Research Paper

Low Very low-Density Lipoprotein Cholesterol but High Very low-Density Lipoprotein Receptor mRNA Expression in Peripheral White Blood Cells: An Atherogenic Phenotype for Atherosclerosis in a Community-Based Population☆

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ABSTRACT

Very low-density lipoprotein cholesterol (VLDL-C), via binding very low-density lipoprotein receptor (VLDLR), can induce the development of atherosclerosis. Besides monocytes, VLDLR expression is detected in various peripheral white blood cells (WBCs), yet its underlying role remains unclear. We thereby aimed to test the hypothesis that VLDLR in all types of peripheral WBCs may be involved in the association between VLDL-C and atherosclerosis. *VLDLR* mRNA expression in peripheral WBC and plasma VLDL-C levels were measured in 747 participants from a community-based study. Plaque prevalence and total plaque area (TPA) were used to evaluate the burden of carotid atherosclerosis. VLDL-C was positively associated with atherosclerosis risk, whereas this association was modified by *VLDLR* mRNA level. In participants with the lowest VLDL-C but the highest *VLDLR* mRNA expression, the risk for plaque prevalence unexpectedly was the highest. This association was also observed for TPA. Moreover, this association remained unchanged after adjusting for WBC or monocytes. Our findings described an atherogenic phenotype characterized by low VLDL-C but high *VLDLR* mRNA expression in peripheral WBCs, which suggested that VLDLR in all types of peripheral WBCs may be involved in lipid deposition, and VLDL-C and VLDLR may co-determine the development of atherosclerosis.

1. Introduction

Very low-density lipoprotein cholesterol (VLDL-C), a component of non-high-density lipoprotein cholesterol, is identified as a risk factor for atherosclerotic cardiovascular disease (ASCVD) (Liu et al., 2006; Ren et al., 2010; Varbo et al., 2013a; Prenner et al., 2014), and its importance as a prevention target for ASCVD has been increasingly acknowledged by several international guidelines (Grundy et al., 2014; Deanfield et al., 2014; Catapano et al., 2016). Elevated VLDL-C is a major type of dyslipidemia, especially in China, and recent epidemiological studies supported the superiority of VLDL-C over low-density lipoprotein cholesterol (LDL-C) in terms of the population-attributable risk proportion

for ASCVD (Ren et al., 2010). However, the molecular mechanisms of VLDL-C underlying ASCVD still remain an open question.

There is compelling evidence that the receptor-mediated uptake of very low-density lipoprotein plays a key role in the pathogenesis of atherosclerosis in experimental study (Gianturco and Bradley, 1988). VLDL has a high-binding affinity to very low-density lipoprotein receptor (VLDLR) in monocyte-derived macrophages (Kosaka et al., 2001). Functional studies have shown that VLDLR can mediate the accumulation and deposition of VLDL-C in macrophages and induce macrophage-derived foam cell formation (Kosaka et al., 2001), and thus have an important pathological function in the initiation and progression of atherosclerosis (Eck et al., 2005; Tacken et al., 2002). However, VLDLR was not exclusively expressed in monocytes, its expression was also detected in other types of peripheral white blood cells (WBCs) (Su et al., 2004; Wu et al., 2009), which altogether accounted for 92–97% of WBCs. It remains uncertain whether VLDLR expression in all types of peripheral WBCs can be involved in the association between VLDL-C and atherosclerosis. To address this uncertainty, we sought to examine whether VLDL-C was associated with atherosclerosis and further

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☆ These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
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whether this association was influenced by the VLDLR expressed in all types of peripheral WBCs in a community-based study.

2. Materials and Methods

2.1. Study Participants

All participants were from the Chinese Multi-Provincial Cohort Study-Beijing Project, as described previously (Liu et al., 2014; Qi et al., 2015; Xie et al., 2016). This project was initiated in 1992 by including 1982 participants via the stratified random sampling method from a community in Beijing. Of 1982 participants, 114 died and 202 were lost to follow-up before September 2012. A total of 1252 participants (75.2% of the remaining participants) aged 55–84 years took part in the examination of cardiovascular risk factors and a carotid B-mode ultrasound measurement in September 2012. After excluding participants with incomplete information (n = 16), absence of blood samples for RNA extraction (n = 277), and RNA of insufficient quality (n = 212), 747 participants with complete data were used for final analysis. This study was approved by the Clinical Research Ethics Committee of Beijing An Zhen Hospital, and written informed consent was obtained from each participant.

2.2. Risk Factor Surveys

A standardized questionnaire was designed to collect information covering demographic characteristics, smoking status, and medical history from each participant. Anthropometric measurements and blood pressure (BP) levels were recorded during physical examinations. BP was measured in the right arm of each participant while seated using an electronic sphygmomanometer after resting for at least 5 min. Three consecutive BP readings were recorded and mean value was calculated. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Smoking referred to current smokers who smoked at least one cigarette per day on average.

