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ARTICLE

Modeling of the Weight Status and Risk of Nonalcoholic Fatty Liver Disease in Elderly Individuals: The Potential Impact of the Disulfide Bond-Forming Oxidoreductase A-Like Protein (*DsbA-L*) Polymorphism on the Weight Status

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Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity. Disulfide bond-forming oxidoreductase A-like protein (DsbA-L) is known to be a key molecule in protection against obesity and obesity-induced inflammation. In the present study, we used a modeling and simulation approach in an attempt to develop body mass index (BMI) and BMI-based NAFLD prediction models incorporating the *DsbA-L* polymorphism to predict the BMI and NAFLD in 341 elderly subjects. A nonlinear mixed-effect model best represented the sigmoidal relationship between the BMI and the logit function of the probability of NAFLD prevalence. The final models for BMI and NAFLD showed that *DsbA-L* rs1917760 polymorphism, age, and gender were associated with the BMI, whereas gender, patatin-like phospholipase 3 rs738409 polymorphism, HbA1c, and high-density and low-density lipoprotein cholesterol levels were associated with the risk of NAFLD. This information may aid in the genetic-based prevention of obesity and NAFLD in the general elderly population.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Although obesity is closely associated with the development and progression of NAFLD, at present, no models incorporating detailed population background information can adequately predict the association between the weight status and NAFLD. Several experimental models showed that DsbA-L is known as a key molecule in protecting against obesity and obesity-induced inflammation and insulin resistance; however, it is totally unclear whether *DsbA-L* polymorphism is associated with the weight status or NAFLD in humans.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ This study addresses the how the weight status is associated with the risk of developing NAFLD, and how functional polymorphism of *DsbA-L* (rs1917760, -1308G>T) is potentially associated with the weight status and the risk of NAFLD in the elderly general population using a NONMEM approach and structural equation modeling.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ This study adds information regarding: (1) a population prediction model for predicting the risk of NAFLD, which can be shown as a sigmoidal maximum response using the BMI as an exposure variable; and (2) the clinical implications of the *DsbA-L* rs1917760 polymorphism, which is associated with the weight status, and which is possibly indirectly associated with the risk of NAFLD in the general elderly population.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS?
☑ The results of this study suggest that the DsbA-L may be an effective target for preventing or treating obesity, especially in *DsbA-L* T/T genotype carriers. In addition, the modeling and simulation procedure of this study may contribute to the further development of genetic-based prediction models for other metabolic diseases.

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome, which is an independent risk factor for type 2 diabetes, cardiovascular disease, and its related future events.[1–3] NAFLD is particularly common in elderly people, and elderly patients with NAFLD have a high risk of strongly aging-related metabolic

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complications (i.e., type 2 diabetes and cardiovascular disease).[1,4] Experimental and epidemiological evidence have shown that obesity is closely associated with the development and progression of NAFLD.[1–3] However, NAFLD can also be observed in subjects with a normal-weight (body mass index (BMI) <25 kg/m^2), especially in Asian populations.[1,5] We recently reported that patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409, a well-known polymorphism that predisposes carriers for a fatty liver,[6] was associated with a risk of developing NAFLD, even in individuals with a normal weight status.[7] Thus, the population background (e.g., genetic polymorphisms, lifestyle, age, gender, and underlying diseases) should be carefully considered when determining the association between the weight status and the risk of NAFLD; however, at present, no population models incorporating detailed population background information can predict this association with sufficient accuracy.

Adiponectin, an adipose tissue-specific hormone, has been reported to be associated with the protection against adipose tissue inflammation, insulin resistance, and mitochondrial dysfunction and to have an important role in the prevention of obesity and its related diseases.[8–10] Plasma adiponectin exists as a low-molecular-weight trimer, medium-molecular-weight hexamer, and high-molecular-weight (HMW) oligomer.[10] HMW oligomers of adiponectin are the major relevant forms for improving insulin sensitivity, anti-inflammatory and antidiabetic activities, and low levels of HMW oligomers represent an independent risk factor for several metabolic diseases.[10] Thus, adiponectin multimerization, which requires disulfide bond formation between two different trimers,[11,12] may play a crucial role in the prevention of metabolic disease.

Disulfide bond-forming oxidoreductase A-like protein (DsbA-L), a renamed protein from glutathione *S*-transferase kappa 1, is highly expressed in the endoplasmic reticulum and mitochondria and plays an important role in disulfide bond formation and antioxidant action.[13–15] DsbA-L is considered to be a key regulator of adiponectin multimerization in 3T3-L1 cells that are most commonly used as an adipocyte differentiation model.[14] In addition, it has been reported that the mRNA level of *DsbA-L* in adipose tissue correlated negatively with obesity in both mice and humans.[13,14] Chen *et al*.[16] showed that the liver-specific knock-out of *DsbA-L* in mice exacerbated high-fat diet-induced hepatosteatosis, and the overexpression of *DsbA-L* protected mice against hepatosteatosis and insulin resistance. Furthermore, the suppression of *DsbA-L* is associated with the impairment of the respiratory capacity in mitochondria and the elevation of cellular oxidative stress.[16] Recently, Bai *et al*.[17] revealed DsbA-L as a key molecule in protecting obesity-induced inflammation and insulin resistance by suppressing the cGMP-AMP (cGAMP) synthase (cGAS)-cGAMP-stimulator of interferon genes (STING) pathway, which mediates DNA sensing and signaling and is involved in the lipotoxic activation of Tank-binding protein kinase 1 and subsequent p62 phosphorylation in hepatocytes.[18,19] Thus, in humans, the *DsbA-L* polymorphisms may play a key role in the development and/or progression of obesity and obesity-induced liver diseases (e.g., NAFLD).

