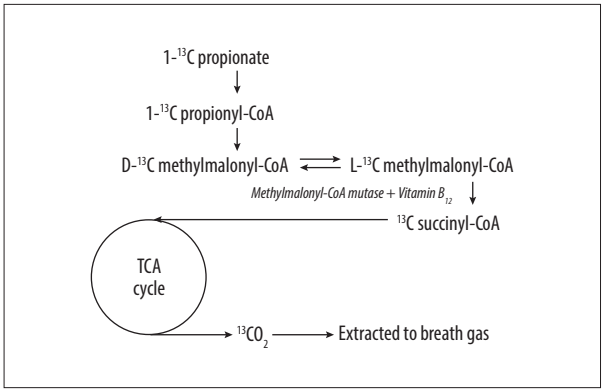

Figure 1. Propionate metabolism and measuring principle of the propionate breath test. Vitamin B_{12} functions as a coenzyme of methylmalonyl-CoA mutase in the conversion from L-methylmalonyl-CoA to succinyl-CoA in propionate metabolism. TCA – tricarboxylic acid.

Material and Methods

This study was approved by the Ethics Committee of Toho University Omori Hospital (approval number: 17-64 and M20278). All patients involved in this study gave written informed consent.

Patients

We performed a 1-h PBT and collected blood samples from patients who visited the Department of General Medicine and Emergency Care, Toho University School of Medicine, Tokyo, Japan, between April 2014 and February 2023, with clinically suspected VB_{12} deficiency according to symptoms such as numbness, muscle cramps, cognitive dysfunction, or macrocytic anemia. The patients who underwent gastrectomy were excluded because gastrectomy causes altered intestinal excretion.

1-h PBT

The patients were asked to fast for more than 8 h before the test. We collected 2.0 L of breath gas in the collecting bag before administering ^{13}C-propionate. We asked the participants to drink 100 mL of 1 mg/mL ^{13}C-propionate aqueous solution that we prepared immediately before administration to the participants. After the participants drank the aqueous solution containing ^{13}C-propionate, we collected 100-200 mL of breath gas every 10 min until 60 min. We recorded the body weight and height of the participants immediately before the 1-h PBT to calculate the body surface area.

Measurement of ^{13}C Recovery Rate (RR)

Because ^{13}C-propionate is metabolized in the liver and finally exhaled as $^{13}CO_2$ (**Figure 1**), we measured the $^{13}CO_2/^{12}CO_2$

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isotope ratio of the collected gas using infrared isotope spectrometry (POCone; Otsuka Electrics Co., Ltd., Hirakata, Japan). The detected $^{13}CO_2$ levels in the exhaled gas were expressed as delta over base line per mil (Δ‰). We converted Δ‰ to $^{13}CO_2$ recovered in the breath per hour (RR% dose/h), based on the body surface area (calculated as 0.0024265 × weight$^{0.5378}$ × height$^{0.3964}$ [m^2]), and assumed CO_2 excretion (VCO_2) with reference to the previous study by Urita et al, as follows [12,13]:

$$RR\ (\%dose/h) = \Delta‰ \times VCO_2 \times 0.001123 \times 10/(Dose \times APE/MW)$$

where MW (molecular weight) is 97.07, VCO_2 is 300 (BSA mmol/h), dose is 100 mg and APE (atom% excess) is 99.0% [13].

Blood Tests

We measured the serum VB_{12} level by chemiluminescent enzyme immunoassay (CLEIA) at SRL (Tokyo, Japan), hemoglobin level, and mean corpuscular volume (MCV) of all patients. In this study, we did not measure the serum MMA level due to the lack of access to serum MMA measurements in Japan and the high cost. Instead, we measured serum the VB_{12} level because we regarded it a feasible and somewhat reliable biomarker in light of the results of a previous systematic review of randomized control trials [10].

Definition of VB_{12} Deficiency and Macrocytosis

Because the lower limit of serum VB_{12} levels measured by CLEIA was 50 pg/mL, we could not analyze serum VB_{12} level as a continuous variable. Thus, we divided the patients into a VB_{12} deficiency group and a normal group. To avoid false-positive results, we defined VB_{12} deficiency as serum VB_{12} level <145 pg/mL, as the lowest cut-off value among those used in previous studies [14,15], and we defined macrocytosis as MCV >100 fL based on previous studies.