2.3. Laboratory Measurements

Fasting blood samples were collected in EDTA and serum tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). One milliliter blood from an EDTA tube was immediately transferred to a new 10 mL tube containing 3 mL RNALock reagent [DP440, TIANGEN (Beijing) Co., Ltd., Beijing, China] and mixed, prior to RNA extraction from peripheral WBCs.

Total cholesterol and triglyceride levels were determined using enzymatic methods (Sekisui Medical Co., Ltd., Tokyo, Japan). High-density lipoprotein cholesterol (HDL-C) and LDL-C were measured using a homogeneous assay (Sekisui Medical Co., Ltd.). High-sensitivity C-reactive protein (hsCRP) levels were measured using a particle-enhanced immunoturbidimetric method (DiaSys Diagnostic Systems, Holzheim, Germany). Fasting blood glucose (FBG) levels were determined using a hexokinase method (Beckman Coulter, Inc., Brea, CA, USA). Information on kits used and range of sensitivity of assays for aforementioned measurement were listed in Supplementary Table S1. WBC count and differentials were measured using an automatic blood cell analyzer (XE-5000, Sysmex, Corporation). Inter- and intra-assay coefficients of variation were <5% for all blood variables. Serum VLDL-C levels were calculated by subtracting serum LDL-C and HDL-C from total cholesterol.

2.4. Assessment of VLDLR mRNA Expression in Peripheral WBC

RNA extraction from peripheral WBCs and VLDLR mRNA expression which was measured as the relative expression of VLDLR, normalized by ACTB for RNA quantity using a modified $2^{-\Delta\Delta CT}$ method were

previously described (Schmittgen and Livak, 2008; Livak and Schmittgen, 2001). In fact, the relative expression of VLDLR mRNA to ACTB mRNA can only reflect the mRNA abundance of VLDLR in 1 μg total RNA from each sample. However, in view of the wide variation of total RNA mass in WBCs per mL blood (range, 2.02–13.54 μg) in the current study, the relative expression of VLDLR mRNA to ACTB mRNA per μg total RNA cannot represent total VLDLR mRNA expression in peripheral WBCs. Therefore, total VLDLR mRNA expression in WBCs per mL blood was calculated as the product of the relative expression of VLDLR mRNA per μg total RNA and the mass of total RNA extracted from WBCs. Moreover, in this study, there was substantial variation in WBC counts among participants with the same levels of total VLDLR mRNA expression. As shown in Supplementary Table S2, there was 5.18-fold variation of WBC counts among participants with total VLDLR mRNA expression < 1.03×10^{-2}/mL, whereas 3.69-fold variation of WBC counts among participants with total VLDLR mRNA expression pants with ≥2.23×10^{-2}/mL. The various levels of average VLDLR mRNA expression in a single cell of WBC (VLDLR mRNA expression per WBC) may exert a diverse impact on the carotid atherosclerosis driven by VLDL-C under the same levels of total VLDLR mRNA expression. Accordingly, the average VLDLR mRNA expression per WBC was calculated by dividing the total VLDLR mRNA expression by WBC counts. Therefore, two indicators were used to assess VLDLR mRNA expression in peripheral WBCs in this study, viz. total VLDLR mRNA expression in WBCs per mL blood and the average VLDLR mRNA expression per WBC.

2.5. Measurement of Atherosclerosis

Ultrasonography of the right and left carotid arteries was performed with a linear array 5–14 MHz probe and a duplex B-mode scanner (Z.One Ultra; Zonare Medical Systems, Inc., Mountain View, CA, USA) according to a standardized protocol. The presence of carotid plaque was determined in each segment of both common carotid arteries, bifurcations and internal carotid arteries, in magnified longitudinal views. The maximal value of the carotid intima-media thickness (cIMT) at each of the six segments was recorded. A carotid plaque was defined as the presence of focal wall thickening 50% greater than that of the surrounding vessel wall, or as a focal region with cIMT > 1.5 mm (Stein et al., 2008). A representative carotid plaque image is shown in Supplementary Fig. S1. Carotid plaque area (mm²) was measured using automated computerized edge tracking software (Carotid Analyzer, Vascular Research Tools 6; Medical Imaging Applications, Coralville, IA, USA). The maximum plaque at each of the six carotid artery segments was measured by tracing the perimeter with a cursor on the screen in a longitudinal view, and the area of the maximum plaque was computed using Carotid Analyzer software packages. Total carotid plaque area (TPA) was defined as the sum of all maximum plaque areas (Johnsen et al., 2005).

The reproducibility of carotid atherosclerosis measurements was also assessed. Images from 20 participants with plaques were randomly selected and measured in duplicate by three observers (at intervals 2 weeks apart). The average intra-class correlation coefficients for inter-observer agreement of cIMT and TPA were 0.937 (95% confidence interval (CI): 0.843–0.975), and 0.944 (95% CI: 0.884–0.976), respectively. The minimum intra-class correlation coefficients for intra-observer agreement of cIMT and TPA were 0.928 (95% CI: 0.810–0.972) and 0.945 (95% CI: 0.864–0.978).

