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Serum low density lipoprotein subclasses in asthma

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Summary
Background: The levels of serum low-density lipoproteins (LDL) have been implicated in the inflammatory cascade in a murine model of asthma. Recent findings suggest that LDL may modulate the inflammatory state of the asthmatic airways in humans.
Objective: We explored whether LDL subclasses are associated with the occurrence and severity of asthma.
Methods: 24 asthmatics (M/F: 11/13) and 24 healthy individuals, with normal BMI and absence of metabolic syndrome, matched for age and gender. Serum concentrations of LDL subclasses were distributed as seven bands (LDL-1 and -2 defined as large, least pro-inflammatory LDL, and LDL-3 to −7 defined as small, most pro-inflammatory LDL), using the LipoPrint© System (Quantimetrix Corporation, Redondo Beach, CA, USA).
Results: LDL-1 was similar in the two groups (56 ± 16% vs. 53 ± 11, p = NS), while LDL-2 was significantly lower in asthmatics as compared to controls (35 ± 8% vs. 43 ± 10%, $p = 0.0074$). LDL-3 levels were two-fold higher in the asthmatics, but the difference did not reach the statistical significance (8 ± 7.3% vs. 4 ± 3%, p = NS). Smaller subclasses LDL-4 to LDL-7 were undetectable in controls. In asthmatics, LDL-1 was positively associated with VC% predicted ($r = +0.572$, $p = 0.0035$) and FEV_1% predicted ($r = +0.492$, $p = 0.0146$). LDL-3 was inversely correlated with both VC% predicted ($r = -0.535$, $p = 0.0071$) and FEV_1% predicted ($r = -0.465$, $p = 0.0222$).
Conclusions: The findings of this pilot study suggest a role of LDL in asthma, and advocate for larger studies to confirm the association between asthma and dyslipidemia.

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Introduction

The pathogenesis of asthma is complex and involves a condition of exaggerated airway response to stimuli, which modulates the occurrence of the clinical manifestations, and the remodeling changes of the airway wall components. The latter are among the main responsible for the non-reversible features of bronchial obstruction and the blunted response to current treatments. These phenomena are promoted and maintained by a condition of chronic airway inflammation, which represents the cardinal feature of asthma. In this regard, the pathways and mediators that modulate the inflammatory response in asthma are still a topic of research.

Recently, an apolipoprotein E (ApoE)-low density lipoproteic receptor pathway has been described as being implicated in the pathogenesis of a murine model of allergic asthma [1]. Although apolipoproteins play a key role in lipid metabolism by serving as structural components of plasma lipoproteins, for the mechanism whereby these proteins modulate asthma pathogenesis and severity has never been explored. In the cited experimental murine model of asthma, airway hyperresponsiveness (AHR) and goblet cell hyperplasia were increased in ApoE knock-out mice, suggesting a role of ApoE as negative regulator of AHR and goblet cell hyperplasia [1]. This novel observation allowed to hypothesize that lipoproteins could play some role in the manifestations of the inflammatory state of the asthmatic airways also in humans.

It is known that cholesterol promotes Th2 immunity and allergic inflammation in rodents [2]; whether this occurs in humans is unclear [3,4]. Emerging evidence from animal and cell experiments have shown anti-inflammatory effects of HDL consistent with a protective role in asthma [5]. Recently, low levels of serum HDL were found to be associated with an increased risk for asthma in adolescence, suggesting a potential role of this lipoprotein in the pathogenesis of pediatric asthma [6]. However, the association between LDL and asthma has not been investigated.

On the basis of density or size, determined by ultracentrifugation or polyacrylamide gradient gel electrophoresis (GGE), up to 7 distinct LDL subclasses can be distinguished [7,8]. Smaller LDL sizes means increased oxidative susceptibility and decreased antioxidant concentrations. Small LDL lead to retarded metabolism and decreased intra-extravascular equilibration compared to medium sized LDL [9]. In addition, it is known that oxidized LDL (oxLDL) directly interacting with proinflammatory signal pathways in vascular cells and monocytes/macrophages [10]. The macrophages "present the antigen" to T lymphocytes and this antigen may be a fragment of oxLDL "digested" by the macrophages [11]. Lately it has been emphasized that adaptive immune responses have an importance in the regulation of inflammation [12] and T cells specifically recognize epitopes in oxLDL [13].

