# Quantification of intramyocardial hemorrhage volume using magnetic resonance imaging with three-dimensional T1-weighted sequence in patients with ischemia-reperfusion injury : a semi-automated image processing technique

Arai, Hideo
Fukuokaken Saiseikai Futsukaichi Hospital

Kawakubo, Masateru
Department of Health Sciences, Faculty of Medical Sciences, Kyushu University: Assistant Professor

Abe, Kou
Fukuokaken Saiseikai Futsukaichi Hospital

Hatashima, Hikaru
Oita Prefectural Hospital

他



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**Title**

# Quantification of intramyocardial hemorrhage volume using magnetic resonance imaging with three-dimensional T1-weighted sequence in patients with ischemia-reperfusion injury—a semi-automated image processing technique

**Hideo Arai, RT**

Fukuokaken Saiseikai Futsukaichi Hospital

**Masateru Kawakubo, PhD**

Department of Health Sciences, Faculty of Medical Sciences, Kyushu University

**Kou Abe, M.D.**

Fukuokaken Saiseikai Futsukaichi Hospital

**Hikaru Hatashima, M.D.**

Oita Prefectural Hospital

**Kenichi Sanui, RT**

Fukuokaken Saiseikai Futsukaichi Hospital

**Hiroshi Nishimura, MD, PhD**

Fukuokaken Saiseikai Futsukaichi Hospital

**Toshiaki Kadokami, MD, PhD**

Fukuokaken Saiseikai Futsukaichi Hospital

**A concise and informative titles**

Quantification of intramyocardial hemorrhage volume using magnetic resonance imaging

**Corresponding author**

Masateru Kawakubo

Department of Health Sciences, Faculty of Medical Sciences, Kyushu University

3-1-1 Maidashi Higashi-ku Fukuoka 812-8582 Japan

Tel: +81-92-642-6728

Fax: +81-92-642-6728

E-mail: k-mstr@hs.med.kyushu-u.ac.jp

**Abstract (243/250)**

**Purpose**

Although intramyocardial hemorrhage (IMH) is a poor prognostic factor caused by ischemia reperfusion injury, little evidence is available regarding the association between IMH volume and biomarkers. In the present study, we measured IMH volume using three-dimensional (3D) T1-weighted magnetic resonance imaging (T1-MRI) and investigated its association with biomarkers. Moreover, the accuracy of semi-automatic measurement of IMH volume was validated.

**Methods**

We retrospectively enrolled 33 consecutive patients (mean age, 67 ± 11 years ) who underwent cardiac MRI after reperfusion therapy for acute myocardial infarction. IMH was observed in 4 patients (12.1 %). Receiver operating characteristics (ROC) analysis of creatine kinase (CK) and CK-muscle/brain (CK-MB) tests for detecting IMH were performed. IMH volume measured using semi-automatic methods by a 2 standard deviation (SD) threshold was compared to manual measurements using the Spearman's correlation coefficient ($\rho$) and Bland-Altman analyses.

**Results**

ROC analysis revealed optimal cutoff values of CK: 2460 IU/L and CK-MB: 231 IU/L (area under the curve: 0.95 and 0.91; sensitivity: 86 % and 79 %; specificity: 100 % for both). IMH volume with the 2SD threshold correlated with that of the manual measurement (5.84 g [3.30 to 9.00] g vs. 8.07 g [5.37 to

9.33]; $\rho$: 0.85, $p < 0.01$; bias [limit of agreement]: -0.01 g [-0.51 to 0.49]; intraclass correlation coefficients (0.84 [0.75 to 0.90]).

**Conclusions**

Our findings could help identify the risk of IMH after reperfusion therapy with biomarkers. 3D T1-MRI can semi-automatically provide accurate IMH volume without being time-consuming.

**Keywords**

Intramyocardial hemorrhage; biomarker; quantification; semi-automated threshold method; magnetic resonance imaging; acute myocardial infarction

## Introduction

Advances in the treatment of acute coronary syndrome (ACS), such as percutaneous coronary intervention (PCI) and thrombus aspiration therapy have allowed early reperfusion in ACS cases leading to an improvement in the survival rate [1]. However, severe myocardial damage after reperfusion therapy, termed as ischemia-reperfusion injury, has received considerable attention in the recent years [2–4]. Ischemia-reperfusion injury is a severe disease damaging the myocardium due to reperfusion [5]. A result of severe ischemia-reperfusion injury is intramyocardial hemorrhage (IMH), which occurs in some cases. Several studies have reported that IMH is associated with the extent of infarct, presence of microvascular obstruction (MVO), increased left ventricular volumes, decreased ejection fraction (EF), the lack of long-term improvement at follow-up, and increasing major adverse cardiac events (MACE) [6–8]. To the best of our knowledge, biomarkers like creatine kinase (CK), CK-Muscle/Brain (CK-MB), and cardiac troponins T and I are useful as indicators of myocardial injury, although no clinical studies have investigated the association between these biomarkers and IMH [9].

Cardiac magnetic resonance (CMR) is the only method that enables accurate imaging of IMH. Most studies pertaining to the imaging of IMH utilized two-dimensional (2D) T2-weighted or T2 star (T2*)-weighted magnetic resonance imaging (MRI) [10, 11]. However, 2D-MRI is limited to the measurement of IMH volume because the hemorrhages occurring outside of the imaging plane are mostly overlooked. Conversely, a previous study reported that the visualization of IMH by three-dimensional

(3D) T1-weighted MRI (T1-MRI), as compared to T2- and T2*-weighted MRI, near-accurately resembled the IMH region corroborating with the pathological findings [12]. Therefore, 3D T1-MRI has the potential for quantifying IMH and image acquisition that covers the entire left ventricle. To measure IMH volume with 3D T1-MRI, manual delineation of IMH region images at multiple planes is needed. Manual analysis is time-consuming in clinical routine, and moreover, variations arising from differences in the observer experience may cause erroneous measurements. Late gadolinium enhancement (LGE) CMR can image myocardial scars with high contrast, and the size of LGE on the image as the major predictor of morbidity and mortality, provides clinical usefulness in myocardial infarction patients [13, 14]. Various image processing protocols for semi-automatic measurements of LGE size have been proposed, such as the standard deviation (SD) based thresholding method, full width half maximum (FWHM) method, and the Otsu method [15–17]. As discussed so far, the purpose of this study was to clarify the association between biomarkers and IMH for ACS patients after reperfusion therapy using IMH imaging and to assess the accuracy of various methods for quantifying IMH in patients with IMH.

## Materials and Methods

### Study population

We retrospectively enrolled 33 consecutive patients who underwent reperfusion therapy for acute myocardial infarction (AMI) and underwent CMR with 3D T1-MRI between June 2015 and December 2017. The creatine kinase CK and CK-MB were calculated from the appropriate source (serum).

According to an earlier animal study (pigs), which compared the pathological analysis and various CMR contrast studies, including 3D T1-MRI, the high signal intensity of the 3D T1-MRI was visually defined as IMH by consensual decisions made by both, the cardiologist and radiologist [12]. The study was approved by the institutional review board, and the requirement for patient consent was waived. An online provision, on the hospital homepage, was prospectively made available to the patients for opting out of the study. The study was conducted in accordance with the Declaration of Helsinki.

**MR imaging protocol**

CMR imaging was performed using a 1.5 T MRI system (Ingenia, Philips Healthcare, Best, The Netherlands), equipped with 33-mT/m maximum gradient strength, 120-T/ms slew rate, and a 32-channel phased-array receiver coil. An ECG-gated steady-state free precession cine imaging was acquired in four-chamber view with 100 reconstructed phases per heartbeat to detect the static periods of the cardiac cycle. The cine sequence parameters were as following: TR/TE, 2.8 ms/1.38 ms; flip angle, 60°; slice thickness, 7 mm; field of view, 350 × 350 $mm^2$; in-plain resolution, 1.09 × 1.09 $mm^2$; acquisition matrix, 192 × 185, and SENSE (sensitivity encoding) factor, 2.5. The 3D T1-MR images were acquired in the transverse axial plane with diaphragm- and cardiac-triggering. The delay time for inversion recovery pulse for blood signal suppression was configured to 500 ms. The 3D T1-MRI parameters were as follows: TR/TE, 4.6 ms/2.1 ms; flip angle, 10°; slice thickness, 1.0 mm; field of view, 200 × 200 $mm^2$; in-plain resolution, 2.0 × 2.0 $mm^2$; acquisition matrix, 100 × 99; SENSE factor, 1, and phase direction, R-L. The data acquisition

periods during the static period was determined by visuomotor analysis of the cine images.

