Serum Anti-Apo B Antibody Level as Residual CVD Marker in DM Patients under Statin Treatment

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Aim: In the pathogenesis of atherosclerosis, autoantibodies have two-facedness of progression and protection. Previous reports have indicated that low autoantibody levels against apolipoprotein B-100 (apo B-100) could increase the risk of atherosclerotic cardiovascular diseases (CVD) in healthy subjects. In this study, we investigated the relationship between circulating anti-apo B-100 autoantibodies and the clinical parameters in Japanese diabetic patients with or without CVD.

Methods: We measured the serum levels of anti-apo B-100 autoantibodies against native and malondialdehyde (MDA)-modified p45 or p210 epitopes, as well as anti-apo E autoantibodies, using enzyme-linked immunosorbent assay.

Results: In patients with CVD, the circulating levels of IgG against native p45, MDA-modified p45, and MDA-modified p210 (IgGN-45, IgGMDA-45, and IgGMDA-210) were significantly lower than those in patients without CVD, whereas no difference was observed in anti-apo E autoantibody levels. In addition, IgM N-45, IgM MDA-45, and IgGMDA-45 were negatively correlated with LDL-C levels, whereas IgG N-45 and IgG N-210 were positively correlated with HbA1c levels. No correlation was observed between autoantibody levels and diabetic microangiopathy. In the statin-treated subgroup, IgGMDA-45 and IgGMDA-210 were significantly lower in patients with CVD than in those without CVD.

Conclusion: Measurement of serum anti-apo B-100 autoantibodies can be useful for the evaluation of CVD risk in patients with diabetes receiving statin treatment.

Key words: Apolipoprotein B-100, Autoantibody, Cardiovascular disease, Diabetes

Abbreviations: apo, apolipoprotein; CVD, cardiovascular diseases; DM: diabetes mellitus; HDL, high-density lipoprotein; Ig, immunoglobulin; LDL, low-density lipoprotein; MDA, malondialdehyde

Introduction

The prevalence of diabetes mellitus is steadily increasing, with more than 400 million people being reported to have type 2 diabetes globally in 20141. Cardiovascular disease (CVD) comprises a major cause of death in patients with diabetes2. Although multiple clinical trials have demonstrated that statin treatment reduces primary and secondary CVD events and mortality3, a considerable residual CVD risk has been noted. Therefore, biomarkers of CVD risk need to be identified for targeted diabetic therapies.

CVD events are developed based on the progression of atherosclerotic plaques, recognized as inflammatory lesions occurring in large- and medium-sized arteries4. Atheromatous plaques contain different
types of inflammatory cells, such as macrophages and lymphocytes, that are involved in the innate recognition of disease-specific antigens, followed by adaptive immunity. At the initiation of atherosclerosis, monocytes attach to the vascular endothelial surface and mature into macrophages. The activated macrophages uptake modified lipoproteins in the arterial intima, subsequently stimulating B cells to generate antibodies against modified lipoproteins.

Accordingly, the roles of B cells on the development of atherosclerosis have been studied in several experimental models. Caligiuri et al. demonstrated that B cell-associated protective immunity reduced atherosclerotic progression, observed as aggravated atherosclerosis in the apolipoprotein (apo) E-deficient, B cell-deprived mice. Similarly, Major et al. showed that B cell depletion increased atherosclerosis in LDL receptor knockout mice, and Doran et al. demonstrated that adoptive B cell-transfer reduced diet-induced atherosclerosis in mice deficient in B cells. In addition, some immunization studies have supported athero-protective roles of B cell-derived humoral immunity.

Of the several antigens involved in these adaptive responses of atherosclerosis, apo B-100, oxidized LDL (oxLDL), heat shock protein 60 (HSP60), and HSP65 are the promising candidates for T cell activation. However, clinical studies have showed conflicting data on the relationship between the serum levels of these antibodies and CVD. Some studies have reported that the plasma antibody titers against HSP60 and oxLDL were elevated in patients with CVD, whereas some have reported an inverse correlation between anti-oxLDL antibody levels and carotid artery atherosclerosis. Apo B-100 is the primary apolipoprotein on LDL, and elevated serum apo B levels are supposed to drive plaque formation. The relationship between anti-apo B autoantibodies and atherosclerosis has been mostly studied in patients with CVD, but the role of these autoantibodies on CVD has not been well clarified in patients with diabetes.

We proposed to investigate the association of serum autoantibody titers against apo B-100 peptides with the macro- and microangiopathies in Japanese patients with diabetes.

**Methods**

**Patients**

We enrolled outpatients with diabetes presenting to the NTT West Osaka Hospital between September and November 2014. In total, 90 patients with records on diabetic complications who provided informed consent were consecutively enrolled. Those aged >85 years or having renal dysfunction (serum creatinine [s-Cr] > 2.0 mg/dL) were excluded from this study. This study was approved by the ethics committees of both NTT West Osaka Hospital and the Osaka University Hospital.

Diabetic retinopathy was diagnosed by the treating ophthalmologist, based on the presence of characteristic microvascular changes in the retina observed by ophthalmoscopy through dilated pupils. Severity of diabetic retinopathy was determined according to the Davis classification, and staged according to “a new classification of diabetic nephropathy 2014” of Japan Diabetes Society. Diabetic neuropathy was diagnosed based on the presence of at least two positive findings among abnormal sensation, vibration abnormality on both sides of the ankle, and ankle tendon reflex abnormality on both sides.

