Changes in serum levels of angiopoietin-like protein-8 and glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 after ezetimibe therapy in patients with dyslipidemia

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Introduction

Ezetimibe reduces serum low-density lipoprotein cholesterol (LDL-C) levels by inhibiting the intestinal absorption of cholesterol through the blockade of Niemann-Pick C1 Like1[1, 2, 3]. In the ZENITH trial, we reported that ezetimibe significantly decreased serum LDL-C levels and may be especially effective in dyslipidemic (DL) males with metabolic syndrome (MetS) and relatively lower ratios of LDL-C to high-density lipoprotein cholesterol (HDL-C)[4].

Angiopoietin-like protein-8 (ANGPTL8) is one of a group of secreted glycoproteins composed of eight members (ANGPTL1-8)[5]. ANGPTL8 has been reported to be associated with lipid metabolism[6-11]. ANGPTL8 levels were significantly positively correlated with triglyceride (TG) and LDL-C levels, and negatively correlated with HDL-C levels[12, 13]. ANGPTL8 promotes ANGPTL3 cleavage and binds to the N-terminus of ANGPTL3. The complex of ANGPTL8 and the N-terminus of ANGPTL3 inhibits lipoprotein lipase (LPL) and suppresses lipolysis of chylomicrons[5]. Abu-Farha *et al.* reported that the ANGPTL8 levels in a MetS group were higher than those in a non-MetS group[14], whereas ANGPTL8 levels at baseline in a newly developed MetS group were lower than those in a non-MetS group during 3.5 years of follow-up[15]. The ANGPTL8 level was inversely correlated with the incidence of MetS even after multivariate adjustments[15]. Thus, the association of the ANGPTL8 level with lipid metabolism in MetS remains unclear[16].

Glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 **(**GPIHBP1) associated with LPL, which is inhibited by the complex of ANGPTL8 and ANGPTL3, is also involved in lipid metabolism. GPIHBP1 is positively correlated with LPL and inversely correlated with remnant-like particle cholesterol (RLP-C) and small-dense LDL-C[17]. GPIHBP1 is a protein in lymphocyte antigen 6 and an essential partner of LPL[18]. GPIHBP1 captures LPL

on the abluminal plasma membrane of capillary endothelial cells, and stabilizes the structure of LPL.

However, the associations between the serum lipid profile and ANGPTL8 or GPIHBP1 levels after ezetimibe therapy in DL patients remain to be elucidated. Therefore, using blood samples from the ZENITH trial, we investigated the effects of ezetimibe on ANGPTL8 and GPIHBP1 in all DL patients, and also compared patients with MetS to those without MetS.

Methods

Study design

The ZENITH trial was prospectively performed from January 2009 to August 2011 at Fukuoka University Hospital and its related hospitals in the Kyushu area of Japan[4]. Untreated DL patients or patients who had been pretreated with statin and had undergone a 4-week washout period were enrolled. Serum samples were collected before and after 16 weeks of treatment with 10 mg/day ezetimibe. Written informed consent was obtained from all patients. In this study, we excluded some patients from the ZENITH trial due to an insufficient volume of blood sample, and finally analyzed ANGPTL8 and GPIHBP1 levels in 38 patients. Various parameters were investigated before and after 16 weeks of ezetimibe treatment in all patients. In addition, the patients were also divided into metabolic syndrome (MetS) (n=22) and Non-MetS (n=16) groups, and various parameters were compared between these groups. The procedures were performed in accordance with the Declaration of Helsinki and the ethical standards of the Independent Review Board of Fukuoka University. This investigation was approved by the Independent Review Board of Fukuoka University (2017M160).

