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Role of dysfunctional HDL and oxidative stress in bladder cancer

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Abstract

Epidemiological studies suggest associations between type 2 diabetes mellitus and bladder cancer. Several potential mechanisms may explain the increased bladder cancer burden in DM patients. Hyperglycaemia is associated with dysregulation of cell intracellular metabolism and alterations of lipoprotein metabolism and oxidative stress. In fact, previous studies have shown that levels and functions of circulating lipoprotein are modified in DM patients. Dysfunctional HDL including glycated and oxidized HDL are described in patients with type 2 diabetes mellitus. We evaluated the effect of normal HDL and glycated HDL on cell proliferation and oxidative stress of J82 bladder cancer cells. We also studied the effect of HDL on cholesterol influx and efflux. In addition, the expression of proteins involved in cholesterol transport (ABCA1, SRB1, ABCG1) by western blot analysis was studied. Our results demonstrate that HDL incubated 7 days at 37^{o}C with increasing concentrations of glucose (30-100mM) have lower levels of free amino groups with respect to untreated HDL. The decrease realized at a higher extent using glucose 100 mM. Therefore, this concentration was used to investigate the effect of glycated HDL on J82 cells. Levels of TBARS and conjugated dienes were higher in G-HDL compared with N-HDL. These results demonstrate that lipid peroxidation occurs during glycation treatment. The enzyme activity of paraoxonase (HDL-PON1) was significantly decreased in G-HDL**.** Higher markers of lipid peroxidation and a decrease of Paraoxonase activity are described in dysfunctional HDL.

The study of the effect of Normal HDL and glycated HDL (G-HDL) on J82 cells in culture has shown that both N-HDL and G-HDL promote cell proliferation and increase the levels of intracellular reactive oxygen species (ROS) during incubation with oxidizing agent tert-butyl hydroperoxide. The increase of intracellular ROS was associated to higher levels of TBARS in cells incubated with G-HDL than N-HDL. The increase in oxidative stress in cells incubated with N-HDL was associated with alterations of cholesterol homeostasis. In detail Cholesterol efflux was increased. On the contrary, cholesterol influx was significantly decreased in cells incubated with G-HDL. Expression of receptor protein SR-B1 and ABCG1 was increased. The higher expression of SR-B1 in cells incubated with G-HDL suggests that dysfunctional HDL could affect cholesterol homeostasis in J82 bladder cancer cells. These results suggest that HDL-based treatments should be considered for treatment of bladder cancer.

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Abbreviations

BC: Bladder Cancer
HDL: High-density lipoprotein
HDL-C: Cholesterol bounded to HDL
LCAT: Lecithin-cholesterol
LDL: Low density lipoprotein
RCT: Reverse cholesterol Transport
AGEs: Advanced glycation product
ApoA-I: Apolipoprotein A-I
ApoA-II: Apolipoprotein A-II
CETP: Cholesteryl ester transfer protein
D-HDL: HDL of diabetes patients
G-HDL: Glycated-HDL
LOOH: Lipid hydroperoxides
N-HDL: Normal HDL
Ox-HDL: oxidized HDL
PKC: Protein kinase C
SR-B1: Scavenger receptor B1
ABCA1: ATP-binding cassette A1
ABCG1: ATP-binding cassette G1
MDA: Malondialdehyde
PON: Paraoxonase
PON1: Paraoxonase-1
PON2: Paraoxonase-2
PON3: Paraoxonase-3
RNS: Reactive nitrogen species
ROS: Reactive oxygen species