A common polymorphism in the *DsbA-L* gene at -1308 bp (rs1917760) can influence the DsbA-L function and/or expression.[20] Among Asians, the rs1917760 polymorphism has previously been described with an allele frequency of ~20%[20]; however, the polymorphism was not detected among European and African populations. Our recent cross-sectional study indicated that the *DsbA-L* rs1917760 polymorphism is associated with decreased levels of *DsbA-L* mRNA in peripheral blood mononuclear cells and an increased prevalence of being overweight among male Japanese patients with schizophrenia.[21] Given these findings, the *DsbA-L* polymorphism may play a critical role in the development of obesity and NAFLD; however, the clinical roles of the *DsbA-L* polymorphism in weight gain and the development of NAFLD, including its relationship to adiponectin, oxidative stress, and environmental factors, remain unknown.

Pharmacometrics is a relatively recently established science that provides quantitative models regarding the pharmacology through the mathematical modeling of clinical efficacy based on multivariable analyses.[22–25] A nonlinear mixed-effect model (NONMEM) is a pharmacometric procedure that is widely applied in pharmacokinetic (PK) and PK/pharmacodynamic (PD) analyses based on longitudinal data, which enables the examination of various base models and the effects of various cofactors, the utilization of all observation points, and the evaluation of validity based on simulation.[22–25] On the other hand, disease progression models are often integrated with PK/PD models using a NONMEM approach to quantify the influence of various factors on disease progression because of the ability to quantify several levels of variability, to address instability data, and to identify individual specific cofactors.[22,23] Thus, a NONMEM approach can be an effective tool for developing a disease prediction model using clinical information and to help clarify the precise effects of various factors on disease development and progression.

Structural equation modeling is a multivariable statistical method that involves the estimation of parameters for a system of simultaneous equations, including regression analyses, pathway analyses, factor analyses, simultaneous econometric equations, and latent growth curve models.[26] Structural equation modeling, which has been widely used in medical sciences, including clinical studies, enables the identification of direct or indirect relationships among predictors, mediators, and clinical outcomes.[26] It is a general and powerful approach that accounts for measurement error and causal pathways by estimating the parameters for a system of simultaneous equations.[27] Thus, structural equation modeling is another useful tool for modeling the associations between massive patient information and complex systems of multiple phenotypes.[27,28]

In the present study, we applied NONMEM using genetic and longitudinal clinical data to characterize the weight status and the risk of developing NAFLD in elderly Japanese subjects who participated in a health screening program. The primary objective of this study was to investigate the potential impact of the *DsbA-L* rs1917760 polymorphism on the weight status and the risk for NAFLD in the elderly general population using the prediction models of BMI and risk for NAFLD

constructed by a NONMEM program. In addition, we analyzed the association among the *DsbA-L* genotype, BMI, NAFLD, and their related covariates (e.g., adiponectin multimerization) using structural equation modeling.

MATERIALS AND METHODS
Subjects and study protocol
All subjects were Japanese participants in the elderly health screening program held by the Japanese Red Cross Kumamoto Health Care Center. A retrospective longitudinal analysis with a follow-up period of 5.5 ± 1.1 years was conducted among 341 subjects who did not have a habitual alcohol intake (consumption of >30 g/day of alcohol in men or >20 g/day in women) and/or were not hepatitis B or C virus-positive, in accordance with the previously reported practical guidelines for NAFLD.[29] The study complies with the Declaration of Helsinki, and was approved by the ethics committees of the Faculty of Life Sciences at Kumamoto University and the Japanese Red Cross Kumamoto Health Care Center. All the subjects provided their written informed consent prior to enrollment in the study. All analyses were performed in accordance with Ethical Guidelines for Epidemiological Research in Japan.

Measurements
Overweight and normal-weight statuses were defined as BMI ≥ 25 kg/m^2 and BMI <25 kg/m^2, respectively. Hepatic ultrasonography scanning was used to diagnose fatty liver disease (FLD). The FLD was diagnosed based on the following four criteria: (1) a diffuse hyperechoic echotexture (bright liver); (2) an increased echo texture in comparison to the kidneys; (3) vascular blurring; and (4) deep attenuation.[29]

Genotyping
Genomic DNA was extracted from whole blood using a DNA purification kit (FlexiGene DNA kit; QIAGEN, Hilden, Germany). *DsbA-L* rs1917760 (-1308G$>$T) and *PNPLA3* rs738409 (c.444C$>$G, encoding I148M) genotypes were determined using a real-time TaqMan allelic discrimination assay (Applied Biosystems, Waltham, MA) in accordance with the manufacturer's protocol (DsbA-L, assay no. C_11980950_10; PNPLA3, assay no. C_7241_10). To ensure the genotyping quality, we included DNA samples as internal controls, hidden samples of a known genotype, and negative controls (water).

Measurement of adiponectin
The levels of total and HMW adiponectin were measured using fasting serum samples that were collected at the end of the observation period. Concentrations of total and HMW adiponectin in fasting serum samples were determined by enzyme-linked immunosorbent assay (ELISA) kits with intra-assay coefficients of variation of 2.5–4.7% and 2.6–3.7%, respectively, and with interassay coefficients of variation of 5.8–6.9% and 8.3–8.6%, respectively (Human Total Adiponectin/Acrp30 and Human HMW Adiponectin/Acrp30 Quantikine ELISA Kits; R&D Systems, Minneapolis, MN). These ELISA assays were performed in accordance with the manufacturer's protocol.

Measurement of oxidized human serum albumin
The redox state of human serum albumin (HSA) was used as a systemic oxidative stress marker, as it reflects the progression of oxidative stress-related chronic diseases.[30,31] The redox state of HSA was analyzed by high-performance liquid chromatography for the fasting serum samples that were collected at the end point of the observation period, as described in a previous study for assessing the level of oxidative stress.[32] Based on the high-performance liquid chromatography profiles of HSA, the values of each of the albumin fractions (for human mercapto-albumin, human non-mercapto-albumin (HNA)1 and HNA2) were estimated by dividing the area of each fraction by the total area corresponding to HSA. A mixture of HNA1 and HNA2 was defined as oxidized HSA.