Statistical Analysis

We compared the means or medians of the RRs between VB_{12} deficient patients (defined as serum VB_{12} levels <145 pg/mL) and normal patients (defined as serum VB_{12} ≥145 pg/mL). To compensate for the lack of asymptomatic healthy controls and comparison between 1-h PBT results and other biomarkers such as serum MMA, we additionally compared the RRs between macrocytic patients (defined as MCV >100 fL) and normocytic patients (MCV ≤100 fL) and between VB_{12}-deficient patients before and after VB_{12} supplementation. We evaluated the normality of all variables using the Kolmogorov-Smirnov test and evaluated statistical differences between groups using the *t* test and Mann-Whitney U test for normally and non-normally distributed variables, respectively. Statistical significance was set at $P<0.05$. If patients received 1-h PBT before and after supplementation with VB_{12}, we separately compared the results before and after supplementation. All statistical analyses were performed using R, version 4.2.0 [16].

Table 1. Complaints or reason for PBT of the participants.

Complaint or reason for PBT	Numbers of patients
Paraesthesia	13
Macrocytic anemia	12
Disequilibrium	7
Cognitive dysfunction	6
Edema	4
Leg cramp	2
Normocytic anemia	2
Lower extremities weakness	1
Polycythemia	1
Generalized pain	1
Total	**49**

PBT – ^{13}C-propionate breath test.

Results

Clinical Characteristics of the Participants

We performed the 1-h PBT in 55 patients. Of them, 6 post-gastrectomy patients were excluded; hence, only 49 patients were finally evaluated. The median age was 70 years (range, 27-89 years). The median MCV was 93.5 fL (range, 78.3-135.6 fL). The frequencies of complaints or reasons for suspected VB_{12} deficiency are listed in **Table 1**.

Comparisons of 1-h PBT Between Patients with Normal and Low Serum VB_{12} Levels

Of the 49 patients, 20 (40.8%) had serum VB_{12} levels <145 pg/mL and were defined as having VB_{12} deficiency. The RRs were not significantly different between patients with VB_{12} deficiency and normal patients (**Table 2, Figure 2**).

Comparisons of 1-h PBT Between Macrocytic and Normocytic Patients

In the comparison of the RRs between 14/49 (28.6%) macrocytic, defined as MCV >100 fL, and 35 normocytic patients, the RR at 20 min after administration of ^{13}C-propionate (RR_{20}) and the RR at 30 min after administration of ^{13}C-propionate (RR_{30}) were significantly lower in macrocytic patients than in normocytic patients, with values of 41.28 vs 50.07 (P=0.026)

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Table 2. Comparisons of RRs (%dose/h) from 1-h PBT between patients with normal and low serum VB_{12} levels.

Time	VB_{12} deficiency	Normal	P-value
All (*n*=49)			
10 min	37.49±20.12	42.51±19.76	0.365
20 min	46.63±16.23	48.34±11.51	0.365
30 min	42.71±10	42.14±8.14	0.824
40 min	36.91±7.63	35.38±8.92	0.495
50 min	31.42±7.69	29.52±6.71	0.351
60 min	27.55±7.81	27.52±14.53	0.992
Male (*n*=31)			
10 min	31.41±15.42	40.17±20.00	0.126
20 min	40.85±13.97	44.81±10.04	0.126
30 min	40.06±9.31	40.73±8.17	0.816
40 min	36.23±8.40	34.29±9.49	0.503
50 min	31.29±8.69	28.76±6.67	0.341
60 min	27.67±8.92	28.13±17.61	0.912
Female (*n*=18)			
10 min	55.71±23.19	46.87±19.25	0.473
20 min	63.97±8.20	54.9±11.50	0.473
30 min	50.69±8.12	44.74±7.70	0.199
40 min	38.95±4.77	37.42±7.65	0.615
50 min	31.84±4.04	30.91±6.79	0.724
60 min	27.2±3.35	26.4±5.90	0.718

VB_{12} deficiency was defined as serum VB_{12} level <145 pg/mL. Means and standard deviations are listed. PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate; VB_{12} – vitamin B_{12}.