Measurements of various parameters

Age, gender, body mass index (BMI), and history of hypertension (HTN), diabetes mellitus (DM), and coronary artery disease (CAD) were evaluated. BMI was calculated as weight/height^2 (kg/m^2). MetS was defined based on the following diagnostic criteria[19]. In Japan, MetS is defined as a waist circumference over 85 centimeters (males) or 90 centimeters (females) and two or more of the following three criteria: the presence of HTN (systolic blood pressure over 130 mmHg and/or diastolic blood pressure over 85 mmHg), DM (fasting blood sugar over 100 mg/dl), or DL (fasting TG level over 150 mg/dl and/or fasting HDL-C level less than 40 mg/dl). In this study, we also defined HTN or DM based on appropriate medical treatment. Serum levels of white blood cells, high-sensitivity C-reactive protein, blood urea nitrogen, creatinine, aspartate aminotransferase, alkaline phosphatase, total cholesterol (TC), TG, LDL-C, HDL-C, RLP-C, and markers of cholesterol synthesis (lathosterol/TC) and absorption (campesterol/TC, sitosterol/TC) were evaluated at the Fukuoka University Hospital Laboratory Unit or by SRL Inc. (Tokyo, Japan), as reported previously[4]. ANGPTL8 and GPIHBP1 were detected using a Human GPIHBP1 Assay Kit (#27179) and a Human ANGPTL8 Assay Kit (#27795) (Immuno-Biological Laboratories Co, Ltd, Gunma, Japan).

Statistical analyses

All data analyses were performed using the SAS (Statistical Analysis System) Software Package (Ver. 9.4, SAS Institute Inc., Cary, NC, USA) at Fukuoka University (Fukuoka, Japan). Continuous variables with a normal distribution were expressed as mean ± standard deviation and compared by Student's t-test. Continuous variables with a non-normal distribution were expressed as median (interquartile range) and compared by the Wilcoxon rank sum test.

Categorical variables were compared by a Chi-square analysis. Paired variables were analyzed by a paired t-test for continuous variables with a normal distribution and by a Wilcoxon signed rank test for continuous variables with a non-normal distribution. The Spearman Rank Correlation Coefficient was used to evaluate associations. We defined p value < 0.05 as significant.

Results

Patient characteristics at baseline in all patients and in the MetS and Non-MetS groups

Patient characteristics at baseline are shown in Table 1. Average age and BMI in all patients were 61±9 years and 26.3±3.4 kg/m^2, respectively. The percentages of (%) male, HTN, DM, and CAD in all patients were 61%, 87%, 58% and 13%, respectively. %DM in the MetS group was significantly higher than that in the Non-MetS group.

Changes in biochemical parameters between before and after ezetimibe treatment in all patients and in the MetS and Non-MetS groups

Changes in biochemical parameters between before and after ezetimibe treatment are shown in Table 1. Although blood urea nitrogen after treatment in the MetS group significantly decreased, there were no significant changes in white blood cells, high-sensitivity C-reactive protein, creatinine, aspartate transaminase or alanine transaminase in all patients or in the MetS and Non-MetS groups.

Changes in the lipid profile between before and after ezetimibe treatment in all patients and in the MetS and Non-MetS groups

Changes in the lipid profile between before and after ezetimibe treatment are shown in Figure 1. Before treatment, TG and RLP-C levels in the MetS group were significantly higher than those in the Non-MetS group. After 16 weeks of treatment, the levels of TC and LDL-C were significantly reduced in all patients and in the MetS and Non-MetS groups. The levels of HDL-C and TG in all patients and in the MetS and Non-MetS groups were not significantly changed after 16 weeks of treatment. The level of RLP-C after 16 weeks was significantly reduced in all patients and in the MetS group, but not in the Non-MetS group.

Changes in ANGPTL8 and GPIHBP1 between before and after ezetimibe treatment in all patients and in the MetS and Non-MetS groups

Changes in ANGPTL8 and GPIHBP1 between before and after ezetimibe treatment are shown in Figure 2. There were no significant differences in the levels of ANGPTL8 and GPIHBP1 at baseline among all patients and the MetS and Non-MetS groups. The level of ANGPTL8 in all patients was significantly reduced by 16 weeks of treatment with ezetimibe. Although the levels of ANGPTL8 in the MetS and Non-MetS groups decreased after treatment, these changes were not significant. The levels of GPIHBP1 in all patients and in the MetS and Non-MetS groups were significantly reduced by 16 weeks of treatment with ezetimibe.

Changes in the sterol/TC ratios after treatment with ezetimibe in all patients and in the MetS and non-MetS groups

Figure 3A-C shows the changes in the sterol/TC ratio after treatment with ezetimibe. Lathosterol/TC in the MetS group was significantly higher than that in the non-MetS group before treatment, whereas there were no significant differences in campesterol/TC or

sitosterol/TC between the MetS and non-MetS groups before treatment. Lathosterol/TC was significantly increased after treatment in all patients and in the MetS and non-MetS groups, and campesterol/TC and sitosterol/TC were significantly decreased.