Statistical analyses
The details of the statistical analyses are shown in the **Supplementary Materials and Methods**.

RESULTS
Clinical characteristics of the subjects at baseline
The observed genotype frequency distributions of the *DsbA-L* and *PNPLA3* were consistent with the Hardy-Weinberg equilibrium ($P > 0.05$). The demographic characteristics at baseline of the subjects stratified by the *DsbA-L* genotypes are shown in **Table 1**. The longitudinal differences in the BMI and the cumulative prevalence of NAFLD between the *DsbA-L* genotypes are shown in **Figure 1**. Although the BMI at baseline did not differ among patients with the *DsbA-L* genotypes (**Table 1**), the BMI was higher in carriers of the *DsbA-L* T/T genotype than in those with the G/G or G/T genotypes during the observation period that was analyzed using the generalized estimating equations approach ($P = 0.013$; **Figure 1**). The prevalence of NAFLD at baseline did not differ among the *DsbA-L* genotypes (**Table 1**), and no significant association was found between the cumulative prevalence of NAFLD and the *DsbA-L* genotypes during the observation period ($P = 0.128$; **Figure 1**).

Development of a model describing the interindividual variability in the BMI values
First, we developed a model describing the interindividual variability in the BMI values. Because the mean value of BMI was nearly unchanged during the observation period (**Figure 1**), we developed a model for BMI using a linear regression model. **Supplementary Table S1** shows the effects of the tested covariates on the objective function of the parameters regarding modeling for the BMI. Age, gender, and *DsbA-L* genotype were significantly associated with the BMI. The final model for BMI was as follows:

$$BMI = 22.6 \times (AGE/70.8)^{-0.071} \times 0.968^{female} + 1.50^{DsbALT/Tgenotype} \quad (1)$$

Female $= 1$ for females, and 0 for males; *DsbA-L* T/T genotype $= 1$ for carriers of the T/T genotype, and 0 for noncarriers (i.e., carriers of *DsbA-L* G/G or G/T genotype). This BMI model indicated that age, female gender, and *DsbA-L* T/T genotype were associated with BMI (Eq. 1).

Table 1 Clinical characteristics of all subjects at baseline

	DsbA-L genotype			
	G/G ($n = 192$)	G/T ($n = 129$)	T/T ($n = 20$)	*P* value
Female (%)	80 (41.7)	56 (43.4)	9 (45.0)	0.928
Age, years	68.0 ± 5.9	67.3 ± 5.8	67.3 ± 6.0	0.513
BMI, kg/m^2	22.5 ± 2.9	22.6 ± 2.7	23.9 ± 3.0	0.112
Waist circumstance, cm	82.2 ± 8.3	82.9 ± 7.3	83.8 ± 6.7	0.691
Fasting blood glucose, mg/dL	99.7 ± 18.8	104.0 ± 23.2	102.9 ± 8.2	0.200
HbA1c, %	5.80 ± 0.63	5.88 ± 0.75	5.91 ± 0.45	0.500
Systolic blood pressure, mmHg	123.4 ± 18.3	121.9 ± 17.3	122.6 ± 13.0	0.762
Diastolic blood pressure, mmHg	72.1 ± 10.3	71.4 ± 11.3	72.7 ± 11.2	0.775
eGFR, mL/min/1.73 m^2	72.1 ± 14.5	72.1 ± 11.6	75.6 ± 15.9	0.541
LDL-C, mg/dL	125.9 ± 27.3	125.5 ± 25.9	121.0 ± 25.6	0.747
HDL-C, mg/dL	69.7 ± 16.1	68.8 ± 16.3	71.2 ± 23.8	0.799
TG, mg/dL	100.9 ± 49.7	100.5 ± 44.1	105.6 ± 52.2	0.907
AST, IU/L	24.4 ± 7.3	23.1 ± 5.9	23.8 ± 5.3	0.221
ALT, IU/L	21.7 ± 10.0	21.2 ± 9.3	23.3 ± 7.0	0.653
GGT, IU/L	30.2 ± 25.6	27.6 ± 22.8	27.1 ± 13.4	0.586
Overweight (%)	35 (18.2)	22 (17.1)	7 (35.0)	0.154
Diabetes (%)	22 (11.5)	19 (14.7)	2 (10.0)	0.644
Hypertension (%)	78 (40.6)	53 (41.1)	9 (45.0)	0.931
Dyslipidemia (%)	96 (50.0)	61 (47.3)	7 (35.0)	0.430
NAFLD (%)	27 (14.1)	19 (14.7)	5 (25.0)	0.435
Ever smoker (%)	57 (29.7)	51 (39.5)	6 (30.0)	0.176
Alcohol intake (g/day)	4.6 ± 6.7	4.7 ± 7.0	3.7 ± 7.2	0.835

The data are the means ± SD or proportions for categorical variables.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DsbA-L, disulfide bond-forming oxidoreductase A-like protein; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; TG, triglyceride.

The results showed that the BMI in the *DsbA-L* T/T genotype was higher by ~1.5 kg/m^2 than that of the G/G or G/T genotype carriers (Eq. 1).