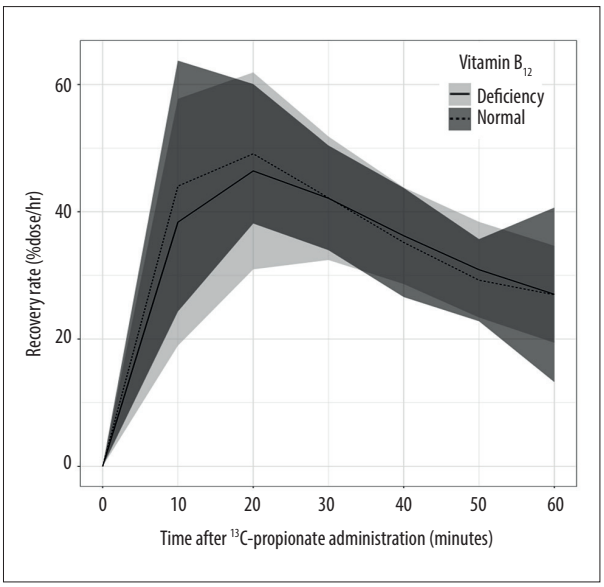

Figure 2. Comparisons of RRs from 1-h PBT between patients with normal and low serum VB_{12} levels. Horizontal axis and vertical axis indicate time (min) after ^{13}C-propionate administration. and RR (%dose/h), respectively. VB_{12} deficiency was defined as serum VB_{12} level <145 pg/mL. The tinted area around each line indicates standard deviation. There were no significant differences of RRs between the 2 groups.
PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate; VB_{12} – vitamin B_{12}.

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Table 3. Comparisons of RRs (%dose/h) from 1-h PBT between macrocytic and normocytic patients.

Time	Macrocytic	Normocytic	P-value
All (*n*=49)			
10 min	42.08±23.07	40.38±19.28	0.795
20 min	41.82±11.45	50.07±13.47	0.026*
30 min	37.82±5.32	43.93±9.29	0.003*
40 min	32.95±5.65	36.88±9.26	0.056
50 min	28.15±4.36	30.87±7.86	0.102
60 min	29.93±21.73	26.68±7.29	0.566
Male (*n*=31)			
10 min	40.55±22.90	34.68±16.96	0.411
20 min	41.49±10.98	44.12±12.47	0.500
30 min	37.36±5.55	41.9±9.53	0.068
40 min	32.25±5.16	36.24±10.61	0.123
50 min	27.86±4.39	30.59±8.88	0.207
60 min	30.35±23.24	26.77±8.47	0.586
Female (*n*=18)			
10 min	52.81±30.05	48.77±19.88	0.881
20 min	44.15±19.62	58.83±9.74	0.479
30 min	41.06±0.36	46.93±8.30	0.010*
40 min	37.84±8.79	37.82±70	0.998
50 min	30.2±5.01	31.27±6.33	0.817
60 min	26.99±6.54	26.56±5.34	0.941

Means and standard deviations are listed. Macrocytosis was defined as mean corpuscular volume >100 fL. * *P*<0.05.
PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate.

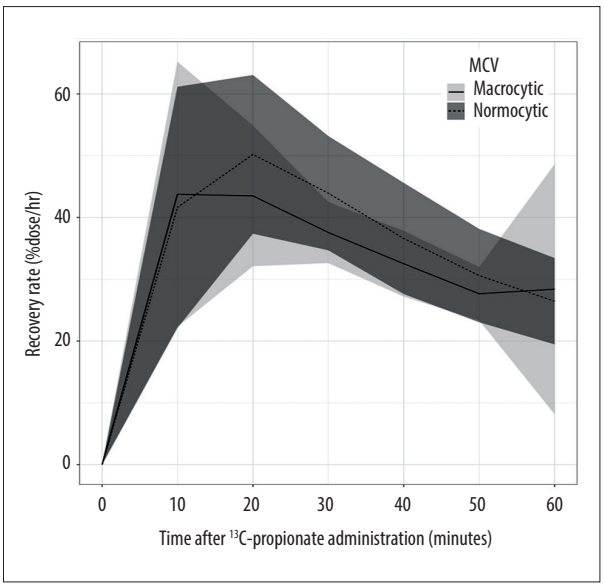

Figure 3. Comparisons of RRs in 1-h PBT between macrocytic and normocytic patients. Horizontal axis and vertical axis indicate time (min) after ^{13}C-propionate administration. and RR (%dose/h), respectively. Macrocytosis is defined as MCV >100 fL. The tinted area around each line indicates standard deviation. RR_{20} and RR_{30} were significantly low in VB_{12} deficiency group compared than in the normal group.
MCV – mean corpuscular volume; PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate; RR_{20} – RR at $_{20}$ min after administration of ^{13}C-propionate; RR_{30} – RR at 30 min after administration of ^{13}C-propionate; VB_{12} – vitamin B_{12}.

