（博士論文表紙様式）

Increased plasma lipoprotein lipase activity in males with autism spectrum disorder

（　自閉スペクトラム症の男性では血漿中のリポ蛋白リパーゼが上昇している　）

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Increased plasma lipoprotein lipase activity in males with autism spectrum disorder

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ABSTRACT

Background: Autism spectrum disorder (ASD) is a neurodevelopmental disorder with complex genetics, characterized by impaired social communication and repetitive behaviors and interests. The involvement of lipid metabolism in ASD pathophysiology has been demonstrated in previous studies; however, the molecular mechanisms of abnormal lipid metabolism are not fully understood. A mutation in *Lipoprotein lipase* (*LPL*), which has central roles in lipid metabolism, has been identified in patients with ASD. We have reported that *Lpl* is downregulated in ASD model mice. Therefore, we explored the role of LPL in lipid metabolism in ASD patients.

Methods: We quantified LPL amount, LPL activity, and glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) amount in the plasma of ASD male subjects (n = 28) compared with typical development (TD) controls (n = 28), using enzyme-linked immunosorbent assay for LPL amount and fluorometric assays for LPL activity. We examined the correlations of plasma LPL with GPIHBP1 and clinical characteristic scores from the Autism Diagnostic Interview-Revised (ADI-R).

Results: There was higher LPL activity, but not LPL amount, in the plasma of ASD subjects compared with controls. Receiver operating characteristics analysis also demonstrated that pure LPL activity (LPL activity/LPL amount) is a useful indicator to distinguish ASD from TD controls. There were no correlations between plasma LPL and ADI-R scores; however, LPL activity was negatively correlated with GPIHBP1 levels in the plasma of ASD subjects.

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Conclusions: Our results demonstrate increased activity of plasma LPL, regulated by GPIHBP1, in ASD, providing novel insights into the lipid metabolism associated with ASD pathophysiology.

1. Introduction

ASD is a complex, pervasive, and heterogeneous neurodevelopmental disorder that is characterized by social communication deficits and restricted and repetitive behaviors (Gillott & Standen, 2007; Lai, Lombardo, & Baron-Cohen, 2014). Recent epidemiological studies reported that the prevalence of ASD in the USA was 1 in 54 children, with a greater proportion of boys (1 in 34) than girls (1 in 145) (Maenner et al., 2020). The diagnosis of ASD is solely based on behavioral characteristics because there are not yet any reliable biomarkers (Walsh, Elsabbagh, Bolton, & Singh, 2011). Although the etiology of ASD is thought to involve genetic, metabolic, immunological, and environmental factors, it remains poorly understood (Volkmar, Lord, Bailey, Schultz, & Klin, 2004).

Recent studies have demonstrated that serum levels of total cholesterol (CHO) lower than average in up to 20 % of ASD individuals (Tierney et al., 2006), and decreased amount of apolipoprotein B100 protein in the serum of children with ASD by proteomic analysis (Corbett et al., 2007). In addition, a decrease in CHO high-density lipoprotein (HDL) and an increase in total triglycerides (TG) have been reported in the plasma of children with ASD (Kim, Neggers, Shin, Kim, & Kim, 2010). Other studies have also demonstrated that Smith–Lemli–Opitz syndrome, which is characterized by impaired CHO synthesis, is associated with ASD (Bukelis, Porter, Zimmerman, & Tierney, 2007; Tierney et al., 2001). Furthermore, in adult males with Asperger's syndrome (AS), the levels of both total CHO and low-density lipoprotein (LDL) are elevated in the peripheral blood (Dziobek, Gold, Wolf, & Convit, 2007). In addition, proteome analysis studies using serum from adult subjects with AS also demonstrated alterations in proteins that are mostly involved in lipid transport and metabolism pathways (Schwarz et al., 2011; Steeb et al., 2014). Together, these studies suggest that lipid metabolism plays distinct roles in ASD pathology.

LPL is an enzyme responsible for the hydrolysis of core TG in chylomicrons and very low-density lipoprotein (VLDL) to produce chylomicron remnants and intermediate-density lipoprotein (IDL), respectively (Nilsson-Ehle, Garfinkel, & Schotz, 1980; Wang & Eckel, 2009). These products play central roles in the energy storage, metabolism, and transport of lipids such as lipoproteins. In addition, the results from several metabolomic and immunological studies using blood or saliva samples suggest that abnormal lipid metabolism in ASD is strongly associated with factors linked to LPL (Ngounou Wetie et al., 2014, 2015; West et al., 2014).

Furthermore, a mutation in *LPL* has been identified in the postmortem brains of patients with ASD (Li et al., 2014). We have also previously reported that *Lpl* gene expression is significantly downregulated in the prefrontal cortex of mice with a conditional knockout of *Foxp1*, an ASD-associated gene (Usui et al., 2017). Together, these findings suggest that LPL may be associated with ASD pathology; however, there are as yet no reports of studies demonstrating the relationship between LPL and ASD.

LPL is regulated at the transcriptional, posttranscriptional, and posttranslational levels in a tissue-specific manner. Nutrient states, hormonal levels, and protein levels all have divergent effects on the regulation of LPL (Wang & Eckel, 2009). In particular, GPIHBP1 is a crucial factor in the regulation of LPL function, because it binds LPL from within the subendothelial spaces and shuttles it to the capillary lumen (Davies et al., 2010); it also stabilizes the structure and catalytic activity of LPL (Mysling et al., 2016).

To improve our understanding of ASD pathophysiology, we therefore investigated LPL using peripheral plasma from male subjects with ASD. We revealed a trend toward significant increase in LPL activity, and a significant increase in pure LPL activity (LPL activity/LPL amount) in male ASD subjects compared with TD male controls. In contrast, there was no significant difference in the amount of LPL between these two groups. There were no correlations between plasma LPL and ADI-R domain scores. There was trend toward significance detected in correlations in that the greater the LPL activity observed, as well as the greater the LPL activity/LPL amount observed, the lower the GPIHBP1 levels in males with ASD, while this was not observed in individuals with TD. Our findings suggest that increased LPL activity may have an important relationship with the metabolic abnormalities of ASD, providing novel insights into lipid metabolism in ASD pathology.

2. Methods

2.1. Participants and diagnosis

We recruited 28 Japanese males with ASD (age = 11.5 ± 6.2 years (mean ± SD); range = 6.5–27.5 years) and 28 age- and sex-matched TD controls (age = 10.0 ± 3.1 years; range = 5.3–14.9 years) who lived in the Aichi, Gifu, Shizuoka, or Fukui prefectures. The diagnosis of ASD was made by an experienced child psychiatrist using the criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM-IV-TR) (Bell, 1994), based on clinical interviews and the Japanese version of the ADI-R (Tsuchiya et al., 2013). The ADI-R gathers comprehensive information regarding five domains of development. The ADI-R domain score A quantifies impairment in social interaction; domain score B quantifies in qualitative abnormalities in communication; domain score C quantifies restricted, repetitive, and stereotyped patterns of behaviors and interests; and domain score D quantifies abnormality of development evident at or before 36 months. The assessment of domain B was further divided into two types of assessments according to verbal skills of the examined individuals; domain BV (domain B for verbal communication) and domain BNV (domain B for non-verbal communication). In this study, we did not use the BNV domain score due to missing some deficiencies in data. In addition, we did not analyze domain D, since this is the summary code for evidence of abnormality within the

first 3 years. Thus, we only analyzed domain A, BV, and C scores for ADI-R. Higher scores indicate worse performance. We also used the Wechsler Intelligence Scale for Children, third edition (WISC-III), to evaluate intelligence quotients (IQs). Participants were excluded from the study if they had inflammation symptoms, liver disease, lipid metabolism abnormality, a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorder, or any additional psychiatric or neurological diagnosis. The Structured Clinical Interview for the DSM-IV (SCID) was also conducted to evaluate any personal or familial history of past or present mental illness. All subjects were drug-naive and were not taking any dietary supplements.