The changes in ANGPTL8 and campesterol/TC before and after ezetimibe treatment (ΔANGPTL8 and Δcampesterol/TC) are shown in Figure 3D. ΔANGPTL8 was negatively associated with Δcampesterol/TC.

Associations between the lipid profile and ANGPTL8 or GPIHBP1 before ezetimibe treatment in all patients and in the MetS and Non-MetS groups

The associations between the lipid profile (LDL-C, HDL-C and TG) and ANGPTL8 before treatment with ezetimibe are shown in Figure 4. ANGPTL8 before treatment was significantly negatively associated with HDL-C and positively associated with TG in all patients (Figure 4B and 4C) and in the MetS group (Figure 4E and 4F), but not in the Non-MetS group (Figure 4H and 4I). There were no associations between ANGPTL8 and LDL-C in any of the groups (Figure 4A, 4D and 4G). In addition, GPIHBP1 was not associated with either LDL-C or RLP-C before treatment with ezetimibe (data not shown).

Discussion

This is the first investigation of whether ezetimibe changes serum ANGPTL8 and GPIHBP1 levels in DL patients and whether there are any differences between MetS and Non-MetS groups. Ezetimibe decreased serum levels of ANGPTL8 and GPIHBP1 in addition to reducing TC and LDL-C. In addition, ezetimibe significantly decreased RLP-C levels in the MetS group, but not in the Non-MetS group.

The most important finding in this study is that ezetimibe decreased serum levels of ANGPTL8 in all patient. This reduction of ANGPTL8 might be a newly recognized effect of ezetimibe therapy. Lower ANGPTL8 levels keep LPL activity high and accelerate the lipolysis of chylomicron[5]. In other words, high LPL activity is not necessary under a lower chylomicron condition as a result of the blockade of cholesterol uptake by ezetimibe, and subsequently ANGPTL8 levels should be increased. Thus, there is a discrepancy between this concept and our results regarding changes in ANGPTL8 levels. ANGPTL8 is predominantly expressed in the liver or white and brown fat tissues[16]. Ezetimibe might affect the reduction of ANGPTL8 in the liver. Ezetimibe is expected to act on the liver to improve nonalcoholic fatty liver disease, insulin sensitivity, and hepatic steatosis[20]. Altmann *et al*. reported that the liver expressed around 90% of the level of NPC1L1 messenger ribonucleic acid relative to that in the small intestine in human[21]. Ezetimibe might suppress ANGPTL8 production via NPC1L1 in the liver. In addition, the inhibition of cholesterol absorption by ezetimibe might induce the reduction of ANGPTL8, since ΔANGPTL8 was negatively associated with Δcampesterol/TC. Further studies will be needed to resolve this issue.

GPIHBP1 was significantly reduced after ezetimibe therapy in all patients, and in the MetS and Non-MetS groups. Ezetimibe reduces plasma chylomicrons[22] and ameliorates intestinal chylomicron overproduction[23] as a result of blockage of cholesterol uptake. GPIHBP1 would become unnecessary under ezetimibe treatment, because GPIHBP1 is needed to carry LPL under hyperchylomicronemia. Moreover, GPIHBP1-deficient mice were sensitive to cholesterol intake. The plasma TG levels in GPIHBP1-deficient mice fed a low-cholesterol diet were significantly higher than those in mice fed a high-cholesterol diet. In wild-type mice, a high-cholesterol diet had little or no effect on plasma TG levels.[24] Thus, the reduction of GPIHBP1 may be a result of

the pharmacological action of ezetimibe.

Next, we discuss the similarities and differences between the MetS and Non-MetS groups in this study. Before ezetimibe treatment, ANGPTL8 was significantly positively associated with TG and negatively associated with HDL-C in all patients and in the MetS group, but not in the Non-MetS group. Although the mechanism of the association between ANGPTL8 levels and lipid metabolism in MetS is unclear, our results at baseline were consistent with those in previous reports[12, 13]. Both the MetS and Non-MetS groups showed significant reductions of LDL-C and GPIHBP1 after treatment with ezetimibe. Although both groups showed a reduction in ANGPTL8 after treatment with ezetimibe, these reductions were not significant. Thus, ezetimibe treatment induced similar changes in LDL-C, ANGPTL8 and GPIHBP1 levels in both groups. On the other hand, the groups differed with respect to reductions in RLP-C after ezetimibe treatment. Ezetimibe significantly decreased RLP-C levels in the MetS group, but not in the Non-MetS group. Before treatment, RLP-C levels in the MetS group were significantly higher than those in the Non-MetS group. In this regard, ezetimibe exerted a stronger anti-atherogenic effect through the reduction of RLP-C levels in the MetS group than in the Non-MetS group.