**Measurement of Serum Parameters**

Morning blood samples were obtained after overnight fast, and the biochemical markers were measured in the hospital laboratory.

**Measurement of Serum Levels of Antibodies against Apolipoprotein B by Enzyme-Linked Immunosorben Assay**

The apo B peptides, p45 (amino acids 661–680; IEIGLEGKGFEPTELEFGLK) and p210 (amino acids 3136–3155; KTTKQSFDSLVSQAYKKKNKH), were synthesized (Sigma-Aldrich; Saint-Louis, MO), and their MDA-modified peptides were produced according to the previously mentioned method. Apo E peptide (amino acids 158–178; HLRLKLKRLRDAADDLQKRLA) containing the LDL receptor-binding domain was also generated (Sigma-Aldrich; Saint-Louis, MO). These peptides were diluted at 4 µg/mL in dimethyl sulfoxide, and 50 µL was dropped into each microtiter plate well for 2 hours using peptide coating kit (TAKARA; Shiga, Japan), according to the manufacturer’s protocol. After washing with distilled water three times, the peptide-coated plates were incubated with blocking solution (TAKARA) for 1 h at room temperature (RT).

The test serum was then diluted at 1:100 with TBS-containing 0.01% Tween-20 (Santa Cruz Biotechnology; Dallas, TX), and 100 µL of the diluted serum was added into each well of the ELISA plate for 2-h incubation at RT. After rinsing with 0.1% Tween 20-containing PBS (pH 7.5) three times, deposition of autoantibodies directed to the peptide was detected using HRP-conjugated rabbit polyclonal secondary antibodies against human IgG, IgM, and IgA (Agilent Technologies; Santa Clara, CA) with an appropriate dilution with TBS-T. After washing the wells using...
lipid levels were in good control with the use of statins and fibrates (Supplemental Table 1); the mean serum triglyceride was 126 ± 75 mg/dL and the mean LDL-C level was 96 ± 21 mg/dL. The diabetic micro- and macroangiopathy complications in patients are listed in Table 2. The number of patients with ischemic heart disease, stroke, and arteriosclerosis obliterans was 23, 8 and 6, respectively. Patients who had at least one of these three diseases were defined as “atherosclerotic patients” (n = 29). Missing data numbers were 4, 4, and 5 in retinopathy, nephropathy, and neuropathy, respectively. There were no missing data for macroangiopathy.

Measurement of Anti-Apo B-100 Autoantibodies by ELISA
The serum levels of autoantibodies against native and MDA-modified apo B peptides, p45 and p210, were measured using a homemade ELISA (Supplemental Table 2). The titers of the autoantibodies against native p45 and MDA-p45 in all immunoglobulin subclasses were significantly and positively correlated (Supplemental Fig. 1). Similarly, positive correlations were observed between the antibody titers to native p210 and MDA-p210 in each subclass (Supplemental Fig. 1).

The serum levels of IgG class antibodies against native p45 (IgGN45) and IgGN210 were both positively correlated with HbA1c levels ($\rho = 0.230, p < 0.05$ and $\rho = 0.300, p < 0.05$, respectively; Supplemental Table 3), whereas no such correlation was observed for MDA-modified antibodies. The IgMN45 and IgMMDA-45 levels were negatively correlated with serum LDL-C.

### Table 1. Characteristics of the patients

|                          | Mean ± SD (range) |
|--------------------------|-------------------|
| Age (years)              | 67 ± 9 (42–83)    |
| Diabetes history (years) | 16 ± 10 (1–43)    |
| Sex (M/F)                | 73/17             |
| BMI (kg/m²)              | 24.7 ± 3.7 (17.4–38.0) |
| Systolic blood pressure (mmHg) | 130 ± 13 (84–161) |
| Smoking (+ / –)          | 51/39             |
| TC (mg/dL)               | 174 ± 26 (122–255) |
| TG (mg/dL)               | 126 ± 75 (31–530) |
| HDL-C (mg/dL)            | 54 ± 13 (33–95)   |
| LDL-C (mg/dL)            | 96 ± 21 (38–160)  |
| RemL-C (mg/dL)           | 8.0 ± 5.5 (1.7–38.4) |
| Fasting plasma glucose (mg/dL) | 129 ± 26 (78–223) |
| HbA1c (%)                | 7.0 ± 0.7 (5.5–9.2) |

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RemL-C, remnant cholesterol; HbA1c, hemoglobin A1c.

Statistical Analysis
JMP version 11.2.2 (SAS Institute Inc., Cary, North Carolina) was used for statistical analysis. Data are presented as mean ± standard deviation (SD) values. Spearman correlation coefficient was used to evaluate the association between two variables. In the stepwise multiple regression analysis, the F-value was set at 2.0 for the inclusion of variables. Mann–Whitney test was used for statistical analyses of independent samples, and a $p$ value of < 0.05 was considered significant.