We first explored whether the serum concentrations of the LDL subclasses were abnormal in untreated asthmatics compared to non-asthmatics. As a secondary aim, we assessed whether changes in the lipoprotein pattern were associated with lung function impairment in asthmatics.

Methods

Study subjects

We enrolled consecutive, not smoker individuals with normal body mass index and absence of metabolic syndrome, who fulfilled the diagnostic criteria for asthma [14]. Asthmatics were recruited from the Outpatient Clinic for Respiratory Diseases section of DiBiMIS of the University of Palermo, Italy. All subjects had history of asthma with a positive reversibility test to confirm the diagnosis. All of them were positive to skin test for perennial aeroallergens. When sensitization to pollens was also present, subjects were tested out of season. None of them had suffered an exacerbation within the three months preceding the study, and all were clinically stable at the time of the study. Subjects were asked to refrain from taking asthma drugs for two weeks before the study visit, to avoid any influence of inhaled corticosteroids on the lipid profile, and only rescue medications were allowed during the wash-out period. Healthy, not smoker individuals, with normal body mass index and matched for age and gender were recruited from co-workers to serve as healthy controls. Their lung function was always within normal values, and all of them denied any respiratory symptom suggestive of chronic respiratory diseases. None of the subjects was receiving statins. The study was performed in accordance with the Good Clinical Practice guidelines recommended by the International Conference on Harmonization of Technical Requirements and was approved by the Ethics Committee of the University of Palermo, Palermo, Italy (April 5, 2012); written informed consent was obtained from each volunteer.

Study design

The study was conducted in one visit, and included clinical and functional assessments, as well as measurements of serum concentrations of the lipid composition (LDL subclasses).

Clinical and functional assessments

We recorded clinical and demographic data including age, sex, duration of disease, frequency of exacerbations in the previous year. Laboratory tests included total cholesterol, LDL- and HDL-cholesterol, and tryglicerides. Functional assessment included measures of static and dynamic lung volumes, using computerized water-sealed spirometry and helium dilution technique (Biomedin; Padua, Italy). Measurements were made in accordance to the European Thoracic Society standardisation of lung volume measurements [15]. FEV_1% predicted and FVC% predicted, as well as TLC % predicted and RV% predicted, were calculated and used for the analysis.

LDL subclass analysis

Blood samples were obtained at rest after fasting overnight; blood was drawn from the subject's antecubital vein into sterile tubes. Serum was separated and stored for

subsequent analysis. Non-denaturing, linear polyacrylamide gel electrophoresis was used to separate and measure LDL subclasses, with the LipoPrint© System (Quantimetrix Corporation, Redondo Beach, CA, USA) [16]. This method has been validated against gradient gel electrophoresis and nuclear magnetic resonance and it is the only Food and Drug Administration-cleared diagnostic tool for lipoprotein subfraction testing in the USA [17]. The electrophoresed gels were scanned to determine the relative area of each lipopoprotein subfraction. The diagnostic procedure was performed for 60 min with 3 mA for each gel tube [18]. Each electrophoresis chamber involved two quality controls. For quantification, scanning was performed with a digital scanner and a Mac personal computer (Apple Computer Inc, USA). After scanning, electrophoretic mobility and the area under the curve were calculated qualitatively and quantitatively. LDL subclasses were distributed as seven bands (LDL-1 to LDL-7, respectively), in consistency with the data that can be obtained by gradient gel electrohoresis [9]. LDL-1 and -2 are defined as large LDL; LDL-3 to −7 are defined as small LDL.[18].

Statistical analysis

Statistical analysis was performed using Statview® 5.0 (SAS Institute Inc., Cary, NC, USA). Univariate analysis was performed using non-parametric Mann−Whitney test for numeric variables, while the differences in the prevalence for nominal variables were analysed by the McNemar test. Correlation analysis was performed using the Spearman rank correlation method. Multivariate analysis was performed to establish whether the significant correlations between LDL subclasses and lung parameters, as assessed by univariate analysis, remained significant after correcting for age, gender and BMI. Given the explorative nature of this pilot study, no power calculation was performed.