This study has several limitations. First, the sample size was small. Notwithstanding this limitation, our results raise the possibility of new mechansims of ezetimibe therapy. Second, it would be helpful to understand the roles played by ANGPTL3 and LPL on the suppressive effect of ezetimibe on ANGPTL8. Third, although we set a 4-week washout period for patients in whom DL had been pretreated with statin, the legacy effect of statin treatment should be considered.

In conclusion, this is the first report to support the possibility of a new effect of ezetimibe

therapy. Ezetimibe significantly decreased the serum level of LDL-C, but not TG or HDL-C, while reducing ANGPTL8 and GPIHBP1. Ezetimibe significantly decreased RLP-C levels in the MetS group, but not in the Non-MetS group.

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Conflict of Interest

None to declare.

CRediT author statement

Akihito Ideishi**:** Conceptualization, Methodology, Validation. Yasunori Suematsu: Validation, Writing - Original Draft. Kohei Tashiro: Investigation, Validation. Hidetaka Morita*:* Investigation**.** Naoko Kumagai-Koyanagi: Validation. Takashi Kuwano**:** Visualization. Shin-ichiro Miura: Supervision, Writing- Reviewing and Editing.

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Figure legends

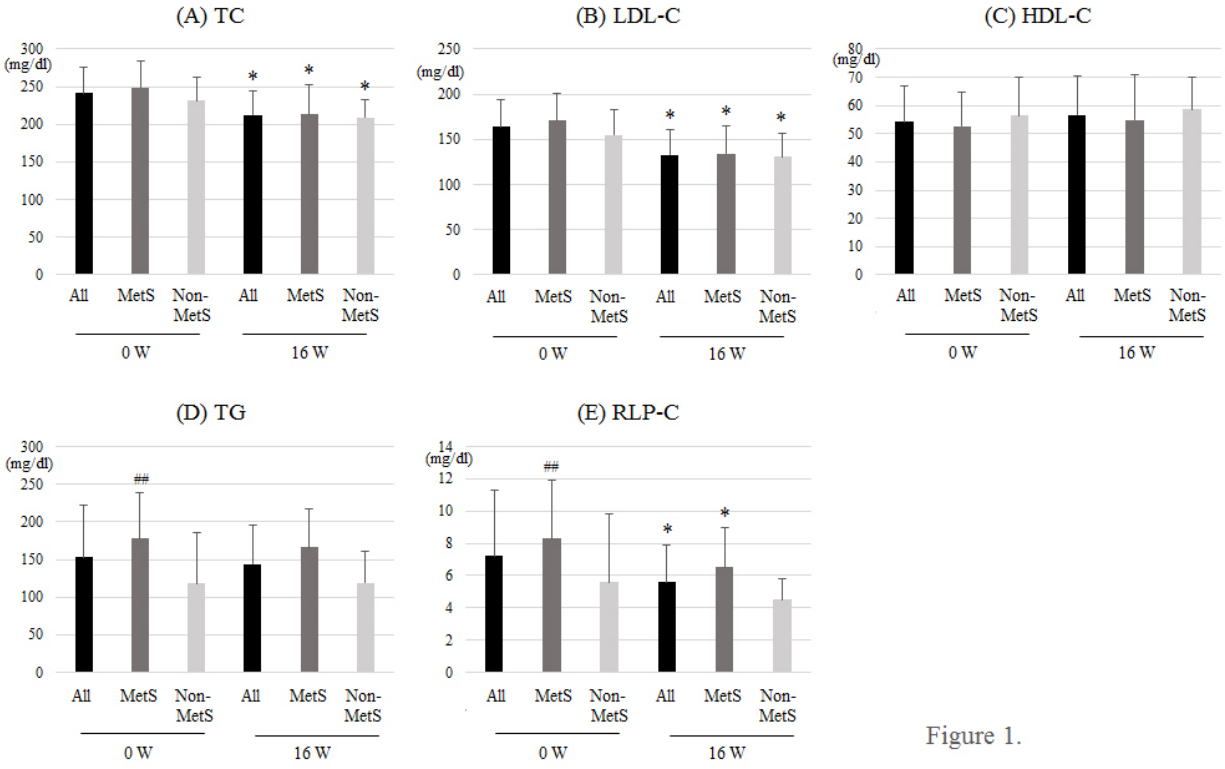

Figure 1.