Results

Patients
The demographic and clinical characteristics of the enrolled patients are detailed in Table 1. The study population was predominantly men, with an average age of 67 ± 9 years. Most patients were not obese, with a mean body mass index of 24.7 ± 3.7 kg/m². Moreover, their blood glucose levels were well controlled with oral hypoglycemic agents and/or insulin (Supplemental Table 1), as HbA1c and FPG were 7.0 ± 0.7% and 129 ± 26 mg/dL, respectively. Serum
Table 2. Complications in the patients with diabetes

| Microangiopathy       | Stage (1/2/3/4/5) | 47/27/11/1/0 |
|-----------------------|-------------------|--------------|
| Nephropathy           | (NDR/SDR/PrePDR/PDR) | 64/13/4/5   |
| Neuropathy            | (+/-)             | 52/33        |

| Macroangiopathy       |                     |              |
|-----------------------|---------------------|--------------|
| Atherosclerotic changes| 29                 |              |
| Ischemic heart disease | 23                 |              |
| Stroke                | 8                   |              |
| Arteriosclerosis obliterans | 6               |              |

NDR, no diabetic retinopathy; SDR, simple diabetic retinopathy; PrePDR, pre-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

Atherosclerotic changes were defined as having at least one previous cardiovascular disease, stroke, or arteriosclerosis obliterans.

Relationship between Autoantibodies against Apo B-100 or Anti-Apo E and Diabetic Complications

We next investigated the association between anti-apo B-100 autoantibodies and diabetic complications (Table 3 and Fig. 1). Compared with the non-atherosclerotic group, the atherosclerotic group had significantly lower serum IgGN-45, IgGMMDA-45, and IgGMMDA-210 levels. The serum IgGN-210 levels tended to be low in the atherosclerotic group. No differences were observed in the titers of the IgM class autoantibodies against apo B-100 between these two groups. There was no association between anti-apo E autoantibodies and diabetic complications (Supplemental Fig. 2). In addition, the antibody titers against both apo B-100 and apo E were similar, regardless of the severity of diabetic microangiopathy (Table 3 and data not shown).

Relationship between Anti-Apo B Autoantibodies and Atherosclerotic Diseases According to Statin Treatment

Interestingly, compared with the atherosclerotic group without statin treatment, the statin-treated atherosclerotic group had significantly lower IgG autoantibody levels, including IgGN-45 (0.223 ± 0.178 vs. 0.306 ± 0.281, p = 0.056); IgGMMDA-45 (0.360 ± 0.284 vs. 0.510 ± 0.345, p < 0.05); IgGN-210 (0.282 ± 0.236 vs. 0.357 ± 0.287, p = 0.092); and IgGMMDA-210 (0.446 ± 0.285 vs. 1.067 ± 1.257, p < 0.05) (Table 4). No significant difference was observed in the group without statin treatment.

Because LDL-C levels are recognized as a risk factor for atherosclerosis and can be reduced by statin treatment, we examined the effects of statin treatment on the relationship between the autoantibody titers and the clinical parameters. Overall, LDL-C level was negatively correlated with IgMN-45 (p = −0.262, p < 0.05; Supplemental Table 3) and IgMMDA-45 (p = −0.259, p < 0.05); in the group without statin treatment, these correlations were stronger (p = −0.358, p < 0.05 and p = −0.410, p < 0.05, respectively; Supplemental Table 4). Moreover, in the group without statin treatment, LDL-C level was significantly and negatively correlated with IgMN-210 (p = −0.473, p < 0.01) and IgMMDA-210 (p = −0.403, p < 0.05) levels. No associations were observed in the statin-treated group. The HbA1c level was positively correlated with the serum levels of IgGN-45 and IgGN-210, but not with the MDA-modified antibodies.

Finally, we conducted stepwise regression analyses to identify independently associated clinical parameters for atherosclerosis in the statin-treated group. These analyses revealed that IgGN-210 and IgGMMDA-210 had stronger association with atherosclerosis, compared with other clinical parameters. IgGMMDA-210 was found to be the strongest explanatory variable for atherosclerosis (data not shown).

Discussion

In this study, we investigated the relationship
Table 3. Relationship between the autoantibody titers and diabetes complications

| Class | Atherosclerosis        | Nephropathy        |                  |
|-------|-------------------------|---------------------|------------------|
|       | ( )                     | (+)                 | Stage <3         | Stage ≥ 3       | p   |
| N-45  | IgG 0.264 ± 0.233       | 0.196 ± 0.150       | < 0.05           | 0.232 ± 0.156  | 0.331 ± 0.434 | n.s. |
|       | IgM 0.732 ± 0.447       | 0.723 ± 0.659       | n.s.             | 0.708 ± 0.547  | 0.813 ± 0.389 | n.s. |
| MDA-45| IgG 0.443 ± 0.329       | 0.313 ± 0.243       | < 0.05           | 0.391 ± 0.269  | 0.492 ± 0.524 | n.s. |
|       | IgM 0.978 ± 0.606       | 0.818 ± 0.455       | n.s.             | 0.915 ± 0.584  | 0.987 ± 0.538 | n.s. |
| N-210 | IgG 0.313 ± 0.242       | 0.248 ± 0.198       | 0.065            | 0.279 ± 0.184  | 0.392 ± 0.438 | n.s. |
|       | IgM 0.754 ± 0.509       | 0.708 ± 0.580       | n.s.             | 0.731 ± 0.559  | 0.788 ± 0.413 | n.s. |
| MDA-210| IgG 0.826 ± 1.051      | 0.384 ± 0.255       | < 0.05           | 0.681 ± 0.919  | 0.761 ± 0.891 | n.s. |
|       | IgM 1.025 ± 0.656       | 0.844 ± 0.690       | n.s.             | 0.967 ± 0.691  | 0.954 ± 0.644 | n.s. |