2.2. Blood sampling

Fasting blood samples were collected by venipuncture from all participants between 6:00 and 8:30 A.M. using plasma-collection tubes containing EDTA. After 30 min incubation at room temperature, the samples were centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was collected into microfuge tubes as 200 μL aliquots and stored at −80 °C until use. To exclude inflammatory diseases and lipid diseases, plasma C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (γ-GTP) were quantified using a routine clinical biochemistry automatic analyzer. All plasma samples were quantified in triplicate, and the mean values of the three measurements were used for analysis.

2.3. LPL assay

Quantification of LPL amounts (Machida et al., 2015) and LPL activity (Navab et al., 2013) were performed as previously described. Briefly, the amounts of plasma LPL were quantified with LPL latex (#50309, IBL, Gunma, Japan) according to the manufacturer's instructions, using a 7170 Clinical Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). This is a quantification method based on enzyme-linked immunosorbent assay (ELISA). In contrast, the plasma levels of LPL activity were fluorometrically quantified with an LPL Activity Assay Kit (# STA-610, Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions, using a SpectraMax M5 (Molecular Devices, San Jose, CA). This quantification method uses a fluorogenic triglyceride analog as a substrate for lipase, and measures the fluorescence intensity released when the substrate is cleaved by lipase. All plasma samples were quantified in triplicate, and the mean values of the three measurements were used for analysis.

2.4. GPIHBP1 assay

Quantification of GPIHBP1 was performed as previously described (Miyashita et al., 2018). Briefly, the amounts of plasma GPIHBP1 were quantified with a GPIHBP1 Assay Kit (#27179, IBL) according to the manufacturer's instructions, using a SpectraMax M5 (Molecular Devices, San Jose, CA). This quantification method is based on ELISA. All plasma samples were quantified in triplicate, and the mean values of the three measurements were used for analysis.

2.5. Statistical analysis

The data are presented as the mean ± standard deviation (SD). To evaluate any differences in continuous variables between subjects with TD and ASD, we used the Student's *t*-test if there were no significant differences in variance (as assessed by the *F*-test). If there were significant differences in variance as assessed by the *F*-test, the Welch's *t*-test was performed. The correlations between plasma levels of LPL amount, LPL activity, or LPL activity/LPL amount and ADI-R scores were evaluated using the Pearson's correlation coefficient. This analysis was also used to analyze the correlation between GPIHBP1 and LPL measurements, and the correlation between LPL measurements or GPIHBP1 and age. Bonferroni correction and false discovery rate by Benjamini-Hochberg (BH) method were used for considering multiplicity (type 1 error). A logistic regression analysis was used for considering type 2 error. In addition, receiver operating characteristics (ROC) analysis was also carried out to distinguish between the two groups. The area under the curve (AUC), optimal cutoff point, sensitivity, and specificity were determined using the maximum value of the Youden index. All statistical analyses were performed using SPSS software version 26.0 (IBM, Armonk, NY). In this study, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Clinical characteristics of participants

The characteristics of the age- and sex-matched participants are summarized in Table 1. We first examined demographic distributions and confirmed that there were no significant differences in age, weight, height, and BMI between the TD and ASD male subjects (Table 1).

Because LPL is known to be involved in lipid metabolism, we next examined liver function and other metabolic factors in all participants. There were no significant differences in AST, ALT, or γ-GTP between the TD and ASD male subjects (Table 1). One ASD male subject had a γ-GTP measurement of 90 U/L; however, the subject did not have any subjective symptoms or a history of lipid and lipid-related disorders. Thus, we did not exclude this plasma sample for the downstream analyses. We also examined the concentration of CRP, as an inflammatory marker, and found no difference in CRP between the TD and ASD male subjects (Table 1).

We next investigated the IQ scores as a clinical characteristic in participants using WISC-III. There were no significant differences

Table 1
Clinical characteristics in TD and ASD male subjects.

	TD (n = 28)	ASD (n = 28)	p-value	FDR-BH
Age (years)	10.0 ± 3.1 (5.3–14.9)	11.5 ± 6.2 (6.5–27.5)	NS	NS
Weight (kg)	35.0 ± 13.4 (16.3–57.6)	35.9 ± 15.5 (18.0–84.3)	NS	NS
Height (cm)	138.2 ± 20.5 (106.1–174.0)	139.2 ± 18.2 (115.0–177.5)	NS	NS
BMI (kg/m^2)	17.6 ± 2.5 (14.0–25.3)	17.7 ± 3.2 (13.4–26.8)	NS	NS
ADI-R				
Domain A score		20.5 ± 6.9 (7–29)	–	–
Domain BV score		15.1 ± 4.9 (3–23)	–	–
Domain C score		4.2 ± 2.9 (0–12)	–	–
WISC-III				
Full-scale IQ	101.4 ± 12.2 (76–129)	95.0 ± 22.6 (41–133)	NS	NS
Verbal IQ	101.1 ± 13.5 (70–128)	99.1 ± 21.1 (60–126)	NS	NS
Performance IQ	101.6 ± 11.2 (76–124)	92.4 ± 22.5 (54–136)	NS	NS
AST (U/L)	25.0 ± 5.3 (14–36)	23.4 ± 6.0 (15–38)	NS	NS
ALT (U/L)	15.0 ± 5.4 (8–36)	15.7 ± 7.6 (8–39)	NS	NS
γ-GTP (U/L)	15.0 ± 3.5 (11–25)	20.7 ± 16.0 (11–90)	NS	NS
CRP (mg/dl)	0.04 ± 0.04 (0.01 to 0.20)	0.03 ± 0.04 (0.01 to 0.17)	NS	NS
LPL amount (ng/mL)	48.8 ± 12.0 (32.8–90.3)	46.0 ± 14.4 (26.8–72.8)	NS	NS
LPL activity (mUnits/mL)	126.7 ± 21.1 (84.2–169.4)	138.7 ± 18.7 (104.6–174.4)	0.028	0.084
LPL activity/LPL amount	2.7 ± 0.8 (1.3–5.0)	3.3 ± 0.9 (1.7–5.0)	0.033	0.049
GPIHBP1 (pg/mL)	879.0 ± 244.4 (466.4–1341.4)	881.2 ± 219.8 (522.7–1353.9)	NS	NS

Values are expressed as mean ± SD (range). BMI: body mass index, ADI-R: Autism Diagnostic Interview-Revised, WISC-III: the third edition of Wechsler Intelligence Scale for Children, IQ: Intellectual Quotient, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ-glutamyltranspeptidase, CRP: C-reactive protein, LPL: lipoprotein lipase, GPIHBP1: glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1, FDR-BH: False Discovery rate obtained by Benjamini-Hochberg method, NS: not significant.

between the two groups in full-scale IQ, verbal IQ, or performance IQ scores in the WISC-III (Table 1). Together, these data indicate that there were no differences in demographic distributions between the TD and ASD male subjects.