Development of the prediction model for NAFLD
Next, we developed the base model of the risk for NAFLD using a logistic regression model. Because the development of NAFLD is closely related to the presence of obesity, the logit (probability (Pr)) value of the model was a sigmoidal maximum response of the prevalence for NAFLD using BMI as the exposure variable. **Supplementary Table S2** shows the effects of the tested covariates on the objective function of the parameters regarding the risk of developing NAFLD. Female gender; levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and HbA1c; and a *PNPLA3* genotype were significantly associated with the risk of developing NAFLD. However, the *DsbA-L* genotype had no effect on the risk of developing NAFLD. The final prediction model for the risk of NAFLD was as follows:

$$Logit(\text{Pr}) = -5 + \frac{Logit(\text{Pr})_{max} \times (BMI - 17)^{3.43}}{(BMI_{50} - 17)^{3.43} + (BMI - 17)^{3.43}} \quad (2)$$

$$Logit(\text{Pr})_{max} = 4.17 + 1.02^{female} - 0.060 \times (HDL - 69.4) + 0.009 \times (LDL - 120) \quad (3)$$

$$BMI_{50} = 17 + 6.42 \times 0.760^{PNPLA3\ C/G\ genotype}$$
$$\times 0.591^{PNPLA3\ C/G\ genotype} \times (HbA1c/5.88)^{-3.34} \quad (4)$$

where Logit(Pr)$_{max}$ is the maximum value of the logit regarding the prevalence of NAFLD; BMI$_{50}$ is the BMI corresponding to 50% of the Logit(Pr)$_{max}$; female = 1 if female, and 0 if male; *PNPLA3* C/G genotype or *PNPLA3* G/G genotype = 1 for carriers of each genotype, and 0 for carriers of other genotypes. This NAFLD model indicated that the risk of developing NAFLD increased with increasing BMI (Eq. 2 and **Figure 2**). Female gender, a low value of HDL-C, and a high value of LDL-C were associated with an increased Logit(Pr)$_{max}$ (Eq. 3 and **Figure 2**). The *PNPLA3* rs738409 polymorphism and a high value of HbA1c were associated with a decreased BMI$_{50}$ (Eq. 4 and **Figure 2**). The *DsbA-L* genotype was not found to be a significant covariate for the Ligit (Pr) of the risk of developing NAFLD. The results of model evaluation and simulation are shown in the **Supplementary Results**. In order to assess the effect of the *DsbA-L* genotype on overweight-induced NAFLD, we also developed models for NAFLD stratified by the weight status (i.e., overweight or normal-weight; **Supplementary Tables S2 and S4**). However, no association was found between the *DsbA-L* genotype and the risk of developing NAFLD in either normal weight or overweight subjects (**Supplementary Tables S2 and S4**).
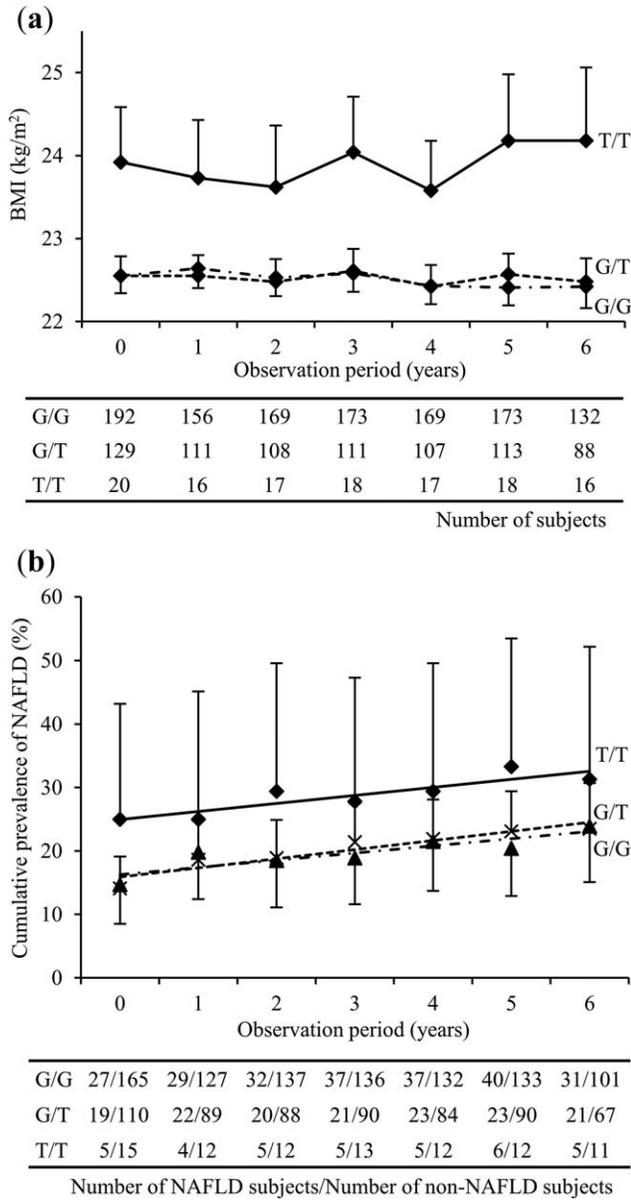
Figure 1 Longitudinal changes in the mean values of body mass index (BMI) and the cumulative prevalence of nonalcoholic fatty liver disease (NAFLD) stratified by the disulfide bond-forming oxidoreductase A-like protein (*DsbA-L*) genotype. The mean values of BMI (**a**) and the cumulative prevalence of NAFLD (**b**) are shown as dashed-dotted, dotted, and solid lines for the subjects with the *DsbA-L* G/G, G/T, and T/T genotypes, respectively. (**a**) The bars represent the mean ± SE. (**b**) The graph provides the best fit linear lines of the cumulative prevalence of NAFLD (%). The bars represent the 95% confidence intervals, and the trigonal, cross, and dimetric plots represent the actual measurements in the *DsbA-L* G/G, G/T, and T/T genotypes, respectively. The coefficient of determinations (R^2) of the best fit linear lines in the *DsbA-L* G/G, G/T, and T/T genotypes were 0.886, 0.740, and 0.783, respectively.

