Peer-reviewed version available at *J. Cardiovasc. Dev. Dis.* **2018**, *5*, 36; doi:10.3390/jcdd5030036

ApoB-100 Lipoproteins Complex Formation with Intima Proteoglycans as Cause of Atherosclerosis and Its Possible E*x vivo* Evaluation as a Disease Biomarker

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Abstract Experimental and clinical data indicates that the initiation and progress of atherosclerosis, and its clinical manifestations, are caused first by circulating apoB-100 lipoproteins that enter and are retained in the arterial intima. Extracellular sulfated proteoglycans (PGs) of the intima are the retention agents. The PGs also initiate physical and biochemical lipoprotein degradation with the production of bioactive, lipid products that trigger an inflammatory response that leads to atherosclerosis. There are many simple methods for measuring abnormalities of circulating lipoproteins and their relation to atherosclerotic cardiovascular disease (ACVD). However, limited research has been aimed to evaluate procedures that could report quantitatively about the contribution of the apo-100 lipoprotein-arterial intima PGs interaction to clinical manifestation of ACVD. In the present review we will discuss observations indicating that simple *ex vivo* evaluation of the affinity of apoB-100 lipoproteins for arterial PGs and glycosaminoglycans (GAGs) can give indication of its association with clinical manifestations of atherosclerosis. In addition, we will discuss molecular and cellular aspect of the apoB-100 lipoproteins association with arterial PGs that are related to atherogenesis and that support the experimental framework behind the current “Response-to-Retention” hypothesis of atherosclerosis.

Key words: ApoB100 lipoproteins-Proteoglycans-Interactions-Arterial intima Atherosclerosis-LDL modifications

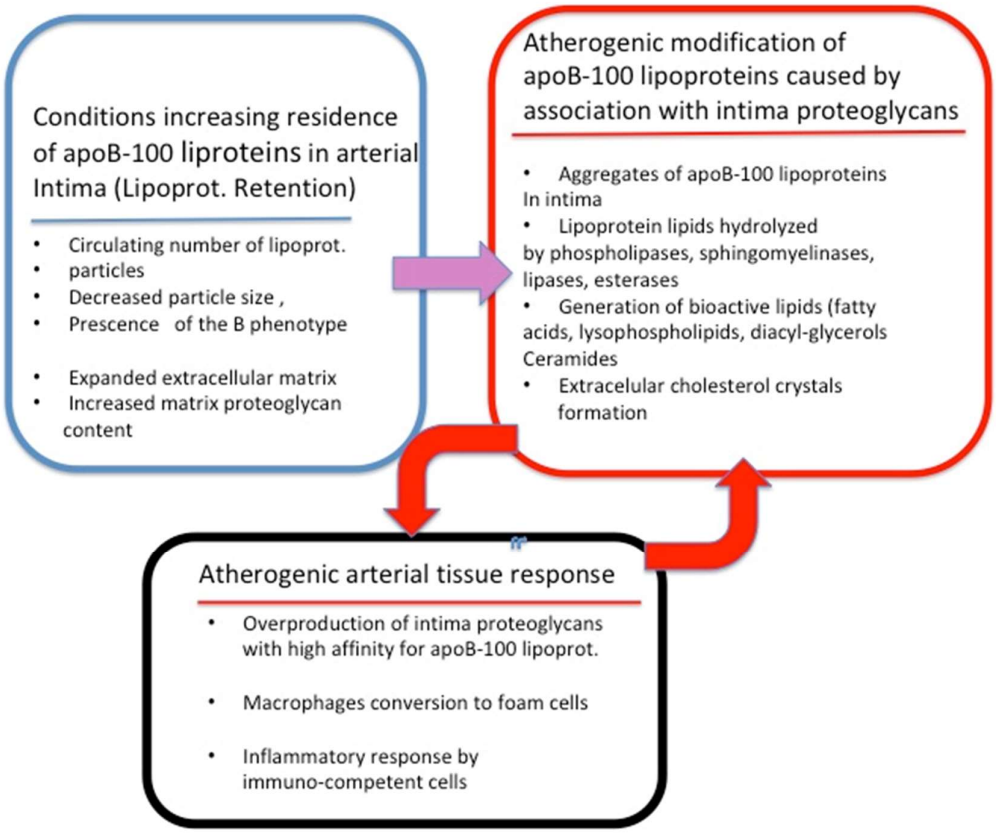
Peer-reviewed version available at J. Cardiovasc. Dev. Dis. 2018, 5, 36; doi:10.3390/jcdd5030036