**Post process of IMH imaging using 3D T1-MRI**

No patients were excluded from the study due to unacceptable image quality due to magnetic-inhomogeneity and motion-artifact. Figure 1 shows the flowchart of the post processing of IMH imaging with 3D T1-MRI. The transverse axial images were reconstructed into short-axis images with 5 mm thickness as described in a previous study [12]. The reconstructed images in the patients with IMH were processed as follows. Firstly, the left myocardium regions from all short-axis images were manually extracted using SYNAPSE VINCENT software (Fujifilm Co, Tokyo, Japan). Secondly, IMH volumes were measured using a manual method, and 6 different methods were applied to the thresholds determined from various image processing protocols. IMH measurements using the manual method was performed using the SYNAPSE VINCENT software. The analyst manually delineated the myocardial high-intensity area, introducing corrections, while varying the contrast windows. All threshold-based measurements for image processing were performed using an off-line software based on MATLAB (The MathWorks, Inc, U.S.). The threshold value of signal intensity was defined similarly to previous LGE studies [15–17]. The area of IMH was defined as the pixels having signal intensity higher than the threshold value. The threshold value, using the Otsu method, was automatically calculated based on the value estimated from the histogram of cumulative myocardial signal intensities [18]. For determining the SD- and FWHM-based threshold, the largest region of interest (ROI) of the normal myocardium was

manually from amongst all myocardial images. Mean signal intensity in the ROI (Mean) and its SD were calculated. The threshold of SD-based method was calculated from the following equation:

$$Threshold\ of\ SDs = Mean + k \times SD\ (with\ k = 1\ to\ 4)$$

The threshold of FWHM was calculated from the following equation using the mean and the maximum signal intensity of all left myocardial ROIs.

$$Threshold\ of\ FWHM = (Max - Mean)/2$$

IMH volume ($mm^3$) of the entire left myocardium was calculated as the summation of IMH volumes from all short-axis slices. The volume was converted to weight (mg) by multiplying the specific gravity of myocardium (1.05 $g/cm^3$) as well as the previous calculation of the size of LGE. Manual processing of all data in this study was performed by an analyst with 10 years of clinical experience in cardiac radiology.

**Statistical analyses**

The Shapiro-Wilk test was employed to assess the normality of data distribution. Due to non-normal data distribution, descriptive statistics are provided as the median and the corresponding interquartile ranges (IQR). The receiver operating characteristics (ROC) analysis was performed to validate the accuracy for detection of IMH with biomarkers (CK and CK-MB). The correlations between IMH volumes from the various threshold methods and a manual method were analyzed using the Spearman's rank-correlation coefficient ($\rho$). The accuracy of various threshold methods with respect to the manual method was

evaluated using Bland-Altman analyses and intraclass correlation coefficient (ICC) with one-way random single measures. ICCs were defined as excellent (ICC ≥ 0.75), good (ICC = 0.60–0.74), moderate (ICC = 0.40–0.59), and poor (ICC ≤ 0.39). All statistical analyses were performed using the software package R version 3.4.1 [19]. A P value less than 0.05 was considered significant.

**Intra- and inter-observer variability**

To evaluate intra- and inter-observer variability of manual myocardial segmentation, measurements based on manual and various threshold methods (manual, 1SD to 4SD, and FWHM) were repeated at least 1 month later in 20 randomly selected slices by the same primary analyst and an additional analyst, both with more than 5 years of clinical experience in cardiac radiology. The analysts were blinded to the results of the initial study to ensure variability of IMH volume measurements. The variability was evaluated using Bland-Altman analyses and ICC with one-way random single measures or two-way random single measures (ICC(1,1) or ICC(2,1)). ICCs were defined as excellent (ICC ≥ 0.75), good (ICC = 0.60–0.74), moderate (ICC = 0.40–0.59), and poor (ICC ≤ 0.39).

**Results**

In the present retrospective study, 33 AMI patients, who underwent reperfusion therapy, were enrolled. The CK and CK-MB were increased in all patients with AMI. The CMR examination was performed 5.4 ± 3.2 days after hospitalization for AMI, resulting in the inclusion of 4 patients with IMH in this study. The detailed clinical characteristics of all 33 patients are shown in Table 1. IMH was observed in 39 of

the 371 slices in the 4 patients.

IMH weight of 4 patients was 8.07 g (5.37 to 9.33), whereas, the myocardial weight was 123 g (110 to 137). The CK and CK-MB biomarkers were significantly higher in patients with IMH, than in patients without IMH, although the other parameters showed no significant differences (Table 1). The ROC analysis was used to describe the optimal cutoff values of CK and CK-MB in order to identify patients with IMH (Fig. 2). The area under the curves (AUC) of the CK and CK-MB were 0.95 (95% CI: 0.87-1.00) and 0.91 (95% CI: 0.79-1.00), respectively. The optimal cutoff value of CK levels at 2460 IU/L showed 86% sensitivity and 100% specificity. That of CK-MB levels at 231 IU/L showed 79% sensitivity and 100% specificity. The results of Spearman's rank-correlation coefficient analysis, Bland-Altman analysis, and ICC, comparing the measurements from various threshold methods with the manual method, are shown in Table 2. The measurements of 1SD, 2SD, 3SD, 4SD, and FWHM showed significant correlations with the manual method; However, no significant correlation was observed between the measurements obtained using the Otsu and the manual method. The correlations of 2SD, 3SD, and 4SD measurements were statistically stronger than those of 1SD and FWHM ($P$ <0.01). Moreover, the correlation of 3SD was statistically the strongest among all comparisons ($P$ <0.01). The respective ICCs obtained for 2SD, 3SD and 4SD were excellent, good, and moderate. We show the results of the measurements of IMH in Figure 3 for a representative case (Case 4: a 54-year-old male).

The manual segmentation of left myocardial weight showed excellent intra- and inter-observer

variability. The bias (limit of agreement) [g] with Bland-Altman analysis for intra- and inter-observer variability were -0.4 (-2.0 to 1.3), and -0.6 (-2.0 to 0.8), respectively, and the standard deviation of the difference [g] were 0.8 and 0.7, respectively. Both ICCs showed excellent variability as highlighted by their respective values of 0.91 and 0.91. The intra- and inter-observer variability for all manual and semi-automatic measurements are shown in Table 3. Excellent intra- and inter-observer variability was observed. Bland-Altman analysis for all methods except 1SD and Otsu indicated small bias, standard deviation of the difference, and a narrow limit of agreement. All ICCs were observed as excellent variability.

**Discussion**

IMH was observed in 4 out of 24 AMI patients in this study. According to a previous report, the mean prevalence of IMH was 35% (95% CI: 31% to 38%) in reperfused AMI patients [7]. In our study, the mean prevalence of IMH was observed to be lower than that reported previously. This may be attributed to the early interventional reperfusion therapy due to technical innovation in later years and the lower prevalence of IMH in our study due to the availability of high-quality medical systems [1, 20]. The high-quality medical systems may also help explain the lower mean values of CK and CK-MB in this study when compared with previous studies.