3.2. Increased LPL activity in males with ASD

To investigate the role of LPL in ASD, we first quantified LPL amount and LPL activity using peripheral plasma samples from ASD and TD male subjects. In order to consider the type 1 error due to multiplicity, BH method was performed in statistical analysis. There was no significant difference in the LPL amount of peripheral plasma of ASD male subjects compared with TD male controls (Fig. 1A, Table 1). In contrast, LPL activity in the plasma of ASD male subjects was significantly higher than in TD male controls and showed a trend toward significance even after correction for multiplicity (t = 2.253, p = 0.028; p = 0.084 after BH correction) (Fig. 1B, Table 1). Furthermore, we examined the LPL activity/LPL amount to determine the concentration-independent increase in pure LPL activity. The plasma levels of LPL activity/LPL amount in ASD male subjects were significantly higher than those of TD male controls (t = 2.191, p = 0.033; p = 0.049 after BH correction) (Fig. 1C, Table 1).

In addition, a logistic regression analysis was also used to examine the independent associations between plasma LPL amount, LPL activity, or LPL activity/LPL amount and ASD status, adjusting for age and BMI, which were potential confounding factors. There was no significantly increased risk for ASD associated with plasma LPL amount (OR = 0.986, 95 % confidence interval (CI) = 0.944–1.023, p = 0.507), which was similar to the results comparing LPL amount between TD and ASD subjects (Fig. 1A). We revealed that a one-unit increase in plasma LPL activity was associated with an approximately 10 % increased risk of ASD (odds ratio (OR) = 1.108, 95 % CI = 1.002–1.023, p = 0.035). In addition, plasma LPL activity/LPL amount showed a trend toward an approximately 90 % increase in the risk of ASD, although this was not a statistically significant result (OR = 1.925, 95 % CI = 0.997–3.720, p = 0.051). Together, these results suggest that there are ASD-specific changes in plasma levels of LPL activity.

3.3. LPL activity/LPL amount can distinguish between TD and ASD male subjects

To assess the usefulness of the plasma LPL levels for the diagnosis of ASD, a ROC analysis was performed. The AUC demonstrated the diagnostic value of LPL, with a higher AUC being correlated with higher sensitivity, specificity, and accuracy.

The optimal cut-off points to distinguish between TD and ASD male subjects were 36.0 ng/ mL for plasma LPL amount and 114.0 mUnits/ mL for plasma LPL activity (Fig. 2). For plasma LPL amount and activity, respectively, these cut-off points were associated with a sensitivity of 92.9 % and a specificity of 71.4 % (AUC = 0.60; 95 % CI = 0.445–0.750, p = 0.210) or a sensitivity of 96.4 % and a specificity of 64.3 % (AUC = 0.65; 95 % CI = 0.505–0.793, p = 0.055), but were not statistically significant. (Fig. 2).

In contrast, the optimal cut-off point for plasma LPL activity/LPL amount was 2.26 (Fig. 2). This cut-off point was associated with

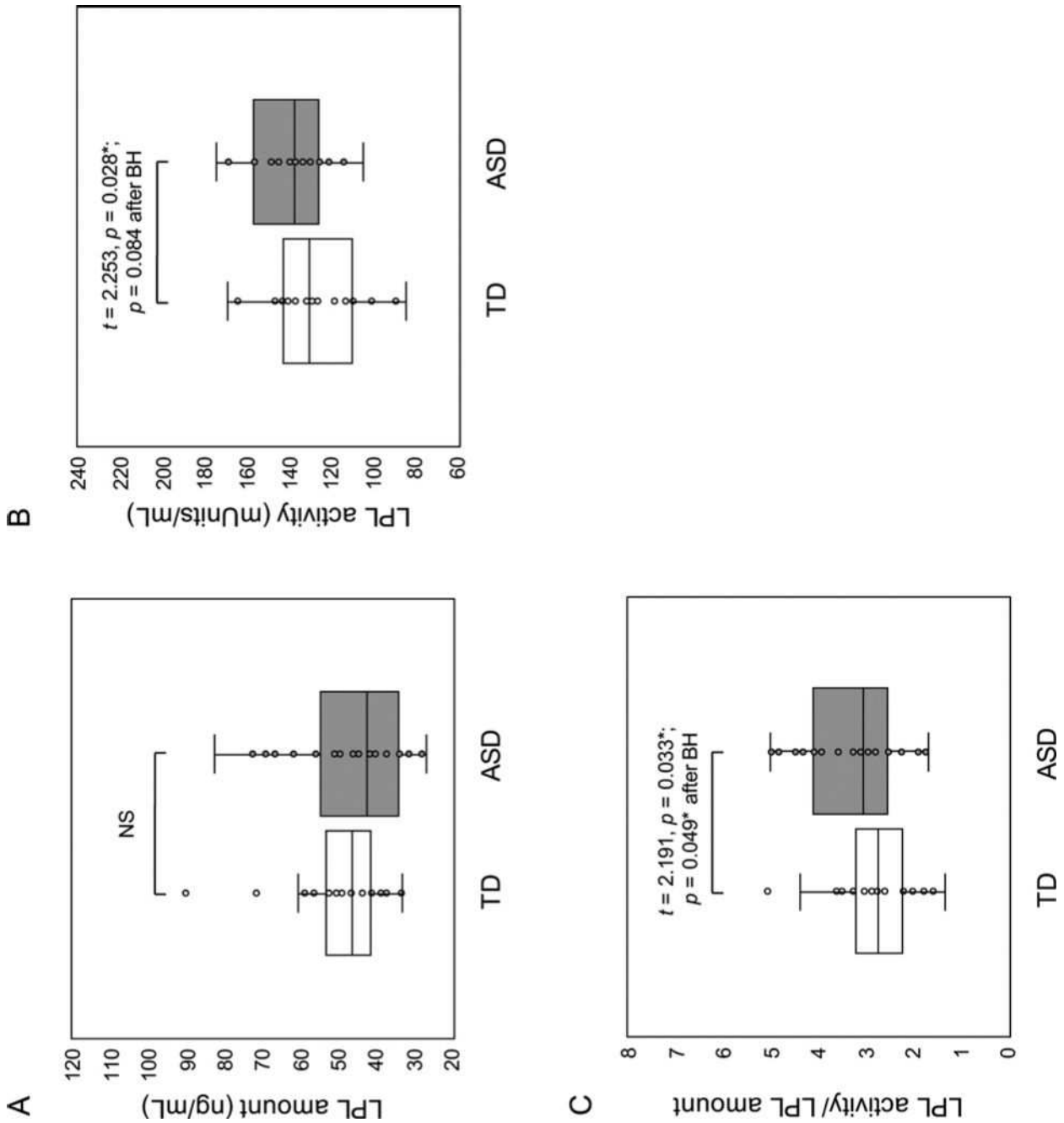

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Fig. 1. Increased plasma LPL activity in ASD male subjects.
(A) Plasma LPL amounts in TD (48.8 ± 12.0 ng/mL) and ASD (46.0 ± 14.4 ng/mL) male subjects. (B) Plasma LPL activities in TD (126.7 ± 21.1 mUnits/mL) and ASD (138.7 ± 18.7 mUnits/mL) male subjects. (C) Plasma levels of LPL activity/LPL amount in TD (2.7 ± 0.8) and ASD (3.3 ± 0.9) male subjects. Data are represented as the mean (± SD). *$p < 0.05$, Student's *t*-test, $n = 28$ per group.

a sensitivity of 89.3 % and a specificity of 67.9 % (AUC = 0.66; 95 % Cl = 0.511–0.800, $p = 0.046$), and these results were statistically significant (Fig. 2). These results indicate that the plasma LPL activity/LPL amount is an appropriate indicator to distinguish between TD and ASD male subjects.

