Controlled attenuation parameter is correlated with actual hepatic fat content in patients having nonalcoholic fatty liver disease with none-to-mild obesity and liver fibrosis

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; MRI, magnetic resonance imaging; VCTE, vibration-controlled transient elastography; CAP, controlled attenuation parameter; LS, liver stiffness; US, ultrasonography; BMI, body mass index; ROC, receiver-operating characteristics.

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Running title: CAP and hepatic fat content

Keywords: nonalcoholic fatty liver disease; controlled attenuation parameter;

liver fat; body mass index; fibrosis

Abstract

Background & Aim: Non-invasive steatosis-quantifying methods are required for nonalcoholic fatty liver disease (NAFLD) patients in order to monitor disease severity and assess therapeutic efficacy. Controlled attenuation parameter (CAP) evaluated with vibration-controlled transient elastography can predict the presence of steatosis, but its application to absolute hepatic fat quantitation remains unclear. The aim of this study was to examine whether CAP is correlated with real hepatic fat content in NAFLD patients.

Methods: Eighty-two NAFLD patients underwent percutaneous liver biopsy were enrolled. CAP was measured using FibroScan® just before liver biopsy. The percentage of fat droplet area to hepatocyte area in biopsied specimen was determined morphometrically using computerized optical image analyzing system. The correlation between CAP and liver histology was examined.

Results: CAP showed an excellent correlation with actual liver fat percentage in the NAFLD patients having body mass index (BMI) < 28 kg/m^2 (r = 0.579, P < 0.0001), especially < 25 kg/m^2 (r = 0.708, P < 0.01), but the meaningful correlation disappeared in the patients with BMI > 28 kg/m^2. In the patients with BMI < 28 kg/m^2, CAP quantitativeness was affected by the presence of stage 2-4

fibrosis, but not the presence of hepatocyte ballooning and severity of lobular inflammation.

Conclusions: CAP may be a promising tool for quantifying hepatic fat content in NAFLD patients having none-to-mild obesity and liver fibrosis. Further improvement of CAP performance is needed for the NAFLD patients having BMI > 28 kg/m^2 or significant hepatic fibrosis.

Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) has been increasing worldwide. NAFLD exhibits a wide spectrum ranging from nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH) and ensuing fibrosis and hepatocellular carcinoma.[1-4] NASH is characterized by the presence of hepatocyte ballooning degeneration, lobular inflammation and/or various degree of fibrosis in addition to macrovesicular steatosis.[1-3] Since the severity of steatosis is one of the essential indicators of NAFLD/NASH activity, objective determination method of hepatic steatosis is needed to assess disease severity and therapeutic response for NAFLD patients. There are several trials to predict NAFLD activity using various serum biomarkers,[5-7] but the accuracy is still unsatisfactory. Liver biopsy is considered as the gold standard method for evaluating NAFLD/NASH activity, but its invasiveness and cost lets the patients be unwilling to receive repeated biopsies. Additionally, it is not realistic that liver biopsy is routinely performed for large numbers of NAFLD patients. Therefore, a simple, non-invasive, accurate, and quantitative method is strongly desired to assess hepatic steatosis.

We previously report a simple, non-invasive, and accurate method to quantify hepatic fat content using magnetic resonance imaging (MRI)

double-echo chemical shift gradient-echo sequence (double-echo fast low-angle shot sequence) on a 1.5-T scanner.[8] The percentage of hepatic steatosis estimated by MRI was highly correlated with the percentage of hepatic fat droplet area calculated using computerized morphometry in biopsied specimens. MRI is very accurate and useful for hepatic fat quantitation, but is somewhat expensive and dependent of imaging facility and equipment.

Recently, transient elastography is widely used to assess hepatic fibrosis.[9,10] By using vibration-controlled transient elastography (VCTE), such as FibroScan®, controlled attenuation parameter (CAP) and liver stiffness (LS) value can be measured simultaneously. CAP is calculated from liver ultrasonic attenuation rate, reflecting the degree of hepatic steatosis.[9] There are some studies regarding the usefulness of CAP for detecting hepatic steatosis and estimating the severity of steatosis semi-quantitatively,[11-14] but these studies lacked quantitative evaluation of hepatic fat content based on objective histopathological assessment. This study aimed to examine if CAP can quantify real hepatic fat content.