1. Introduction

The deposition of cholesterol-containing apoB-100 lipoproteins in the extracellular arterial intima is a critical initial step in the development of atherosclerotic lesions. A recent consensus document has reviewed the evidence that connects the levels of circulating apoB-100 lipoproteins, their interaction with the intima and with the clinical events caused by atherosclerosis [1]. M. Faber in pioneering, studies already in 1949, suggested the possible mechanisms that linked plasma lipoproteins with atherosclerosis in humans using histochemical observations [2]. Faber proposed that the cholesterol in human atherosclerotic lesions originates from circulating plasma lipoproteins. Furthermore, he found that the extracellular lipoprotein cholesterol deposits in the arterial intima-media were associated with extracellular sulfated polysaccharides [2]. Iverius in 1972 showed that the molecules that bind LDL lipoproteins in connective tissue, including the arterial intima, were the sulfated polysaccharides (glycosaminoglycans, GAGs) of the PGs and he characterized the ionic nature of the association [3]. The biochemical nature of the lipoproteins-GAGs complex in human atherosclerotic lesion was firmly established by Srinivasan and collaborators in 1972 [4] After these studies, the biochemical and molecular details of the processes generated by the interaction of apoB-100 lipoproteins and the intima PGs have been fitted into a coherent pathogenic sequence that is currently known as the “response-to-retention” hypothesis of atherosclerosis. Williams and Borén published recently an in-depth review of the experimental support for this concept [5,6]. A short version of the hypothesis can be described as follows: the “retention” part is related to the entrapment of apoB-100 plasma lipoproteins in the arterial intima by PGs of the extracellular matrix. Once retained, the cholesterol-rich apoB-100 lipoproteins coalesce as large lipid-protein aggregates that are partially degraded by enzymatic and oxidative pathways. Several of the byproducts of the retained apoB-100 lipoproteins are pro-inflammatory and can act on macrophages, smooth muscle cells and other immune- competent cells triggering a complex inflammatory process that evolves into atherosclerotic plaques. This is the “response” part of the hypothesis [1,5-8]. Being the interaction of apoB-100 lipoproteins with arterial intima PGs such a central biochemical and pathogenic mechanism, it seems important to explore if the basic biochemical mechanism can be used as an additional *in vitro* marker of the lipoproteins atherogenicity. In the present review we will summarize data suggesting that measurements of the affinity of apoB-100 lipoproteins for arterial PGs could be a biomarker of atherogenesis.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 June 2018 doi:10.20944/preprints201806.0239.v1

Peer-reviewed version available at J. Cardiovasc. Dev. Dis. 2018, 5, 36; doi:10.3390/jcdd5030036



2. Ex vivo evaluation of the apoB-100 lipoproteins proteoglycans interaction and its possible relation with ACVD

Our laboratory found that soluble extracts of dissected human aortic intimas obtained from young accident victims contain a macromolecular component that formed an insoluble complex with LDL at near physiological conditions. The specific LDL precipitation by the arterial macromolecule also occurred when human plasma or serum are used. The arterial macromolecule was found to be versican, the most common chondroitin sulfate-rich PG of the human intima [9]. Using purified versican solutions, a standard procedure was developed in which the amount of complex of serum LDL with the arterial versican was measured *ex vivo.* This simple procedure can be used to compare the amount of complex formation with isolated LDL or with that LDL present in human serum or plasma samples. The results can be expressed as micrograms of LDL cholesterol (LDL-C) precipitated. The ionic conditions selected for this *ex vivo* measurement were based on studies of Berenfeld and collaborators, that established the required ionic composition of buffers needed for specific LDL precipitation with biological and synthetic sulfated polysaccharides [10]. We confirmed that in the conditions of pH and with the buffer composition selected more then 95 % of the cholesterol precipitated by the versican is present as LDL (density range 1.019-1063 g/mi) when examined by density gradient ultracentrifugation after re-solubilizing the LDL-PG complex. In a first study with humans sera we evaluated the LDL-PG insoluble complex formation, measured as µg of insolubilized LDL-C, using 50 µl of serum or plasma added to 1 ml of the PG