Associations of the adiponectin levels with the *DsbA-L* genotype

The median values (range) of total and HMW adiponectin and the ratio of HMW to total adiponectin (i.e., index of adiponectin multimerization) were 123.0 (7.9–542.6) ng/mL,
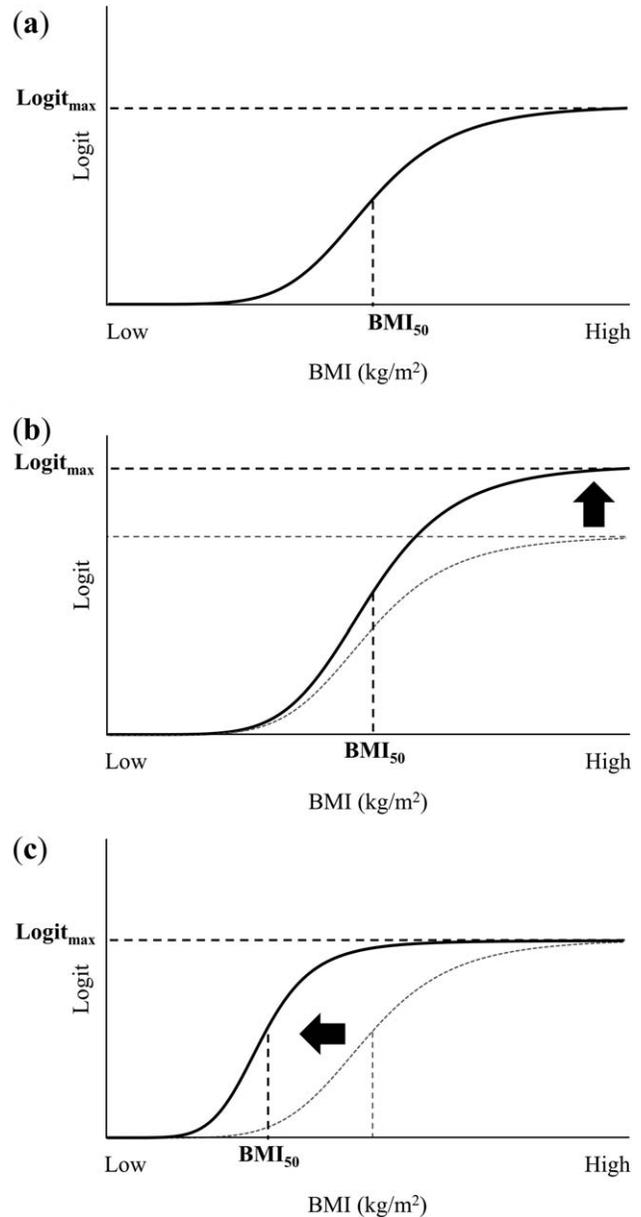

Figure 2 The relationships between the body mass index (BMI) and the logit of nonalcoholic fatty liver disease (NAFLD) prevalence based on the prediction model for NAFLD. The effects of covariates on the relationships between the BMI and the logit of NAFLD prevalence are indicated by arrows. (**a**) The relationship between the BMI and the logit of NAFLD prevalence is shown as a solid curve for average subjects. (**b**) The relationships between the BMI and the logit of NAFLD prevalence are shown as a solid curve for female subjects or subjects with a low value of high-density lipoprotein cholesterol and/or a high value of low-density lipoprotein cholesterol, and are shown as a dotted curve for average subjects. (**c**) The relationship between the BMI and the logit of NAFLD prevalence are shown as a solid curve for subjects with patatin-like phospholipase domain-containing 3 (*PNPLA3*) C/G or G/G genotypes and/or a high value of HbA1c and are shown as a dotted curve for average subjects.

74.9 (3.4–350.7) ng/mL, and 0.65 (0.09–0.99), respectively. The ratio of HMW to total adiponectin was significantly lower in carriers of *DsbA-L* T/T genotype than in those with
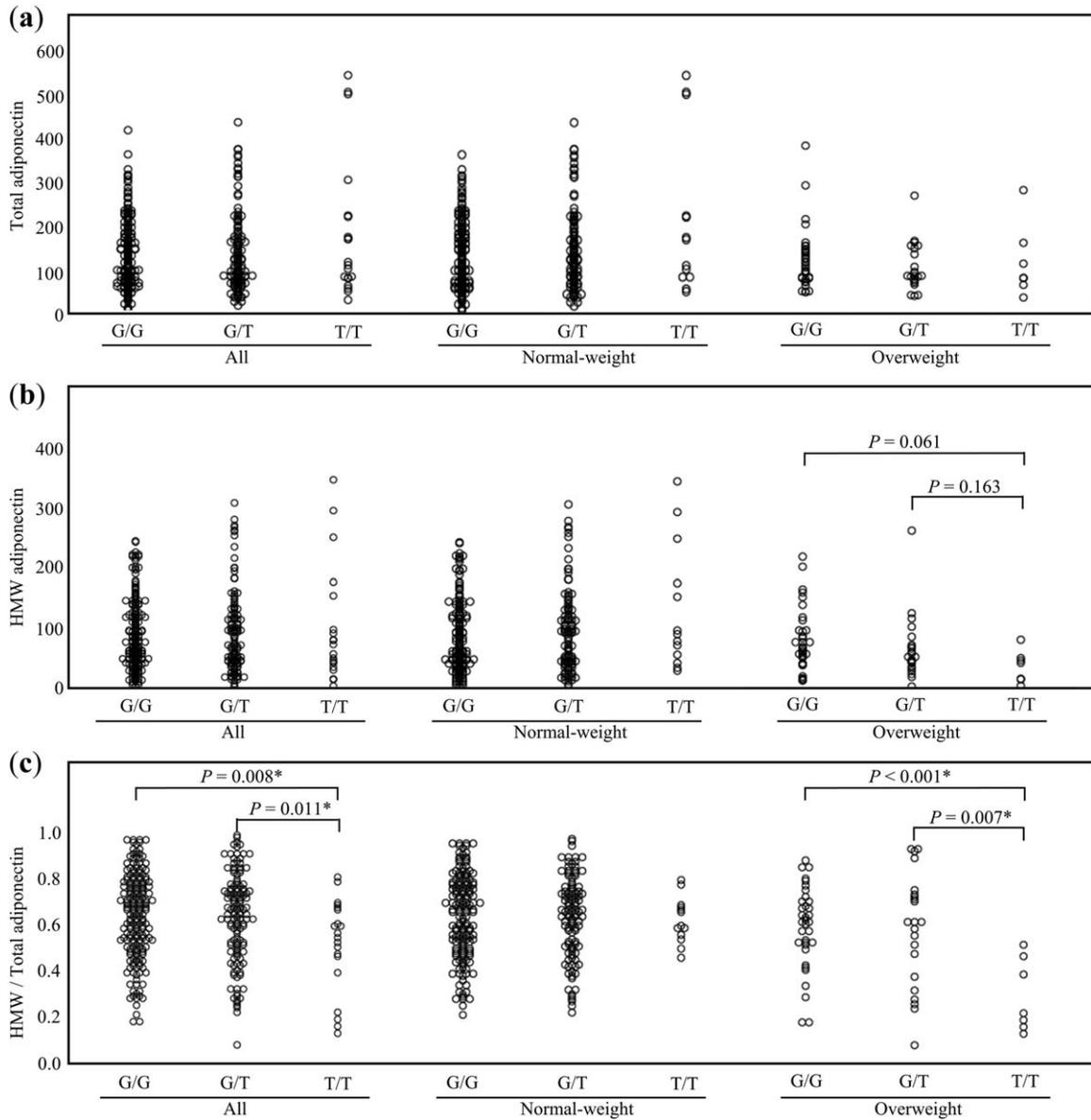
Figure 3 The effect of the disulfide bond-forming oxidoreductase A-like protein (*DsbA-L*) genotype on the adiponectin levels. Plots of the values of total adiponectin (**a**), high-molecular-weight (HMW) adiponectin (**b**), and the ratio of HMW to total adiponectin (**c**) stratified by the *DsbA-L* genotype are shown for all, overweight, and normal weight subjects. The *P* values were calculated by Mann-Whitney U-test. *The significance remained after Bonferroni's test.