Methods

Patients

This study was approved by the Committee for Medical Ethics of Shinshu University School of Medicine (the approved ID number is 2276) and were in accordance with the Helsinki declaration of 1975, as revised in 1983. Informed consent was obtained from all patients. Eighty-two NAFLD patients who admitted to Shinshu University Hospital from 2013 April to 2015 December for percutaneous liver biopsy were enrolled. The possibility of NAFLD was considered according to the following criteria: (1) the presence of hepatorenal contrast and increased hepatic echogenicity on abdominal ultrasonography (US), (2) ethanol consumption of < 20 g/day, (3) the absence of other causes of liver dysfunction, such as viral hepatitis, drug-induced liver injury, autoimmune liver diseases, primary sclerosing cholangitis, Wilson's disease, hereditary hemochromatosis, and citrin deficiency.[15,16] The diagnosis of NAFLD/NASH was confirmed based on histological findings of biopsied specimens. All patients received VCTE examination before liver biopsy in the same day.

Body weight and height were measured before liver biopsy in the fasting state. The presence of obesity was defined as having a body mass index (BMI)

of ≧ 25 kg/m^2 based on criteria released by the Japan Society for the Study of Obesity.[17] Medical information was also recorded. The presence of hypertension and hyperlipidemia, and diabetes was judged as described elsewhere.[18,19] All laboratory data in a fasting state were obtained in the liver biopsy day.

VCTE and CAP measurement

LS and CAP were determined using FibroScan® (Echosens, Paris, France) prior to liver biopsy. After the patient lay down in the dorsal decubitus position, the tip of the probe was placed on the patient's skin between the ribs over the right lobe (segment 5 or 8) of the liver. Only a 3.5-MHz standard M probe was used because other probes, such as XL probe, are not available for CAP measurement at present. CAP was computed only when the LS measurement was valid and measured in the same volume of liver parenchyma between 25 and 65 mm in depth. The final CAP was calculated as the median of each value obtained from 10 measurements and expressed in dB/m. Obtaining no value even after 10 measurements was judged as measurement failure.

Histopathological analysis

Liver specimens were obtained from segment 5 or 8 using 14-G needles as described previously and immediately fixed in 10% neutral formalin. Sections were cut in 4-μm thickness and stained by means of hematoxylin and eosin and Azan-Mallory methods. The histological activity of NAFLD was assessed by an independent expert pathologist (KS) in a blinded manner according to the scoring system proposed by Kleiner et al.[20] NASH is defined as the presence of macrovesicular steatosis (≥ 5% of hepatocyte affected) and hepatocyte ballooning with and without lobular inflammation and fibrosis. A BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) and Dynamic cell count BZ-II analysis application (Keyence) were used to automatically determine the percentage of fat droplet area to hepatocyte area in the biopsied specimens. Briefly, hematoxylin and eosin-stained liver sections were observed under x100 magnification (Fig. 1A). The area of liver parenchyma was marked in pink and the areas of big vasculature and bile duct were manually excluded (Fig. 1B). The pink background was regarded as hepatocyte area. Subsequently, the areas of lipid droplets were marked in blue (Fig. 1C) and the percentage of fat droplet area to hepatocyte area was calculated in each field (Fig. 1D). The entire area or more than 10 fields in the section (if the specimen is too big) were examined and

the final value was expressed as the percentage of total fat areas to total hepatocyte areas.

Statistical analysis

Clinical data were expressed as a number (percentage) or median (range). Pearson's test was adopted to examine the correlation between CAP and optical image analysis results using StatFlex Ver6.0 (Artech Co., Ltd., Osaka, Japan). Receiver operating characteristic (ROC) curves were plotted and optimal LS cut-off points to detect stage 2-4 fibrosis were determined as the values showing maximum sensitivity plus specificity. A P value < 0.05 was considered to be statistically significant.