Figure 1.

Serum levels of total cholesterol (TC) (A), low-density lipoprotein cholesterol (LDL-C) (B), high-density lipoprotein cholesterol (HDL-C) (C), triglyceride (TG) (D) and remnant-like particle cholesterol (RLP-C) (E) at weeks 0 and 16 after ezetimibe treatment in all patients and in the metabolic syndrome (MetS) and Non-MetS groups.

*$p<0.05$ vs. week 0 in each group. $^{\#\#}p<0.01$ vs. Non-MetS group.

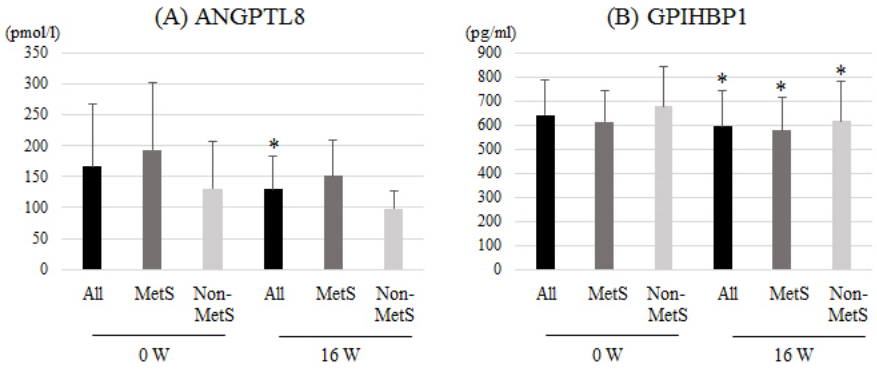

Figure 2.

Figure 2.

Serum levels of angiopoietin-like protein-8 (ANGPTL8) (A) and glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) (B) at weeks 0 and 16 after ezetimibe treatment in all patients and in the metabolic syndrome (MetS) and Non-MetS groups. *p<0.05 vs. week 0 in each group.

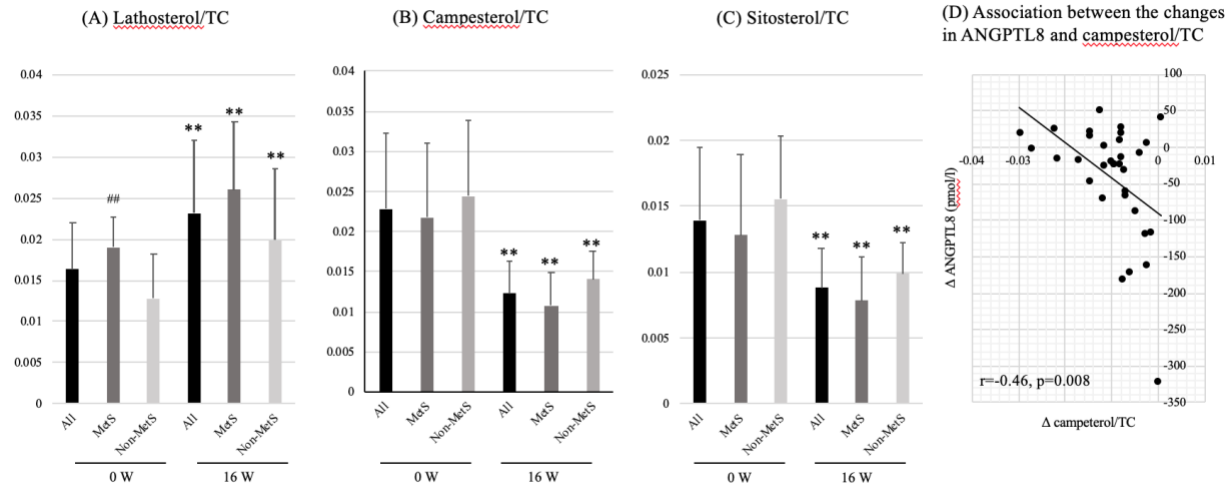

Figure 3.

