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Evaluation of sub-clinical atherosclerosis and plasma levels of minimally modified LDL in patients with ankylosing spondylitis and its correlation with disease activity

Fernanda Teles Ceccon[a], *Valderílio Feijó Azevedo*[b,*], *Carlos A. Engelhorn*[a], *Dulcinéia S. P. Abdalla*[c], *Tanize E. S. Faulin*[c], *Luis Cesar Guarita-Souza*[a], *Roberto Pecoits-Filho*[a], *José Rocha Faria-Neto*[a]

[a]*Pontifícia Universidade Católica do Paraná, Curitiba, PR, Brazil*
[b]*Hospital de Clínicas, Universidade Federal do Paraná, Curitiba, PR, Brazil*
[c]*Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, SP, Brazil*

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ABSTRACT

Introduction: Accelerated atherosclerosis has been shown in some autoimmune diseases, mainly in Systemic Lupus Erythematosus and Rheumatoid Arthritis. Although high prevalence of corticosteroids use may be a confounding factor due to their detrimental effects on several risk factors, systemic inflammation per se is supposed to play an important role in atherogenesis in these patients.

Methods: We have evaluated sub-clinical atherosclerosis and plasma levels of circulating electronegative LDL, which represents the fraction of LDL that is minimally modified, in patients with ankylosing spondylitis (AS). Fourteen patients who fulfilled the modified New York criteria for AS were compared with 13 paired controls. Carotid intimal-media thickness (IMT) was assessed by ultrasonography bilaterally in common carotid artery, internal carotid artery and in the bifurcation. Groups were homogeneous regarding cardiovascular risk factors. Only a single patient in AS group was in use of corticosteroid.

Results: The presence of active inflammation was demonstrated by elevated BASDAI and higher CRP levels and in patients versus controls (12.36 vs. 3.45 mg/dl, P = 0.002). No difference was found in carotid IMT between both groups, in any site of artery. Averaged IMT (6 measurements, at 3 pre-specified sites bilaterally) was 0.72 ± 0.28 in AS group and 0.70 ± 0.45 mm in controls (P = 0.91). Minimally modified LDL did not differ significantly either between patients and controls (14.03 ± 17.40 vs. 13.21 ± 10.21; P = 0.88).

Conclusions: Patients with AS did not show increased carotid IMT in comparison to controls. In the same way, circulating plasma levels of LDL (-), did not differ significantly in both groups.

* *Corresponding author.*
E-mail: valderilio@hotmail.com (V. F. Azevedo).

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Avaliação da aterosclerose subclínica e de níveis plasmáticos de LDL minimamente modificada em pacientes com espondilite anquilosante e sua correlação com a atividade da doença

Palavras-chave:
Aterosclerose
Inflamação
Espondilite anquilosante
LDL minimamente modificada
Espessura da íntima-média da carótida

RESUMO

Introdução: A aterosclerose acelerada foi demonstrada em algumas doenças autoimunes, principalmente lúpus eritematoso sistêmico e artrite reumatóide. Embora a alta prevalência do uso de corticosteróides possa ser um fator complicador, por causa de seus efeitos prejudiciais em diversos fatores de risco, acredita-se que, nesses pacientes, a inflamação sistêmica *per se* desempenhe papel importante na aterogênese.

Métodos: Avaliamos a aterosclerose subclínica e os níveis plasmáticos de LDL eletronegativa circulante em pacientes com espondilite anquilosante (EA). Catorze pacientes que atendiam aos critérios de Nova York modificados para EA foram comparados com 13 controles equiparados. Avaliamos a espessura da íntima-média (EIM) na carótida por ultrassonografia bilateral da artéria carótida comum, artéria carótida interna e na bifurcação. Os grupos foram homogêneos, no que tange a fatores de risco cardiovasculares. Apenas um paciente no grupo de EA estava sendo medicado com corticosteróide.

Resultados: A presença de inflamação ativa foi demonstrada por BASDAI elevado e níveis mais elevados de PCR em pacientes *versus* controles (12,36 vs. 3,45 mg/dl, P=0,002). Não observamos diferença na EIM da carótida entre os dois grupos, em qualquer local da artéria. A média de EIM (6 mensurações em 3 locais pré-especificados, bilateralmente) foi 0,72 ± 0,28 no grupo de EA e 0,70 ± 0,45 mm nos controles (P=0,91). Também não observamos diferença significativa na LDL minimamente modificada entre pacientes e controles (14,03 ± 17,40 vs. 13,21 ± 10,21; P=0,88).