2.6. Sample Size Estimation

In current study, the prevalence of carotid plaque older than 55 years old was 71.4%. Based on the previous report, the hazard ratio for coronary heart disease was 1.44 in participants with VLDL-C ≥ 30 mg/dL relative to those with VLDL-C < 20 mg/dL in Chinese (Ren et al., 2010), the estimated sample size was 383, assuming α (probability of type I error)

equals 0.05 and power equals 0.90. The actual sample size of 747 in this study exceeded these limits, lending confidence to our findings.

2.7. Statistical Analysis

Continuous data are expressed as mean ($\pm$ standard deviation or SD) in case of normal distributions and as median (interquartile range, IQR) otherwise, and the between-group differences were determined by Student's t-test or Kruskal–Wallis H test where appropriate. Categorical data are presented as number (percentage), and the between-group differences were determined by the Chi-square test. Partial correlation was used to assess the relationship between *VLDLR* mRNA expression and VLDL-C while controlling for age and sex. For variables with skewed distributions, log-transformed data were used in the correlation analysis. As recommended by International Atherosclerosis Society (Grundy et al., 2014), the value > 0.78 mmol/L was thought to be dyslipidemia for VLDL-C.

VLDL-C and TPA levels were categorized using the fixed-increment method. For VLDL-C, the cutoff point for low levels was 0.61, equivalent to the cutoff point of the lowest tertile. A fixed increment of 0.21 mmol/L was used, and study participants were classified into three groups (<0.61, 0.61 to 0.81, and ≥0.82 mmol/L). For TPA, participants with no carotid plaque were set at 0 mm^2 (Alsulaimani et al. 2013). The cutoff point for the low level of plaque area was set at 15, equivalent to the cutoff point of the lowest tertile of participants with carotid plaque. A fixed increment of 15 mm^2 was used to classify study participants into four ordinal groups (0, 0 to 14.9, 15 to 29.9, and ≥30.0 mm^2).

The two indicators of *VLDLR* mRNA expression were classified into the lowest, medium and highest levels in order to examine the impact of *VLDLR* expression in WBC on the association of VLDL-C with carotid plaque. For *VLDLR* mRNA expression per WBC, the cutoff values were 1.60×10^{-9}/WBC and 3.20×10^{-9}/WBC. For total *VLDLR* mRNA expression in WBCs per mL of blood, the cutoff values were 1.03×10^{-2}/mL and 2.23×10^{-2}/mL. Both estimates of *VLDLR* mRNA expression in peripheral WBC were separately combined with the three groups of VLDL-C level to form 9 (3 × 3) subgroups. The associations of combined subgroups of *VLDLR* mRNA expression and VLDL-C with prevalence of plaque and TPA were estimated using binary and ordinal logistic regression, respectively, adjusted for age, sex, BMI, systolic BP, FBG, current smoking, LDL-C, HDL-C, antidyslipidemic drugs, and antihypertensive drugs. Bonferroni adjustment has been applied to account for multiple comparisons. The subgroups with lowest levels of both *VLDLR* mRNA expression in peripheral WBC (<1.60×10^{-9}/WBC or <1.03×10^{-2}/mL) and VLDL-C (<0.61 mmol/L) were set as the references respectively. We further adjusted for WBC counts, counts or percentages of monocyte, separately in the multivariable models to evaluate the impact of WBC or monocyte on the association of *VLDLR* mRNA expression and VLDL-C with atherosclerosis. Additional adjustment was performed for exploring the impact of hsCRP or the duration of lipid-lowering treatment on the association between *VLDLR* mRNA expression, VLDL-C and carotid atherosclerosis.

Furthermore, to test whether the unavailability of data would lead to any potential bias affecting the validity of our findings, we compared the major risk factors and levels of lipids between the 747 participants who were eligible in the final analyses and those for whom no RNA data were available. No significant differences were observed except in HDL-C and hsCRP levels (Supplementary Table S3).

Statistical analyses and graphics were performed using the SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). Sample size estimation was calculated using the PASS software for Windows (version 08.0.3, Hintze, J. PASS 2008; NCSS, LLC, Kaysville, UT, USA). All statistical tests were two-tailed, and P < 0.05 was considered statistically significant unless otherwise indicated.

3. Results

3.1. Characteristics of Study Population

A total of 747 eligible participants (mean age: 69.2 years; males: 46.6%) were analyzed, and their characteristics are summarized in Table 1. The median (IQR) level of VLDL-C was 0.70 (0.57, 0.91) (range, 0.28–4.16) mmol/L, and 38.2% of participants had VLDL-C levels ≥ 0.78 mmol/L. Estimated *VLDLR* mRNA expression per WBC was 2.61 (1.46, 5.25) × 10^{-9}/WBC with range (0.25–87.34) × 10^{-9}/WBC, and total *VLDLR* mRNA expression in WBCs per mL of blood was 1.40 (0.82, 2.88) × 10^{-2}/mL with range (0.12–62.78) × 10^{-2}/mL. VLDL-C and *VLDLR* mRNA expression across all study participants had no significant correlation after adjusting for age and sex.