| Class | Retinopathy        | Neuropathy        |                  |
|-------|---------------------|-------------------|------------------|
|       | ( )                 | (+)               |                  |
| N-45  | IgG 0.251 ± 0.229   | 0.228 ± 0.172     | n.s.             | 0.231 ± 0.150  | 0.266 ± 0.294 | n.s. |
|       | IgM 0.725 ± 0.521   | 0.673 ± 0.496     | n.s.             | 0.692 ± 0.547  | 0.786 ± 0.499 | n.s. |
| MDA-45| IgG 0.418 ± 0.324   | 0.368 ± 0.283     | n.s.             | 0.371 ± 0.229  | 0.450 ± 0.415 | n.s. |
|       | IgM 0.925 ± 0.498   | 0.889 ± 0.746     | n.s.             | 0.879 ± 0.501  | 1.016 ± 0.678 | n.s. |
| N-210 | IgG 0.297 ± 0.250   | 0.292 ± 0.189     | n.s.             | 0.271 ± 0.184  | 0.331 ± 0.302 | n.s. |
|       | IgM 0.712 ± 0.487   | 0.795 ± 0.665     | n.s.             | 0.689 ± 0.491  | 0.832 ± 0.608 | n.s. |
| MDA-210| IgG 0.700 ± 0.961  | 0.533 ± 0.515     | n.s.             | 0.526 ± 0.505  | 0.959 ± 1.295 | n.s. |
|       | IgM 0.984 ± 0.670   | 0.847 ± 0.642     | n.s.             | 0.972 ± 0.717  | 0.971 ± 0.634 | n.s. |

Values are presented as mean ± SD.

n.s. = not significant.

Fig.1. Association between serum levels of anti-apolipoprotein B autoantibodies and atherosclerosis in patients with diabetes

The serum levels of anti-apolipoprotein B autoantibodies in the IgG or IgM class were compared between the atherosclerotic (+) or non-atherosclerotic (−) group (n = 29 and 60, respectively).
considerably low LDL-C levels identified that diabetes mellitus, hypertension, low HDL-C levels, and high apo B levels were independent risk factors\(^27\). It is conceivable that there are unknown risk factors in addition to these risk factors.

Recently, the role of immune response by autoantibodies against self-antigens in the pathogenesis of atherosclerosis has been the focus of many studies. Several immunohistochemical studies have revealed that oxidized LDL epitopes, anti-oxidized LDL autoantibodies, and T cells recognizing oxidized LDL were found in atherosclerotic plaques\(^28\). These Th1 immune responses to self-antigens could accelerate the process of atherosclerosis. On the contrary, anti-atherosclerotic immune responses induced by autoantibodies have been reported by some studies; for example, reduced atherosclerosis was found in immunized animals with anti-LDL or anti-apo B-100 antibodies\(^29\). Circulating autoantibodies against oxidized LDL are commonly detected in almost all individuals\(^30\). These immunogenic targets are generated from lipids and apolipoproteins contained in the oxidized

| Statin (+) | Class | Atherosclerosis | | | |
|-----------|-------|-----------------|---|---|---|
| N-45      | IgG   | 0.306 ± 0.281   | 0.223 ± 0.178 | 0.056 |
|           | IgM   | 0.727 ± 0.397   | 0.769 ± 0.804 | n.s. |
| MDA-45    | IgG   | 0.510 ± 0.345   | 0.360 ± 0.284 | <0.05 |
|           | IgM   | 1.009 ± 0.541   | 0.848 ± 0.522 | n.s. |
| N-210     | IgG   | 0.357 ± 0.287   | 0.282 ± 0.236 | 0.092 |
|           | IgM   | 0.745 ± 0.444   | 0.736 ± 0.684 | n.s. |
| MDA-210   | IgG   | 1.067 ± 1.257   | 0.446 ± 0.285 | <0.05 |
|           | IgM   | 1.077 ± 0.684   | 0.950 ± 0.823 | n.s. |

| Statin (-) | Class | Atherosclerosis | | | |
|------------|-------|-----------------|---|---|---|
| N-45       | IgG   | 0.217 ± 0.152   | 0.145 ± 0.050 | n.s. |
|            | IgM   | 0.737 ± 0.504   | 0.641 ± 0.221 | n.s. |
| MDA-45     | IgG   | 0.368 ± 0.239   | 0.231 ± 0.098 | n.s. |
|            | IgM   | 0.943 ± 0.681   | 0.768 ± 0.323 | n.s. |
| N-210      | IgG   | 0.262 ± 0.170   | 0.189 ± 0.069 | n.s. |
|            | IgM   | 0.764 ± 0.584   | 0.655 ± 0.341 | n.s. |
| MDA-210    | IgG   | 0.551 ± 0.675   | 0.281 ± 0.130 | n.s. |
|            | IgM   | 0.966 ± 0.630   | 0.636 ± 0.253 | n.s. |

Values are presented as mean ± SD. n.s. = not significant.

between circulating anti-apo B-100 autoantibodies and the clinical parameters in 90 Japanese diabetic patients with or without CVD. The serum levels of IgG class anti-apo B-100 antibodies were significantly lower in patients with CVD than in those without CVD, but there was no correlation between autoantibody levels and any diabetic microangiopathy. Even in statin-treated patients with diabetes (\(n=52\)), serum levels of IgG class anti-apo B-100 autoantibodies were significantly lower in those with CVD complications.