Results

A total of 24 asthmatics (M/F: 11/13) and 24 healthy subjects (M/F: 11/13) were enrolled. Five asthmatics were receiving inhaled corticosteroids, which were stopped two weeks prior to the study visit. In the asthmatic group, FEV_1 % predicted was 91 ± 18% (mean ± SD, range: 52−121%), VC % predicted was 100 ± 14% (range: 82−112%), and FEV_1/FVC was 74 ± 9% (range: 58−91%). Individual lung function characteristics of the asthmatics are presented in Table 1.

Fig. 1 shows the single bands of LDL subclasses obtained with the applied method in asthmatics and healthy subjects. By LDL subclass analysis we found that LDL-1 was similar in the two groups (56 ± 16% vs. 53 ± 11, p = NS), while LDL-2 was significantly lower in asthmatics as compared to controls (35 ± 8% vs. 43 ± 10%, $p = 0.0074$). Conversely, LDL-3 levels were two-fold higher in the asthmatics, but the difference did not reach the statistical significance (8 ± 7.3% vs. 4 ± 3%, p = NS). Furthermore, smaller subclasses LDL-4 to LDL-7 were undetectable in controls, while the asthmatics had little presence of LDL-4 (2 ± 4%) and traces of LDL-5 and LDL-7 (0.2 ± 0.7% and 0.2 ± 0.8%, respectively). This is summarized in Table 2.

Table 1 Demographic and lung function characteristics of the asthmatics.

	Gender	Age	VC % pred.	FEV_1 % pred.	FEV_1 /VC	RV % pred.	TLC % pred.	RV/TLC % pred.
1	F	45	86	71	70.9	92	86	107
2	F	22	111	99	77.6	134	118	113
3	F	35	113	116	89.5	139	133	115
4	M	33	82	79	76.5	83	82	98
5	M	58	108	105	74.7	152	120	119
6	F	38	109	105	83.8	87	100	87
7	M	40	73	67	73.1	58	68	84
8	M	55	65	52	61.8	87	71	118
9	M	59	102	110	82.7	86	94	89
10	M	61	92	105	85.9	82	86	90
11	F	48	114	89	67.0	72	95	77
12	M	74	101	109	79.4	163	122	127
13	F	36	120	102	73.4	142	126	112
14	M	61	89	72	62.2	91	88	105
15	F	53	111	101	78.4	95	99	97
16	F	64	111	77	58.5	99	98	100
17	M	44	99	94	74.6	94	97	96
18	F	40	117	107	79.1	102	110	93
19	F	57	88	79	76.5	123	96	133
20	F	55	107	83	66.1	86	94	89
21	M	31	94	74	63.5	95	94	100
22	F	59	112	121	91.0	99	100	98
23	M	64	102	78	58.2	108	102	106
24	F	52	98	93	80.7	137	108	125

In the asthmatic group, we performed correlation analysis between LDL subclasses and lung function parameters. LDL-1 was positively associated with VC% predicted ($r = +0.572$, $p = 0.0035$) and FEV_1% predicted ($r = +0.492$, $p = 0.0146$) (Fig. 2, panels A and C). Conversely, LDL-3 was inversely correlated with VC% predicted ($r = -0.535$, $p = 0.0071$) and FEV_1% predicted ($r = -0.465$, $p = 0.0222$) Fig. 2, panels B and D). In addition, TLC % predicted positively correlated with LDL-1 ($r = +0.585$, $p = 0.0026$) and inversely with LDL-2 and LDL-3 ($r = -0.412$, $p = 0.0237$ and $r = -0.485$, $p = 0.0163$, respectively). Neither RV nor RV/TLC significantly correlated with LDL subclasses (Table 3). Finally, no

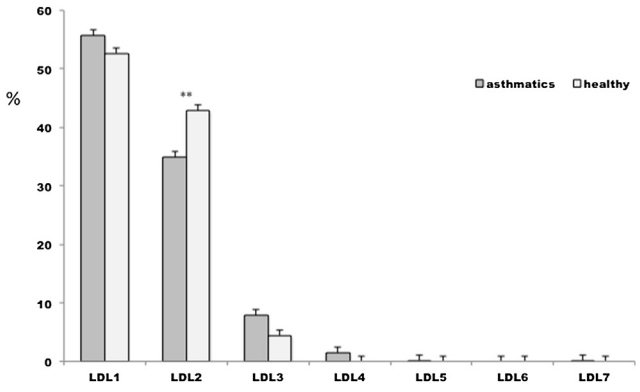

Figure 1 Histograms showing the single bands of LDL subclasses in asthmatic and healthy subjects. **$p < 0.01$.