3.2. Association Between VLDL-C and Carotid Atherosclerosis

Of 747 participants, 71.4% had carotid plaque. VLDL-C was probably positively associated with plaque prevalence after multiple adjustments (Table 2). Participants with the highest levels of VLDL-C might have 1.71-fold [odds ratio (OR) = 1.71, 95% confidence interval (CI): 0.91–3.23, P = 0.095] higher plaque prevalence than those with the lowest levels of VLDL-C. Among the 533 participants with carotid plaque, the median (IQR) level of carotid plaque area (TPA) was 22.2 (11.8, 44.0) mm^2. The association of VLDL-C with TPA was similar to those with plaque prevalence, and the OR for the highest levels of VLDL-C with TPA reached statistical significance (OR = 1.98, 95% CI: 1.22–3.23, P = 0.006).

3.3. Association Between VLDL-C, VLDLR mRNA Expression in WBC and Carotid Atherosclerosis

ORs were calculated for carotid atherosclerosis within combined subgroups of VLDL-C levels and *VLDLR* mRNA expression (Fig. 1). With

Table 1
Characteristics of study participants.

Variables	Total (n = 747)	Males (n = 348)	Females (n = 399)
Age, years	69.2 ± 8.0	70.6 ± 7.7	68.0 ± 8.0
BMI, kg/m^2	24.3 ± 3.4	24.5 ± 3.1	24.2 ± 3.7
Systolic BP, mm Hg	137.8 ± 16.1	137.8 ± 15.4	137.8 ± 16.7
Diastolic BP, mm Hg	78.5 ± 8.8	79.3 ± 8.4	77.7 ± 8.7
FBG, mmol/L	5.69 ± 1.16	5.67 ± 0.94	5.70 ± 1.32
Total cholesterol, mmol/L	5.08 ± 1.05	4.80 ± 1.00	5.32 ± 1.04
Triglyceride, mmol/L	1.36 (0.94, 1.88)	1.32 (0.90, 1.80)	1.43 (0.96, 1.96)
LDL-C, mmol/L	2.96 ± 0.88	2.85 ± 0.84	3.05 ± 0.90
HDL-C, mmol/L	1.33 ± 0.31	1.23 ± 0.27	1.42 ± 0.31
Non-HDL-C, mmol/L	3.75 ± 1.02	3.57 ± 0.98	3.90 ± 1.04
hsCRP, mg/L	0.80 (0.40, 1.71)	0.74 (0.38, 1.46)	0.87 (0.43, 1.89)
VLDL-C, mmol/L	0.70 (0.57, 0.91)	0.64 (0.52, 0.82)	0.75 (0.62, 0.98)
VLDLR mRNA (10^{-9}/WBC)	2.60 (1.43, 5.26)	2.46 (1.44, 4.38)	2.66 (1.50, 5.70)
VLDLR mRNA (10^{-2}/mL)	1.40 (0.82, 2.88)	1.18 (0.79, 2.55)	1.39 (0.83, 3.16)
WBC count (10^9/L)	5.67 ± 1.41	5.85 ± 1.48	5.52 ± 1.32
Current smoking, n (%)	52 (7.0)	51 (14.7)	1 (0.3)
Antidyslipidemic drugs, n (%)	232 (31.1)	95 (27.3)	137 (34.3)
Antihypertensive drugs, n (%)	387 (51.8)	187 (53.7)	200 (51.7)
Antidiabetic drugs	113 (15.1)	54 (15.5)	59 (14.8)
Plaque prevalence, n (%)	533 (71.4)	262 (75.3)	271 (67.9)
TPA, mm^{2a}	22.2 (11.8, 44.0)	27.1 (14.6, 58.0)	17.1 (9.8, 35.2)

Values are expressed as number (percent) for categorical variables, as mean ± SD for continuous variables in case of normal distributions and medians (IQR) otherwise.
BMI, body mass index; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; hsCRP, high-sensitivity C-reaction protein; VLDL-C, very low-density lipoprotein cholesterol; *VLDLR*, very low-density lipoprotein receptor; WBC, white blood cell; TPA, total plaque area; SD, standard deviation; IQR, interquartile ranges.
[a] Values are for participants with plaques.

Table 2
Logistic regression analysis of the association between VLDL-C and plaque prevalence and total plaque area in the carotid artery.