Results

Correlation between CAP and liver fat content

To examine whether CAP reflects actual fat content in the liver, CAP was measured just prior to liver biopsy in 82 NAFLD patients and a correlation between CAP and fat area percentage in biopsied specimens was analyzed. Clinicopathological features of the patients enrolled are summarized in Table 1. Fifty-four patients (66%) had obesity and 63 (77%) were diagnosed as having NASH. CAP was obtained from all patients underwent VETC. CAP showed a significant correlation with the area of hepatic fat globules determined by computerized optical image analysis (r = 0.480, P < 0.0001, Fig. 2), but the correlation coefficient was less than that obtained from the previous MRI study (r = 0.91).[7] Median CAP of the patients with liver fat area of 5-10, 10-15, 15-20, and > 20 % was 288, 306, 340, 329 dB/m, respectively. When the patients were divided into 3 groups based on BMI (< 25, 25-30, and > 30 kg/m^2), a significant correlation was detected in the NAFLD patients having BMI < 25 kg/m^2 (r = 0.708, P < 0.01, Fig. 3A), but the meaningful positive correlation was not found in the patients with BMI $\geq$ 25 kg/m^2 (Fig. 3B and C). It was reported that LS measurement using FibroScan® is very difficult for the patients with BMI > 28

kg/m^2.[21] When the patients were divided into 2 groups with BMI of more or less of 28 kg/m^2, a positive correlation was detected in the patients with BMI < 28 kg/m^2 (r = 0.579, P <0.0001, Fig. 4A), but not in those with BMI ≧ 28 kg/m^2 (Fig. 4B). These results indicate that CAP is correlated with actual liver fat content in the patients with BMI < 28 kg/m^2, especially BMI < 25 kg/m^2.

The impact of lobular inflammation and ballooning on the correlation between CAP and liver fat content

We examined in the patients with BMI < 28 kg/m^2 whether the presence of lobular inflammation, ballooning, and fibrosis affects the correlation between CAP and liver fat content. In these patients, significant correlations were detected regardless of the absence/presence of ballooning (r = 0.780, P < 0.01 in grade 0 and r = 0.435, P < 0.01 in grade 1-2, respectively, Fig. S1) and severity of lobular inflammation (r = 0.492, P < 0.05 in grade 1 and r = 0.512, P < 0.05 in grade 2-3, respectively, Fig. S2).

The impact of fibrosis on the correlation between CAP and liver fat content

The excellent correlation was also detected in the patients with stage 0 fibrosis (r

= 0.955, $P < 0.01$), but the correlation was attenuated in the patients with stage 1 fibrosis and disappeared in the patients with 2-4 fibrosis (Fig. 5). In all patients, there was no significant relationship between BMI and fibrosis stage ($r = 0.210$, $P = 0.06$).

ROC curve analysis revealed that area under the ROC curve of LS values for stage 2-4 fibrosis detection was as high as 0.84, and the best LS cutoff value was calculated as 10.2 kPa. When the NAFLD patients with BMI < 28 kg/m^2 were divided into 2 groups, higher or lower than 10.2 kPa, a significant correlation between CAP and liver fat content was found in the patients with LS < 10.2 kPa ($r = 0.672$, $P < 0.0001$, Fig. 6A), but the positive correlation diminished in those with LS $\geqq$ 10.2 kPa (Fig. 6B). Collectively, CAP is not correlated with real liver fat content in the NAFLD patients having stage 2-4 fibrosis, even in the patients with BMI < 28 kg/m^2.

The impact of sample size on the correlation

We previously analyzed the correlations in 39 (biopsied between 2013 April to 2014 December) and 59 (biopsied between 2013 April to 2015 April) NAFLD patients and obtained similar results to those in final 82 patients (between 2013

April to 2015 December) (Table S1). Judging from the good reproducibility, we considered that the number of patients did not affect the accuracy of the findings.