The present study was aimed at clarifying association between biomarkers and IMH and to identify the most appropriate image processing protocol for quantifying IMH volume. Excellent variability

was observed using manual segmentation of the left myocardium. Therefore, it can be concluded that the dataset prepared before the comparison of various image processing methods is reliable. The mean values of CK and CK-MB in this study were lower than those in previous studies; however, the mean values of those in patients with IMH were considerably higher than the values of previous studies [21, 22]. This result indicated that the IMH was caused by severe myocardial injury [7]. Based on these findings, the severe AMI patients with high values of biomarkers is high likely to have the IMH leading to adverse event. For this reason, if the AMI patients have over our cutoff value of the biomarkers, it is necessary to determine the severity by presence or absence of IMH using CMR. It has been reported that severe myocardial damage due to AMI may cause MVO, and the presence or absence MVO, which is detected with CMR, is an importance sign of reperfusion status after reperfusion therapy [23]. Recent reports suggested that accurate detection of MVO was independently associated with adverse ventricular remodeling and patient prognosis [24]. IMH, which is caused by severe reperfusion injury, only exists in the presence of MVO [8]. IMH is a structural and functional disorder of the microcirculation, similar to MVO. Consequently, the myocardial damage caused by IMH is more severe, than that caused by MVO. Some reports have mentioned that presence of IMH and residual IMH in the chronic phase were associated with left ventricular remodeling and persistently elevated T2 values in the surrounding infarct tissue [25]. Knowledge of the presence and severity of IMH may potentially increase the probability of myocardial damage severity assessment. As is evident from the above, our study may provide significant aid in clinical practice.

In the correlation analysis, of all methods, the 3SD correlated the strongest with manual measurements, whereas, the ICC of 2SD was deemed excellent. The variability of IMH measurements were found to be excellent in all methods. According to these results, 2SD or 3SD is the most appropriate image processing method for measuring IMH volume. Additional statistical analysis found the ICC of 2SD method to be excellent with a high correlation coefficient value ($\rho$ = 0.85). Consequently, we concluded that the 2SD method is the most appropriate for quantifying IMH volume with 3D T1-MRI. The volume of IMH calculated with 2SD methods was in agreement to that obtained with manual measurements. The usefulness of Otsu method for measuring LGE size with appreciable accuracy was previously reported [15]. However, our results were in contrast to the previous observations. This discrepancy may be attributed to the mismatch caused by the differences of signal intensities of LGE and IMH. The high signal intensity of LGE represents a significant shortening of the T1 value (425.4±68.1 ms) due to the distribution of the contrast medium in the injured myocardium [26]. On the contrary, the image signal of IMH with 3D T1-MRI is lower reflecting the relatively smaller shortening of T1 values compared with the contrast medium. Moreover, IMH includes components with varying densities of paramagnetic origin i.e. the breakdown products of oxygenated hemoglobin (deoxyhemoglobin, methemoglobin) or blood degradation product (ferritin and hemosiderin) [8], and IMH expresses varied signal intensity in T1-MRI with temporal changes [27]. The Otsu method is superior in automatically detecting a high signal such as that from the contrast medium in LGE. However, for detecting lower intensity signals such as IMH, despite the addition of a

manual process, determination of the threshold with the delineation of normal myocardial region is necessary.

The limitation of our study should be acknowledged. The lack of T2*W imaging, which is a standard CMR technique for detecting IMH is a big limitation in this study, and the lack of association between 3D T1-MRI and T2*W, and other contrast-enhanced CMR imaging studies is also a limitation. Further studies are required to investigate the association between high-signal intensity on 3D T1-MRI and CMR imaging findings. The second limitation is a single-center study with limited patient population. The other limitation is that the lack of validation methods for assessing the accuracy of the quantified IMH volumes, since physically weighing IMH density and measuring IMH volume from cardiac tissue is impossible. However, this study revealed that 3D T1-MRI can detect IMH in humans as a high-signal intensity. 3D T1-MRI with full cardiac coverage overcomes the issue of restricted coverage faced by the established 2D imaging for IMH. The detection and location of IMH using 3D T1-MRI aids in selecting the appropriate slice, in order to further assess IMH using various CMR sequences in the future. Moreover, this study revealed that IMH volume can be accurately measured with minimal manual labor. Therefore, our future studies shall be aimed at investigating the clinical feasibility of IMH volume as a diagnostic biomarker for AMI.

**Conclusion**

It is necessary for the AMI patients with higher cutoff values of CK and CK-MB to perform CMR to check

for myocardial bleeding because of the high risk of IMH. With respect to quantifying IMH, the threshold determined by 2SD method is most appropriate for processing images to quantify the IMH volume in the compiled left myocardium of 3D T1-MR images. On obtaining further confirmatory evidence, this approach may prove to be an improved, cost-effective, and potentially useful tool for the clinical examination of patients with IMH following myocardial reperfusion injury.

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A declares that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the institutional review board, and the requirement for patient consent was waived. An online provision, on the hospital homepage, was prospectively made available to the patients for opting out of the study.

**Conflict of interest**

The other authors have no conflict of interest to disclose.

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**Legends for Figures and Table**

**Fig. 1**

Flowchart of the study protocol. Description from acquired images to various segmentation methods.

**Fig. 2**

Receiver operating characteristic (ROC) curve of CK and CK-MB for detecting patient with IMH. Both of area under the curve (AUC) of the biomarkers show high sensitivity and specificity. The cutoff values of CK and CK-MB are 2460 IU/L and 231 IU/L, respectively.

**Fig. 3**

Assessment of IMH volume using different quantification methods in acute myocardial infarction (AMI) patient with IMH.

Short-axis 3D T1-MRI images in patients with AMI of segment 6. IMH size shows that 1SD is larger than manual method and Otsu is considerably larger than manual method. Whereas, IMH size of 3SD, 4SD and FWHM show smaller values than that obtained from manual method. IMH size of calculated by 2SD is congruent to the values obtained by manual method.

**Table 1**

Patient Characteristics.

Values are mean ± standard deviation or median (IQR). Characteristics of all 33 patients are shown.

IMH = intramyocardial hemorrhage; STEMI = ST elevation myocardial infarction; CK = creatine kinase;

CK-MB = creatine kinase MB; IQR = interquartile range; TIMI = thrombolysis in myocardial infarction; PCI = percutaneous coronary intervention; EDV = end-diastolic volume; ESV = end-systolic volume; EF = ejection fraction.

**Table 2**

Spearman rank-correlation coefficient, Bland-Altman analysis and ICC of different quantification methods compared to the manual method for IMH measuring.

The myocardial weight is expressed in grams (g), computed slice-by-slice.

IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.

**Table 3**

Bland-Altman analysis and ICC for intra- and inter-observer variability of different quantification methods for IMH measurement.

The myocardial weight is expressed in milligrams (mg), computed slice-by-slice.

IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.

**Title**

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**Hideo Arai, RT**

Fukuokaken Saiseikai Futsukaichi Hospital

**Masateru Kawakubo, PhD**

Department of Health Sciences, Faculty of Medical Sciences, Kyushu University

**Kou Abe, M.D.**

Fukuokaken Saiseikai Futsukaichi Hospital

**Hikaru Hatashima, M.D.**

Oita Prefectural Hospital

**Kenichi Sanui, RT**

Fukuokaken Saiseikai Futsukaichi Hospital

**Hiroshi Nishimura, MD, PhD**

Fukuokaken Saiseikai Futsukaichi Hospital

**Toshiaki Kadokami, MD, PhD**

Fukuokaken Saiseikai Futsukaichi Hospital

**A concise and informative titles**

Quantification of intramyocardial hemorrhage volume using magnetic resonance imaging

**Corresponding author**

Masateru Kawakubo

Department of Health Sciences, Faculty of Medical Sciences, Kyushu University

3-1-1 Maidashi Higashi-ku Fukuoka 812-8582 Japan

Tel: +81-92-642-6728

Fax: +81-92-642-6728

E-mail: k-mstr@hs.med.kyushu-u.ac.jp

**Abstract (243/250)**

**Purpose**

Although intramyocardial hemorrhage (IMH) is a poor prognostic factor caused by ischemia reperfusion injury, little evidence is available regarding the association between IMH volume and biomarkers. In the present study, we measured IMH volume using three-dimensional (3D) T1-weighted magnetic resonance imaging (T1-MRI) and investigated its association with biomarkers. Moreover, the accuracy of semi-automatic measurement of IMH volume was validated.