Numerous large-scale trials have demonstrated that statin therapy reduces the rates of primary and secondary cardiovascular events. However, several statin-treated patients continue to experience life-threatening vascular events, usually described as “residual risk.” An important effect of statin treatment is the reduction of LDL-C, which is clearly associated with decreased cardiovascular events and plaque regression. However, statin-mediated risk reduction was only about 30%, indicating the presence of unidentified residual risks other than LDL-C\(^3\). Analysis of patients whose cardiac plaque progressed despite considerably low LDL-C levels identified that diabetes mellitus, hypertension, low HDL-C levels, and high apo B levels were independent risk factors\(^27\). It is conceivable that there are unknown risk factors in addition to these risk factors.

Recently, the role of immune response by autoantibodies against self-antigens in the pathogenesis of atherosclerosis has been the focus of many studies. Several immunohistochemical studies have revealed that oxidized LDL epitopes, anti-oxidized LDL autoantibodies, and T cells recognizing oxidized LDL were found in atherosclerotic plaques\(^28\). These Th1 immune responses to self-antigens could accelerate the process of atherosclerosis. On the contrary, anti-atherosclerotic immune responses induced by autoantibodies have been reported by some studies; for example, reduced atherosclerosis was found in immunized animals with anti-LDL or anti-apo B-100 antibodies\(^29\). Circulating autoantibodies against oxidized LDL are commonly detected in almost all individuals\(^30\). These immunogenic targets are generated from lipids and apolipoproteins contained in the oxidized...
The correlation between atherosclerosis and serum autoantibody levels against p45 or p210 has been investigated in previous reports. Serum IgGMDA-45 and IgGN-210 levels were significantly lower in patients with coronary artery disease. IgGMDA-210 levels were also negatively correlated with coronary plaque size, although further investigation is necessary. In addition, a positive correlation was observed between IgG autoantibodies and HbA1c levels. In patients with type 1 diabetes, an inverse correlation between the levels of the anti-oxLDL antibody and HbA1c was reported, but their mechanism of action remains to be elucidated.

In this study, serum LDL-C levels were negatively correlated with anti-apo B autoantibody levels, especially with the IgM class autoantibody against p45 and p210, but not with the IgG-class. The negative correlation between IgM autoantibodies and LDL-C levels was observed more strongly in the subgroup without statin treatment. Previous reports suggested that statin treatment itself might lead to various alterations in the serum levels of anti-oxLDL autoantibody by modulating adaptive immunity.

We observed that IgM class antibodies (N-45, MDA-45, N-210, and MDA-210) were correlated with LDL-C and TC, as well as HDL-C levels. Because apo B-100 is a major apolipoprotein on LDL particle, the correlation between LDL-C and anti-apo B autoantibodies can be derived from direct effects of the autoantibodies on LDL metabolism. On the contrary, previous reports have shown the mutual correlation between LDL and HDL metabolism, suggesting that an indirect effect of anti-apo B-100 autoantibodies on HDL metabolism. The decreased production and increased catabolism of apo A-1 were reported in familial hypercholesterolemia (FH). Similarly, defective apo B-100 affected both catabolism and production rate of apo A-1 in patients with familial defective apo B-100 (FDB). Although the cross-talk mechanism between LDL and HDL metabolism is still unclear, anti-apo B-100 autoantibodies could influence not only LDL metabolism but also HDL metabolism.

This study has some limitations. We conducted a cross-sectional, single-center study and the sample size was markedly small. We did not measure hydroxyxenonal- and methylglyoxal-modified forms of anti-apo B-100 antibodies. Further larger case-control studies are required to clarify the detailed mechanism.
are necessary to determine whether the anti-apo B-100 autoantibodies IgGMDA-45 and IgGMDA-210 are risk factor of CVD in patients with diabetes.

In conclusion, low serum levels of the anti-apo B-100 autoantibodies IgGMDA-45 and IgGMDA-210 could be a CVD risk factor in Japanese patients with diabetes. The measurement of these autoantibodies might be useful to identify patients with high residual CVD risk under statin treatment.

Contribution Statement

H.Y., M.K., I.K., M.I. and Y.M. performed research and clinical data analysis; T.S., Y.C., and K.H. performed clinical work and analysis; H.Y., K.H., and S.K. designed research, and H.Y. and S.K. wrote the manuscript.

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Disclosure Summary

There are no financial conflicts of interest to disclose.