Discussion

CAP was significantly correlated with actual hepatic fat content calculated by computerized morphometry in the patients having BMI < 28 kg/m^2, especially BMI < 25 kg/m^2, and stage 0-1 fibrosis. The excellent correlation was independent of the absence/presence of hepatocyte ballooning and severity of lobular inflammation in the liver. However, CAP was not correlated with the amount of liver fat droplets in the patients having BMI > 28 kg/m^2 or stage 2-4 fibrosis. These results suggest that CAP measurement may be useful to quantify actual hepatic fat in humans with none-to-mild obesity and liver fibrosis. As far as we know, this is the first study to examine the accuracy and utility of CAP to quantify liver fat content using morphometry-based objective determination.

It has been widely accepted that high CAP can predict the presence of hepatosteatosis.[11-14] The degree of ultrasonic attenuation may be increased in hepatic parenchyma when fat accumulation is severe, which is consistent with the principle of deep attenuation in routine US examination. CAP appeared to be increased as steatosis became severe,[11-14] making us to hypothesize whether CAP reflects the absolute amount of hepatic fat. In the previous studies to compare CAP with liver fat content, histological evaluation was limited to

subjective semi-quantitative grading by pathologists. However, reliability and accuracy of pathological grading is poor and inter-observer differences may occur even between pathologists.[22] For example, steatosis grading is sometimes problematic when approximately 30-40% of hepatocytes are affected. Since the range of steatosis grade is relatively broad (e.g., grade 1 means 5 to 33% of hepatocytes affected), the semi-quantitative grading system is not sensitive to monitor less drastic changes during follow-up and after therapy. To address these issues, we determined the percentage of fat droplet area to hepatocyte area in the liver tissue using computerized optical imaging analyzing system and examine the correlation with CAP. This study demonstrated a nice correlation between CAP and actual hepatic fat content in the NAFLD patients with BMI < 28 kg/m^2. Therefore, CAP may be useful to evaluate the severity of hepatic steatosis and therapeutic response for none-to-mildly-obese NAFLD/NASH patients.

However, obesity is frequently accompanied by NAFLD/NASH. CAP was not correlated with morphometrically-measured hepatic fat content in the NAFLD patients with BMI $\geq$ 28 kg/m^2. This is consistent with the results of the previous studies that the correlation between CAP and steatosis grade is poorer in obese

patients compared with non-obese ones and CAP is increased according to increased BMI even in the absence of hepatic steatosis.[23,24] Discrepancy between CAP and liver fat content in obese NAFLD patients is likely derived from thick subcutaneous adipose tissue. Indeed, it was reported that skin capsular distance of more than 25 mm may overestimate hepatic steatosis.[25] For severely obese patients, LS can be determined more accurately using XL probe than a conventional M probe. If CAP can be measured using XL probe in the future, this shortcoming might be overcome.

Another intriguing finding in the present study is the lack of correlation between CAP and liver fat content in the presence of significant liver fibrosis even in the NAFLD patients having BMI < 28 kg/m^2. The impact of liver fibrosis on the correlation between CAP and hepatic fat content has not been investigated previously. Because fibrosis develops around hepatocytes in NAFLD/NASH livers, it might be reasonable that the presence of significant fibrosis interferes ultrasonic attenuation through hepatocytes causing disruption of CAP performance.

A good correlation between CAP and fat area percentage is independent of severity of hepatic inflammation and the presence of hepatocyte ballooning. LS

is reported to be affected by acute liver injury that is characterized by marked hepatocyte degeneration, such as swelling and coagulation necrosis, and massive inflammatory cell infiltration.[26] Since these pathological changes are much milder in NAFLD/NASH compared with acute hepatitis, the contribution of hepatitis and ballooning degeneration to CAP performance is likely minor in the context of NAFLD/NASH.

In the present study, there were no patients with more than 40% of hepatic steatosis by morphological determination. The previous report indicated poor CAP performance for discrimination between moderate (33-67%) and severe steatosis (> 67%).[12,14] If severe steatosis is suspected, other imaging modalities (e.g., computed tomography, MRI) may be more useful to evaluate hepatic fat content accurately. Comparison of steatosis-quantifying ability between MRI double-echo chemical shift gradient-echo sequence and CAP deserves further investigation.

Since fat deposition is sometimes focal in NAFLD livers with minimal-to-mild steatosis[5,27], sampling variability of liver biopsy may occur. However, CAP was measured in segment 5 or 8 and liver sample was obtained from the same lobe. Thus, the influence of sampling error was considered to be very few in the

present study.