Variables	Plaque prevalence		TPA	
	OR (95%CI)	P	OR (95%CI)	P
VLDL-C low	Ref		Ref	
VLDL-C medium	1.20 (0.75, 1.91)	0.452	1.23 (0.85, 1.77)	0.280
VLDL-C high	1.71 (0.91, 3.23)	0.095	1.98 (1.22, 3.23)	0.006
P trend of VLDL-C		0.099		0.006
Age, years	1.09 (1.06, 1.12)	0.000	1.07 (1.05, 1.09)	0.000
Female	0.95 (0.64, 1.42)	0.815	0.67 (0.49, 0.91)	0.010
HDL-C, mmol/L	0.46 (0.22, 0.93)	0.032	0.44 (0.25, 0.78)	0.008
LDL-C, mmol/L	1.34 (1.07, 1.67)	0.010	1.34 (1.13, 1.60)	0.001
Antidyslipidemic drugs	1.55 (1.02, 2.36)	0.039	1.63 (1.19, 2.23)	0.002

All substantial models are additionally adjusted for systolic BP, FBG, BMI, smoking status, and antihypertensive drugs. VLDL-C level was categorized as <0.61 (low), 0.61 to 0.81(medium),≥0.82 (high) mmol/L.
VLDL-C, very low-density lipoprotein cholesterol; TPA, total plaque area; OR, odds ratio; CI, confidence interval; Ref, reference; BP, blood pressure; FBG, fasting blood glucose; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; BMI, body mass index.

respect to atherosclerotic plaque, participants with the lowest levels of both VLDL-C and *VLDLR* mRNA expression per WBC had the lowest plaque prevalence. Among those with the highest levels of VLDL-C, plaque prevalence risk was elevated, regardless of *VLDLR* mRNA expression per WBC, compared with those with the lowest levels of both variables (Fig. 1a). However, participants with the lowest levels of VLDL-C but the highest *VLDLR* mRNA expression per WBC had the highest risk of

plaque prevalence in the multivariable regression model (OR = 3.76, 95% CI: 1.70–8.30, P = 0.001) (Fig. 1a). Data in Fig. 1c showed the impact of total *VLDLR* mRNA expression in WBCs per mL of blood on the association between VLDL-C and plaque prevalence. Similar to *VLDLR* mRNA expression per WBC, the reference group had the lowest plaque prevalence, and participants in the subgroup with the lowest levels of VLDL-C but the highest total *VLDLR* mRNA expression had the highest risk of plaque prevalence in the multivariable regression model (OR = 3.66; 95% CI: 1.57–8.52, P = 0.003) (Fig. 1c).

For plaque area, participants with the lowest levels of both VLDL-C and *VLDLR* mRNA expression per WBC had the lowest risk of having larger TPA, which was also the reference. In those with the highest levels of VLDL-C, the risk of having larger TPA was elevated, regardless of levels of *VLDLR* mRNA expression per WBC, when compared with the reference (Fig. 1b). However, those with the lowest levels of VLDL-C but the highest *VLDLR* mRNA expression per WBC also had a greater risk (OR = 1.80, 95% CI: 1.01–3.21, P = 0.046) of having larger TPA (Fig. 1b). Moreover, similar results were observed in the analysis of the impact of total *VLDLR* mRNA expression in WBCs per mL of blood on the association between VLDL-C and TPA (Fig. 1d).