**Methods**

We retrospectively enrolled 33 consecutive patients (mean age, 67 ± 11 years ) who underwent cardiac MRI after reperfusion therapy for acute myocardial infarction. IMH was observed in 4 patients (12.1 %). Receiver operating characteristics (ROC) analysis of creatine kinase (CK) and CK-muscle/brain (CK-MB) tests for detecting IMH were performed. IMH volume measured using semi-automatic methods by a 2 standard deviation (SD) threshold was compared to manual measurements using the Spearman's correlation coefficient ($\rho$) and Bland-Altman analyses.

**Results**

ROC analysis revealed optimal cutoff values of CK: 2460 IU/L and CK-MB: 231 IU/L (area under the curve: 0.95 and 0.91; sensitivity: 86 % and 79 %; specificity: 100 % for both). IMH volume with the 2SD threshold correlated with that of the manual measurement (5.84 g [3.30 to 9.00] g vs. 8.07 g [5.37 to

9.33]; $\rho$: 0.85, $p < 0.01$; bias [limit of agreement]: -0.01 g [-0.51 to 0.49]; intraclass correlation coefficients (0.84 [0.75 to 0.90]).

**Conclusions**

Our findings could help identify the risk of IMH after reperfusion therapy with biomarkers. 3D T1-MRI can semi-automatically provide accurate IMH volume without being time-consuming.

**Keywords**

Intramyocardial hemorrhage; biomarker; quantification; semi-automated threshold method; magnetic resonance imaging; acute myocardial infarction

## Introduction

Advances in the treatment of acute coronary syndrome (ACS), such as percutaneous coronary intervention (PCI) and thrombus aspiration therapy have allowed early reperfusion in ACS cases leading to an improvement in the survival rate [1]. However, severe myocardial damage after reperfusion therapy, termed as ischemia-reperfusion injury, has received considerable attention in the recent years [2–4]. Ischemia-reperfusion injury is a severe disease damaging the myocardium due to reperfusion [5]. A result of severe ischemia-reperfusion injury is intramyocardial hemorrhage (IMH), which occurs in some cases. Several studies have reported that IMH is associated with the extent of infarct, presence of microvascular obstruction (MVO), increased left ventricular volumes, decreased ejection fraction (EF), the lack of long-term improvement at follow-up, and increasing major adverse cardiac events (MACE) [6–8]. To the best of our knowledge, biomarkers like creatine kinase (CK), CK-Muscle/Brain (CK-MB), and cardiac troponins T and I are useful as indicators of myocardial injury, although no clinical studies have investigated the association between these biomarkers and IMH [9].

Cardiac magnetic resonance (CMR) is the only method that enables accurate imaging of IMH. Most studies pertaining to the imaging of IMH utilized two-dimensional (2D) T2-weighted or T2 star (T2*)-weighted magnetic resonance imaging (MRI) [10, 11]. However, 2D-MRI is limited to the measurement of IMH volume because the hemorrhages occurring outside of the imaging plane are mostly overlooked. Conversely, a previous study reported that the visualization of IMH by three-dimensional

(3D) T1-weighted MRI (T1-MRI), as compared to T2- and T2*-weighted MRI, near-accurately resembled the IMH region corroborating with the pathological findings [12]. Therefore, 3D T1-MRI has the potential for quantifying IMH and image acquisition that covers the entire left ventricle. To measure IMH volume with 3D T1-MRI, manual delineation of IMH region images at multiple planes is needed. Manual analysis is time-consuming in clinical routine, and moreover, variations arising from differences in the observer experience may cause erroneous measurements. Late gadolinium enhancement (LGE) CMR can image myocardial scars with high contrast, and the size of LGE on the image as the major predictor of morbidity and mortality, provides clinical usefulness in myocardial infarction patients [13, 14]. Various image processing protocols for semi-automatic measurements of LGE size have been proposed, such as the standard deviation (SD) based thresholding method, full width half maximum (FWHM) method, and the Otsu method [15–17]. As discussed so far, the purpose of this study was to clarify the association between biomarkers and IMH for ACS patients after reperfusion therapy using IMH imaging and to assess the accuracy of various methods for quantifying IMH in patients with IMH.

## Materials and Methods

### Study population

We retrospectively enrolled 33 consecutive patients who underwent reperfusion therapy for acute myocardial infarction (AMI) and underwent CMR with 3D T1-MRI between June 2015 and December 2017. The creatine kinase CK and CK-MB were calculated from the appropriate source (serum).

According to an earlier animal study (pigs), which compared the pathological analysis and various CMR contrast studies, including 3D T1-MRI, the high signal intensity of the 3D T1-MRI was visually defined as IMH by consensual decisions made by both, the cardiologist and radiologist [12]. The study was approved by the institutional review board, and the requirement for patient consent was waived. An online provision, on the hospital homepage, was prospectively made available to the patients for opting out of the study. The study was conducted in accordance with the Declaration of Helsinki.

**MR imaging protocol**

CMR imaging was performed using a 1.5 T MRI system (Ingenia, Philips Healthcare, Best, The Netherlands), equipped with 33-mT/m maximum gradient strength, 120-T/ms slew rate, and a 32-channel phased-array receiver coil. An ECG-gated steady-state free precession cine imaging was acquired in four-chamber view with 100 reconstructed phases per heartbeat to detect the static periods of the cardiac cycle. The cine sequence parameters were as following: TR/TE, 2.8 ms/1.38 ms; flip angle, 60°; slice thickness, 7 mm; field of view, 350 × 350 $mm^2$; in-plain resolution, 1.09 × 1.09 $mm^2$; acquisition matrix, 192 × 185, and SENSE (sensitivity encoding) factor, 2.5. The 3D T1-MR images were acquired in the transverse axial plane with diaphragm- and cardiac-triggering. The delay time for inversion recovery pulse for blood signal suppression was configured to 500 ms. The 3D T1-MRI parameters were as follows: TR/TE, 4.6 ms/2.1 ms; flip angle, 10°; slice thickness, 1.0 mm; field of view, 200 × 200 $mm^2$; in-plain resolution, 2.0 × 2.0 $mm^2$; acquisition matrix, 100 × 99; SENSE factor, 1, and phase direction, R-L. The data acquisition

periods during the static period was determined by visuomotor analysis of the cine images.

**Post process of IMH imaging using 3D T1-MRI**

No patients were excluded from the study due to unacceptable image quality due to magnetic-inhomogeneity and motion-artifact. Figure 1 shows the flowchart of the post processing of IMH imaging with 3D T1-MRI. The transverse axial images were reconstructed into short-axis images with 5 mm thickness as described in a previous study [12]. The reconstructed images in the patients with IMH were processed as follows. Firstly, the left myocardium regions from all short-axis images were manually extracted using SYNAPSE VINCENT software (Fujifilm Co, Tokyo, Japan). Secondly, IMH volumes were measured using a manual method, and 6 different methods were applied to the thresholds determined from various image processing protocols. IMH measurements using the manual method was performed using the SYNAPSE VINCENT software. The analyst manually delineated the myocardial high-intensity area, introducing corrections, while varying the contrast windows. All threshold-based measurements for image processing were performed using an off-line software based on MATLAB (The MathWorks, Inc, U.S.). The threshold value of signal intensity was defined similarly to previous LGE studies [15–17]. The area of IMH was defined as the pixels having signal intensity higher than the threshold value. The threshold value, using the Otsu method, was automatically calculated based on the value estimated from the histogram of cumulative myocardial signal intensities [18]. For determining the SD- and FWHM-based threshold, the largest region of interest (ROI) of the normal myocardium was

manually from amongst all myocardial images. Mean signal intensity in the ROI (Mean) and its SD were calculated. The threshold of SD-based method was calculated from the following equation:

$$Threshold\ of\ SDs = Mean + k \times SD\ (with\ k = 1\ to\ 4)$$

The threshold of FWHM was calculated from the following equation using the mean and the maximum signal intensity of all left myocardial ROIs.

$$Threshold\ of\ FWHM = (Max - Mean)/2$$

IMH volume ($mm^3$) of the entire left myocardium was calculated as the summation of IMH volumes from all short-axis slices. The volume was converted to weight (mg) by multiplying the specific gravity of myocardium (1.05 $g/cm^3$) as well as the previous calculation of the size of LGE. Manual processing of all data in this study was performed by an analyst with 10 years of clinical experience in cardiac radiology.