3.4. No correlations between LPL and ADI-R domain scores

We next assessed whether LPL values reflect the clinical symptoms of ASD. We examined the correlations between plasma LPL amount, plasma LPL activity, or plasma LPL activity/LPL amount and ADI-R clinical scores in ASD male subjects. There were no significant correlations between any LPL levels and ADI-R domain A, BV, or C scores (Fig. 3, Table 2). Although there were no statistically significant correlations, we observed trends toward correlations between LPL amount and ADI-R domain BV score as well as LPL activity and ADI-R domain A score (Fig. 3, Table 2). Since these results have been repeated at least nine times for the correlation analyses, multiple comparisons analyses were using for those correlations. There were no correlations between plasma LPL amount, plasma LPL activity, or plasma LPL activity/LPL amount and ADI-R clinical scores in ASD male subjects after BH correction (Fig. 3, Table 2).

There were also no correlations between plasma LPL amount, plasma LPL activity, or plasma LPL activity/LPL amount and any clinical variables, such as weight, height, BMI, AST, ALT, γ-GTP, CRP, full-scale IQ, verbal IQ, or performance IQ (data not shown).

These results suggest that LPL levels are not useful as direct indicators of ASD clinical symptoms; however, our other results suggest that LPL does play an important role in the pathology of ASD, such as in lipid metabolism.

3.5. Correlations between LPL activity and GPIHBP1

To understand the molecular mechanism of increased LPL activity in ASD male subjects, we investigated the plasma amount of GPIHBP1, an essential partner for LPL. There was no significant difference in plasma GPIHBP1 amount between ASD male subjects and TD male controls (Fig. 4A, Table 1).

We next investigated the correlation between GPIHBP1 and LPL, but there were no significant correlations between plasma GPIHBP1 and LPL amounts in TD or ASD male subjects (Fig. 4B, Table 2). In TD male subjects, neither LPL activity nor LPL activity/LPL amount were correlated with plasma GPIHBP1 amount (Fig. 4C, D, Table 2). In contrast, both LPL activity and LPL activity/LPL

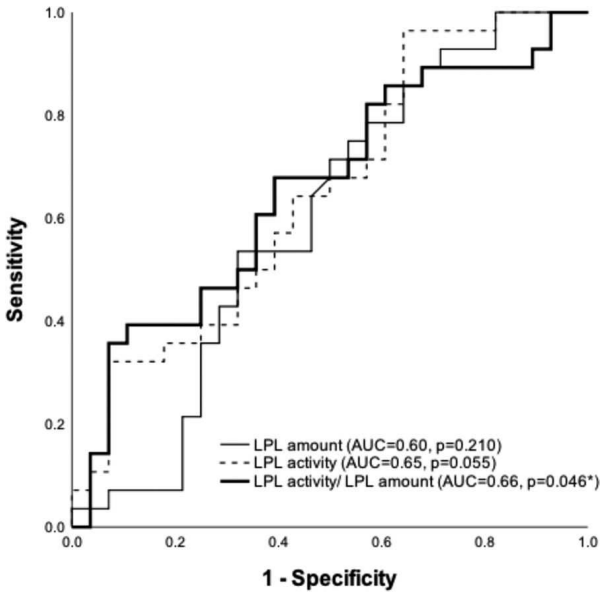

Fig. 2. Receiver operating characteristic (ROC) curves for LPL.
The level of LPL activity/LPL amount (AUC = 0.66, $p = 0.046$) significantly distinguished between the TD and ASD male subjects; however, there were no correlations in LPL amount (AUC = 0.60, $p = 0.210$) or LPL activity (AUC = 0.65, $p = 0.055$) by themselves. The optimal cut-off points using LPL amount, activity, and activity/amount were 36.0 ng/mL, 114.0 mUnits/mL, and 2.26, respectively. These cut-off points were associated with sensitivities of 92.9 %, 96.4 %, and 89.3 %, and specificities of 71.4 %, 64.3 %, and 67.9 %, respectively, for LPL amount, activity, and activity/amount. *$p < 0.05$.