References

1) WHO: Global report on diabetes. 2016
2) Naslafkih A and Sestier F: Diabetes mellitus related morbidity, risk of hospitalization and disability. J Insur Med, 2003; 35: 102-113
3) Cholesterol Treatment Trialists C, Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keetch A, Simes J and Collins R: Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet, 2010; 376: 1670-1681
4) Ross R: Atherosclerosis—an inflammatory disease. N Engl J Med, 1999; 340: 115-126
5) Koltsouva EK, Hedrick CC and Ley K: Myeloid cells in atherosclerosis: a delicate balance of anti-inflammatory and proinflammatory mechanisms. Curr Opin Lipidol, 2013; 24: 371-380
6) Ley K, Miller YI and Hedrick CC: Monocyte and macrophage dynamics during atherogenesis. Arterioscler Thromb Vasc Biol, 2011; 31: 1506-1516
7) Tse K, Tse H, Sidney J, Sette A and Ley K: T cells in atherosclerosis. Int Immunol, 2013; 25: 615-622
8) Lichtman AH, Binder CJ, Tsimikas S and Witztum JL: Adaptive immunity in atherogenesis: new insights and therapeutic approaches. J Clin Invest, 2013; 123: 27-36
9) Witztum JL and Lichtman AH: The influence of innate and adaptive immune responses on atherosclerosis. Annu Rev Pathol, 2014; 9: 73-102
10) Caligiuri G, Nicoletti A, Poirier B and Hansson GK: Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J Clin Invest, 2002; 109: 745-753
11) Major AS, Fazio S and Linton MF: B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler Thromb Vasc Biol, 2002; 22: 1892-1898
12) Doran AC, Lipinski MJ, Oldham SN, Garney JC, Campbell KA, Skafien MD, Cutchins A, Lee DJ, Glover DK, Kelly KA, Galkina EV, Ley K, Witztum JL, Tsimikas S, Bender TP and McNamara CA: B-cell aortic homing and atheroprotection depend on Id3. Circ Res, 2012; 110: e1-e12
13) Kimura T, Tse K, Sette A and Ley K: Vaccination to modulate atherosclerosis. Autoimmunity, 2015; 48: 152-160
14) Fredrikson GN, Hedblad B, Berglund G, Alm R, Ares M, Cerneck B, Chyu KY, Shah PK and Nilsson J: Identification of immune responses against aldehyde-modified peptide sequences in apoB associated with cardiovascular disease. Arterioscler Thromb Vasc Biol, 2003; 23: 872-878
15) Steinberg D and Witztum JL: Oxidized low-density lipoprotein and atherosclerosis. Arterioscler Thromb Vasc Biol, 2010; 30: 2311-2316
16) Wick G, Jakic B, Buszko M, Wick MC and Grundtman C: The role of heat shock proteins in atherosclerosis. Nat Rev Cardiol, 2014; 11: 516-529
17) Grundtman C, Kreutmayer SB, Almanzar G, Wick MC and Wick G: Heat shock protein 60 and immune inflammatory responses in atherosclerosis. Arterioscler Thromb Vasc Biol, 2011; 31: 960-968
18) Ravandi A, Bookholdt SM, Mallat Z, Talmud PJ, Kastelein JJ, Wareham NJ, Miller ER, Benessiano J, Tedgui A, Witztum JL, Khaw KT and Tsimikas S: Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. J Lipid Res, 2011; 52: 1829-1836
19) Karvonen J, Paivansalo M, Kesiemi NY and Horkko S: Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. Circulation, 2003; 108: 2107-2112
20) Shapiro MD and Fazio S: Apolipoprotein B-containing lipoproteins and atherosclerotic cardiovascular disease. F1000Res, 2017; 6: 134
21) Wilkinson CP, Ferris FL, 3rd, Klein RE, Lee PP, Agardh CD, Davis M, Dills D, Kimpik A, Pararajasegaram R, Verdaguer JT and Global Diabetic Retinopathy Project G: Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology, 2003; 110: e1-12
22) Haneda M, Utsunomiya K, Koya D, Babazono T, Moriya T, Makino H, Kimura K, Suzuki Y, Wada T, Ogawa S, Inaba M, Kanno Y, Shigematsu T, Masakane I, Tsuchiya K, Honda K, Ichikawa K, Shide K and Joint Committee on Diabetic N: A new Classification of Diabetic Nephropathy 2014: a report from Joint Committee on Diabetic Nephropathy. J Diabetes Investig, 2015; 6: 242-246
23) Palinski W, Yla-Herttuala S, Rosenfeld ME, Butler SW, Socher SA, Parthasarathy S, Curtiss LK and Witztum JL: Antibodies to oxidized low density lipoprotein. Arteriosclerosis, 1990; 10: 325-335

24) Group. SSSS: Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet, 1994; 344: 1383-1389

25) LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK and Treating to New Targets I: Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med, 2005; 352: 1425-1435

26) Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ and Group JS: Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med, 2008; 359: 2195-2207

27) Bayturan O, Kapadia S, Nicholls SJ, Tuzcu EM, Shao M, Uno K, Shreevatsa A, Lavoie AJ, Wolksi K, Schenk C, P and Nissen SE: Clinical predictors of plaque progression despite very low levels of low-density lipoprotein cholesterol. J Am Coll Cardiol, 2010; 55: 2736-2742

28) Gistera L and Hansson GK: The immunology of atherosclerosis. Nat Rev Nephrol, 2017; 13: 368-380

29) Chyu KY, Dimayuga PC and Shah PK: Vaccine against arteriosclerosis: an update. Ther Adv Vaccines, 2017; 5: 39-47

30) Tinahones FJ, Gomez-Zumaquero JM, Garrido E, Garcia-Fuentes E, Rojo-Martinez G, Esteve I, Ruiz de Adana MS, Cardona F and Soriguier F: Influence of age and sex on levels of anti-oxidized LDL antibodies and anti-LDL immune complexes in the general population. J Lipid Res, 2005; 46: 452-457

31) Palinski W and Witztum JL: Immune responses to oxidative neoepitopes on LDL and phospholipids modulate the development of atherosclerosis. J Intern Med, 2000; 247: 371-380

32) Boullier A, Hamon M, Walters-Laporte E, Martin-Nizart F, Mackereel R, Fruchart JC, Bertrand M and Duriez P: Detection of autoantibodies against oxidized low-density lipoproteins and of IgG-bound low density lipoproteins in patients with coronary artery disease. Clin Chim Acta, 1995; 238: 1-10