**Statistical analyses**

The Shapiro-Wilk test was employed to assess the normality of data distribution. Due to non-normal data distribution, descriptive statistics are provided as the median and the corresponding interquartile ranges (IQR). The receiver operating characteristics (ROC) analysis was performed to validate the accuracy for detection of IMH with biomarkers (CK and CK-MB). The correlations between IMH volumes from the various threshold methods and a manual method were analyzed using the Spearman's rank-correlation coefficient ($\rho$). The accuracy of various threshold methods with respect to the manual method was

evaluated using Bland-Altman analyses and intraclass correlation coefficient (ICC) with one-way random single measures. ICCs were defined as excellent (ICC ≥ 0.75), good (ICC = 0.60–0.74), moderate (ICC = 0.40–0.59), and poor (ICC ≤ 0.39). All statistical analyses were performed using the software package R version 3.4.1 [19]. A P value less than 0.05 was considered significant.

**Intra- and inter-observer variability**

To evaluate intra- and inter-observer variability of manual myocardial segmentation, measurements based on manual and various threshold methods (manual, 1SD to 4SD, and FWHM) were repeated at least 1 month later in 20 randomly selected slices by the same primary analyst and an additional analyst, both with more than 5 years of clinical experience in cardiac radiology. The analysts were blinded to the results of the initial study to ensure variability of IMH volume measurements. The variability was evaluated using Bland-Altman analyses and ICC with one-way random single measures or two-way random single measures (ICC(1,1) or ICC(2,1)). ICCs were defined as excellent (ICC ≥ 0.75), good (ICC = 0.60–0.74), moderate (ICC = 0.40–0.59), and poor (ICC ≤ 0.39).

**Results**

In the present retrospective study, 33 AMI patients, who underwent reperfusion therapy, were enrolled. The CK and CK-MB were increased in all patients with AMI. The CMR examination was performed 5.4 ± 3.2 days after hospitalization for AMI, resulting in the inclusion of 4 patients with IMH in this study. The detailed clinical characteristics of all 33 patients are shown in Table 1. IMH was observed in 39 of

the 371 slices in the 4 patients.

IMH weight of 4 patients was 8.07 g (5.37 to 9.33), whereas, the myocardial weight was 123 g (110 to 137). The CK and CK-MB biomarkers were significantly higher in patients with IMH, than in patients without IMH, although the other parameters showed no significant differences (Table 1). The ROC analysis was used to describe the optimal cutoff values of CK and CK-MB in order to identify patients with IMH (Fig. 2). The area under the curves (AUC) of the CK and CK-MB were 0.95 (95% CI: 0.87-1.00) and 0.91 (95% CI: 0.79-1.00), respectively. The optimal cutoff value of CK levels at 2460 IU/L showed 86% sensitivity and 100% specificity. That of CK-MB levels at 231 IU/L showed 79% sensitivity and 100% specificity. The results of Spearman's rank-correlation coefficient analysis, Bland-Altman analysis, and ICC, comparing the measurements from various threshold methods with the manual method, are shown in Table 2. The measurements of 1SD, 2SD, 3SD, 4SD, and FWHM showed significant correlations with the manual method; However, no significant correlation was observed between the measurements obtained using the Otsu and the manual method. The correlations of 2SD, 3SD, and 4SD measurements were statistically stronger than those of 1SD and FWHM ($P$ <0.01). Moreover, the correlation of 3SD was statistically the strongest among all comparisons ($P$ <0.01). The respective ICCs obtained for 2SD, 3SD and 4SD were excellent, good, and moderate. We show the results of the measurements of IMH in Figure 3 for a representative case (Case 4: a 54-year-old male).

The manual segmentation of left myocardial weight showed excellent intra- and inter-observer

variability. The bias (limit of agreement) [g] with Bland-Altman analysis for intra- and inter-observer variability were -0.4 (-2.0 to 1.3), and -0.6 (-2.0 to 0.8), respectively, and the standard deviation of the difference [g] were 0.8 and 0.7, respectively. Both ICCs showed excellent variability as highlighted by their respective values of 0.91 and 0.91. The intra- and inter-observer variability for all manual and semi-automatic measurements are shown in Table 3. Excellent intra- and inter-observer variability was observed. Bland-Altman analysis for all methods except 1SD and Otsu indicated small bias, standard deviation of the difference, and a narrow limit of agreement. All ICCs were observed as excellent variability.

**Discussion**

IMH was observed in 4 out of 24 AMI patients in this study. According to a previous report, the mean prevalence of IMH was 35% (95% CI: 31% to 38%) in reperfused AMI patients [7]. In our study, the mean prevalence of IMH was observed to be lower than that reported previously. This may be attributed to the early interventional reperfusion therapy due to technical innovation in later years and the lower prevalence of IMH in our study due to the availability of high-quality medical systems [1, 20]. The high-quality medical systems may also help explain the lower mean values of CK and CK-MB in this study when compared with previous studies.

The present study was aimed at clarifying association between biomarkers and IMH and to identify the most appropriate image processing protocol for quantifying IMH volume. Excellent variability

was observed using manual segmentation of the left myocardium. Therefore, it can be concluded that the dataset prepared before the comparison of various image processing methods is reliable. The mean values of CK and CK-MB in this study were lower than those in previous studies; however, the mean values of those in patients with IMH were considerably higher than the values of previous studies [21, 22]. This result indicated that the IMH was caused by severe myocardial injury [7]. Based on these findings, the severe AMI patients with high values of biomarkers is high likely to have the IMH leading to adverse event. For this reason, if the AMI patients have over our cutoff value of the biomarkers, it is necessary to determine the severity by presence or absence of IMH using CMR. It has been reported that severe myocardial damage due to AMI may cause MVO, and the presence or absence MVO, which is detected with CMR, is an importance sign of reperfusion status after reperfusion therapy [23]. Recent reports suggested that accurate detection of MVO was independently associated with adverse ventricular remodeling and patient prognosis [24]. IMH, which is caused by severe reperfusion injury, only exists in the presence of MVO [8]. IMH is a structural and functional disorder of the microcirculation, similar to MVO. Consequently, the myocardial damage caused by IMH is more severe, than that caused by MVO. Some reports have mentioned that presence of IMH and residual IMH in the chronic phase were associated with left ventricular remodeling and persistently elevated T2 values in the surrounding infarct tissue [25]. Knowledge of the presence and severity of IMH may potentially increase the probability of myocardial damage severity assessment. As is evident from the above, our study may provide significant aid in clinical practice.