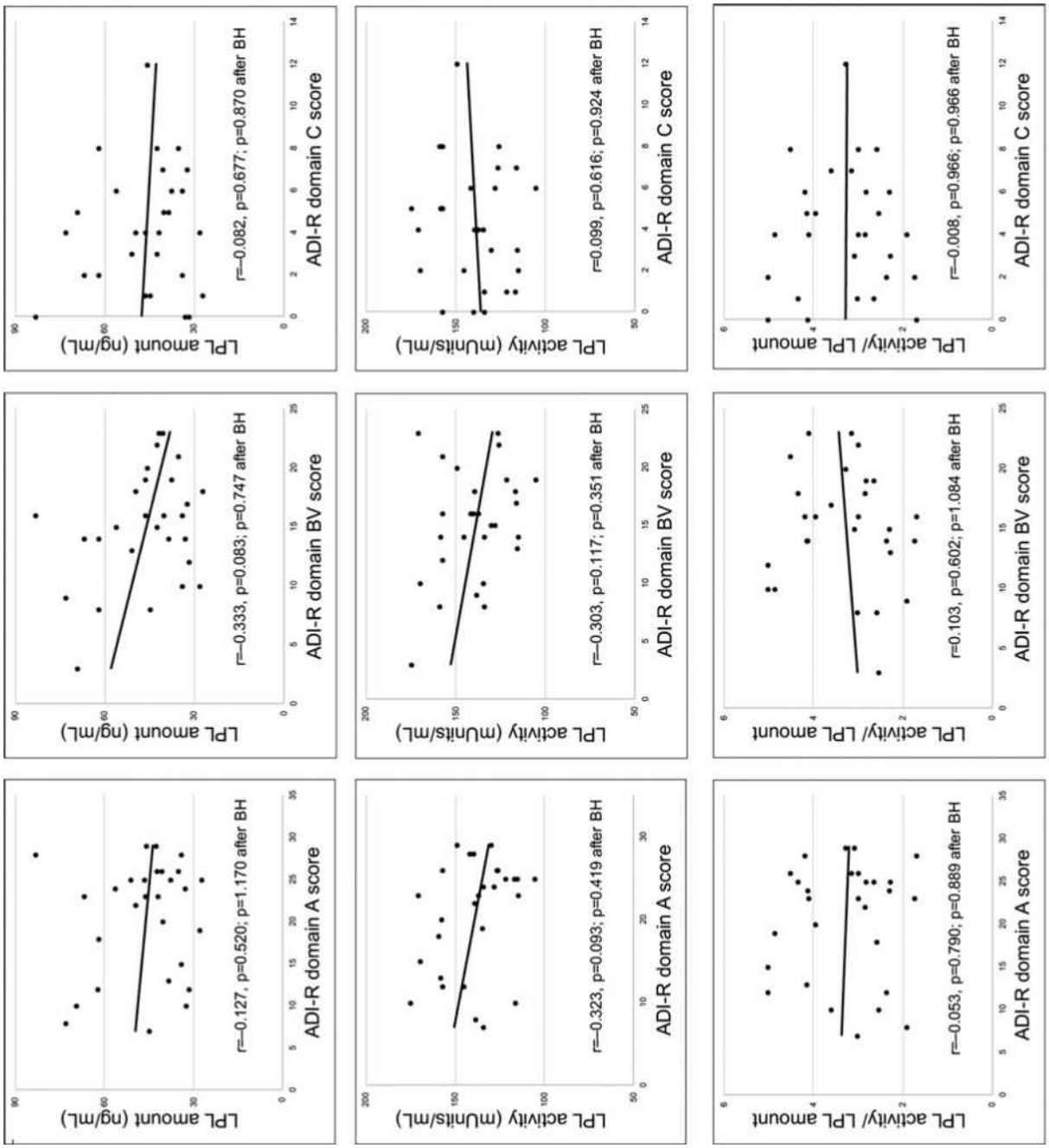

Fig. 3.

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Fig. 3. No correlations between LPL and ADI-R domain scores.
There were no significant correlations between ADI-R domain scores and plasma LPL amount, LPL activity, or LPL activity/LPL amount, as follows: A score (amount $r = -0.127$, $p = 0.520$, $p = 1.170$ after BH correction; activity $r = -0.323$, $p = 0.093$, $p = 0.419$ after BH correction; activity/amount $r = -0.053$, $p = 0.790$, $p = 0.889$ after BH correction), BV score (amount $r = -0.333$, $p = 0.083$, $p = 0.747$ after BH correction; activity $r = -0.303$, $p = 0.117$, $p = 0.351$ after BH correction; activity/amount $r = 0.103$, $p = 0.602$, $p = 1.084$ after BH correction), and C score (amount $r = -0.082$, $p = 0.677$, $p = 0.870$ after BH correction; activity $r = 0.099$, $p = 0.616$, $p = 0.924$ after BH correction; activity/amount $r = -0.008$, $p = 0.966$, $p = 0.966$ after BH correction).

amount had a trend toward significant negative correlations with plasma GPIHBP1 amount in the ASD subjects, even after corrected for multiplicity by BH method ($r = -0.439$, $p = 0.020$; $p = 0.060$ after BH correction and $r = -0.463$, $p = 0.013$; $p = 0.078$ after BH correction; respectively) (Fig. 4C, D, Table 2). These results suggest that the increased LPL activity in the plasma of ASD males may be regulated by GPIHBP1, providing a novel insight into ASD metabolism.

3.6. Correlations between age and LPL or GPIHBP1

Finally, we also examined whether the plasma amounts of LPL and GPIHBP1 were regulated by age-dependent manner and BH correction for multiplicity was used. We found that the positive correlations of age in LPL activity or LPL activity/LPL amount before correction ($r = 0.381$, $p = 0.046$; $p = 0.184$ after BH correction and $r = 0.453$, $p = 0.016$; $p = 0.128$ after BH correction; respectively), but not in LPL amount of TD male subjects (Fig. 5A-C, Table 2). There was also a positive correlation trend of age with GPIHBP1 in TD male subjects, especially before correction was found ($r = 0.361$, $p = 0.083$; $p = 0.221$ after BH correction) (Fig. 5D, Table 2). Although these results have a multiplicity issue, the correlation between LPL and GPIHBP1 suggest that it may be influenced by both age and disease-specific status factors.

4. Discussion & implications

In the current study, we found significantly increased LPL activity/LPL amount and a trend toward significance in LPL activity in the plasma of ASD male subjects compared with TD male subjects; however, there was no difference in LPL amount. In addition, ROC analysis demonstrated that plasma LPL activity/LPL amount is a useful indicator to distinguish ASD subjects from TD controls. Although LPL was not correlated with any ADI-R domain scores, a negative correlation of plasma LPL activity/LPL amount with plasma GPIHBP1 amount in ASD male subjects was found. Our findings suggest the possibility that LPL activity, regulated by GPIHBP1, might play a critical role in the lipid metabolism associated with ASD pathology.

Table 2
Summary of the Pearson's correlation.

	TD				ASD			
	r	*p* (unadjusted)	*p* (Bonferroni correction)	FDR-BH	*r*	*p* (unadjusted)	*p* (Bonferroni correction)	FDR-BH
LPL vs ADI-R								
LPL amount vs Domain A					–0.127	0.520	4.680	1.170
LPL amount vs Domain BV					–0.333	0.083	0.747	0.747
LPL amount vs Domain C					–0.082	0.677	6.093	0.870
LPL activity vs Domain A					–0.323	0.093	0.837	0.419
LPL activity vs Domain BV					–0.303	0.117	1.053	0.351
LPL activity vs Domain C					0.099	0.616	5.544	0.924
LPL activity/LPL amount vs Domain A					–0.053	0.790	7.110	0.889
LPL activity/LPL amount vs Domain BV					0.103	0.602	5.418	1.084
LPL activity/LPL amount vs Domain C					–0.008	0.966	8.694	0.966
GPIHBP1 vs LPL								
GPIHBP1 vs LPL amount	–0.129	0.548	3.288	0.548	0.155	0.432	2.592	0.648
GPIHBP1 vs LPL activity	0.158	0.460	2.760	0.552	–0.439	0.020	0.120	0.060
GPIHBP1 vs LPL activity/amount	0.211	0.323	1.938	0.646	–0.463	0.013	0.078	0.078
Age vs LPL or GPIHBP1								
Age vs LPL amount	–0.226	0.247	1.976	0.494	–0.214	0.274	2.192	0.438
Age vs LPL activity	0.381	0.046	0.368	0.184	–0.164	0.405	3.240	0.540
Age vs LPL activity/LPL amount	0.453	0.016	0.128	0.128	0.048	0.808	6.464	0.808
Age vs GPIHBP1	0.361	0.083	0.664	0.221	0.150	0.445	3.640	0.509