solution containing 10 µg PGs as hexuronate. The subjects were 291 adult males with no history of myocardial infarct and that were not taken lipid-lowering drugs. The subjects were classified as 214 apparent healthy and 77 probably ischemic using standardized exercise electrocardiography. It was found that the serum from the apparent ischemic subjects showed a higher prevalence of high values for LDL-PG complex formation (14-16 µg LDL-C) than the non-ischemic (6-8 µg LDL-C) [11]

In a following study, in order to discard the possibility that the differences measured in the amount of LDL-PG complex formation were just the product of differences in LDL serum content, Lindén et al. compared the *ex vivo* LDL-PG affinity in myocardial young infarct patients with that of apparently healthy controls that were matched for age, sex, and levels of serum cholesterol, triglycerides, LDL-cholesterol, apoB and HDL-cholesterol [12]. In these well-characterized groups the sera from myocardial infarct patients sera showed significant higher values of precipitated µg of LDL-C (23.7 ± 5.3 vs. 15.6 ±4.4, $p < 0.0001$,). These differences remained highly significant also when expressed as percentage of the cholesterol present in the serum aliquots added to the PG solution. Furthermore, LDL-PG complex formation appeared as an independent contributor in multiple regression analysis that together with serum triglycerides could discriminate patients and controls. There are other important observations in the study by Lindén et al [12]. They show that freezing at -80 ^{0} C and thawing has minor effects on the LDL-PG precipitation measured value, and that the analysis has approximately 15 % coefficient of variation. Finally, and importantly, using density gradient ultracentrifugal analysis, the authors also confirmed that with the used protocol in the LDL-PG complex, once dissolved, more than 90 % of the cholesterol was associated with lipoproteins with the density range of LDL, 1.019-1.063 g/ml. The gradient profiles showed no HDL in the dissolved pellets and less than 5 % of the precipitated cholesterol was associated with the density of VLDL (< 1.019 g/ml).

Analysis of the LDL-PG complex formation has been applied also to serum from patients at high cardiovascular risk (obese, hypertensive, with high triglycerides and hypercholesterolemia) that were subjected to multifactorial treatment [13]. The randomized patients (61 in the intervention group and 51 in the usual care group) were evaluated after 3 years of treatment compliance. The LDL-PG test, expressed as % of added serum cholesterol, was -4.9 % in the intervention group compared with the usual care group ($p<0.05$). In a subsequent study, the effect of lipid lowering drugs on the LDL-PG association was analyzed in moderate hypercholesterolemic patients by Wiklund et al. [14]. The patients were randomized to pravastatin (40 mg), gemfibrozil (600 mg b.i.d), gemfibrozil + pravastatin (same doses) and placebo for 12 weeks. The drugs treatments showed the expected significant effects on serum triglycerides, total cholesterol, LDL-C and apoB and there were no changes in the placebo group. Differences between before and after treatments in measurements of the serum LDL-PG precipitation were very significant when expressed as absolute values or as percentage of serum cholesterol, of apoB or LDL-cholesterol added. These results also indicate that the LDL-PG precipitation test measures changes in affinity of LDL for the arterial PG and not only differences in LDL levels. A general observation in some of the previous studies is that the serum LDL of subjects with type 2 diabetes (T2D) or insulin resistance produce more aggregates with arterial versican PG than subjects without these conditions. Garces et al. [15] explored this observation in an study in which LDL-PG affinity was

measured in subjects with obesity but not type 2 diabetes (T2D), in subjects with obesity with TD2 , in subjects with T2D but no diabetes and in apparently healthy controls with no obesity or T2D. The results show that obesity, with or without T2D was associated with significant higher values of plasma LDL-PG precipitation (18 µg LDL-C precipitated /mg apoB added vs. 11.7 µg of LDL-C precipitated / mg apoB added). Interestingly, the elevated levels of LDL-C-PG complex were strongly associated with levels of serum phospholipase A_2 and with high prevalence of smaller LDL particles. We will discuss this aspect below. The summarized clinical studies indicate that increased LDL affinity for arterial versican is associated with increased clinical markers of atherosclerosis and cardiovascular risk.