33) Festa A, Kopp HP, Schernthaner G and Menzel EJ: Autoantibodies to oxidised low density lipoproteins in IDDM are inversely related to metabolic control and microvascular complications. Diabetologia, 1998; 41: 350-356

34) Hulthe J, Bokemark L and Fagerberg B: Antibodies to oxidized LDL in relation to intima-media thickness in carotid and femoral arteries in 58-year-old subjectively healthy men. Arterioscler Thromb Vasc Biol, 2001; 21: 101-107

35) Rouhi RP, van Oostenbrugge RJ, Theunissen RO, Knotternus IL, Staals J, Henskens LH, Kroon AA, de Leeuw PW, Lodder J, Tervaert JW and Damoiseaux JG: Autoantibodies against oxidized low-density lipoprotein in cerebral small vessel disease. Stroke, 2010; 41: 2687-2689

36) Engelbertsen D, Anand DV, Fredrikson GN, Hopkins D, Corder R, Shah PK, Lahiri A, Nilsson J and Bengtsson E: High levels of IgM against methylglyoxal-modified apolipoprotein B100 are associated with less coronary artery calcification in patients with type 2 diabetes. J Intern Med, 2012; 271: 82-89

37) Fagerberg B, Pahl Gullberg U, Alm R, Nilsson J and Fredrikson GN: Circulating autoantibodies against the apolipoprotein B-100 peptides p45 and p210 in relation to the occurrence of carotid plaques in 64-year-old women. PLoS One, 2015; 10: e0120744

38) Fredrikson GN, Anand DV, Hopkins D, Corder R, Alm R, Bengtsson E, Shah PK, Lahiri A and Nilsson J: Associations between autoantibodies against apolipoprotein B-100 peptides and vascular complications in patients with type 2 diabetes. Diabetologia, 2009; 52: 1426-1433

39) Fredrikson GN, Hedblad B, Berglund G, Alm R, Nilsson JA, Schiopu A, Shah PK and Nilsson J: Association between IgM against an aldehyde-modified peptide in apolipoprotein B-100 and progression of carotid disease. Stroke, 2007; 38: 1495-1500

40) Goncalves I, Gronholt ML, Soderberg I, Ares MP, Nordestgaard BG, Bentzon JF, Fredrikson GN and Nilsson J: Humoral immune response against defined oxidized low-density lipoprotein antigens reflects structure and disease activity of carotid plaques. Arterioscler Thromb Vasc Biol, 2005; 25: 1250-1255

41) McLeod O, Silveira A, Fredrikson GN, Gertow K, Baldassarre D, Veglia F, Sennblad B, Strawbridge RJ, Larsson M, Leander K, Gigante G, Kauhanen J, Rauramaa R, Smit AJ, Mannanino E, Giral P, Humphries SE, Tremoli E, de Faire U, Ohrvik J, Nilsson J and Hamsten A: Plasma autoantibodies against apolipoprotein B-100 peptide 210 in subclinical atherosclerosis. Atherosclerosis, 2014; 232: 242-248

42) Sjogren P, Fredrikson GN, Samnegard A, Ericsson CG, Ohrvik J, Fisher RM, Nilsson J and Hamsten A: High plasma concentrations of autoantibodies against native peptide 210 of apoB-100 are related to less coronary atherosclerosis and lower risk of myocardial infarction. Eur Heart J, 2008; 29: 2218-2226

43) Zhang X, Zhang X, Lei M, Lin Y, Megson IL, Wei J, Yu B and Jin Y: Detection of circulating IgG antibodies to apolipoprotein B100 in acute myocardial infarction. FEBS Open Bio, 2015; 5: 712-716

44) Fredrikson GN, Soderberg I, Lindholm M, Dimayuga P, Chyu KY, Shah PK and Nilsson J: Inhibition of atherosclerosis in apoE-null mice by immunization with apoB-100 peptide sequences. Arterioscler Thromb Vasc Biol, 2003; 23: 879-884

45) Fredrikson GN, Schiopu A, Berglund G, Alm R, Shah PK and Nilsson J: Autoantibody against the amino acid sequence 661-680 in apoB-100 peptide 210 of apoB-100 peptide 210 in acute myocardial infarction. FEBS Open Bio, 2015; 5: 712-716

46) Zeng Z, Cao B, Guo X, Li W, Li S, Chen J, Zhou W, Zheng C and Wei Y: Apolipoprotein B-100 peptide 210 antibody inhibits atherosclerosis by regulation of macrophages that phagocytize oxidized lipid. Am J Transl Res, 2018; 10: 1817-1828