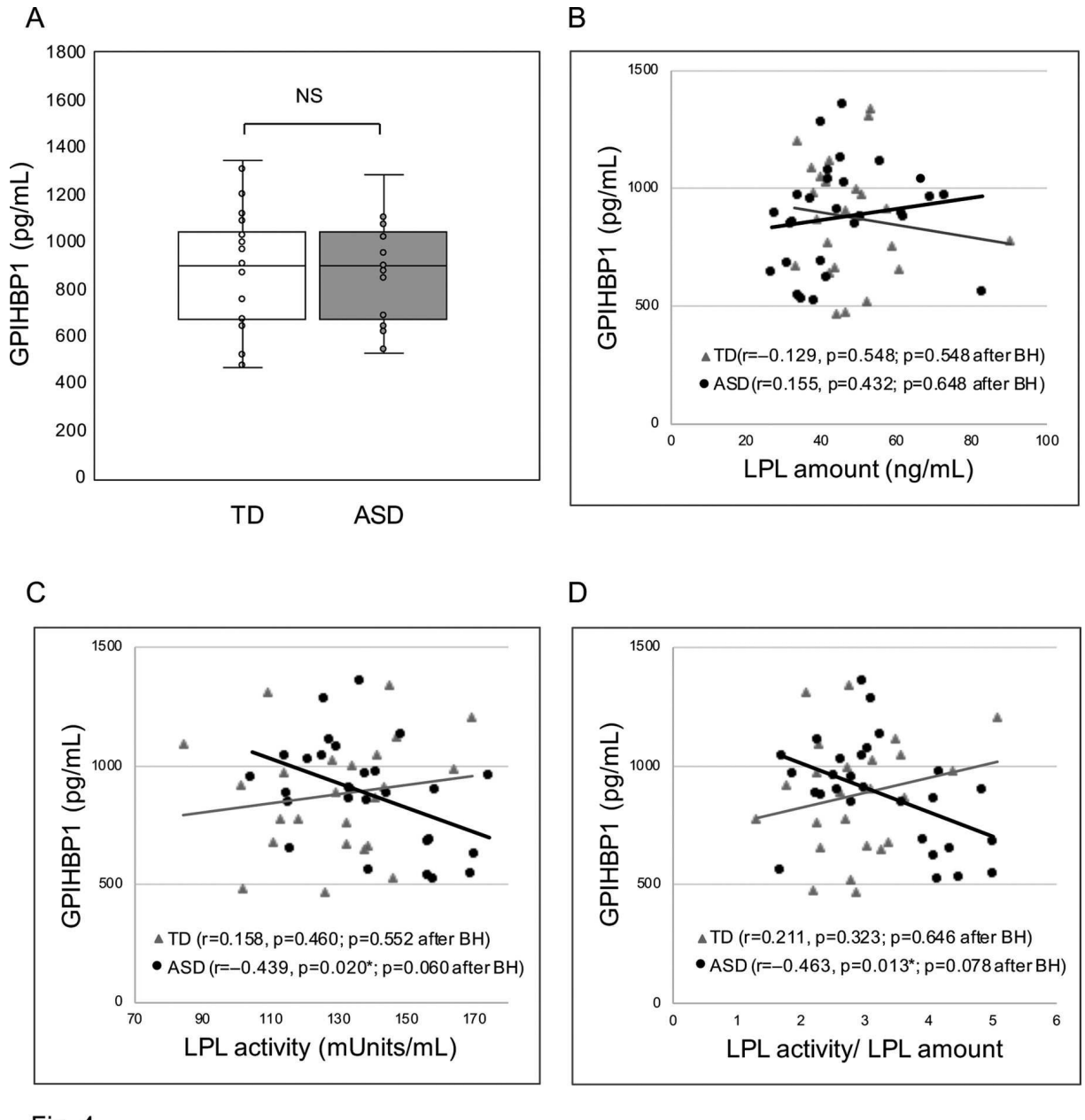

Fig. 4.

Fig. 4. LPL activity/LPL amount is negatively correlated with GPIHBP1 in ASD.
(A) Plasma amounts of GPIHBP1 in TD (879.0 ± 244.4 pg/mL) and ASD (881.2 ± 219.8 pg/mL) male subjects ($t = 0.035$, $p = 0.973$). Data are represented as the mean (± SD). Student's *t*-test, $n = 24$–28 per group. (B–D) The correlations between plasma amounts of GPIHBP1 and LPL in TD and ASD male subjects. There were no significant correlations between plasma GPIHBP1 and LPL amount (B), LPL activity (C), or LPL activity/LPL amount (D) in TD male subjects ($r = -0.129$, $p = 0.548$, $p = 0.548$ after BH correction; $r = 0.158$, $p = 0.460$, $p = 0.552$ after BH correction; and $r = 0.211$, $p = 0.323$, $p = 0.646$ after BH correction; respectively). In contrast, there were negative correlations between GPIHBP1 and LPL activity ($r = -0.439$, $p = 0.020$, $p = 0.060$ after BH correction) (C) and LPL activity/LPL amount ($r = -0.463$, $p = 0.013$, $p = 0.078$ after BH correction) (D), but not LPL amount ($r = 0.155$, $p = 0.432$, $p = 0.648$ after BH correction) (B) in ASD male subjects. *$p < 0.05$.

4.1. Roles of LPL in ASD

A previous study has reported a mutation in *LPL* from the postmortem brains of ASD patients (Li et al., 2014). This mutation forms a missense variant of *LPL* (c.106 G > A; p.Asp36Asn) (Li et al., 2014), suggesting that the mutation may lead to defective lipase activity. If LPL expression or its lipase activity is altered by a mutation, or for any other reason, it may have a large effect on lipid metabolism, because LPL has central roles in the energy storage, metabolism, and transport of lipids (Nilsson-Ehle et al., 1980; Wang & Eckel, 2009). In addition, in *Lpl* knockout mice studies, abnormalities in lipid metabolism (such as increases in weight, CHOs, and