Aim of the study

Dysregulation of cholesterol metabolism in cancer, is the object of extensive discussion. Intracellular cholesterol levels and its compartmentalization are modulated by complex mechanisms and are integrated by a metabolic network involving de novo biosynthesis, LDL-cholesterol entry cholesterol distribution by sterol transferring proteins, and cholesterol efflux by ABCA1 and ABCG1 proteins.
HDL exerts a key role in cholesterol efflux and regulation of cell proliferation. This PhD projects aims to investigate the interaction between bladder cancer cells and normal and glycated HDL Bladder cancer (BC), one of the most common malignancy of the urinary tract for both men and women. BC represents a crucial public health hazards due to its high aggressiveness and poor prognosis. BC has the highest recurrence rate of any other solid tumour, most of them, exceed relapses or progresses from non-muscle invasive to muscle invasive disease. Diabetes Mellitus (DM) is associated with elevated bladder cancer or cancer mortality risk, especially in men. Several potential mechanisms may explain increased bladder cancer burden in DM patients. Hyperglycaemia is associated with dysregulation of cell intracellular metabolism and alterations of lipoprotein metabolism, and oxidative stress. In fact, previous studies have shown that levels and functions of circulating lipoprotein are modified in DM patients, and may reflect in alterations of tissue cholesterol metabolism. Dysfunctional HDL including glycated and oxidized HDL are described in patients with type 2 diabetes mellitus (T2DM).
In vitro studies have shown that HDL behaves as potential growth factor and promote cell proliferation. Even glycation and/or oxidation of HDL promote cell proliferation and alter cholesterol metabolism. Furthermore, glycation of HDL triggers oxidative stress and promotes the proliferation and migration of cells in culture. The role of normal and dysfunctional HDL in bladder cancer cholesterol and oxidative stress has not been investigated till today.
Aim of the study was to investigate the effect of normal HDL, glycated HDL and oxidized HDL on cell proliferation and oxidative stress of J82 bladder cancer cells. We also studied the effect of HDL on cholesterol influx and efflux and expression of cellular protein SR-BI, ABCG1 and ABCA1. The interest of the study is supported by previous studies which have shown that bladder cancer is associated with oxidative stress and alterations of HDL with a significant increase in markers of lipid oxidative stress.

Introduction

Bladder cancer (BC) is one of the most common malignancy of the urinary tract for both men and women. The most common type is transitional cell carcinoma. Other types include squamous cell carcinoma and adenocarcinoma. BC represents a crucial public health hazards due to its high aggressiveness and poor prognosis [1]. BC is the 3rd most common cancer in men and 11th most common cancer in women worldwide. Transitional bladder tumours originating from urothelial cells are classified as non-muscle invasive (NMI) (pTa or pT1) or invasive (pT2, pT3 or pT4) with latter carrying a worse prognosis. BC has the highest recurrence rate of any other solid tumour, most of them, exceed relapses or progresses from non-muscle invasive to muscle invasive disease. Newly diagnosed patients, approximately (70% to 80%) have non-muscle invasive tumours while 20% have muscle-invasive tumours have been documented from world health organization [2-3].

Genetic and environmental factors have been implicated in BC etiology as recently reviewed [4-6]. Dietary factors such as arsenic and/or environmental xenobiotics can be metabolized in the human body, and carcinogenic by-products reach the urinary bladder via urinary excretion [5, 6]. The molecular mechanisms by which metalloids, smoking and xenobiotics are correlated with increased oxidative stress and are potentially involved in BC have been previously studied [4-6].

The relationship between Bladder cancer and oxidative stress is supported by alterations of ROS/RNS production, modifications of antioxidant enzymes, and higher markers of oxidative stress in tissues and blood of patients affected by bladder cancer [7]. Markers of lipid peroxidation, protein and nucleic acid oxidation are significantly higher in tissues of patients with BC compared with control groups. A decrease activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione, and paraoxonase) has also been demonstrated as reviewed [7]. The imbalance between oxidants and antioxidants could have a potential role in the etiology and progression of bladder cancer [7].

The dysregulation of cholesterol metabolism in cancer, is the object of extensive discussion [8]. Epidemiological studies have reported an association between cancer, serum cholesterol levels and HDL-C. A positive association between elevated serum cholesterol levels and increased cancer risk has been found in melanoma, prostate cancer, non-Hodgkin's lymphoma, endometrial and breast cancer [8,9,10-12]. However contrasting results also have been reported. The differences in the results might partly depend on the investigated tumour [8,9]. Interestingly, cancer development and progression are closely related to cholesterol metabolism at the cellular level [10]. Recent studies also have demonstrated that solid tumours accumulate greater amounts of cholesterol when compared to the host healthy tissues. In several cancer types, elevated mitochondrial cholesterol levels induced resistance to apoptotic signals [10-12].