Table 2 Demographic and laboratory parameters in asthmatics and controls.

	Asthmatics (n = 24)	p=	Controls (n = 24)
Females, n (%)	13 (54)	/	13 (54)
Age (years)	49 ± 13	0.8	50 ± 11
Body Mass Index (kg/m^2)	25 ± 3	0.4	26 ± 4
Smoking, n (%)	9 (38)	0.8	10 (42)
Total Cholesterol (mg/dL)	184 ± 31	0.0009	151 ± 28
Triglycerides (mg/dL)	119 ± 62	0.1	95 ± 38
LDL-cholesterol (mg/dL)	108 ± 25	0.0002	79 ± 22
HDL-cholesterol (mg/dL)	50 ± 13	0.6	53 ± 13
LDL-1 (%)	56 ± 16	0.3	53 ± 11
LDL-2 (%)	35 ± 8	0.0074	43 ± 10
LDL-3 (%)	8 ± 7	0.09	4 ± 3
LDL-4 (%)	2 ± 4	0.2	0 ± 0
LDL-5 (%)	0.2 ± 0.7	0.8	0 ± 0
LDL-6 (%)	0 ± 0	–	0 ± 0
LDL-7 (%)	0.2 ± 0.8	0.8	0 ± 0

relationship was detected between LDL subclasses and the rate of exacerbation in the previous year. The significant correlations involving LDL-4 and LDL-5 (Table 3), should be taken into account with caution since these LDL subclasses were mostly undetectable in the asthmatic group (Table 2).

Multivariate analysis was performed to establish whether the significant correlations between LDL subclasses and lung parameters remained significant after correcting for age, gender and BMI. The analysis confirms the positive association of LDL-1 with VC % predicted and the negative association of LDL-3 with FEV_1 % predicted, as well as the association of TLC% predicted with LDL-1 and LDL-2.

Discussion

The lung has never been considered as an organ sensitive to circulating lipoproteins and their cholesterol cargo. However, recent findings have suggested an important and, perhaps, unique role for lipoproteins and cholesterol in pulmonary physiology and pathophysiology. The findings of this pilot study suggest a role of low density lipoproteins in asthma. In asthmatics, the least pro-inflammatory LDL (LDL-1 and LDL-2) appear to be lower, and the most pro-inflammatory (LDL-3 and LDL-4) higher, compared to healthy controls, although the difference did not always reached significance. In addition, the serum concentrations