After additionally adjusting for WBC counts, counts or percentages of monocyte, the above associations of VLDL-C and *VLDLR* mRNA expression in peripheral WBC with the risk of carotid atherosclerosis were not influenced (Supplementary Tables S4 and S5). We also additionally adjusted for hsCRP or the duration of lipid-lowering treatment in the multivariable regression model and the above associations of VLDL-C and *VLDLR* mRNA expression in peripheral WBC with the risk of carotid atherosclerosis were not influenced (data not shown).
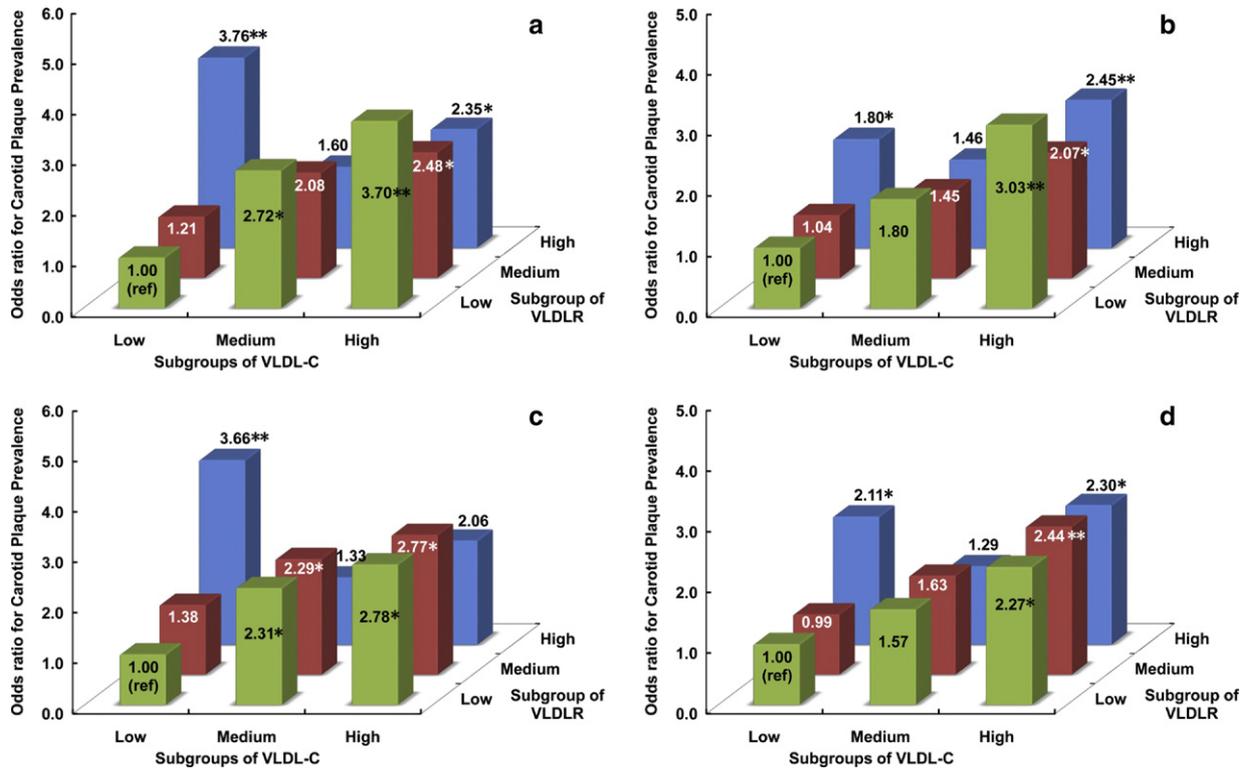

Fig. 1. Multivariable adjusted odds ratio for carotid atherosclerosis among combined subgroups of *VLDLR* mRNA expression and VLDL-C. Values are expressed as multivariable adjusted ORs for prevalence of carotid plaque (a, c) and TPA (b, d) among the combined subgroups of *VLDLR* mRNA expression and VLDL-C, estimated using binary and ordinal logistic regression, respectively. All models were adjusted for age, sex, smoking status, BMI, systolic BP, FBG, LDL-C, HDL-C, use of antidyslipidemic and antihypertensive drugs. VLDL-C level was categorized as <0.61 (low), 0.61 to 0.81(medium), ≥0.82 (high) mmol/L. *VLDLR* mRNA expression per WBC was categorized as <1.60 (low), 1.60 to 3.19 (medium),≥3.20 (high) $\times 10^{-9}$/WBC (a, b). Total *VLDLR* mRNA expression in WBCs per mL of blood was categorized as <1.03 (low), 1.03 to 2.22 (medium), ≥ 2.23 (high) $\times 10^{-2}$/mL (c, d). The subgroup with both the lowest level of *VLDLR* mRNA expression and VLDL-C was set as a reference. *P < 0.05, **P < 0.01. OR, odds ratio; TPA, total plaque area; VLDL-C, very low-density lipoprotein cholesterol; VLDLR, very low-density lipoprotein receptor; TPA, total plaque area; BMI, body mass index; BP, blood pressure; FBG, fasting blood glucose; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; WBC, white blood cell.

3.4. Distribution of WBC Subtypes Among Combined Subgroups of VLDL-C and VLDLR mRNA Expression in WBC

Insignificant differences were found in counts (data not shown) or percentages (Fig. 2) of neutrophils, lymphocytes and monocytes among all subgroups. A similar distribution of WBC subtypes was observed among the combined subgroups of VLDL-C and total *VLDLR* mRNA expression in WBCs per mL of blood (data not shown).

4. Discussion

The present study reported the impact of *VLDLR* mRNA expression in peripheral WBCs on the association between VLDL-C and carotid atherosclerosis in a generally healthy population. The key finding is that VLDL-C was positively associated with the risk of carotid atherosclerosis, but the risk of carotid atherosclerosis remained still high among participants with low VLDL-C if combined with high *VLDLR* mRNA expression. Moreover, the impact of *VLDLR* mRNA expression on the relationship between VLDL-C and carotid atherosclerosis might be independent of the counts or percentages of WBC subtypes, especially monocytes. Our findings highlighted a new atherogenic phenotype characterized by low VLDL-C but high *VLDLR* mRNA expression in peripheral WBCs, which may be of clinical importance to identify those individuals with low VLDL-C levels, low risk of ASCVD, who will be more likely to develop ASCVD events.