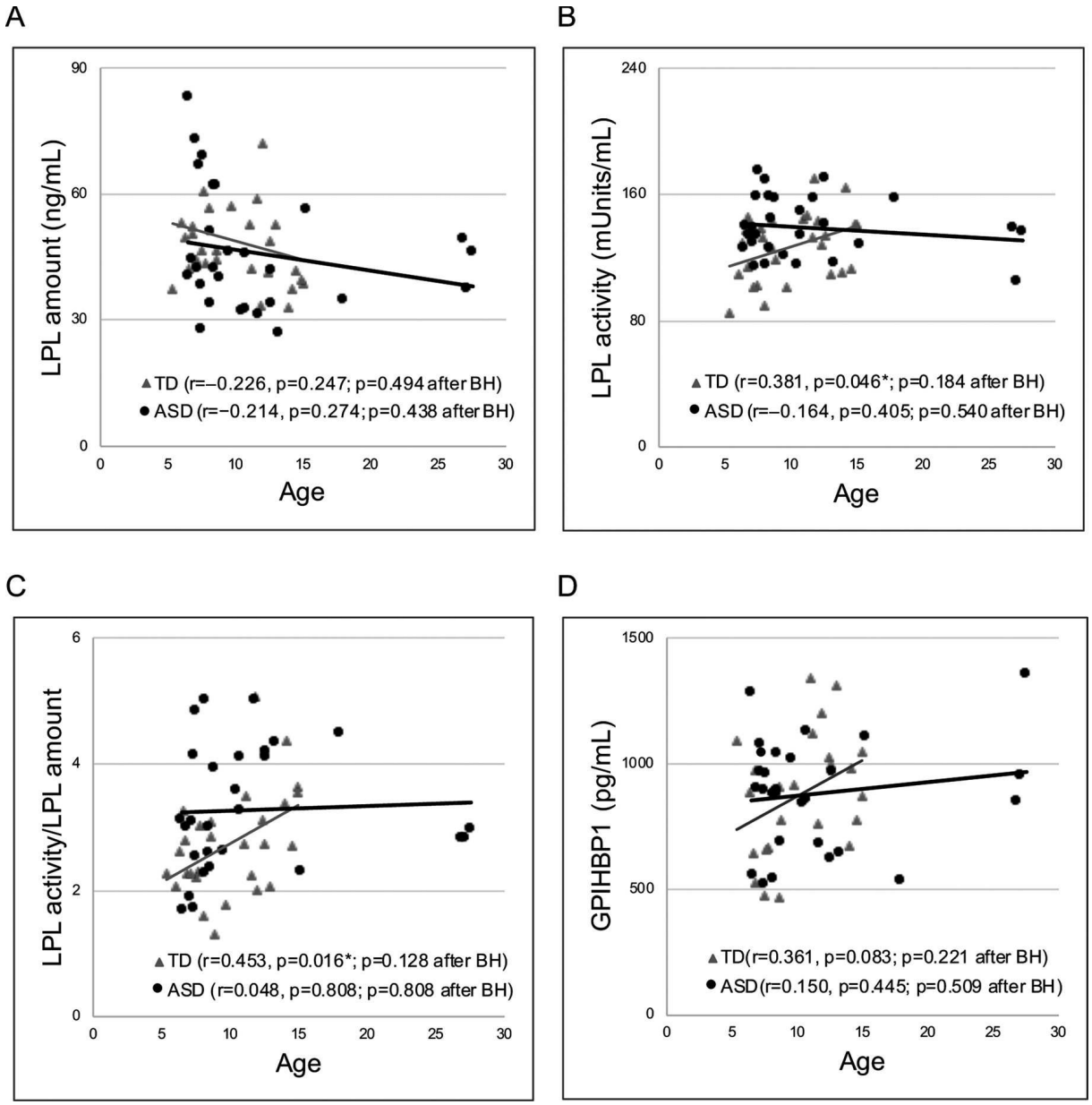

Fig. 5. Age and LPL activity/LPL amount is positively correlated in TD, but not in ASD.
(A–D) The correlations between age and plasma LPL or GPIHBP1 in TD and ASD male subjects. There were significant correlations between age and plasma LPL activity (B), or LPL activity/LPL amount (C) ($r = 0.381$, $p = 0.046$, $p = 0.184$ after BH correction; and $r = 0.453$, $p = 0.016$, $p = 0.128$ after BH correction; respectively), but not LPL amount ($r = –0.226$, $p = 0.247$, $p = 0.494$ after BH correction) (A) in TD male subjects. Moreover, there was a positive correlation tendency of age with GPIHBP1 ($r = 0.361$, $p = 0.083$, $p = 0.221$ after BH correction) (D) in TD male subjects. In contrast, there were not correlations between age and plasma LPL amount (A), LPL activity (B), LPL activity/LPL amount (C), or GPIHBP1 (D) in ASD male subjects ($r = –0.214$, $p = 0.274$, $p = 0.438$ after BH correction; $r = –0.164$, $p = 0.405$, $p = 0.540$ after BH correction; $r = 0.048$, $p = 0.808$, $p = 0.808$ after BH correction; $r = 0.150$, $p = 0.445$, $p = 0.509$ after BH correction; respectively). *$p < 0.05$.

TGs), lipid homeostasis, and adipose tissue morphology have been reported (Chen et al., 2008; Coleman et al., 1995; Weinstock et al., 1995).

A well-known clinical feature of patients with ASD is slenderness, and high levels of LPL activity may influence such clinical features. Specifically, the BMI of boys with ASD is significantly lower than those of age-matched reference populations (Mouridsen, Rich, & Isager, 2008), and ASD patients with *CHD8* mutations are also reportedly slender (Bernier et al., 2014). Underweight phenotypes have also been reported in ASD mouse model studies, including those with *Arid1b, Shank3*, and 16p11.2 deletions (Arbogast et al., 2016; Drapeau, Dorr, Elder, & Buxbaum, 2014; Shibutani et al., 2017). However, not only underweight but also overweight and obesity have been reported in adult patients with ASD (Sedgewick, Leppanen, & Tchanturia, 2020). Further study such as follow-up study is needed to discuss the direct relationship between LPL and weight features. Again, these studies suggest that

LPL may play an important role in ASD pathology, particularly in lipid metabolism and physical features.

Previous studies have investigated the role of LPL in the brain. A study using neuron-specific LPL-deficient mice has reported that LPL not only has a role in regulating energy balance, but is also important for cognitive function (Yu et al., 2015). It is well known that cognitive impairment is present in ASD (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014), and LPL may be important for regulating neuronal survival and differentiation in ASD. Other studies have suggested a relationship between LPL and oxidative stress (Cruciani-Guglielmacci & Magnan, 2017; Paradis, Clement, Julien, & Ven Murthy, 2003), which is involved in central nervous system (CNS) development and the pathophysiology of ASD. Thus, LPL may also have a role in CNS development via the oxidation process (Bjørklund et al., 2020; Islam, 2017). Based on these findings, we hypothesize that LPL may regulate brain development through metabolic and oxidative processes, and that abnormalities in these processes may cause immature brain development and neurodevelopmental disorders such as ASD.

In the present study, we demonstrated that plasma LPL activity/LPL amount is a useful indicator to distinguish between TD and ASD male subjects (Fig. 1,2). However, there were no correlations between LPL measurements and clinical scores in ASD (Fig. 3, Table 2). In this regard, there is a possibility that the present study may reflect only free LPL in the blood, because they were measured in the pre-heparin condition. Heparin administration releases LPL from the surface of microvascular endothelial cells, and affects LPL amount and LPL activity (Vilella et al., 1993). Thus, future studies should investigate LPL in both pre- and post-heparin conditions and sufficient sample size is also needed.

It remains unclear whether plasma LPL activity reflects LPL activity in the brain. In general, lipid metabolism and transport in the CNS are distinct because of the different regulatory mechanisms in peripheral tissues. Studies using plasma (Wang & Eckel, 2014) and cerebrospinal fluid (Koch et al., 2001; Wang & Eckel, 2014) samples reported that only a few small circulating HDL lipoproteins can cross the blood–brain barrier; however, there are no reports in the case of LPL. To understand the mechanisms connecting central and peripheral functions, it is necessary to investigate whether LPL amount and LPL activity in the cerebrospinal fluid and/or blood in the brain are altered in ASD patients. Moreover, because it is possible that LPL in the brain may have a role other than in lipid metabolism, further investigation is required to uncover the function of LPL in the CNS.

4.2. Mechanisms controlling LPL activity

We demonstrated higher LPL activity in ASD male subjects compared with TD controls, but there was no difference in LPL amount. To better understand this phenotype, we speculated that LPL activation may be caused by the molecules that control LPL homodimerization. LPL is only activated when it forms a homodimer, and dissociation from homodimer to monomer results in a loss of the catalytic function (Kobayashi, Nakajima, & Inoue, 2002; Lookene, Zhang, Hultin, & Olivecrona, 2004). Such modulation of LPL activity is tightly regulated by multiple mechanisms in a tissue-specific manner in response to nutritional conditions (Enerback, Semb, Tavernier, Bjursell, & Olivecrona, 1988; Otarod & Goldberg, 2004).