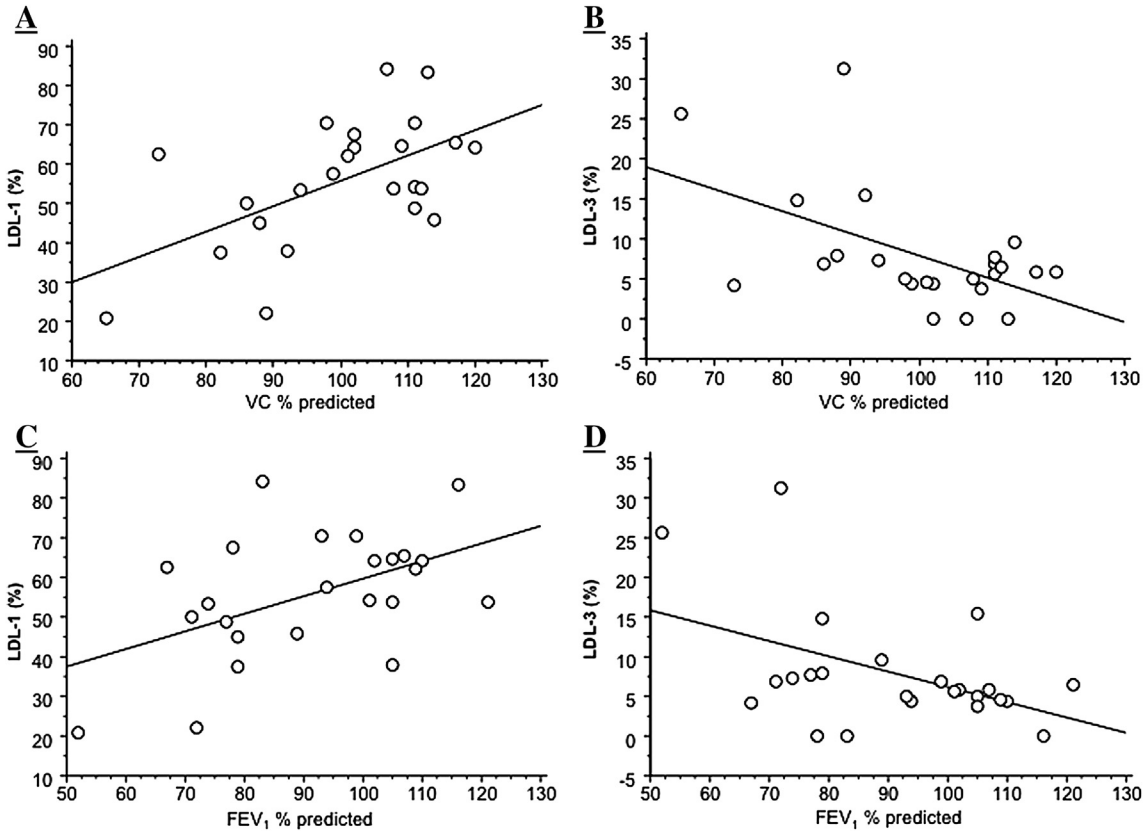

Figure 2 Spearman correlations between VC % predicted and LDL-1 (Panel A: $r = +0.572$, $p = 0.0035$) and LDL-3 (Panel B: $r = -0.535$, $p = 0.0071$), as well as between FEV_1 % predicted and LDL-1 (Panel C: $r = +0.492$, $p = 0.0146$) and LDL-3 (Panel D: $r = -0.465$, $p = 0.0222$).

Table 3 Spearman correlations between LDL subclasses and lung volumes. In bold the relationships that reached statistical significance ($p < 0.05$). *This relationship remained significant when adjusted for gender, age and body mass index.

Parameter	LDL-1	LDL-2	LDL-3	LDL-4	LDL-5	LDL-6	LDL-7
VC % predicted	**0.572***	−0.331	**−0. 535**	**−0.539**	**−0.529**	/	0.133
FEV_1 % predicted	**0.492**	−0.288	**−0.465***	**−0.473**	**−0.471**	/	0.166
FEV_1/VC	0.326	−0.195	−0.302	−0.341	−0.288	/	0.215
RV % predicted	0.346	−0.231	−0.278	−0.290	−0.138	/	0.138
TLC % predicted	**0.585***	**−0.419***	**−0.485**	**−0.468**	−0.375	/	0.007
RV/TLC % predicted	−0.031	0.010	0.067	0.042	0.215	/	−0.237

of the LDL-1 (least pro-inflammatory) positively correlated with lung function, whereas the serum concentrations of LDL-3 (most pro-inflammatory) were negatively associated with lung function.

The activation of the innate immune system by cholesterol overload has only recently been examined in the context of the lung [2,19—23]. Circulating LDL and HDL are both taken up by the lung through specific receptors, and supply cholesterol to lung-resident cells, thereby inhibiting local pulmonary cholesterol biosynthesis. Although cholesterol is essential for type II cell function, excessive amounts of cholesterol impair surfactant function, suggesting the critical importance of alveolar cholesterol homeostasis to normal lung physiology [24—27]. Several experimental studies describing the pathogenic role of Th1 immunity based on induction of disease by hypercholesterolemia suggest that the relevant autoantigen is a lipoprotein or possibly a protein modified by lipids. Most attention has focused on the role of LDL in these processes and strong evidence for the existence of autoimmune responses against LDL has been provided [28]. In this context, Akdis et al. have reported that in allergy and asthma, the balance between the Th1, Th2 and T regulatory cytokine responses appears to be shifted towards Th2, and that synthetic lipopeptides (which contain the typical lipid part of the lipoprotein of gram-negative bacteria) stimulate a particular regulatory cytokine pattern (abundant levels of IL-10 and IFN- γ) and inhibit several Th2 cell-related phenomena (including IgE production and eosinophilia) [29]. Similarly, ongoing studies point out to the T cells as the most likely candidate for the alternative protective immune pathway and the possibility of developing immune-modulating therapy for prevention of cardiovascular disease [28].