Changes in the lathosterol/total cholesterol (TC) (A), campesterol/TC (B) or sitosterol/TC (C) ratios before and after treatment with ezetimibe in all patients and in the metabolic syndrome (MetS) and non-MetS groups.

Association between the changes in angiopoietin-like protein-8 (ANGPTL8) and campesterol/TC at weeks 0 and 16 after ezetimibe treatment (ΔANGPTL8 and Δcampesterol/TC) (D).

**p<0.01 vs. week 0 in each group. ##p<0.01 vs. Non-MetS group.

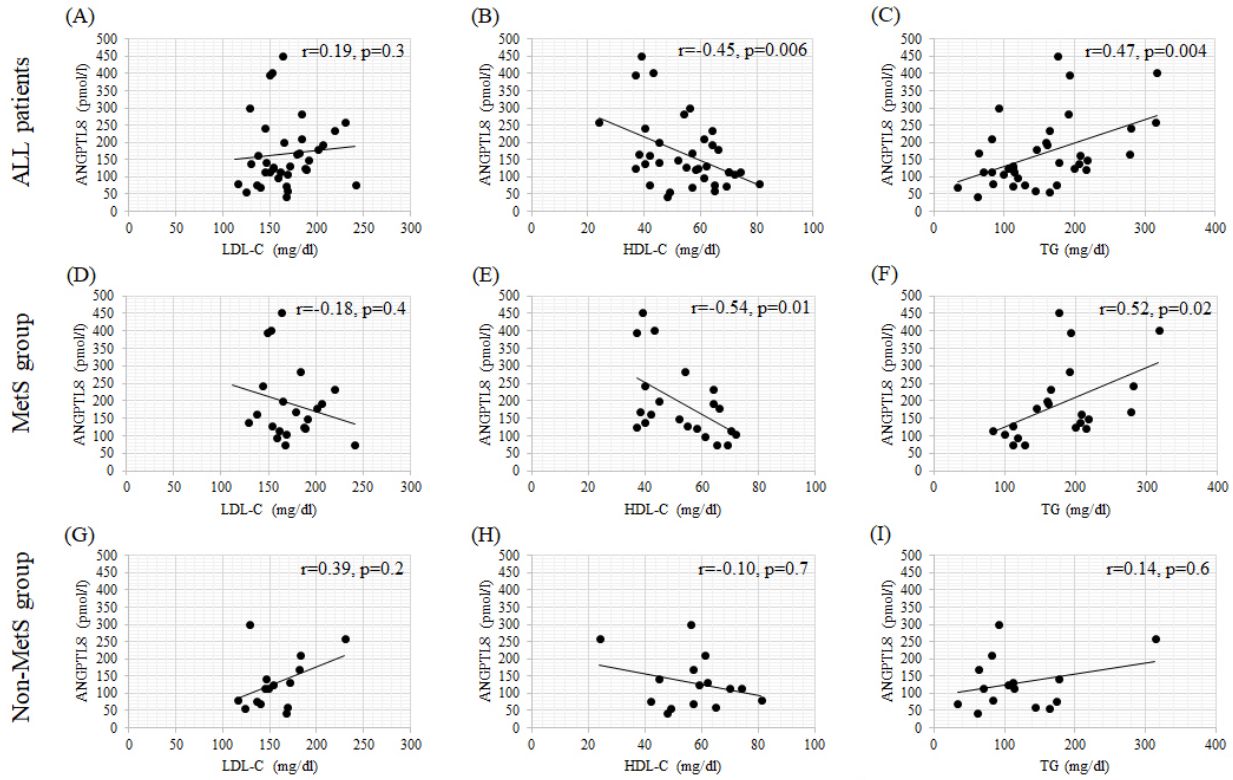

Figure 4.

Figure 4.

Associations of serum angiopoietin-like protein-8 (ANGPTL8) levels with low-density lipoprotein cholesterol (LDL-C) (A, D, G), high-density lipoprotein cholesterol (HDL-C) (B, E, H) or triglyceride (TG) (C, F, I) levels before ezetimibe treatment in all patients (A-C) and in the metabolic syndrome (MetS) (D-F) and Non-MetS groups (G-I).