the G/G or G/T genotype (**Figure 3**), and this association was also observed in the multiple regression analysis (**Supplementary Table S5**). An interactive effect of *DsbA-L* genotype and overweight status (end point) on the ratio of HMW to total adiponectin was observed ($P < 0.05$). Therefore, we also analyzed the associations of the *DsbA-L* genotype with the values of total and HMW adiponectin and their ratio among normal weight and overweight subjects in end point (**Figure 3**). Among overweight subjects, the ratio of HMW to total adiponectin was significantly lower in carriers of *DsbA-L* T/T genotype than in those with the G/G or G/T genotype (**Figure 3**), and this association was also observed in the multiple regression analysis (**Supplementary Table S5**).

In contrast, the *DsbA-L* T/T genotype was not associated with the ratio of HMW to total adiponectin value among normal-weight subjects (**Figure 3 and Supplementary Table S5**).

Association of the oxidized HSA with the *DsbA-L* genotype

The median value (range) of oxidized HSA was 1.99 (1.53–2.64) g/dL. The median value (range) of oxidized HSA was higher in subjects who were overweight than in those with normal weight (2.05 (1.66–2.54) g/dL vs. 1.97 (1.53–2.64) g/dL; $P = 0.005$; **Supplementary Figure S2**). In addition, the median value (range) of oxidized HSA was also higher in subjects with NAFLD than in those with
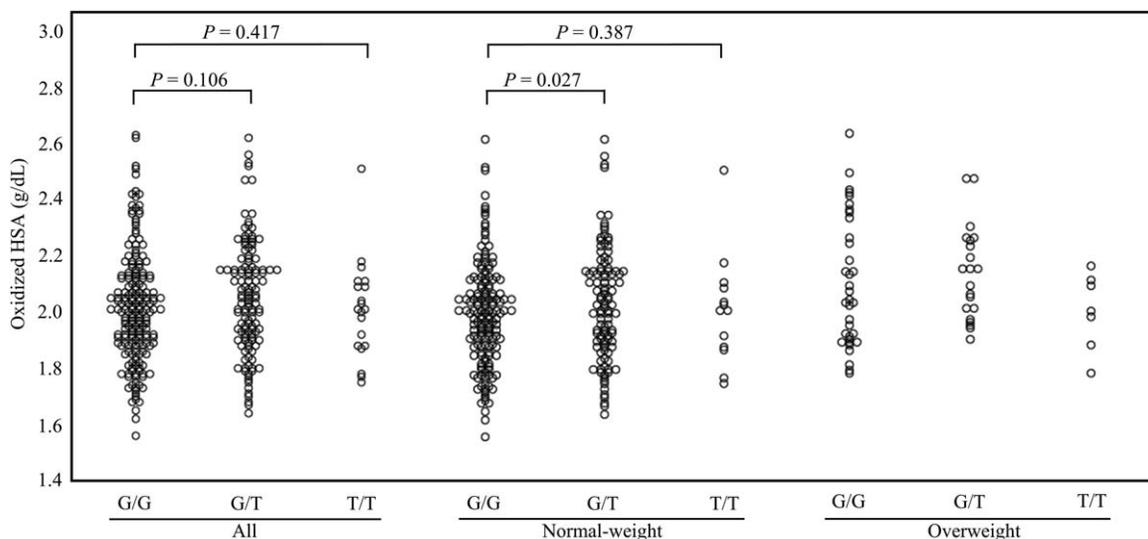
Figure 4 The effect of the disulfide bond-forming oxidoreductase A-like protein (*DsbA-L*) genotype on the oxidized human serum albumin (HAS) level. Plots of the values of total oxidized HSA stratified by the *DsbA-L* genotype are shown for all, overweight, and normal weight subjects. The *P* values were calculated by Mann-Whitney U-test. *The significance disappeared after Bonferroni's test.

non-NAFLD (2.07 (1.71–2.54) g/dL vs. 1.98 (1.53–2.64) g/dL; $P = 0.003$; **Supplementary Figure S2**). Although the differences were not significant, the median values (ranges) of oxidized HSA tended to be higher in the *DsbA-L* G/T or T/T genotypes carriers (2.01 (1.64–2.64) g/dL or 2.06 (1.67–2.26) g/dL) than in the G/G genotype carriers (1.96 (1.53–2.57) g/dL; $P = 0.106$ and $P = 0.417$, respectively; **Figure 4**). Among the normal weight subjects, the median value of oxidized HSA was higher in the *DsbA-L* G/T than in the G/G genotype carriers (1.99 (1.64–2.64) g/dL vs. 1.93 (1.53–2.57) g/dL; $P = 0.027$) but the significance disappeared after Bonferroni's adjustment (**Figure 4**).