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Table 4. Comparisons of RRs (%dose/h) from 1-h PBT between pre- and post- supplementation.

Time	Pre-supplementation	Post-supplementation	P-value
10 min	34.56±24.89	42.23±21.33	0.501
20 min	37.24±17.11	46.93±10.61	0.188
30 min	32.84±7.90	41.93±7.18	0.024*
40 min	31.1±9.87	34.01±5.20	0.468
50 min	26.34±8.09	29.84±5.65	0.319
60 min	22.67±6.96	35.27±26.92	0.184

Means and standard deviations are listed. Macrocytosis is defined as mean corpuscular volume >100 fL. * P<0.05. PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate.

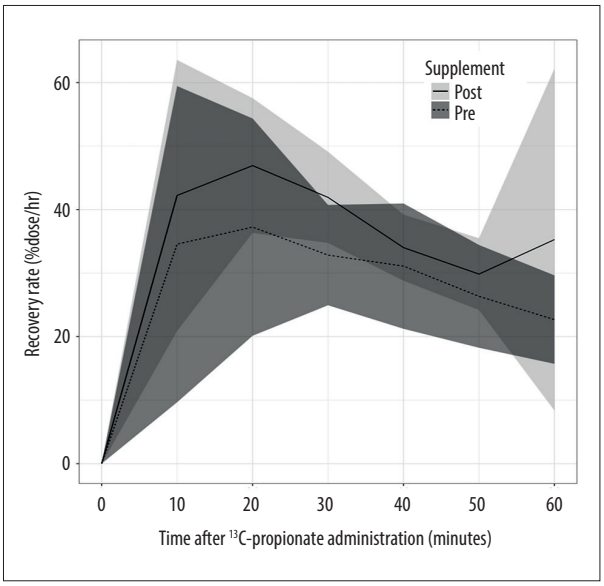

Figure 4. Comparisons of RRs in 1-h PBT between post- and pre-VB_{12} supplementation. Horizontal axis and vertical axis indicate time (min) after ^{13}C-propionate administration and RR (%dose/h), respectively. The tinted area around each line indicates standard deviation. The RR_{30} was significantly higher in the post-supplement group than in the pre-supplement group. MCV – mean corpuscular volume; PBT – ^{13}C-propionate breath test; RR – ^{13}C recovery rate; RR_{30} – RR at 30 min after administration of ^{13}C-propionate; VB_{12} – vitamin B_{12}.

and 37.82 vs 43.93 (P=0.003), respectively (**Table 3, Figure 3**, respectively).

Comparisons of 1-h PBT Between Pre- and Post-Supplementation

Nine patients received the test before and after oral or intramuscular supplementation of VB_{12} for more than 3 months. We compared the RRs of 11 post-treatment data and 9 pre-treatment data (2 patients underwent the 1-h PBT twice after supplementation). As shown in **Table 4** and **Figure 4**, the RRs at all time points were higher in the post-treatment group, although only the RR_{30} was significantly higher (mean: 41.93 vs 32.84, P=0.024).

Discussion

This study evaluated whether the 1-h PBT can be used as an easier, noninvasive method of diagnosis of VB_{12} deficiency by comparing the results of 1-h PBT results of patients with or without low serum VB_{12} levels, macrocytosis, and VB_{12} supplementation. We did not find a significant difference in RRs between the patients with normal and low serum VB_{12} levels; however, significant differences were found between the with and without macrocytosis groups and between the before and after VB_{12} supplementation groups. Furthermore, the RRs peaked within 30 min after propionate administration, suggesting that 1 h is sufficient for measuring the RRs.