47) Reblin T, Meyer N, Labeur C, Henne-Bruns D and
Beisiegel U: Extraction of lipoprotein(a), apo B, and apo E from fresh human arterial wall and atherosclerotic plaques. Atherosclerosis, 1995; 113: 179-188
48) Fraley AE, Schwartz GG, Olsson AG, Kinlay S, Szarek M, Rifai N, Libby P, Ganz P, Witztum JL, Tsimikas S and Investigators MS: Relationship of oxidized phospholipids and biomarkers of oxidized low-density lipoprotein with cardiovascular risk factors, inflammatory biomarkers, and effect of statin therapy in patients with acute coronary syndromes: Results from the MIRACL (Myocardial Ischemia Reduction With Aggressive Cholesterol Lowering) trial. J Am Coll Cardiol, 2009; 53: 2186-2196
49) Goncalves I, Cherfan P, Soderberg I, Nordin Fredrikson G and Jonsson I: Effects of simvastatin on circulating autoantibodies to oxidized LDL antigens: relation with immune stimulation markers. Autoimmunity, 2009; 42: 203-208
50) Laczik R, Szodoray P, Veres K, Szomjak E, Csiipo I, Sipka S, Jr., Shoefeld Y, Szekanecz Z and Soltesz P: Assessment of IgG antibodies to oxidized LDL in patients with acute coronary syndrome. Lupus, 2011; 20: 730-735
51) Resch U, Tatzber F, Budinsky A and Sinzinger H: Reduction of oxidative stress and modulation of autoantibodies against modified low-density lipoprotein after rosuvastatin therapy. Br J Clin Pharmacol, 2006; 61: 262-274
52) Zhang B, Noda K, Matsunaga A, Kumagai K and Saku K: A comparative crossover study of the effects of fluvastatin and pravastatin (FP-COS) on circulating autoantibodies to oxidized LDL in patients with hypercholesterolemia. J Atheroscler Thromb, 2005; 12: 41-47
53) Korpinen E, Groop PH, Akerblom HK and Vaarala O: Immune response to glycated and oxidized LDL in IDDM patients with and without renal disease. Diabetes Care, 1997; 20: 1168-1171
54) Schafer JR, Rader DJ, Ikewaki K, Fairwell T, Zech LA, Kindt MR, Davignon J, Gregg RE and Brewer HB, Jr.: In vivo metabolism of apolipoprotein A-I in a patient with homozygous familial hypercholesterolemia. Arterioscler Thromb, 1992; 12: 843-848
55) Schafer JR, Winkler K, Schweer H, Hoffmann MM, Soufi M, Scharnagl H, Maisch B, Wieland H, Steinmetz A and Marz W: Increased production of HDL ApoA-I in homozygous familial defective ApoB-100. Arterioscler Thromb Vasc Biol, 2000; 20: 1796-1799
**Supplemental Table 1.** Medical treatment of the patients

|                          | n (%)   |
|--------------------------|---------|
| Statins                  | 52 (58%)|
| Fibrates                 | 8 (9%)  |
| Anti-diabetic agents     |         |
| OHA only                 | 51 (57%)|
| Insulin only             | 13 (14%)|
| OHA + Insulin            | 12 (13%)|
| Anti-hypertensive agents | 57 (63%)|
| Anti-platelet agents     | 28 (31%)|

**Supplemental Table 2.** Titres of antibodies against apo B-100

|               | IgG      | IgM      |
|---------------|----------|----------|
| N-45          | 0.244 ± 0.210 | 0.726 ± 0.516 |
| MDA-45        | 0.404 ± 0.306 | 0.920 ± 0.560 |
| N-210         | 0.295 ± 0.229 | 0.734 ± 0.526 |
| MDA-210       | 0.682 ± 0.887 | 0.961 ± 0.664 |

**Supplemental Fig. 1.** Correlation of the titres of native and MDA-modified apo B-100 autoantibodies

The serum levels of anti-apo B-100 autoantibodies against native and MDA-modified apo B-100 autoantibodies in the IgG or IgM class were compared.
### Supplemental Table 3. Correlation between autoantibodies against apolipoprotein B-100 and the clinical parameters

| Class  | IgG     | IgM     | Age   | BMI   | FBS   | HbA1c | LDL-C | HDL-C | TC    |
|--------|---------|---------|-------|-------|-------|-------|-------|-------|-------|
| N-45   | n.s.    | n.s.    | n.s.  | n.s.  | 0.230* | n.s.  | n.s.  | n.s.  | n.s.  |
| MDA-45 | n.s.    | n.s.    | n.s.  | n.s.  | 0.185  | -0.219* | n.s.  | n.s.  | n.s.  |
| N-210  | n.s.    | n.s.    | n.s.  | n.s.  | 0.300*** | n.s.  | n.s.  | n.s.  | n.s.  |
| MDA-210| n.s.    | n.s.    | n.s.  | n.s.  | 0.301* | n.s.  | -0.182| n.s.  | n.s.  |

Values are presented as Spearman's correlation coefficient (ρ)

n.s., not significant

*ρ < 0.05, **ρ < 0.01, ***ρ < 0.005

### Supplemental Fig. 2. Association between serum levels of anti-apolipoprotein E autoantibodies and atherosclerosis in the diabetic patients

The serum levels of anti-apolipoprotein E autoantibodies in the IgG or IgM class were compared between the atherosclerotic (+) or non-atherosclerotic (−) group (n=29 and 60, respectively).
**Supplemental Table 4.** Relationship of the autoantibodies with HbA1c or LDL-C according to the intake of statins

| Class | IgG | IgM | HbA1c | LDL-C |
|-------|-----|-----|-------|-------|
|       |     |     | statin (−) | statin (+) | statin (−) | statin (+) |
| N-45  | n.s. | 0.231 | n.s. | −0.358* | n.s. | n.s. |
|       | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| MDA-45 | 0.319 | n.s. | n.s. | −0.410* | n.s. | n.s. |
|       | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| N-210  | n.s. | 0.337* | n.s. | −0.473** | n.s. | n.s. |
|       | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| MDA-210 | n.s. | n.s. | n.s. | −0.403* | n.s. | n.s. |
|       | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

Values are presented as Spearman's correlation coefficient (ρ)

Statin (−), n=37; Statin (+), n=52

n.s. = not significant, *ρ < 0.05, **ρ < 0.01