Structural equation modeling
Finally, we evaluated the relationships among the *DsbA-L* genotypes, BMI, the prevalence of NAFLD, and their related covariates. **Figure 5** shows the structural equation model using the clinical data at the end point of the observation period. The *P* value for the model fit to a χ^2 (18.40, degree of freedom = 17) was 0.364, and the goodness of fit index, adjusted goodness of fit index, and root mean square error of approximation were 0.987, 0.972, and 0.016, respectively. Taken together, these fitness statistics indicated a good fit for the structural equation model. The *DsbA-L* T/T genotype seems to influence a high BMI both directly and indirectly through a decreased HMW-to-total adiponectin ratio, whereas the *DsbA-L* T/T genotype was not directly associated with the risk of developing NAFLD (**Figure 5**). A high BMI and lower adiponectin multimerization were directly and/or indirectly associated with the risk of NAFLD (**Figure 5**).

DISCUSSION

In the present study, we used a NONMEM program to develop population prediction models incorporating population background characteristics (e.g., the *DsbA-L* T/T

genotype) to predict the BMI and the risk of NAFLD in the general elderly population. In this study, the population prediction model for predicting the risk of NAFLD could be shown as a sigmoidal maximum response using the BMI as an exposure variable, and the interindividual variability in the BMI values was described using a linear regression model. The models we developed described the data adequately (**Supplementary Results, Supplementary Figure S1, and Supplementary Tables S3 and S4**). We expect that these findings and/or the procedure of this study will aid in the further prevention/treatment of obesity and obesity-related diseases, such as NAFLD, in the general elderly population based on the population background.

This is the first study to show that the *DsbA-L* T/T genotype is associated with an increased BMI partially by decreasing adiponectin multimerization. Although the *DsbA-L* polymorphism did not affect the risk for NAFLD directly in the prediction model of NAFLD, the structural equation model suggested that the *DsbA-L* T/T genotype might affect the risk of developing NAFLD through a high BMI in relation to lowering adiponectin multimerization. A previous study reported that DsbA-L was able to protect mice from high-fat-diet-induced obesity, and the expression of *DsbA-L* was inversely correlated with the BMI in both mice and humans.[13] *DsbA-L* rs1917760 polymorphism was also associated with increased insulin secretion and fat deposition in humans.[33] Thus, early intervention to upregulate *DsbA-L* or enhance adiponectin multimerization (e.g., dietary intervention[34]) in *DsbA-L* T/T genotype carriers may be an effective approach for preventing obesity in the elderly population.

Low circulating levels of HMW adiponectin are a strong risk factor for the development of visceral obesity.[10] DsbA-L is a key regulator of the adiponectin multimerization in 3T3-L1 cells[14] and protects mice from diet-induced obesity and insulin resistance.[13] Liu *et al.*[35] showed that DsbA-L
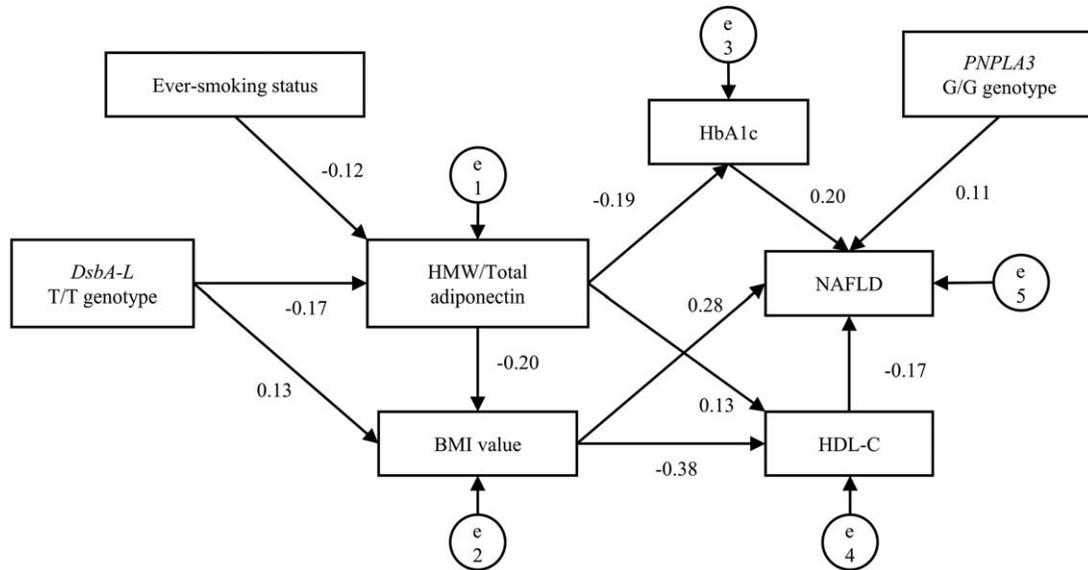
Figure 5 The structural equation modeling diagram of nonalcoholic fatty liver disease (NAFLD) and the disulfide bond-forming oxidore-ductase A-like protein (*DsbA-L*) genotype. Lines with numbers indicate significant paths with standardized partial regression (β) coefficients ($P < 0.05$). The e1, e2, e3, e4, and e5 indicate latent factors. Arrows represent an association between two factors. The β values ranged from -1 to 1, with a positive value representing a positive correlation and negative value representing a negative correlation. HDL-C, high-density lipoprotein cholesterol.