High density lipoproteins (HDL) exert a key role in cholesterol transport and modulate intracellular cholesterol content [13]. Intratumor accumulation of cholesterol appears to be a consequence of lipoprotein uptake mediated by HDL receptor such as scavenger receptor class B type I (SR-B1). Recent evidence has suggested that HDL and its cellular receptor (SR-B1) may play a critical role in cancer progression [10-12]. Since cholesterol regulates cellular proliferation, migration and cell survival, a growing interest is devoted to study the role of the interactions between HDL and SR-B1 and to the mechanisms involved in cellular cholesterol homeostasis in tumour cells. *In vitro* studies using cells in culture have shown that HDL modulate proliferation and migration of normal and cancer cells [12].The activation of signalling pathways is dependent on HDL binding to the HDL receptor (SR-B1), and subsequent lipid transfer to the cell. HDL/SR-BI interaction can also activate both PI3K and Erk1/2 pathways in breast cancer cell lines [10-12]. Intracellular cholesterol levels and its compartmentalization are modulated by complex mechanisms and are integrated by a metabolic network involving de novo biosynthesis, LDL-cholesterol entry, cholesterol distribution by sterol transferring proteins, and cholesterol efflux by ABCA1 and ABCG1 proteins [8,13,14].

A potential role of alterations of intracellular cholesterol metabolism in bladder cancer (BC) cells and signalling pathways has been proposed by Wang G, et al. [15]. However, the interaction between normal and dysfunctional HDL on bladder cancer cells has not been previously investigated till today. This PhD projects aims to study some interactions between normal and dysfunctional HDL on Bladder cancer cells with particular attention to the effect of glycated HDL on J82 cells in culture.

Literature data on HDL lipid and protein composition will be summarized in the first part of the thesis. The pleiotropic roles of HDL and their alterations also will be described. The effects of normal and glycated HDL will be studied using J82 cells. J82 cells in culture have been widely used as cell model to investigate bladder cancer cells metabolism. The interest of the study is supported by previous studies which have demonstrated that diabetes Mellitus (DM) is associated with elevated bladder cancer or cancer mortality risk, especially in men [16]. Several potential mechanisms may explain increased bladder cancer burden in DM patients. Hyperglycaemia is associated with dysregulation of cell intracellular metabolism and alterations of lipoprotein metabolism and oxidative stress. In fact, previous studies have shown that levels and functions of circulating lipoprotein are modified in DM patients. Dysfunctional HDL including glycated and oxidized HDL are described in patients with type 2 diabetes mellitus (T2DM) [17,18].

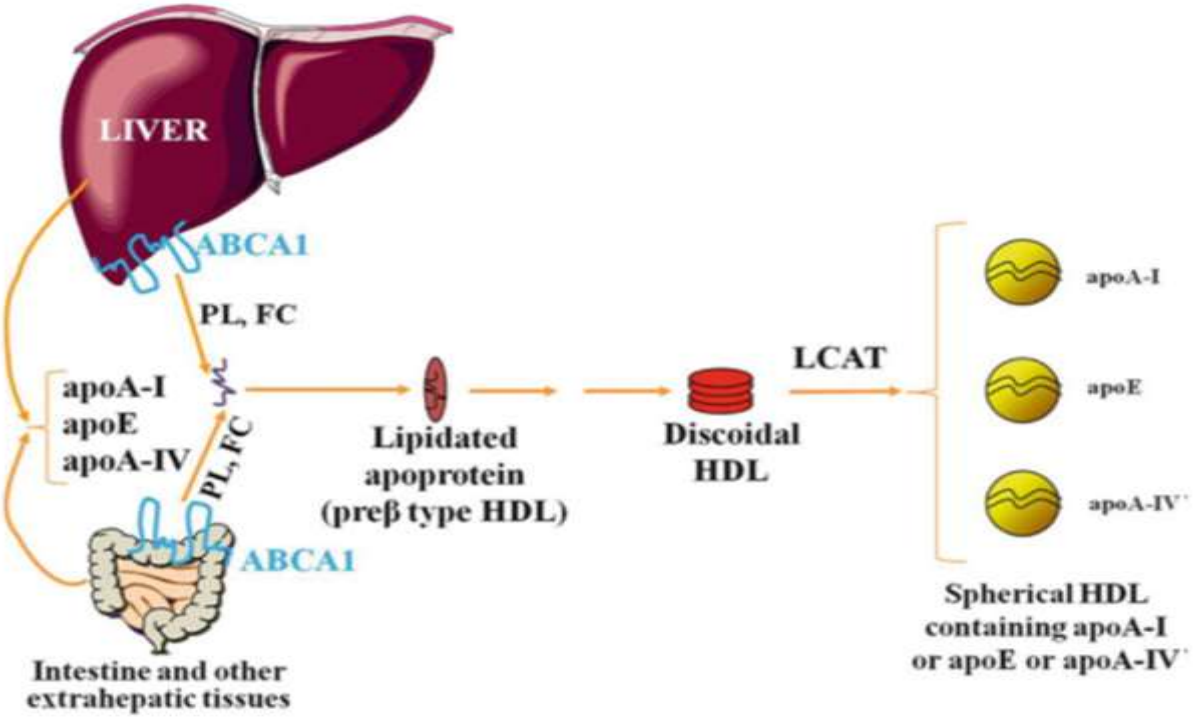
The effect of untreated and glycated HDL on proliferation as well as reactive oxygen species (ROS) production will be studied. We also will investigate the effect of HDL on cholesterol influx and efflux and expression of cellular protein SR-BI, ABCG1 and ABCA1.