In the correlation analysis, of all methods, the 3SD correlated the strongest with manual measurements, whereas, the ICC of 2SD was deemed excellent. The variability of IMH measurements were found to be excellent in all methods. According to these results, 2SD or 3SD is the most appropriate image processing method for measuring IMH volume. Additional statistical analysis found the ICC of 2SD method to be excellent with a high correlation coefficient value ($\rho$ = 0.85). Consequently, we concluded that the 2SD method is the most appropriate for quantifying IMH volume with 3D T1-MRI. The volume of IMH calculated with 2SD methods was in agreement to that obtained with manual measurements. The usefulness of Otsu method for measuring LGE size with appreciable accuracy was previously reported [15]. However, our results were in contrast to the previous observations. This discrepancy may be attributed to the mismatch caused by the differences of signal intensities of LGE and IMH. The high signal intensity of LGE represents a significant shortening of the T1 value (425.4±68.1 ms) due to the distribution of the contrast medium in the injured myocardium [26]. On the contrary, the image signal of IMH with 3D T1-MRI is lower reflecting the relatively smaller shortening of T1 values compared with the contrast medium. Moreover, IMH includes components with varying densities of paramagnetic origin i.e. the breakdown products of oxygenated hemoglobin (deoxyhemoglobin, methemoglobin) or blood degradation product (ferritin and hemosiderin) [8], and IMH expresses varied signal intensity in T1-MRI with temporal changes [27]. The Otsu method is superior in automatically detecting a high signal such as that from the contrast medium in LGE. However, for detecting lower intensity signals such as IMH, despite the addition of a

manual process, determination of the threshold with the delineation of normal myocardial region is necessary.

The limitation of our study should be acknowledged. The lack of T2*W imaging, which is a standard CMR technique for detecting IMH is a big limitation in this study, and the lack of association between 3D T1-MRI and T2*W, and other contrast-enhanced CMR imaging studies is also a limitation. Further studies are required to investigate the association between high-signal intensity on 3D T1-MRI and CMR imaging findings. The second limitation is a single-center study with limited patient population. The other limitation is that the lack of validation methods for assessing the accuracy of the quantified IMH volumes, since physically weighing IMH density and measuring IMH volume from cardiac tissue is impossible. However, this study revealed that 3D T1-MRI can detect IMH in humans as a high-signal intensity. 3D T1-MRI with full cardiac coverage overcomes the issue of restricted coverage faced by the established 2D imaging for IMH. The detection and location of IMH using 3D T1-MRI aids in selecting the appropriate slice, in order to further assess IMH using various CMR sequences in the future. Moreover, this study revealed that IMH volume can be accurately measured with minimal manual labor. Therefore, our future studies shall be aimed at investigating the clinical feasibility of IMH volume as a diagnostic biomarker for AMI.

**Conclusion**

It is necessary for the AMI patients with higher cutoff values of CK and CK-MB to perform CMR to check

for myocardial bleeding because of the high risk of IMH. With respect to quantifying IMH, the threshold determined by 2SD method is most appropriate for processing images to quantify the IMH volume in the compiled left myocardium of 3D T1-MR images. On obtaining further confirmatory evidence, this approach may prove to be an improved, cost-effective, and potentially useful tool for the clinical examination of patients with IMH following myocardial reperfusion injury.

**Acknowledgement**

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A declares that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the institutional review board, and the requirement for patient consent was waived. An online provision, on the hospital homepage, was prospectively made available to the patients for opting out of the study.

**Conflict of interest**

The other authors have no conflict of interest to disclose.

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**Legends for Figures and Table**

**Fig. 1**

Flowchart of the study protocol. Description from acquired images to various segmentation methods.

**Fig. 2**

Receiver operating characteristic (ROC) curve of CK and CK-MB for detecting patient with IMH. Both of area under the curve (AUC) of the biomarkers show high sensitivity and specificity. The cutoff values of CK and CK-MB are 2460 IU/L and 231 IU/L, respectively.

**Fig. 3**

Assessment of IMH volume using different quantification methods in acute myocardial infarction (AMI) patient with IMH.

Short-axis 3D T1-MRI images in patients with AMI of segment 6. IMH size shows that 1SD is larger than manual method and Otsu is considerably larger than manual method. Whereas, IMH size of 3SD, 4SD and FWHM show smaller values than that obtained from manual method. IMH size of calculated by 2SD is congruent to the values obtained by manual method.

**Table 1**

Patient Characteristics.

Values are mean ± standard deviation or median (IQR). Characteristics of all 33 patients are shown.

IMH = intramyocardial hemorrhage; STEMI = ST elevation myocardial infarction; CK = creatine kinase;

CK-MB = creatine kinase MB; IQR = interquartile range; TIMI = thrombolysis in myocardial infarction; PCI = percutaneous coronary intervention; EDV = end-diastolic volume; ESV = end-systolic volume; EF = ejection fraction.

**Table 2**

Spearman rank-correlation coefficient, Bland-Altman analysis and ICC of different quantification methods compared to the manual method for IMH measuring.

The myocardial weight is expressed in grams (g), computed slice-by-slice.

IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.

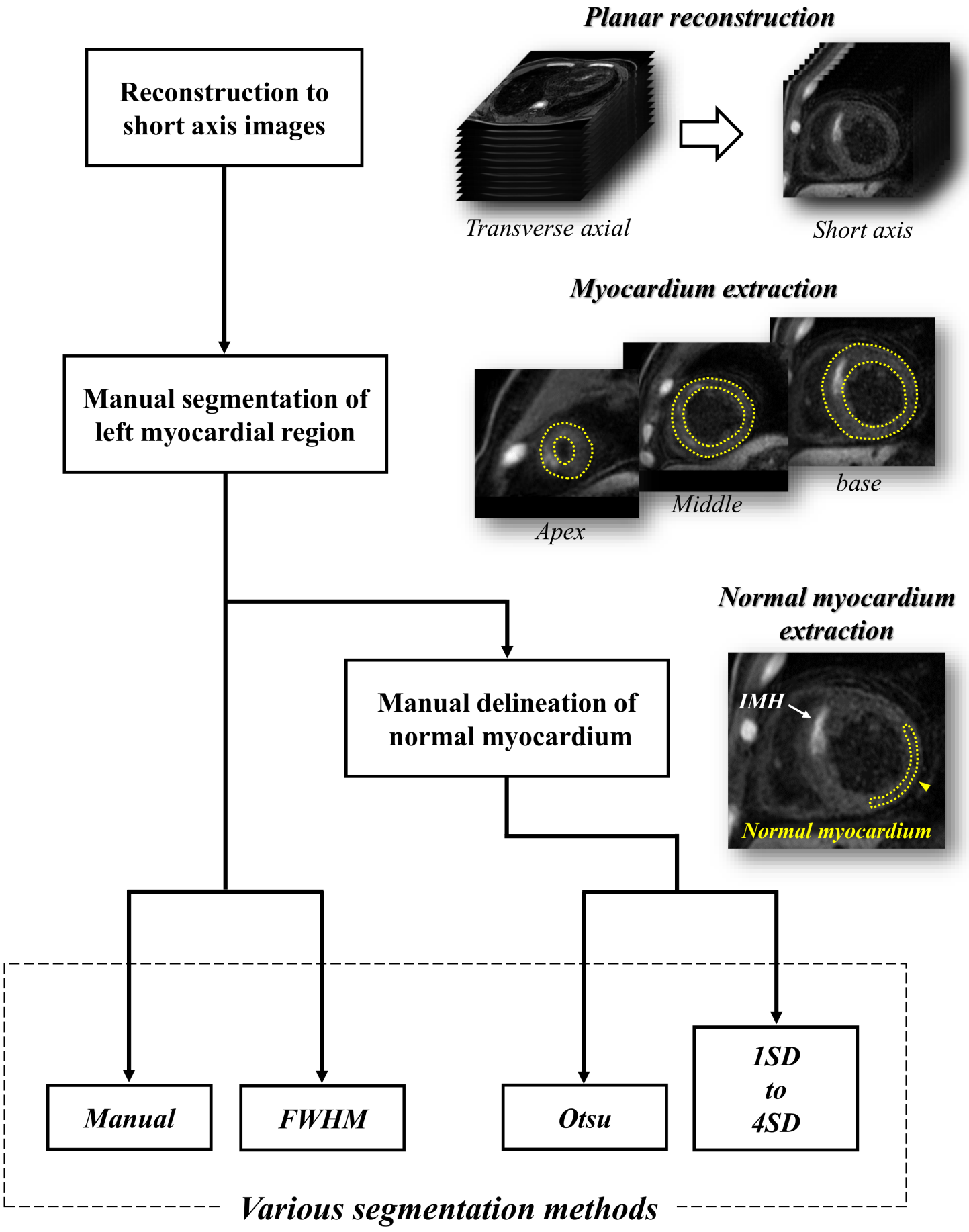
**Table 3**

Bland-Altman analysis and ICC for intra- and inter-observer variability of different quantification methods for IMH measurement.