localized in the endoplasmic reticulum, and this localization was critical for suppressing endoplasmic reticulum stress and promoting HMW adiponectin biosynthesis and secretion. The present study revealed that the *DsbA-L* T/T genotype was associated with a high BMI as well as a low HMW-to-total adiponectin ratio in relation to overweight status in the general Japanese population (Eq. 1, **Figures 1, 3, and 5, and Supplementary Table S5**); however, this genotype did not have any significant impact on the cumulative prevalence of NAFLD (**Figure 1**) or the BMI-based prediction model for the risk of NAFLD (Eqs. 2, 3, and 4). Meanwhile, we found that a high BMI and lower adiponectin multimerization were directly and/or indirectly associated with the risk of NAFLD in our structural equation model (**Figure 5**). Taken together, we hypothesize that the *DsbA-L* T/T genotype may be indirectly and partially associated with the risk of NAFLD through a high BMI and low adiponectin multimerization.

Chen *et al*.[16] showed that hepatic DsbA-L protects mice from high-fat diet-induced fatty liver and insulin resistance. More recently, the cGAS-cGAMP-STING pathway has been identified as a cytosolic DNA sensor of pathogen-derived DNA, which mediates the innate immune response.[18] Bai *et al*.[17] showed that obesity-induced mtDNA is released into cytosol resulting in inflammatory responses through the activation of the cGAS-cGAMP-STING pathway. Moreover, they identified *DsbA-L* as an important regulator of the mitochondrial integrity and function, which suppresses the activation of the cGAS-cGAMP-STING pathway resulting in obesity-induced inflammation and insulin resistance.[17] Thus, it is considered that *DsbA-L* may play a key role in the development and progression of NAFLD, especially in obese populations. In the present study, the association

between *DsbA-L* polymorphism and the median ratio of HMW to total adiponectin, which is related to protection against adipose tissue inflammation and insulin resistance,[8–10] was more pronounced in overweight subjects than in the overall study population (**Figure 3**). However, we did not find any direct effects of *DsbA-L* polymorphism on the risk of NAFLD, even in overweight subjects (Eqs. 2, 3, and 4, and **Supplementary Tables S2 and S4**). In this study, we could not investigate the association between *DsbA-L* polymorphism and the risk of NAFLD in the obese population, because the prevalence of obesity (BMI ≥ 30 kg/m^2) among the study subjects (1.2%) was small. Further larger studies will be needed to elucidate the potential impact of this polymorphism on the obesity-induced development and progression of NAFLD in humans.

DsbA-L is expressed in both the mitochondria and the endoplasmic reticulum in adipocytes,[35] and it plays a role in not only adiponectin multimerization but also antioxidant protection.[35,36] DsbA-L exerts activities against a number of substrates associated with oxidative stress (e.g., l-chloro-2,4-dinitrobenzene, ethacrynic acid, cumene hydroperoxide, and *t*-butyl hydroperoxide).[36] Furthermore, the knockdown of *DsbA-L* in *Caenorhabditis elegans* resulted in a significant decrease in the respiration rate and a change in the fatty acid metabolism in mitochondria.[37] The redox state of HSA has been proposed as a plasma marker of chronic oxidative stress-related diseases,[30] including the progression of chronic hepatitis and cirrhosis.[31] The results of this study showed that the oxidized HSA level was increased in subjects who were overweight and/or with NAFLD (**Supplementary Figure S2**). However, the oxidized HSA level was not markedly different among the *DsbA-L* rs1917760 genotypes (**Figure 4**); as such, the *DsbA-L* T/T genotype might

be associated with being overweight due to the decreased adiponectin multimerization rather than the increased oxidative stress, although further studies are needed to verify the relationship of the *DsbA-L* genotype with oxidative stress using other more sensitive oxidative stress-related markers.

Several limitations associated with the present study warrant mention. The subjects' alcohol consumption and smoking status were evaluated through face-to-face interviews, which might have lacked reliability. The diagnosis of FLD was performed by hepatic ultrasonography scanning and was not confirmed by a liver biopsy. Another limitation of the present study was the study design. The present study was retrospective in nature and investigated a relatively small number of subjects, especially of the subjects stratified by the *DsbA-L* genotypes. Furthermore, some of the subjects did not attend the health screening program annually throughout the observation period; thus, the number of study subjects varied at some points of the observation period. Notably, the decrease in the number of subjects with the T/T genotype ($\sim$20%) was less than that in the patients with the G/G or G/T genotypes ($>$30%) during the observation period (**Figure 1**). In the present study, we modeled the BMI and the risk of NAFLD using a NONMEM procedure, which enabled the quantification of several levels of variability and allowed us to address unstable data (e.g., a limited number of samples per individual at different time points). Thus, the variation in the number of study subjects during the observation period may not have had a significant impact on the results of this study. Nevertheless, a further large longitudinal investigation is required to verify the present findings.

In conclusion, our population prediction model of BMI indicated that the *DsbA-L* T/T genotype was significantly associated with a high BMI. The BMI-based NAFLD prediction model showed that the *DsbA-L* T/T genotype did not have any direct impact on the risk of NAFLD, whereas our structural equation model suggested that this genotype effect might be indirectly or partially associated with the risk of developing NAFLD through a high BMI and low adiponectin multimerization. Thus, genotyping to investigate *DsbA-L* polymorphism and determine the patients with the highest risk of developing obesity may help to prevent obesity, and possibly NAFLD, by facilitating targeted prevention and treatment programs in patients with a high risk of obesity; however, further larger studies are needed to verify our findings.

Conflict of Interest. The authors declared no competing interests for this work.

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