The previous study on conventional PBT, performed by Wagner et al, comparing the RRs for 2 h between 26 participants who were at risk for VB_{12} deficiency and 32 healthy patients showed significantly lower $RR_{10\text{-}30}$ in patients at risk of VB_{12} deficiency compared to healthy participants [12]. In the present study, we confirmed that the RRs peaked within 30 min after propionate administration, as reported by Wagner et al. Thus, we believe that 1 h, which is half the time for a conventional PBT, is sufficient for measuring the RRs. Besides the different measuring period (1 h vs 2 h), there are 2 other differences between the present study and the study by Wagner et al: (1) different reference biomarkers and (2) different comparison groups.

Because we could not measure the serum MMA level as mentioned above, we used serum VB_{12} level as a reference biomarker and defined serum VB_{12} deficiency as serum VB_{12} level <145 pg/mL; therefore, we did not find the expected difference in the 1-h PBT associated with low serum VB_{12} levels. We could not compare and analyze the association of PBT results

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and serum VB_{12} levels between our study and the study by Wagner et al because they did not directly analyze PBT results and serum VB_{12} levels [12]. As mentioned in the Background section above, many studies have pointed out that serum VB_{12} level is unreliable as a diagnostic marker for VB_{12} deficiency; therefore, serum MMA levels but not serum VB_{12} levels were used to diagnose deficiency in a previous study using PBT to diagnose VB_{12} deficiency [6]. Thus, we believe that the lack of changes in 1-h PBT associated with serum VB_{12} level was caused by the poor association between the serum VB_{12} level and actual VB_{12} deficiency rather than by the sensitivity of the 1-h PBT. Given the unreliability of serum VB_{12} levels in the diagnosis of VB_{12} deficiency, we intended to evaluate the diagnostic performance of 1-h PBT by comparing it with serum MMA levels; however, we did not go ahead with using serum MMA levels due to the lack of access to serum MMA measurement methods in Japan and the research cost, and we judged serum VB_{12} level to be a feasible and somewhat reliable biomarker according to the results of a previous systematic review of randomized control trials [10].

In this study, we evaluated the relationship between MCV, a marker of macrocytosis caused by megaloblastic anemia, a typical disease resulting from VB_{12} deficiency, and the RRs in 1-h PBT to compensate for the lack of comparison between 1-h PBT and other reliable biomarkers. As expected, the results showed that the RRs were significantly lower in the macrocytosis group 20-30 min after propionate administration.

For the same purpose of compensation, we additionally compared the RRs of post-treatment data and pre-treatment. As expected, the RR_{30} was significantly higher in the treated group. Although we cannot compare these findings with previous studies because no previous study on PBT evaluated the association between MCV and before and after treatment [12,17], we believe that the decrease in the RR in the macrocytosis group and the increase in the RR after VB_{12} supplementation reflect the propionate metabolic disturbances associated with VB_{12} deficiency, as in previous studies [12,17].

The present study has 3 major limitations: (1) As described above, we could not compare the findings of 1-h PBT with serum MMA levels due to lack of access to serum MMA measurement in Japan and the research cost. We believe that this is a major limitation that limits the significance of this study. To compensate for the lack of comparison between 1-h PBT and reliable biomarkers, we evaluated the relationship between MCV in this study. (2) However, this study did not exclude other causes of macrocytosis, such as folate deficiency, hypothyroidism, bone marrow disease, drug adverse effects, or alcoholic liver disease [14], nor did it determine the presence of iron-deficiency anemia, chronic inflammation, or thalassemia that cause low MCV [18]. Thus, the possibility of these comorbid conditions affecting the MCV values cannot be ignored. (3) We could not enroll asymptomatic healthy controls in this study because we could use the 1-h PBT only for patients with clinically suspected VB_{12} deficiency due to ethical restriction. Despite these limitations, since the peaks of the RRs were within 30 min after propionate administration, we believe that RR measurements for 1 h are sufficient to evaluate the abnormal propionate metabolism caused by VB_{12} deficiency. Therefore, the 1-h PBT may be a more convenient noninvasive diagnostic modality for VB_{12} deficiency than conventional PBT.

Conclusions

The 1-h PBT can be used as a convenient noninvasive diagnostic modality for VB_{12} deficiency because 1 h was found to be a sufficient collection time and expected differences were observed in comparisons between the groups with and without macrocytosis and before and after supplementation. Further studies comparing the 1-h PBT results with serum MMA levels are needed.

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Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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