Table 1. Patient characteristics and biochemical parameters in all patients, MetS and Non-MetS groups.

	All patients (n=38)		MetS group (n=22)		Non-MetS group (n=16)	
Weeks	0	16	0	16	0	16
Age, yrs	61±9		61±10		60±8	
Male, n (%)	23 (61)		16 (73)		7 (44)	
BMI, kg/m^2	26.3±3.4		27.0±3.3		25.1±3.3	
HTN, n (%)	32 (87)		18 (82)		14 (93)	
DM, n (%)	22 (58)		17 (77) ##		5 (31)	
CAD, n (%)	5 (13)		3 (14)		2 (13)	
White blood cell, /μl	5571±1527	5577±1527	5705±1383	5621±1098	4850 (4150-6300)	5525±1581
High-sensitivity C-reactive protein, μg/dl	646 (276-1350)	590 (308-1070)	1027±882	596 (296-1200)	517 (240-1335)	532 (334-896)
Blood urea nitrogen, mg/dl	13.6±3.4	13.1±3.4	13.8±3.2	12.8±3.5 *	13.4±3.6	13.6±3.3
Creatinine, mg/dl	0.79 (0.66-0.87)	0.78±0.15	0.79 (0.71-0.87)	0.80±0.13	0.76±0.18	0.76±0.17
Aspartate transaminase, IU/l	23 (19-31)	22 (18-30)	23 (19-31)	23 (19-36)	23 (19-27)	22 (18-25)
Alanine transaminase, IU/l	22 (17-38)	24 (18-43)	28 (19-47)	29 (18-53)	19 (16-36)	23 (17-30)

MetS, metabolic syndrome; BMI, body mass index; HTN, hypertension; DM, diabetes mellitus; CAD, coronary artery disease. *p<0.05 at week 0 in each group. ##p<0.01 vs. MetS group at week 0.

Erratum:

1. In Table 1 footnote: please change "##p < 0.01 vs. at week 0 in the MetS group" to "##p < 0.01 vs. at week 0 in the Non-MetS group".

2. The paragraph of 2.2: Measurements of various parameters in the Methods section, please change "aspartate aminotransferase, alkaline phosphatase" to "aspartate transaminase, alanine transaminase".

3. We added the unit in Figure 3. And in Figure 3 (B): The 16 weeks-averages in All, MetS, and Non-Mets groups were same as 0 week-data in each group by mistakes. The correct figure is attached by PPT.

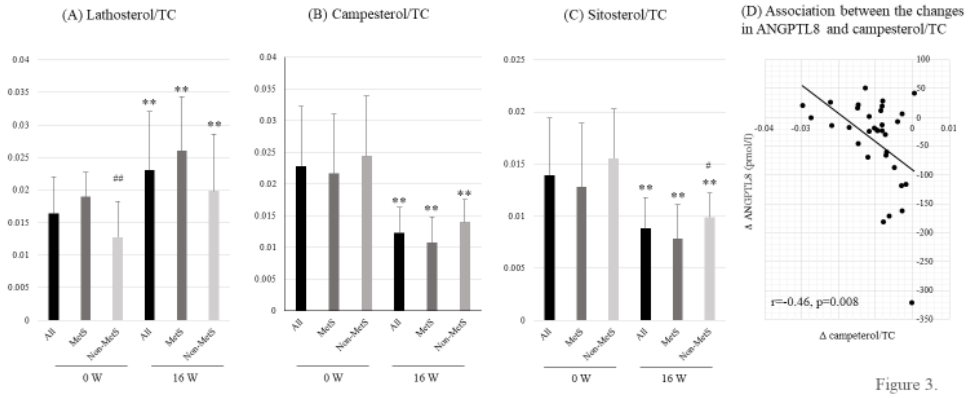
(A) Lathosterol/TC
(B) Campesterol/TC
(C) Sitosterol/TC
(D) Association between the changes in ANGPTL8 and campesterol/TC
All
MetS
Non-MetS
0 W
16 W
r=-0.46, p=0.008
Δ ANGPTL8 (pmol/l)
Δ campeterol/TC

Figure 3.