Two solid phase procedures have been described that use a microtiter format for measuring apoB-100 lipoproteins binding to PGs [16, 17]. In this procedures the PGs are attached to the plates and the solutions of isolated lipoproteins, or in plasma, are incubated in a buffer similar to the one used in the liquid phase procedure. The plates are washed of not bound lipoproteins and the PG-retained apoB-100 lipoproteins are determined with an immune-assay. These solid phase methods can be easily automatized thus increasing its application to many samples.

2. ApoB-100 lipoproteins entry and its proteoglycan-mediated retention in the arterial intima

The steady state concentrations of soluble lipoproteins in the arterial intima depend of their rate of entry and exit [1]. The endothelial barrier appears to exclude particles with diameter above 70 nm in diameter, as very large VLDL and chylomicrons. VLDL remnants, LDL and HDL can cross the endothelium are present in the intima in amounts that are inversely proportional to their size and directly proportional to their concentration in human serum and plasma [1, 6, 7,18] Because of its higher plasma concentration LDL exist at the highest concentration in the extracellular arterial intima, probably at 20 times the value for VLDL remnants [1,19]. There is a linear correlation between the levels of circulating cholesterol-rich apoB-100 lipoproteins and the content of immune-detectable LDL in early atherosclerosis in humans, as shown by Smith and Slater [18]. Thus, the strong association between plasma apoB-100 lipoproteins and the clinical manifestations of atherosclerosis is to be expected [1]. In human early coronary atherosclerosis, lipoprotein deposition in proteoglycan-rich regions of the intima occurs before macrophage infiltration [20] These results support the importance of interactions between apoB-100, cholesterol-rich, lipoproteins intima PGs and early atherogenesis.

Retention of lipoprotein particles in the intima occurs first by association of specific basic segments of the apoB-100 protein in the particle surface that are rich in lysine and arginine. These charged amino-acid sequences can form soluble and insoluble complexes with the sulfate groups of the glycosaminoglycan (GAG) moiety of PGs. We used frontal affinity chromatography and competition experiments with synthetic peptides of the apoB-100 to identify the main GAG-binding sequences of the protein. Two sequences: 3145-3157 (-Seer-Val-**Lys**-Ale-Gln-Gly-Trp-**Lys**-**Lys**-Asn-His-**Arg**-His) and 3359-3367 (-**Arg-**Leu-Thr-**Arg**-**Lys**-**Arg**-Gly-Leu-**Lys**-) showed the highest affinity for versican, the main PG of the human intima. Both sequences have 5 positive charged residues and they have similar affinities for versican. The affinity was 6-7 µm/l when expressed as inhibition constant (IC50) [6,21]. Borén et al.

in a series of important experiments used mutagenesis in the human apoB-100 gene within the coding region for the 3359-3367 PG-binding sequence. They demonstrated, in a hypercholesterolemic rmouse model, how important is this basic segment for the *in vivo* PG-mediated LDL retention and consequently for early atherosclerosis progress [7, 16].

LDL in plasma can be associated with other apolipoproteins like apoE, apoAI and apoCIII [22] that can modulate its affinity for PGs. Davidson et al. [22] studied with a proteomic approach, that in subjects with peripheral atherosclerosis and type 2 diabetes (T2D) that also have LDL, with high prevalence of small, dense LDL, these particles contained elevated levels of all apoCIII isoforms, when compared with the LDL of healthy controls. Furthermore, the apoCIII content in their small, dense LDL were positively correlated with LDL binding to PG [22]. Olin-Lewis et al. found that that the affinity of LDL subclasses for the PG biglycan increased with the decreasing diameter of the particles and this trend was associated with the apoC-III content Hiukka et al. [23] examined the PG-binding of LDL rich in apoC-III from patients with T2D. These lipoproteins also show an increased binding for the small PG biglycan. It is not clear why apoC-III augmented content increase LDL-PG binding. It may be that this occurs because smaller LDLparticle have more ApoC-III copies in their surface and that their intrinsic, size-related affinity is the real cause.