Our findings showed that very high levels of VLDL-C were independently associated with the increased risk of carotid atherosclerosis among asymptomatic individuals. This finding is in accordance with the observational studies of Ren et al. (Ren et al., 2010) and Liu et al. (Liu et al., 2006), who reported that elevated VLDL-C was significantly associated with elevated 15-year coronary heart disease risk in Chinese (Ren et al., 2010) and American population (Liu et al., 2006). Moreover, Varbo et al. found that elevated cholesterol content of triglyceride-rich lipoprotein particles, mainly VLDL, caused ischemic heart disease (Varbo et al., 2013a). Our study supports the atherogenic role of VLDL-C in atherosclerosis.

Previous studies have suggested that the VLDLR pathway might be a major route for monocyte-derived macrophage foam cell formation and atherosclerotic lesion development by VLDL (Kosaka et al., 2001; Eck et al., 2005). In this study, we found that *VLDLR* mRNA could be detected in neutrophils and lymphocytes (Supplementary Table S6), consistent with previous gene expression studies (Su et al., 2004; Wu et al.,

2009). However, a literature search failed to reveal any evidence for the impact of VLDLR in all type of peripheral WBCs on VLDL-C induced atherosclerosis. Our study, investigated the impact of *VLDLR* mRNA expression in peripheral WBC on the association between VLDL-C and carotid atherosclerosis, and we found that VLDL-C was positively associated with atherosclerosis risk, whereas this association may be hinged on *VLDLR* mRNA levels.

Usually, participants with low levels of VLDL-C and other optimized risk factors are categorized into the lowest cardiovascular risk. However, in this study, those with lowest VLDL-C but highest *VLDLR* mRNA expression in peripheral WBC were found to have the highest risk of plaque prevalence and an elevated risk of having larger TPA, after adjusting for other cardiovascular risk factors. This new atherogenic phenotype prompted us to ponder the relationship of the "gate" VLDLR and the "cargo" VLDL-C. The levels of serum VLDL-C were determined by VLDL-C secretion and VLDL-C turnover. VLDL-C turnover includes the transformation of VLDL-C into LDL-C and the intake of VLDL-C into cells. Moreover, *VLDLR* gene expression is not down-regulated by sterols (Sakai et al., 1994; Tacken et al., 2001), indicating that VLDLR could mediate uptake of VLDL-C independently of intracellular cholesterol. One possible reason for the high risk of carotid atherosclerosis in the newly-identified phenotype is the increased intake of VLDL-C by VLDLR on WBCs, rather than the decreased secretion of VLDL-C, inducing the formation of macrophage-derived foam cell and promoting inflammatory response (Nguyen et al., 2014; Varbo et al., 2013b). Another possibility is that when VLDLR is high enough, the VLDL-C has already been uptake into cell, masking the real risk for individuals with low levels of serum VLDL-C. Despite all this, it is so far difficult to speculate on the underlying molecular mechanism causing these responses, and further studies are warranted. The present significant findings inspired us to further interrogate the molecular mechanisms of VLDLR in all types of peripheral WBCs, especially neutrophils and lymphocytes, in VLDL-induced atherosclerosis.

This study has several clear strengths. First, it is the largest study conducted to date in a general population that measures the mRNA expression of *VLDLR* (Clemente-Postigo et al., 2011; Suzuki et al., 2008; Suzuki et al., 2010; Kim et al., 2012). Second, to the best of our knowledge, this is the largest study to explore the impact of peripheral WBC *VLDLR* mRNA expression on the association between VLDL-C and atherosclerosis in humans. Moreover, we used two estimates to assess *VLDLR* mRNA expression in WBC, and both variables indicated that very high *VLDLR* mRNA expression increased the risk of carotid
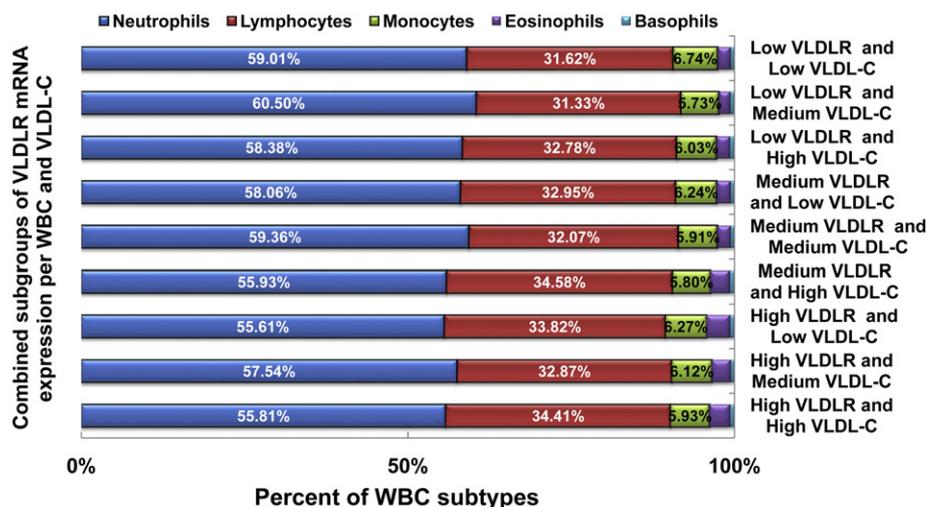
Fig. 2. Distribution of WBC subtypes among combined subgroups of *VLDLR* mRNA expression per WBC and VLDL-C. Values are expressed as means of WBC subtype percentage, including neutrophil, lymphocyte, monocyte, eosinophil and basophil. VLDL-C, very low-density lipoprotein cholesterol; VLDLR, very low-density lipoprotein receptor; WBC, white blood cell.