1.HDL lipoprotein structure, classification and functions

1.1 HDL structure and biogenesis

The structure of HDL is like all lipoproteins [19-21]. Mature HDL consist of a hydrophobic nucleus of non-polar lipids (triglycerides and esterified cholesterol) surrounded by a monolayer of phospholipids and free cholesterol, with inclusions of protein molecules named apoproteins. In human plasma, HDL is comprised of a highly heterogeneous population of particles ranging in size from 5 to 17 nm and with a density between 1.063–1.210 g/mL [19-21]. The biogenesis of HDL is a complex process and involves several membranes bound and plasma proteins. The first step in HDL biogenesis involves secretion of apoA-I mainly by the liver and the intestine [22]. Secreted apoA-I interacts functionally with ABCA1, and this interaction leads to transfer of cellular phospholipids and cholesterol to lipid-poor apoA-I. The lipidated apoA-I is gradually converted to discoidal particles enriched in unesterified cholesterol. The esterification of free cholesterol by the enzyme lecithin/cholesterol acyltransferase (LCAT) [22,23] converts the discoidal to spherical HDL particles **(Figure 1)**. Specific mutations in apoA-I disrupt specific steps in the pathway of HDL biogenesis and generate distinct aberrant HDL phenotypes.

Figure 1 Schematic representation of the pathway of the biogenesis of HDL.



1.2 HDL classification and heterogeneity

HDL particles which can be fractionated and divided into various subtypes by different techniques according to their physicochemical properties as summarized in Table 1. Using analytic ultracentrifugation for HDL separation, two subclasses based on density, the less dense HDL2 (1.063–1.125 g/mL) and denser HDL3 (1.125–1.21 g/mL) can be separated [21,26-29].

A precipitation method has also been proposed [24] for the separation and quantitation of HDL2 and HDL3 which is inexpensive and easier, but with a high degree of variability. HDL2 and HDL3 can be further sub-classified on the basis of their particle size by gradient gel electrophoresis into two HDL2 (HDL2b(10.6 nm), HDL2a(9.2 nm)) and three HDL3 (HDL3a(8.4 nm), HDL3b(8.0 nm), and HDL3c (7.6 nm)) subclasses [24]. Based on electrophoretic mobility, HDL can also be subdivided into two main subpopulations: α-HDL and pre-β HDL [25]. Moreover, using immunoaffinity methods, HDL can be separated into particles containing apolipoprotein A1 (apoA-1) with or without apoA-2 (LpA-1: A-2 or LpA-1, respectively) based on its apolipoprotein composition [26-29].

Table 1: Major HDL subclasses (Isolation and separation technique)

Density (ultracentrifugation)	-HDL2 (1.063–1.125 g/mL) -HDL3 (1.125–1.21 g/mL)
Size (GGE)	-HDL2b (9.7–12.0 nm) -HDL2a (8.8–9.7 nm) -HDL3a (8.2–8.8 nm) -HDL3b (7.8–8.2 nm) -HDL3c (7.2–7.8 nm)
Size (NMR)	-Large HDL (8.8–13.0 nm) -Medium HDL (8.2–8.8 nm) -Small HDL (7.3–8.2 nm)
Shape and charge (agarose gel)	-α-HDL (spherical) -Preβ-HDL (discoidal)
Charge and size (2D electrophoresis)	-Preβ-HDL (preβ1 and preβ2) -α-HDL (α1, α2, α3 and α4) -Preα-HDL (preα1, preα2, preα3)
Protein composition (electro immunodiffusion)	-LpA-I -LpA-I: A-II

HDL lipid composition

Using lipidomic techniques, it has been demonstrated that more than 200 different lipids as well as over 85 proteins can be present in HDL particles [19,20]. Lipid composition of HDL isolated from healthy normolipidemic controls has been studied by FPLC. Heterogeneity based on phospho- and sphingolipidomic of five major HDL subpopulations isolated from healthy normolipidemic subjects have been reported [30-33].