Conclusões: Pacientes com EA não demonstraram aumento na EIM da carótida, em comparação com controles. Do mesmo modo, os níveis plasmáticos circulantes de LDL(-) não diferiram significativamente nos dois grupos.

Introduction

Atherosclerosis is a progressive disease of the large and medium size arteries involving inflammation, lipid accumulation, cell death, and thrombosis in vessel wall.[1] The "response to injury hypothesis" postulates that long-term endothelial cell injury alters endothelial permeability to low density lipoproteins (LDL) and induces leukocyte adhesion and migration to sub-endothelial space.[2] Regardless of the risk factor inducing endothelial disfunction, the following inflammation process will lead to plaque formation. The uptake of oxidized LDL (OxLDL), but not native LDL, by macrophages in vessel wall will lead to the formation of foam cells that are not only a reservoir of modified lipids, but also a source of proinflammatory mediators contributing to plaque progression.[3]

Therefore, proinflammatory OxLDL may be a unifying link between lipid accumulation and inflammation.[4] Although most of the oxidation of LDL occurs in the vessel wall, lipoproteins can be minimally modified in plasma becoming more prone to oxidation on a subsequent entry into the intima.[5] A more electronegative subfraction of LDL, called LDL(-) has been subfractionated by high resolution ion exchange chromatography (IE-HPLC) and seems to represent circulating minimally modified LDL in plasma.[6]

The recognition that inflammation is the main feature of atherosclerotic disease has led to a series of studies reporting high prevalence of atherosclerosis in chronic inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[7,8] There is a twofold increased risk for myocardial infarction and stroke in patients with RA, with risk increasing to nearly threefold in those who have the disease for 10 years or more.[9] These increased morbidity and mortality due to atherosclerosis seem to depend not only on traditional RF, that may be negatively affected by corticosteroid use. Inflammatory mechanisms seem to be associated with worse cardiovascular outcomes in this patients.[10-12] Although some controversial results have been published, another plausible mechanism for accelerated atherosclerosis in these patients may be an increased level of OxLDL.[13]

Ankylosing spondylitis (AS) is a chronic rheumatic disease that compromises mainly the spine and sacroiliac joints. Despite its inflammatory origin like AR and SLE, it is not totally clear if atherosclerosis accounts for higher mortality in these patients.[14,15] As steroids are not commonly part of clinical treatment of these patients, AS may be a better model to evaluate the role of inflammation in atherosclerosis. In this study we evaluated subclinical atherosclerosis (carotid intimal-media thickness – IMT) and plasma levels of minimally modified LDL (LDL(-)) in patients with AS in comparison to controls.

Methods

Study population

In this cross-sectional study we enrolled 14 patients who fulfilled the modified New York[16] criteria for AS and 13 controls.

Written informed consent was obtained from all subjects and the research protocol was approved by the Ethical Committee of the Catholic University of Paraná.

The essentials of diagnosis of AS were inflammatory back pain (IBP) in young adults, generally worst in the morning; progressive limitation of back motion and chest expansion; peripheral arthritis; anterior uveitis; diagnostic radiographic changes in sacroiliac joints and elevated erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP). Patients were recruited in the ADORE Association, a Brazilian association of rheumatic patients. All enrolled patients had axial disease without peripheral arthritis or extra-articular manifestations. Controls were enrolled in a 1:1 ratio matched for age, sex and CVD risk factors status. Subjects were also questioned about lifestyle and clinical information. Age, gender, BASDAI and BMI were recorded. Blood samples were collected for the biochemical measurements of complete lipid profile, fasting blood glucose, hematology evaluation, erythrocyte sedimentation rate, CRP and minimally modified LDL. Samples were collected in the morning, after a fasting period of 10-12h; serum and plasma were separated and frozen at -22°C. Cholesterol and triglyceride levels were determined by fully enzymatic techniques. LDL cholesterol was calculated as described by Friedwald formula. High sensitivity CRP was assessed by turbidimetry methodology.