We therefore investigated GPIHBP1 as an essential regulator of LPL activity. GPIHBP1 is known to stabilize LPL structure and catalytic activity by preventing the expansion of its catalytic domain (Mysling et al., 2016). We quantified the plasma GPIHBP1 amount in both TD and ASD male subjects, but there was no significant difference between the two groups (Fig. 4A, Table 1). Previous studies have reported that the plasma and serum levels of LPL are positively correlated with GPIHBP1 levels in healthy Japanese adult men (16–26 years old) (Matsumoto et al., 2019; Miyashita et al., 2018), but in the present study, there were no correlations between GPIHBP1 and LPL amount, LPL activity, or LPL activity/LPL amount in TD male subjects (Fig. 4B–D, Table 2). Our result was therefore not consistent with those of the previous reports (Matsumoto et al., 2019; Miyashita et al., 2018); this discrepancy may be because our plasma samples were from much younger male subjects (5–14 years old). However, it is noteworthy that plasma levels of LPL activity and LPL activity/LPL amount were negatively correlated with plasma GPIHBP1 amount in only the ASD subjects, and not the TD subjects. Even after we have corrected for multiplicity, these negative correlations demonstrated trend toward significance (Fig. 4C, D, Table 2). Other than age, it may also be that a functional impairment of GPIHBP1 is affected. The two essential domains of GPIHBP1 have been reported as the Ly6 domain (a three-fingered domain specified by 10 cysteines) for LPL binding (Beigneux et al., 2009) and the acidic domain for LPL activity (Mysling et al., 2016). The importance of the acidic domain of GPIHBP1 in LPL activity is suggested; however, no mutations have been reported in the acidic domain of GPIHBP1 (Young et al., 2019). Therefore, it is speculated that the pathophysiological state underlying ASD may affect the function of GPIHBP1, or may promote the activity of LPL. For example, it may be the result of subtle changes in blood pH, caused by abnormal lipid metabolism or the actions of metabolites in ASD. Together, our data suggested that the correlation between LPL activity and GPIHBP1 may be influenced not only by age, but also by the disease-specific state, such as by ASD. Our findings also suggest that the ASD-specific activation of LPL activity may be responsible for the negative correlation between GPIHBP1 and LPL activity in the present study; this provides a key to understanding the molecular mechanisms of increased LPL activity in ASD.

We further explored the essential regulators controlling LPL function. For example, angiopoietin-like protein 3 (ANGPTL3) and ANGPTL4 have both been reported to regulate LPL activity in studies using plasma samples (Ono et al., 2003; Shimizugawa et al., 2002; Yoshida, Shimizugawa, Ono, & Furukawa, 2002). ANGPTL3 modulates plasma TG-VLDL levels via the inhibition of plasma LPL activity (Shimizugawa et al., 2002), while ANGPTL4 prevents LPL dimerization and thereby suppresses LPL activation (Yau et al., 2009). In addition, *Angptl3*- and *Angptl4*-deficient mice studies have shown that there is increased plasma LPL activity in these mice (Koishi et al., 2002; Koster et al., 2005; Wang et al., 2015). These previous studies suggest the involvement of ANGPTL3 and/or ANGPTL4 in the increased LPL activity that we observed in ASD male subjects in the current study. Thus, we also quantified the plasma amounts of ANGPTL3 and ANGPTL4 in our samples; however, there were no significant differences in their amounts between the two groups, and there were also no significant correlations with LPL activity in either TD or ASD male subjects (data not shown).

Lipase maturation factor 1 (LMF1) and suppressor of lin-12-like (SEL-1) are both potential candidates for controlling LPL activity. LMF1 positively regulates LPL activity by controlling the kinetics of its structural folding and assembly (Doolittle, Ehrhardt, & Peterfy, 2010). In mice studies, tissue LPL activity is increased in *Lmf1*-overexpressing transgenic mice, while LPL activity is reduced in *Lmf1* heterozygous mice at neonatal stages (Peterfy, 2012). SEL-1 forms a functional complex with LPL and LMF1, and this complex then stabilizes nascent LPL dimers on the ER membrane (Sha et al., 2014; Sun et al., 2014). However, the latest plasma-based study reported that LPL has lipase activity as a monomer, overturning the long-standing hypothesis that LPL is only activated as a homodimer (Beigneux et al., 2019). Furthermore, hormones such as insulin and cortisol have also been reported to be involved in regulating LPL activity (Kuchay et al., 2017; Sakayama et al., 2008). Thus, future investigations are also needed to understand the molecular mechanisms underlying LPL activity. Our findings provide novel insights into ASD pathogenesis; however, further studies are required to investigate the cause of increased LPL activity in ASD.

4.3. Limitations

There were a number of limitations in this study: 1) the sample size (and thus statistical power) of both groups was small and some cases showing no statistically significant differences by multiple comparisons that accounted for type I errors; 2) LPL quantification was carried out in the pre-heparin state, although LPL is bound and anchored to heparan sulfate proteoglycan on the vascular endothelium; 3) the diagnosis of ASD should have been made using the more objective Autism Diagnostic Observation Schedule, second edition (ADOS-2), rather than ADI-R; 4) the BNV domain (nonverbal communication) of ADI-R should also have been evaluated.

We understand that increasing the sample size is preferable, but we conclude that there is increased LPL activity in ASD because we revealed a statistically significant difference in LPL activity in our small sample size. On the other hand, it is necessary to consider type 1 and type 2 errors in discussing our results. The present study addresses these issues by also showing the results after correction for multiple comparisons, but caution should be exercised in interpreting the results. Moreover, as we have mentioned, most LPL in the blood is present in the vascular endothelium, and the amount of free LPL is very small. Therefore, heparin administration is essential before blood collection for accurate quantification of LPL. However, because our study targeted children with ASD, it was difficult to administer heparin before blood collection.

Taken together, it is hard to conclude whether the results of this study accurately reflect *in vivo* values; however, we reliably evaluated the plasma levels of LPL amount and activity that were free in the blood. To the best of our knowledge, this is the first report demonstrating increased plasma LPL activity in males with ASD.

Ethical statement

The experimental protocols were approved by the ethics committee of the University of Fukui (approval no. 20130034) to adhere to the Declaration of Helsinki (World Medical Association, 2013), and were conducted in accordance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects of the Ministry of Health, Labour and Welfare of Japan. All participants were given a complete description of the study, and written informed consent was obtained from their parent and/or legal guardian before beginning the study.

CRediT authorship contribution statement

Takaharu Hirai: Writing - original draft, Formal analysis, Investigation. **Noriyoshi Usui:** Data curation, Writing - review & editing, Funding acquisition. **Keiko Iwata:** Resources. **Taishi Miyachi:** Resources. **Kenji J. Tsuchiya:** Resources. **Min-Jue Xie:** Resources. **Kazuhiko Nakamura:** Resources. **Masatsugu Tsujii:** Resources. **Toshiro Sugiyama:** Supervision. **Hideo Matsuzaki:** Conceptualization, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.rasd.2020.101630.

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