To follow the clinical course and therapeutic response in NAFLD/NASH patients, simple, non-invasive, accurate, and quantitative methods to evaluate hepatic fat content and fibrosis are needed. VCTE is superior to other imaging modalities with respect to simplicity, inexpensiveness, and simultaneous assessment of liver steatosis and fibrosis. If its accuracy will be improved especially for obese patients, VCTE can be performed easily and repeatedly and will be useful not only for routine follow-up and evaluation of therapeutic response for NAFLD/NASH patients, but also NAFLD screening and annual health check-up in healthy individuals. Therefore, further large-scale studies are needed to improve CAP performance for NAFLD patients with obesity and/or significant liver fibrosis and to confirm the utility of CAP for quantifying hepatic fat content.

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Table 1. Clinicopathological features of the patients (n = 82)

Parameter	Value	
Clinical findings		
Age (years)	56	(14 - 76)
Male	38	(46%)
Obesity (BMI ≧ 25 kg/m^2)	54	(66%)
Diabetes	25	(30%)
Hypertension	36	(44%)
Hyperlipidemia	48	(59%)
BMI (kg/m^2)	26.7	(20.4 - 37.5)
Ethanol (g/day)	0	(0-18)
Platelet (×10^4/μL)	24.1	(9.5 - 45.4)
AST (U/L)	48	(13 - 200)
ALT (U/L)	72	(17 - 281)
γGT (U/L)	54	(14 - 544)
Total cholesterol (mg/dL)	202	(131 - 294)
Triglycerides (mg/dL)	128	(49 - 563)
HDL-cholesterol (mg/dL)	49.5	(32 - 88)
Glucose (mg/dL)	108	(87 - 215)
Insulin (μU/mL)	11.7	(2.9 - 140.4)
HbA1c (%)	5.9	(5.1 - 10.9)
HOMA-IR	3.2	(0.7 - 42.6)
Hyarulonic acid (mg/dL)	55.5	(9 - 742)
Type 4 collagen 7S (mg/dL)	5.1	(3.0 - 11.0)
Histological findings		
Steatosis 0/1/2/3	0/19/42/21	
Lobular inflammation 0/1/2	5/39/38	
Ballooning 0/1/2	19/34/29	
Fibrosis 0/1/2/3/4	13/44/6/16/3	

Data are expressed as a number (percentage) or median (range). Histological findings were scored according to criteria proposed by Kleiner et al.[20] BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; HDL, high-density lipoprotein; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment for insulin resistance.

Figure legends

Fig. 1. Principle of computerized morphometric measurement of the fat percentage. Hematoxylin and eosin-stained liver sections were observed under x100 magnification (**A**). The area of liver parenchyma was marked in pink and the areas of big vasculature and bile duct were manually excluded (**B**). The pink area was considered as hepatocyte area. Subsequently, the areas of lipid droplets were marked in blue (**C**) and the percentage of total fat droplet areas to total hepatocyte areas was calculated (**D**).

Fig. 2. Relationship between CAP and liver fat content in all NAFLD patients (n = 82). A correlation efficient (*r*) and *P* value were calculated using Pearson's test.

Fig. 3. The effect of obesity on the correlation between CAP and liver fat content. A correlation efficient (*r*) and *P* value were calculated using Pearson's test.

A, Non-obese NAFLD patients (BMI < 25 kg/m^2, n = 28): **B**, Mildly-to-moderately-obese NAFLD patients (BMI 25-30 kg/m^2, n = 36): **C**, Severely-obese NAFLD patients (BMI > 30 kg/m^2, n = 18).

Fig. 4. Correlation between CAP and liver fat content in the NAFLD patients with BMI < 28 kg/m^2 (**A**, n = 46) or ≧ 28 kg/m^2 (**B**, n = 36). A correlation efficient (*r*) and *P* value were calculated using Pearson's test.

Fig. 5. The effect of fibrosis on the correlation between CAP and liver fat content in the patients with BMI < 28 kg/m^2. A correlation efficient (*r*) and *P* value were calculated using Pearson's test.