3 Atherogenic structural and biochemical apoB-100 lipoproteins alterations caused by PG retention

The transition from retention in the intima to lipoprotein-mediated alterations of cellular constituents of the intima leading to the “response” phase is very fast. These rapid alterations are initiated by the same interaction of apoB-100 lipoproteins with the extracellular and peri-cellular sulfated PGs. In this situation it is difficult to separate the processes contributing to the “response” and those contributing to the “retention”. Low angle X-ray diffraction and scanning calorimetry experiments indicate that once bound to the GAGs of arterial versican the apoB-100 lipids in the particles in the complex become rapidly disorganized and can form large soluble and insoluble fused lipoprotein aggregates [24]. Furthermore the surface exposure of the apoB-100 basic segments in the surface particles is increased and the protein becomes more susceptible to proteolysis [25] Large aggregates of apoB-100 lipoproteins have been detected with high-resolution electron microcopy in the arterial intima of rabbits after a human LDL bolus infusion [26]. This indicates that the association of LDL with the extracellular matrix takes place very rapidly, as well as it is the formation of large aggregates. The biochemical changes in the apoB-100 particles taken place after apoB-100 lipoproteins association with the arterial matrix has been documented in human intima-media segments with different type of atherosclerotic lesions. Most studies show that lipid and protein moieties of the extractable apoB-lipoproteins from the intima had suffer physical and hydrolytic modifications, see review by Hoff and Hoppe [27]. Our laboratory showed that lipoproteins that can be extracted from human lesion and purified with anti-apoB-100 antibodies affinity chromatography contained particles with the size of LDL and large apoB-100 aggregates that also contained GAGs. Analysis of the fatty acids of phosphatidyl choline, cholesterol esters and triglycerides from the arterial lipoprotein showed approximately 50 % depletion of linoleic acid in all the lipids. This suggest that apoB-100 lipoproteins, with still immuno-reacting apoB are associated with PGs and are targets of different phospholipases, cholesteryl esterases and for possible oxidative processes of fatty acids [28]. Lipolytic hydrolysis of phospholipids, and

cholesterol esters from retained apoB-100 lipoproteins will generate fatty acids that can be targets of free-radical mediated reactions thus producing further pro-inflammatory products. In addition, *in situ* production of lysophospholipids and diacyl-glycerol can also mediate activation of inflammatory cascades in macrophages, endothelial cells and muscle cells [29].

4. Cellular consequences of the association of apoB-100 lipoproteins with intima proteoglycans

As already discussed, interaction of LDL with the human arterial PG versican alters the physico-chemical structure of the particles [6] without necessarily inducing aggregation. One of the most significant effects of such modification is how human macrophages increase the binding and uptake of the soluble PG-modified LDL. Furthermore, the PG-modified LDL causes appreciable intracellular accumulation of free cholesterol, cholesterol esters, triglycerides and phospholipids resulting in appreciable formation of intracellular lipid vacuoles [30]. This increase in lipid cell content is driven by the uptake of LDL via the apoB/E receptor and by endogenous lipid synthesis by the macrophages. We observed that LDL preparations from different blood donors were internalized at different rates in the macrophage experiments. Thus, we explored if different LDL subclasses with dissimilar affinity for PGs could have unique structural properties and furthermore if the macrophages internalized the subclasses at different rates after their association with the human arterial versican [31]. These experiments were prompted by the results showing that LDL subspecies with different density and sizes have specific lipid composition and different conformations of the apoB-100 [28]. The tests showed that LDL subclasses selected by their increasing affinity for human arterial versican were associated with decreased diameter and volume due to an augmented ratio of surface components to core lipids (cholesteryl esters). Also the size decrease was associated with increasing basic isoelectric point. In addition, the smaller LDL subclasses were internalized more efficiently by the human macrophages were found more susceptible to free-radical mediated oxidation [6,31]. These properties of the PG-selected LDL can be considered atherogenic [6]. The dissimilar atherogenicity of small, dense LDL particles and large, less dense ones have been ascribed to easier entry into the intima, to a higher affinity for intima PGs and to higher susceptibility to hydrolytic and oxidative modification of the small, dense LDL [32, 33]. We have hypothesized that all these potentially atherogenic properties are related to increased exposure of the PG-binding, polar segments of apoB-100 in the particle surface [6, 31]. More recently, Flood et al [34], using recombinant protein experiments, showed that conformational changes in the apoB-100 sequences 3145-3157 (PG-binding site A) and 3359-3967 (PG binding site B) in small, dense LDL act cooperatively to increase its affinity for intimal PGs. LDL treatment with $sPLA_2$ that reduces the surface components of the particle also increase the affinity for PGs. Furthermore, extended action of phospholipases on circulating apoB-100 lipoproteins is a probable generator of small, dense LDL with higher PG affinity [29, 33].