atherosclerosis at lowest levels of VLDL-C. Finally, we assessed the atherosclerosis burden by measuring TPA, a measure of carotid plaque area, which was associated with the risk of ASCVD (Spence et al., 2002; Spence, 2002). We used this measure along with the prevalence of plaque to assess carotid atherosclerosis as a reflection of atherosclerosis burden.

Several possible limitations of our study also deserve comments. First, except VLDLR, VLDL may be internalized by scavenger receptors including class A scavenger receptors, scavenger receptor class B type 1 and CD36 (Van Eck et al., 2000; Herijgers et al., 2000; Calvo et al., 1998; Calvo et al., 1997). Scavenger receptors are multi-ligand receptors which can bind to other lipoproteins such as acetyl LDL or oxidized LDL. Although we found that the relationship between VLDL-C, *VLDLR* mRNA expression in WBC, and the risk of carotid atherosclerosis remained unchanged after adjusting for count or percentage of monocytes (Supplementary Tables S4 and S5), we still could not exclude the impact of other receptors that bind VLDL, the intensity of dyslipidemic drugs and unknown residual confounding factors. Second, the present study obtained the level of serum VLDLC by total cholesterol minus HDL-C minus LDL-C in the fasting states. However, the cholesterol content obtained by this formula actually represents remnant cholesterol, include VLDL-C and intermediate-density lipoprotein (IDL) cholesterol. Although IDL indeed exists in circulation, its concentration is scarce and its retention period is short, leading to relatively little impact on atherosclerosis. Moreover, the aim of this study is to explore the role VLDLR in all types of peripheral WBCs in lipid driven atherosclerosis, but the IDL-transformed LDL, triggering the development of atherosclerosis, is via the scavenger receptor class B type 1 pathway rather than VLDLR. Therefore, cholesterol transferred by VLDLR into the subendothelial of the arterial vessel wall can promote the development of atherosclerosis, mainly carried by VLDL particle. Meanwhile, the cholesterol content of VLDL is not VLDL particle per se. It is VLDL particle rather than cholesterol that can bind to VLDL receptor. Use of VLDL-C as the representative of VLDL particle might induce imprecise conclusion. However, using other samples we measured the VLDL particle numbers by nuclear magnetic resonance spectroscopy (Qi et al., 2015), but not measured the VLDL receptor mRNA expression simultaneously, and the correlation analysis of VLDL-C and VLDL particle numbers showed that VLDL-C and VLDL particle numbers was highly correlated. Therefore, we used the cholesterol loading of VLDL particles (VLDL cholesterol) to partly reflect the concentration of VLDL particle, needing further investigations in the future. Third, because of the cross-sectional study design, we could not infer causality from the association between VLDLR, VLDL-C and carotid atherosclerosis. However, results from previous studies suggest that VLDLR is involved in VLDL-C uptake (Kosaka et al., 2001; Liu et al., 2009) and subsequent atherogenesis (Eck et al., 2005; Crawford et al., 2008), as well as the inflammatory response. Our results indicate a role for peripheral WBC VLDLR in the pathophysiology of VLDL-induced atherosclerosis in humans, which requires further experimental investigation. Fourth, the sample size in our subgroup analyses may not have been large enough to draw a firm conclusion, necessitating extensive and clinical validation of the current results in larger well-designed studies in the future. Finally, the observation that our study participants were of Chinese descent may limit the generalization of our findings, calling for further confirmation in other ethnic populations.

In conclusion, our study identified a new atherogenic phenotype characterized by low VLDL-C and high *VLDLR* mRNA expression in peripheral WBCs. This finding indicates that combined patterns of VLDL-C and VLDLR might be exploited in clinical practice to determine the divergent degree of cardiovascular disease risk, thus providing a personalized medicine approach to select optimal treatment strategy for individual patients. The focus of future intervention studies should therefore include decreasing VLDLR expression and the mechanisms of receptor-mediated atherogenic processes in all types of peripheral WBCs.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and (or) publication of this work.

Author Contributions

Y.Q. and D.Z. designed the study and direction of analysis. F.Z., Y.Q., J.L., W.W., W.X, J.S., M.W., Y.H. and Y.L. performed data collection. F.Z., and Y.Q. performed literature search, data analysis, figures, data interpretation and writing. J.L. and D.Z. advised for statistical analysis of data and discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.08.019.

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