The myocardial weight is expressed in milligrams (mg), computed slice-by-slice.

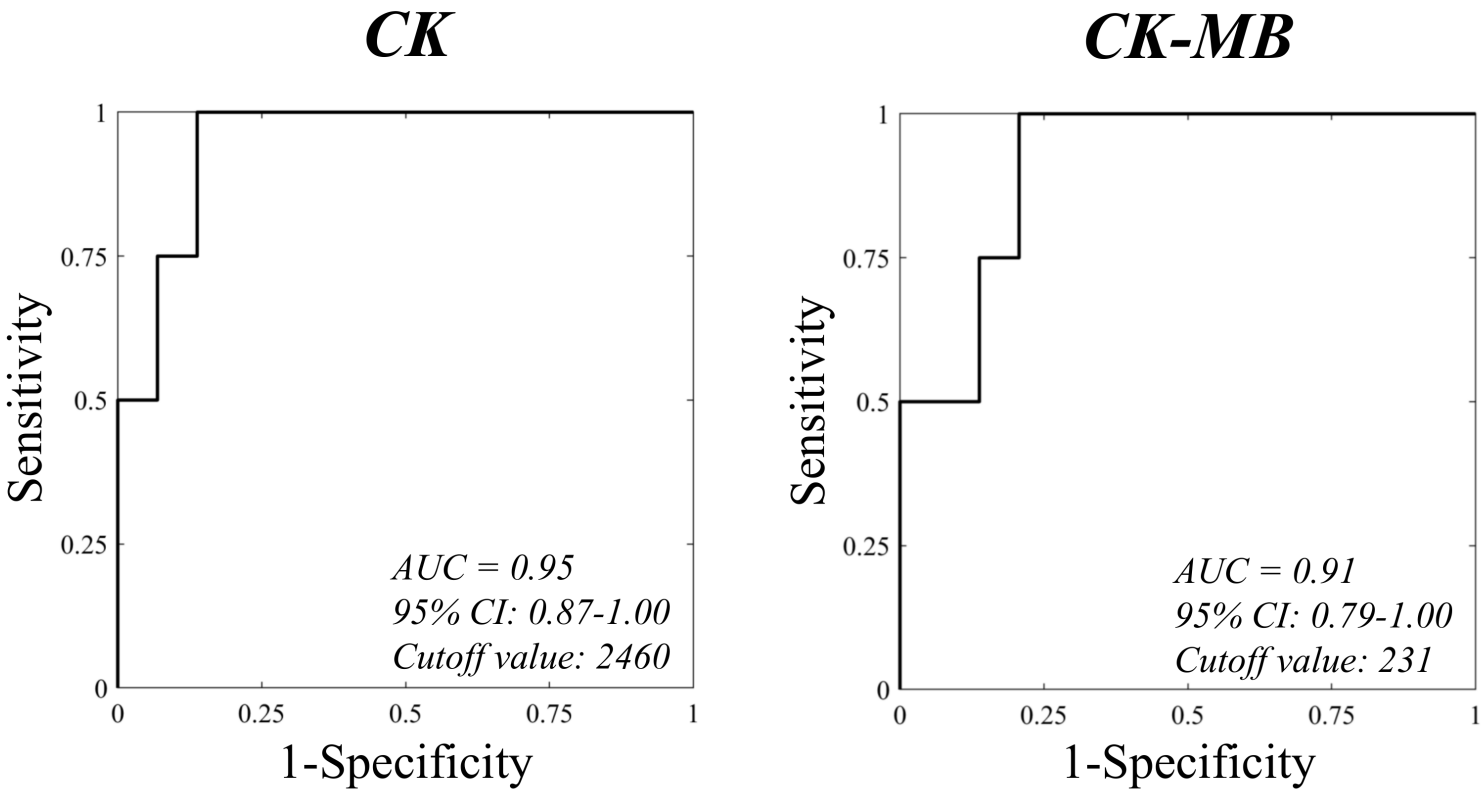
IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.

# Fig. 1




Flowchart of the study protocol. Description from acquired images to various segmentation methods.

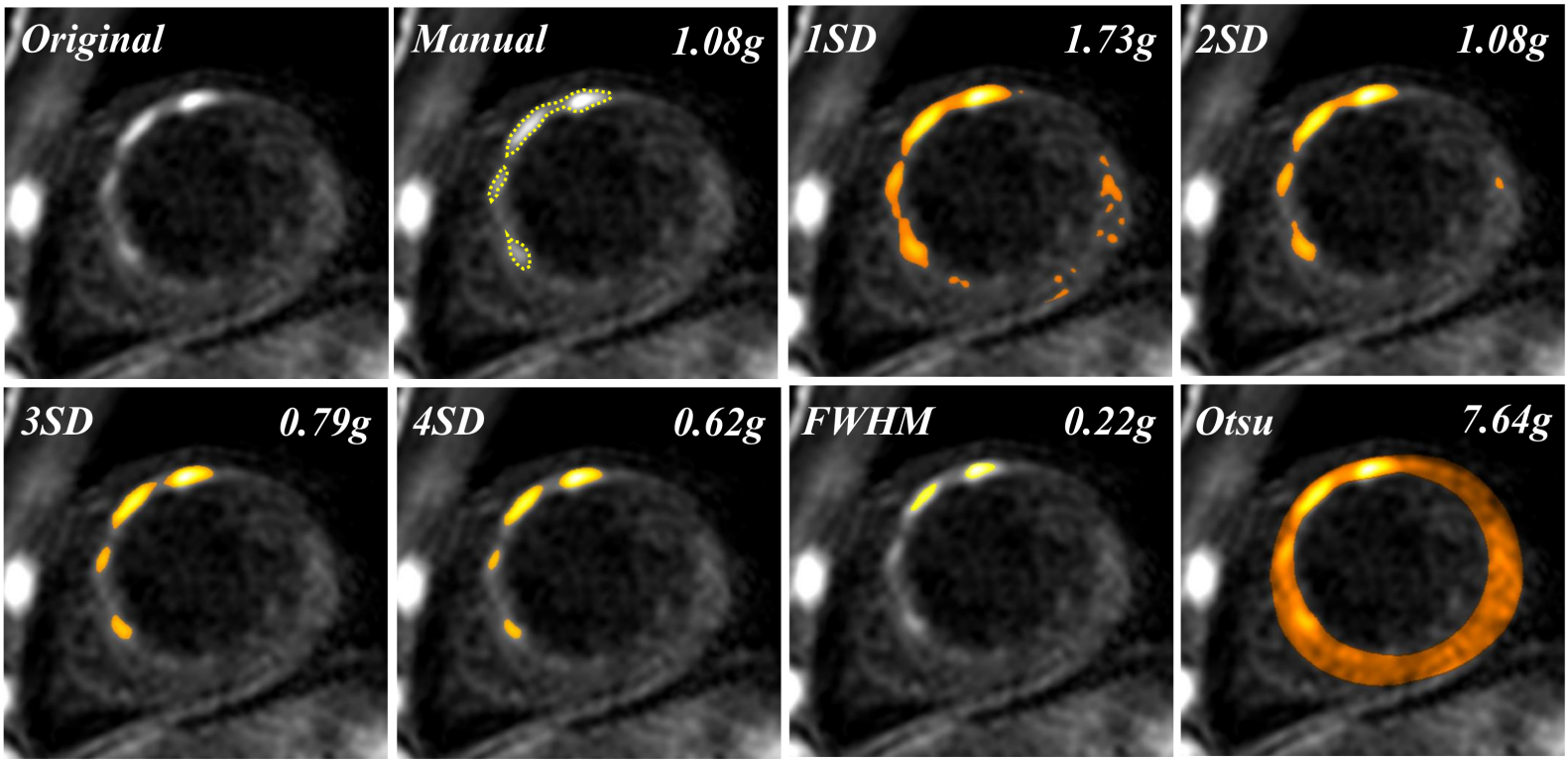
**Fig. 2**




Receiver operating characteristic (ROC) curve of CK and CK-MB for detecting patient with IMH. Both of area under the curve (AUC) of the biomarkers show high sensitivity and specificity. The cutoff values of CK and CK-MB are 2460 IU/L and 231 IU/L, respectively.