Minimally modified LDL (LDL(-))

The plasma LDL fraction is comprised of a heterogeneous population of particles that varies with respect to charge,[17] density, size, antioxidant content, presence of apoproteins other than apoB and degree of oxidative modification.[18] Concentrations of LDL(-) in blood plasma were determined by ELISA using two anti-minimally human LDL monoclonal antibodies (MAb 1A3 and MAb 2C7).

Microplates (EIA/RIA, Costar, Cambridge, Mass., USA) were coated with 50µl MAb 1A3 (1µg/well) in carbonate-bicarbonate buffer (pH 9.4, 0.1M) and incubated overnight at 4°C. Then, each microplate was washed three times with phosphate-buffered saline (PBS; Tris-HCl 50mM and NaCl 150mM, pH 7.4) containing tween 20 (0.5%) and blocked with 5% nonfat dry milk for 2 hours at 37°C. The microplates were washed again and incubated with 50µL plasma for 2 hours at 37°C. The plates were washed and incubated with anti-LDL MAb 2C7 biotinylated for 2 hours at 37°C. After washing, the microplates were incubated with streptavidin-HRP (horseradish peroxidase) conjugate (Rockland Immunochemicals for Research, Gilbertville, Pa., USA) for 1 hour at 37°C. Then, the OPD substrate solution was added to each well. The absorbance was determined immediately using a microplate reader (SpectralCount Microplate Photometer, Packard Instruments Company, Downers Grove, IL, USA). The calibration curve was made with LDL obtained from human plasma. All samples and standards were run in triplicate. The intra-assay and interassay variations for this ELISA test were 8% and 15%, respectively.

Carotid intima-media thickness assessment

Carotid IMT is associated with the risk of coronary artery disease, stroke and myocardial infarction and predicts the progression of CVD19. Measurement of IMT was taken at the common distal carotid (1-2cm proximal to carotid bifurcation), bilaterally in the bifurcation and in the internal carotid, as well as at the origin of the right subclavian artery.

During the analysis, the greatest right and left carotid IMT values were considered, as well as the value measured at the origin of the right subclavian artery. The right subclavian artery was easily evaluated since it is more superficial than the contra-lateral subclavian artery; however, this does not denote advantages or technical limitations relative to the carotid arteries. The measurement of the intima-media complex was performed with Siemens Sonoline Elegra® vascular ultrasonography equipment. A 7.5 mHz linear transducer was used, with a frequency range of 7-9 mHz, longitudinal section and B-mode images. Thickness measurement was performed at the anterior or posterior artery wall, as the distance between two echogenic lines corresponding to the lumen-intima and media-adventitia interfaces of the artery wall.

Statistical analysis

All analyses were performed with GraphPad Prism (Version 3.02, GraphPad Software Incorporated). Continuous variables are presented as means ± standard deviation, and categorical variables as number and percentages. The two groups were compared using the Student t-test. Chi-square statistics were used to assess differences between categorical variables. Pearson's correlation analysis was used to test univariate relations. Prediction of independent variables was obtained by stepwise, forward, multiple regression model including potential confounders. A P value of < 0.05 was considered significant.

Results

Initially 33 subjects were screened for the study. Twenty seven of those were found eligible and willing to participate. The baseline characteristics of patients and controls are presented in Table 1.

Subjects were predominantly male, and mean age of the patients was 45.14 years ± 8.99 vs 46.75 years ± 8.40 of the controls (P = 0.642). Systolic and diastolic blood pressure was similar in both groups. Body mass index (kg/m^2), was 25.60 ± 5.02 for AS group and 28.34 ± 4.09 for controls (P = 0.139). The abdominal circumference in patients and controls was, respectively, 94.0 ± 14.3 cm vs. 93.8 ± 14.3 (P = 0.976). As expected, BASDAI was higher in AS patients than in controls (5.6 ± 1.2 vs 1.3 ± 0.7; P = 0.047). Prevalences of hypertension and diabetes were similar in both groups. Mean time of diagnosis was 12 years for the patients with AS. Only a single patient was in use of corticosteroids. No patient was using anti-TNFs.