A, NAFLD patients with no fibrosis (n = 11): **B**, NAFLD patients with stage 1 fibrosis (n = 22): **C**, NAFLD patients with stage 2-4 fibrosis (n = 13).

Fig. 6. Correlation between CAP and liver fat content in the patients with BMI < 28 kg/m^2 according to LS ≧ 10.2 kPa, an indicator of stage 2-4 fibrosis, or not. A correlation efficient (*r*) and *P* value were calculated using Pearson's test.

A, NAFLD patients with LS < 10.2 kPa (n = 34): **B**, NAFLD patients with LS ≧ 10.2 kPa (n = 12).

Figure 1

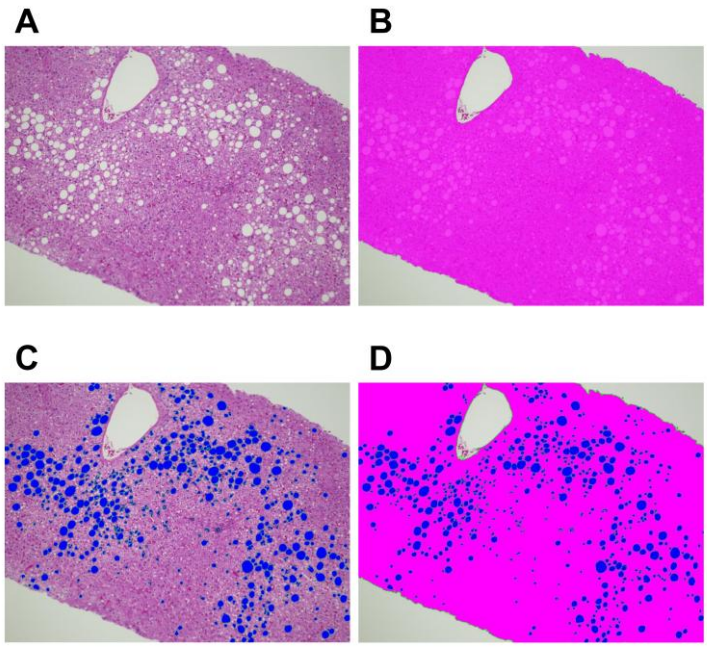


Figure 2

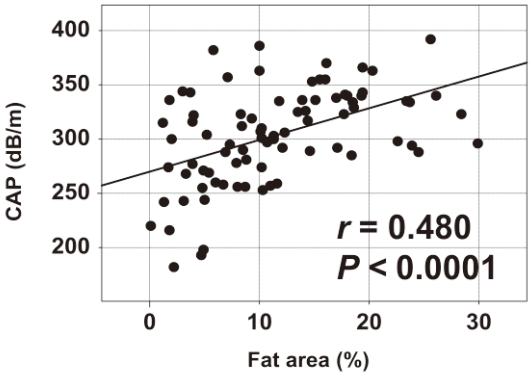
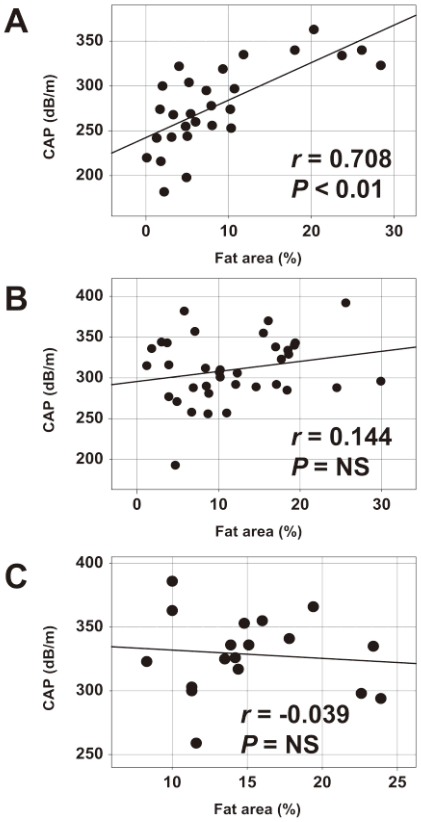