5, Alterations of the arterial intima extracellular matrix that may contribute to increase retention of apoB-100 lipoproteins

One of the characteristics of initial atherosclerotic lesions is the intimal thickening. These are intimal regions, rich in PGs, hat are the preferred site for extracellular apoB-100 lipoproteins retention and lipid accumulation [7,20,36]. The

cells generating the extracellular matrix of these regions are smooth muscle cells, and possible macrophages [20,36]. Biochemical and cell studies shown that phospholipase A_2 (PLA_2), than can enter these PGs-rich regions when bound to lipoproteins or when being secreted by intimal cells, form tight association with PGs and in this form increases its activity towards the phospholipids of also PGs-attached lipoproteins [29,35]. This situation can further potentiate the generation of pro-inflammatory active lipids as non-esterified fatty acids, oxidized fatty acids and lysophospholipids and increase the affinity of apoB-100 lipoproteins for the intima PGs [29,35]. Increased exposure of arterial smooth muscle cells to non-esterified fatty acids, as it may occur in insulin resistance or T2D, may have further atherogenic consequences since it was found that this can augment the secretion of extracellular matrix proteoglycans with increased affinity for apoB-100 lipoproteins [37, 38]. Thus, the PGs-mediated "retention" of apoB-100 lipoproteins can initiate a self-perpetuating process resulting in an enhanced atherogenic "response" of augmented matrix production with more capacity to retain apoB-100 lipoproteins [38].

There other conditions that can cause matrix production with increased capacity to retain apoB-100 lipoproteins mediated by PGs. Recently Kijani et al. found that vascular interventions in the mouse common carotid, resulting in intimal hyperplasia, induce deposition of apoB-100 lipoproteins and rapid atherosclerosis [39]. This matrix changes and circulating antibodies that bind glycosaminoglycans significantly decreased atherogenesis. Another set of recent results further confirm the importance of the extracellular matrix proteoglycan structure on retention of apoB lipoproteins in atherogenesis. Fog Bentzon and collaborators [40] used fluorescently labeled LDL and HDL to first locate the natural sites of lipoprotein retention in mice arteries, the inner aortic arch and nearby branches. In the other hand straight artery segments showed minimal labeled protein retention. The authors then anatomically induced disturbed laminar flow in the straight segments of arteries. This intervention caused hyperplasia and early plaques in 6 weeks. These sites became also locations for massive retention of the labeled lipoproteins. And interestingly, the sites increased the expression of genes associated with smooth muscle cells phenotype and of genes for the core proteins of lipoprotein-retaining PGs and for enzymes responsible for GAGs biosynthesis [40].

Conclusions

Extensive experimental and clinical data consistently supports the premises on which the "response–to-retention" hypothesis of atherosclerosis is based. Moreover, the bases of the hypothesis provide the rationale for further developments in current and new anti-aterosclerotic treatments centered in correction of dyslipidemias. Specially, those contributing to deposition of cholesterol-rich apoB-100 lipoproteins in the arterial intima-media and that are causal of atherosclerotic cardiovascular disease. The discussed preliminary studies about the possibility of identifying biomarkers of the molecular interactions between atherogenic apoB-lipoproteins and the arterial intima are encouraging. We believe that potential high value of non-invasive simple markers that could be used for measuring the predisposition of lipoproteins to be retained in the intima and consequently their triggering of the atherogenic response merits further research and evaluation.

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