Biochemical parameters are presented in Table 2. Lipid profile was similar in both groups, with no difference regarding total cholesterol, HDL cholesterol, triglycerides and LDL cholesterol. Most inflammatory markers were elevated in patients with AS. Patients had higher levels of CRP (12.36 ± 7.99 vs. 3.45 ± 4.81; P = 0.002) and total leukocyte count (9550 ± 2256 vs. 6915 ± 1721; P = 0.003). ESR did not differ significantly in both groups. Platelet count was also higher in patients with

Table 1 – Clinical characteristics of the ankylosing spondylitis and control groups.

Characteristics	AS (n = 14)	Control group (n = 13)	P
Age (years)	45.14 ± 8.99	46.75 ± 8.40	0.642
Male(%)	78.57	76.92	
Body mass index (kg.m^{-2})	25.60 ± 5.02	28.34 ± 4.09	0.139
Abdominal circumference (cm)	94 ± 14.34	93.83 ± 14.31	0.976
DM (%)	7.14	7.70	
Hyperlipidemia (%)	21.43	30.77	
Diastolic blood pressure (mmHg)	78.93 ± 12.12	76.67 ± 6.51	0.552
Hypertension (%)	21.43	30,77	
BASDAI	5.6 ± 1.2	1.3 ± 0.7	0.0712

AS, ankylosing spondylitis; DM, diabetes mellitus; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index.

Table 2 – Biochemical parameters of the ankylosing spondylitis and control groups.

Biochemical measurement	AS (n = 14)	Control group (n = 13)	P
Cholesterol (mg/dL)	185.40 ± 34.88	221.60 ± 59.46	0.080
HDL (mg/dL)	46.79 ± 10.64	44.25 ± 11.11	0.559
Triglycerides (mg/dL)	131.20 ± 66.53	196.40 ± 105.20	0.080
LDL (mg/dL)	112.10 ± 29.10	140.70 ± 47.80	0.099
LDL(-) (U/L)	14.03 ± 17.40	13.21 ± 10.21	0.880
Glucose (mg/dL)	95.43 ± 10.53	109.10 ± 13.33	0.009
Leukocytes	9550 ± 2256	6915 ± 1721	0.003
Platelets (x10^3)	334 ± 106	252 ± 66	0.028
ESR (mm)	21.64 ± 15.01	15.64 ± 12.42	0.285
CRP (mg/L)	12.36 ± 7.99	3.45 ± 4.81	0.002

AS, ankylosing spondylitis; DM, diabetes mellitus; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index.

AS (334,000 ± 106,000 vs. 252,000 ± 66,000; P = 0.028), as have been previously demonstrated in other rheumatic diseases.[20] Although subjects were matched for diabetes, glucose level was higher in control than in AS patients (95.43 ± 10.53 vs. 109.1 ± 13.33; P = 0.009). LDL(-) did not differ significantly between patients with AS and controls (14.03 ± 17.40 vs. 13.21 ± 10.21; P = 0.88).

Data regarding carotid IMT are summarized in Table 3. Regardless of the location (internal, bifurcation or common carotid) and side (left or right) of the artery where IMT was assessed, no difference was found between the two groups. As previously described, right subclavian was also evaluated and no difference was found either.

Table 3 – Doppler ultrasonography assessment of intima-media thickness (IMT) of left (L) and right (R) carotid and right subclavian artery.

Biochemical measurement	AS (n = 14)	Control group (n = 13)	P
Left common carotid (mm)	0.58 ± 0.13	0.69 ± 0.53	0.461
Left bifurcation (mm)	0.86 ± 0.42	0.85 ± 0.35	0.982
Left internal carotid (mm)	0.74 ± 0.54	0.67 ± 0.15	0.623
Right common carotid (mm)	0.55 ± 0.16	0.55 ± 0.12	0.944
Right bifurcation (mm)	0.82 ± 0.37	0.80 ± 0.42	0.889
Right internal carotid (mm)	0.79 ± 0.53	0.70 ± 0.45	0.652
Right subclavian (mm)	1.16 ± 0.62	0.95 ± 0.30	0.287

AS, ankylosing spondylitis.

Discussion

Atherosclerosis is an inflammatory condition and inflammation experienced in chronic immune diseases may contribute to accelerate atherosclerosis in this situations.[21] In this study, despite of active inflammation as demonstrated by high CRP levels and BASDAI, patients with AS did not show higher prevalence of sub-clinical atherosclerosis, as demonstrated by carotid IMT. In the same way, circulating plasma levels of LDL(-), which represents the fraction of LDL that is minimally modified, did not differ significantly in both groups.