These observations led us to explore the role of lipoproteins in asthma. By discriminating among different subclasses, we were able to demonstrate that larger LDL subclasses, specifically LDL-2, were diminished in asthmatic subjects, as opposed to healthy controls, perhaps inducing a shift towards the production of the atherogenic, small LDL, leading to the amplification of the inflammatory cascade in asthma. At this stage, we can only infer on causative relationships between pro-inflammatory components and impairment in lung function. The positive relationship between the least pro-inflammatory LDL subclasses and lung function, and the negative association between the most pro-inflammatory LDL and lung function strongly suggest that LDL are a potent trigger for the inflammatory changes of the airways. The apparent contradictory behavior of LDL-2 in relation to lung function deserves speculation. The relatively small number of asthmatics included might be a reason for the difference with regard to lung function parameters (LDL-1 and -2). On the other hand, the different association with lung function parameters might indicate a shift towards the production of the atherogenic, small LDL, leading to the amplification of the inflammatory cascade in asthma, or might envisage a 'transition state' with disease progression. However, these explanations need further specifically designed studies.

The available literature indicates that severe hypercholesterolemia can induce a switch of autoimmune responses from Th1 to Th2 effector type in apoE knockout (E0) mice [30]. This could either be due to a direct effect of the hypercholesterolemic state on immune activity or to a metabolic change caused by the high-cholesterol diet [31]. The diet-induced dyslipidemia has been shown to induce a dysregulated compartmentalization of neutrophils and cytokines between serum and airspace, enhancing host defenses in the former but compromising host defense in the latter [32,33]. Recently, Yao et al. [1,34] applied an experimental model of house dust mite (HDM)-induced murine asthma to identify pulmonary genes that are upregulated in response to HDM challenge, and remain persistently upregulated despite corticosteroid treatment. In HDM-challenged mice, genome-wide expression profiling of the asthmatic lung transcriptome identified ApoE as a steroid-unresponsive gene with persistently upregulated expression despite steroid treatment. ApoE knockout mice develop hypercholesterolemia and spontaneous atherosclerosis. Interestingly, the dyslipidemic murine strains were recently reported to display increased airway hyperresponsiveness and mucus production but normal airway inflammatory cell counts. These observations could carry clinical implications. Administration of an ApoE mimetic peptide prevented the induction of airway inflammation, airway hyperreactivity, and goblet cell hyperplasia in HDM-challenged E0 mice. Schafer et al. [35] investigated, in a case—control fashion, the relationship between serum cholesterol levels and clinical manifestation of atopy. The total and LDL levels were inversely associated with the frequency of allegic rhinoconjunctivitis and allergen sensitization, whereas positive associations were found between HDL levels and frequency of allergic rhinoconjunctivitis and atopic eczema.

Our findings could be supported by the suggested benefit of statins on asthma symptoms through their anti-inflammatory effect [36,37]. Accordingly, a large prospective population-based cohort study (the Rotterdam Study) reported the lower risk of death in chronic

obstructive pulmonary disease (COPD) patients using statins [38]. The recent meta-analysis shown that even a subpopulation of asthmatics, including smokers and obese individuals who respond poorly to inhaled corticosteroids may respond preferentially to statins [39]. However, it seems that statins do not to have any additional benefit in asthma control or steroid-sparing effect in asthma treatment [40]. Nevertheless, most recent *in vitro* study shown that lovastatin attenuates both TGF-β1-induced phenotypic transdifferentiation of asthmatic bronchial fibroblasts into myofibroblasts in culture and human bronchial fibroblasts proliferation [41]. Also, rosuvastatin is a potent inhibitor of human airway smooth muscle cells growth induced by different mitogenic stimuli [42]. Both simvastatin and atorvastatin treatment reduced leukocytes and leukotrienes in sputum, as well an improvement in FEV1 [43,44]. Some authors highlighted the mevalonate-dependent and -independent pathways that may be modulated by statins as an opportunity to develop new treatments for asthma [45].

In conclusion, the findings of the current study propose a role of LDL in asthma. The elucidation of the mechanism by which this occurs, and the relationship between dyslipidemia and asthma need specifically designed studies.

Competing interest

The authors declare no conflict of interest.

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