# Fig. 3

Assessment of IMH volume using different quantification methods in acute myocardial infarction (AMI) patient with IMH.




Short-axis 3D T1-MRI images in patients with AMI of segment 6. IMH size shows that 1SD is larger than manual method and Otsu is considerably larger than manual method. Whereas, IMH size of 3SD, 4SD and FWHM show smaller values than that obtained from manual method. IMH size of calculated by 2SD is congruent to the values obtained by manual method.

**Table 1**

Patient Characteristics.

| | All (n = 33) | Without IMH (n = 29) | With IMH (n = 4) | P value |
|---|---|---|---|---|
| **Clinical data** | | | | |
| STEMI, n (%) | 27 (82) | 24 (83) | 3 (75) | 1.00 |
| Age (years) | 67 ± 11 | 67 ± 11 | 65 ± 11 | 0.47 |
| Male, n (%) | 27 (82) | 23 (79) | 4 (100) | 1.00 |
| Body mass index (kg/m$^2$) | 23.6 ± 3.9 | 23.8 ± 4.1 | 23.1 ± 2.1 | 0.44 |
| Systolic blood pressure (mmHg) | 143.4 ± 28.2 | 143.2 ± 28.4 | 144.3 ± 30.7 | 0.87 |
| Diastolic blood pressure (mmHg) | 82.7 ± 21.7 | 81.6 ± 22.3 | 90.5 ± 17.0 | 0.19 |
| Systemic hypertension, n (%) | 25 (76) | 21 (72) | 4 (100) | 0.55 |
| Hyperlipidemia, n (%) | 16 (48) | 14 (48) | 2 (50) | 1.00 |
| Diabetes mellitus, n (%) | 11 (33) | 11 (38) | 0 (0) | 0.28 |
| Current smoker, n (%) | 9 (27) | 7 (24) | 2 (50) | 0.30 |
| **Biochemical data** | | | | |
| Peak CK (IU/L) | 2487 ± 3051 | 1713 ± 1640 | 8093 ± 5146 | <0.01 |
| Peak CK-MB (IU/L) | 187 ± 161 | 152 ± 125 | 438 ± 185 | <0.01 |
| **Angiographic data** | | | | |
| Culprit lesion, n (%) | | | | |
| RCA | 15 (45) | 15 (52) | 0 (0) | 0.11 |
| LAD | 14 (42) | 11 (38) | 3 (75) | 0.29 |
| LCX | 4 (12) | 3 (10) | 1 (25) | 0.42 |
| Median (IQR) minutes from OTB | 247 (131-1335) | 247 (131-1440) | 226 (137-564) | 0.72 |
| TIMI flow grade 0/1 pre-PCI, n (%) | 21 (64) | 18 (62) | 3 (75) | 1.00 |
| TIMI flow grade 3 post PCI, n (%) | 31 (94) | 27 (93) | 4 (100) | 1.00 |
| **Echocardiographic data** | | | | |
| EDV (ml) | 84.0 ± 24.1 | 82.4 ± 23.5 | 95.9 ± 28.6 | 0.39 |
| ESV (ml) | 38.1 ± 15.3 | 37.5 ± 14.5 | 42.1 ± 23.0 | 0.93 |

| | | | | |
|---|---|---|---|---|
| EF (%) | 56.9 ± 10.4 | 57.0 ± 10.8 | 56.1 ± 8.3 | 0.98 |

Values are mean ± standard deviation or median (IQR). Characteristics of all 33 patients are shown.
IMH = intramyocardial hemorrhage; STEMI = ST elevation myocardial infarction; CK = creatine kinase; CK-MB = creatine kinase MB; IQR = interquartile range; TIMI = thrombolysis in myocardial infarction; PCI = percutaneous coronary intervention; EDV = end-diastolic volume; ESV = end-systolic volume; EF = ejection fraction.

**Table 2**

Spearman rank-correlation coefficient, Bland-Altman analysis and ICC of different quantification methods compared to the manual method for IMH measuring.

| | | Correlation analysis | | Bland-Altman analysis | | |
|---|---|---|---|---|---|---|
| Semi-automated method | IMH volume (g) vs. Manual measurements (8.07 g (5.37 to 9.33)) | ρ | P value | Bias (LOA) | SDD | ICC (95% CI) |
| 1SD | 19.96 (14.79 to 26.68) | 0.63 | <0.0001 | 0.90 (-0.62 to 2.42) | 0.78 | 0.28 (0 – 0.56) |
| 2SD | 5.84 (3.30 to 9.00) | 0.85 | <0.0001 | -0.01 (-0.51 to 0.49) | 0.26 | 0.84 (0.75 – 0.90) |
| 3SD | 3.00 (1.19 to 5.07) | 0.91 | <0.0001 | -0.21 (-0.71 to 0.30) | 0.26 | 0.67 (0.24 – 0.84) |
| 4SD | 1.90 (0.65 to 3.20) | 0.84 | <0.0001 | -0.28 (-0.89 to 0.32) | 0.31 | 0.46 (0.02 – 0.71) |
| FWHM | 0.46 (0.33 to 0.67) | 0.54 | <0.0001 | -0.37 (-1.17 to 0.44) | 0.41 | 0.11 (0 – 0.31) |
| Otsu | 122.57 (109.97 to 136.55) | 0.30 | 0.01 | 7.11 (2.82 to 11.40) | 2.19 | 0.01 (0 – 0.06) |

The myocardial weight is expressed in grams (g), computed slice-by-slice.

IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.

**Table 3**

Bland-Altman analysis and ICC for intra- and inter-observer variability of different quantification methods for IMH measurement.

| Method | Intra-observer | | | Inter-observer | | |
|---|---|---|---|---|---|---|
| | Bias (LOA) | SDD | ICC (95% CI) | Bias (LOA) | SDD | ICC (95% CI) |
| Manual | -11 (-189 to 167) | 91 | 0.97 (0.93 – 0.99) | 50 (-187 to 288) | 121 | 0.95 (0.87 – 0.98) |
| 1SD | -113 (-698 to 472) | 299 | 0.92 (0.81 – 0.97) | -204 (-615 to 207) | 210 | 0.96 (0.71 – 0.99) |
| 2SD | -22 (-118 to 72) | 48 | 0.99 (0.98 – 1.00) | -52 (-180 to 76) | 65 | 0.98 (0.91 – 0.99) |
| 3SD | -4 (-15 to 7) | 6 | 1.00 (1.00 – 1.00) | -18 (-81 to 45) | 32 | 0.99 (0.97 – 1.00) |
| 4SD | -0.8 (-14 to 12) | 6 | 1.00 (1.00 – 1.00) | -10 (-40 to 21) | 15 | 0.99 (0.97 – 1.00) |
| FWHM | -0.5 (-4 to 3) | 2 | 0.99 (0.97 – 1.00) | -0.9 (-4 to 3) | 2 | 1.00 (0.99 – 1.00) |
| Otsu | -615 (-2339 to 1109) | 880 | 0.91 (0.79 – 0.96) | -522 (-1563 to 519) | 531 | 0.96 (0.70 – 0.99) |

The myocardial weight is expressed in milligrams (mg), computed slice-by-slice.

IMH = intramyocardial hemorrhage; FWHM = full width at half maximum; LOA = limits of agreement; SSD = standard deviation of difference; ICC = intraclass correlation coefficients; CI = confidence interval.