Our study is in accordance with a previous study that also demonstrated lack of correlation between AS and increased IMT.[22] In the study by Sari et al., young age of patients (mean age, 37 years old) could have explained, at least in part, the lack of correlation if inflammation was really assumed as an important factor for atherosclerosis development. In the same study, a compromised endothelial function, as demonstrated by impaired flow mediated dilation of brachial artery, was also shown. This could be interpreted as evidence that maybe patients were evaluated in a very initial phase of atherosclerosis development, since endothelial dysfunction precedes the morphological changes in arterial wall.[23]

Whether age (mean, 45 years old) was a limiting factor in our study is not known. Considering patients with other rheumatologic conditions, not only IMT is already increased in the fourth decade,[24] but also the length of disease (the mean time of diagnosis in our population was 12 years) is a determinant of thickening.[25]

In a publication where IMT was also assessed in patients with AS, only a trend towards increased thickness was seen.[26] Similarly to our study, patients in control group had a slight worse metabolic profile than patients in AS, specially glucose levels.[26] An interesting finding in this study was that arterial stiffness, another marker of subclinical atherosclerosis, was not modified by TNF-α inhibitors, suggesting that modulation of inflammatory response has no effect on arterial wall injury.[26] None of our patients were being treated with TNF-α inhibitors.

This was the first study to evaluate plasma levels of LDL(-) in patients with AS. Although elevated levels of oxidized LDL have been previously reported in patients with other autoimmune diseases,[27] little has been published regarding minimally modified LDL. These moieties are more prone to be oxidized when into the sub-endothelial space, where most of oxidation process occurs.

Although not as pro-atherogenic as oxidized LDL, minimally modified forms induce monocyte adhesion to endothelial cells and MCP-1 production, critical steps in early atherogenesis.[28,29] The source of these minimally modified forms remains unclear; however, there are possible sources:[30] oxidation of LDL in the arterial wall followed by egress

into the circulation, ingestion of oxidized fats and/or generation from postprandial lipoprotein remnants or direct oxidation in blood plasma. Regardless of the source, our findings do not support any role of inflammation in this initial modification of LDL particle. Not only plasma levels were similar in AS and control group, but also no correlation was found between minimally modified LDL and CRP levels (data not shown). Whether the same concept can be applied to oxidized LDL levels in patients with AS remains to be exploited. In patients with SLE and RA, not only oxidized LDL, but also their autoantibodies, are associated to atherosclerosis.[31,32]

It is not clear why most of the data from patients with AS are in the opposite direction of studies with SLE and RA regarding atherosclerosis. It's worth mentioning that corticosteroids treatment is always a confounding factor in these studies. While commonly prescribed in patients with RA and SLE, this is not a first line treatment in AS. In our study, a single patient was in use of prednisone. Mainly when used in high doses, corticosteroids have an important detrimental effect on several cardiovascular risk factors. These effects can be in part responsible for the accelerated atherosclerosis seen in patients with SLE and RA.[33,34] Further studies including higher number of patients with AS taking steroids could clarify this issue.

This study has potential limitations. First, a small number of patients were included, and results might be seen as preliminary. The aim of this study was mainly to evaluate differences in minimally modified LDL plasma levels between patients and controls. As this biochemical parameter is only used for research purposes, with no "normal" values even in healthy individuals, a sample calculation was not performed by the absence of reference values or even of previous studies in patients with AS.

Another limitation was the fact that patients with more physical impairment, although invited during the screening phase, did not participate. Refusal was always justified by difficulties in transportation. This is a frequent bias, reported also in studies with RA and SLE. Once patients with worst clinical conditions were not included, we may have lost those with more complications, including atherosclerosis due to their inflammatory condition.

In conclusion, despite active inflammation and higher disease activity, there were no differences regarding plasma levels of minimally modified LDL and carotid IMT assessed by ultrasonography between patients with AS and controls. These findings are in accordance with previous studies in AS, but further and larger studies are necessary to explore the role of inflammation per se as an accelerating factor of atherosclerosis in patients with spondyloarthritis.

Conflicts of interest

The authors declare no conflicts of interest.

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