Figure 3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CAP (dB/m)
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Fat area (%)
r = 0.708
P < 0.01
B
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Fat area (%)
r = 0.144
P = NS
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CAP (dB/m)
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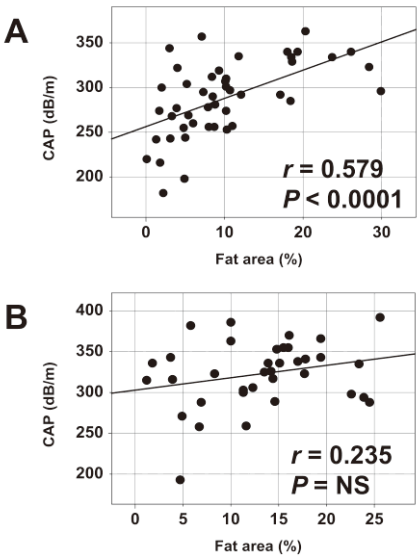
25
Fat area (%)
r = -0.039
P = NS

Figure 4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CAP (dB/m)
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r = 0.579
P < 0.0001
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Fat area (%)
B
CAP (dB/m)
400
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300
250
200
r = 0.235
P = NS
0
5
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20
25
Fat area (%)

Figure 5

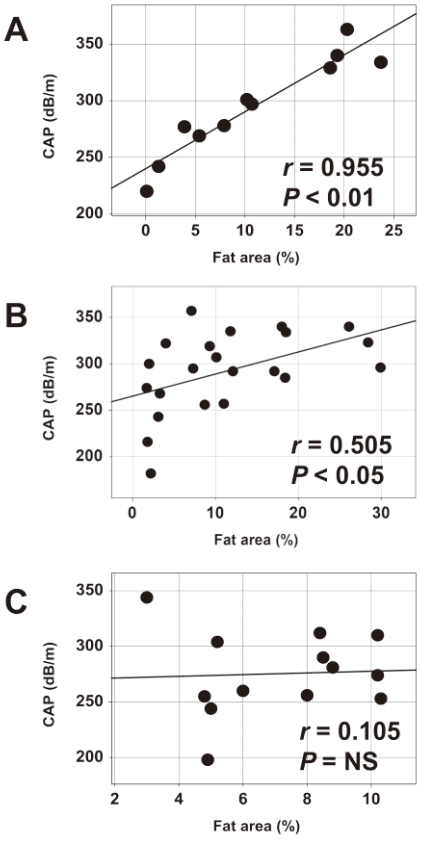
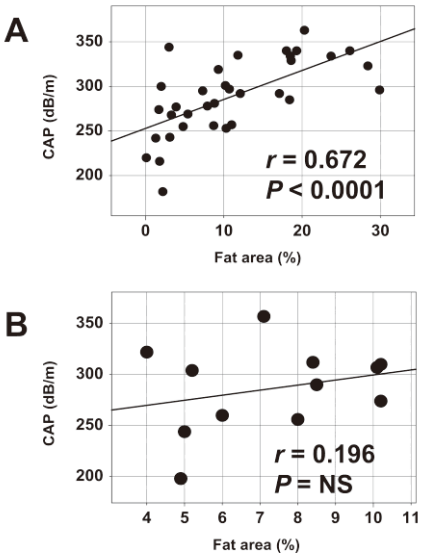

A
CAP (dB/m)
350
300
250
200
0
5
10
15
20
25
Fat area (%)
r = 0.955
P < 0.01
B
CAP (dB/m)
350
300
250
200
0
10
20
30
Fat area (%)
r = 0.505
P < 0.05
C
CAP (dB/m)
350
300
250
200
2
4
6
8
10
Fat area (%)
r = 0.105
P = NS

Figure 6


A
CAP (dB/m)
350
300
250
200
0
10
20
30
Fat area (%)
r = 0.672
P < 0.0001
B
CAP (dB/m)
350
300
250
200
4
5
6
7
8
9
10
11
Fat area (%)
r = 0.196
P = NS