Table 2: Principal components of HDL lipidome

Lipid class	**HDL composition in mol % of total lipids**
Phospholipids	37.4–49.3
Phosphatidylcholine	32–35
PC-plasmalogen	2.2–3.5
LysoPC	1.4–8.1
Phosphatidylethanolamine	0.70–0.87
PE-plasmalogen	0.54–0.87
Phosphatidylinositol	0.47–0.76
Cardiolipin	0.077–0.201
Phosphatidylserine	0.016–0.030
Phosphatidylglycerol	0.004–0.006
Phosphatidic acid	0.006–0.009
Sphingolipids	5.7–6.9
Sphingomyelin	5.6–6.6
Ceramide	0.022–0.097
Neutral lipids	46.7–54.0
Cholesteryl esters	35–37
Free cholesterol	8.7–13.5
Triacylglycerides	2.8–3.2
Diacylglycerides	0.17–0.28
Minor lipids	
Free fatty acids	16:0, 18:0, 18:1[a]
Isoprostane-containing PC	ND (IPGE2/D2-PC (36:4)

Phospholipids

Phosphatidylcholine (PC) is the major plasma phospholipid (about 32–35 mol % of total lipids) in HDL [33]. PC is a structural lipid that distributes across HDL subpopulations and is enriched in polyunsaturated fatty acid moieties. LysoPCis a product of degradation of PC by phospholipases, including LCAT, and Lp-PLA2.

Other lipid molecules are Phosphatidylethanolamine (PE) and Plasmalogens.

Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol(PG), phosphatidic acid (PA) are negatively charged phospholipids of HDL which may significantly impact on net surface charge [34].The content of these lipids can modulate lipoprotein interactions with lipases, membrane proteins, extracellular matrix and other protein components.

Cardiolipin is a minor anionic phospholipid in trace amounts in HDL (0.08–0.2 mol). This lipid with potent anticoagulant properties may contribute to the effects of lipoproteins on coagulation and platelet aggregation [35].

Among bioactive lipids associated to HDL there are Isoprostanes, biomarkers of oxidative stress [36].

Sphingolipids

Sphingomyelin is the major sphingolipid in circulating HDL (5.6– 6.6 mol % of total lipidosis) [37]. It largely originates from triacyl glyceride-rich lipoproteins and minor extent from nascent HD]. Unlike negatively charged PL, sphingomyelin is depleted by up to 30% in small, dense relative to large, light HDL. Among lysosphingolipids, S1P as a bioactive lipid plays key roles in vascular biology [38]. More than 90 % of circulating sphingoid base phosphates found in HDL and albumin-containing fractions where S1P associates with small, dense HDL particles (up to tenfold enrichment compare to light HDL) [37-39].

Neutral Lipids

Unesterified sterol is located on surface lipid monolayer of HDL particles and regulate its fluidity. Cholesteryl esters (CE) are largely (up to 80 %) formed in plasma HDL and transesterification of PL and cholesterol catalysed by LCAT.

1.3 Pleotropic role of HDLs. Roles of HDL associated enzyme and proteins

HDL carries many different proteins as compared to other lipoprotein classes. The high number of associated HDL proteins, along with the wide spectrum of functions, determine the multifunctionality of HDL and their contribution to various biological processes **(figure 2)**. Besides their contribution to Reverse cholesterol transport (RCT), HDL have been shown to have antioxidant properties, modulate inflammatory responses, vasomotor reactions, and blood clotting, as well as mediate immune responses. The proteins and enzymes involved in the pleotropic role of HDLs are summarized in **(Table 3)** [40]. Whereas apolipoproteins and enzymes are widely recognised as key functional HDL components, the role of minor proteins, primarily those involved in complement regulation, has received increasing attention only in recent years, mainly as a result of advances in proteomic technologies [41-44]. Importantly, the composition of the HDL proteome may depend on the method of HDL isolation. Indeed, ultracentrifugation in highly concentrated salt solutions of high ionic strength can remove some proteins from HDL, whereas other methods of HDL isolation (gel filtration, immunoaffinity chromatography, precipitation) provide HDL extensively contaminated with plasma proteins or subject HDL to unphysiological conditions capable of modifying its structure and/or composition (e.g. extreme pH and ionic strength involved in immunoaffinity separation).
(Table 3) summarizes apoproteins and enzymes associated to HDL surface.

Figure 2 - Pleiotropic roles of HDL

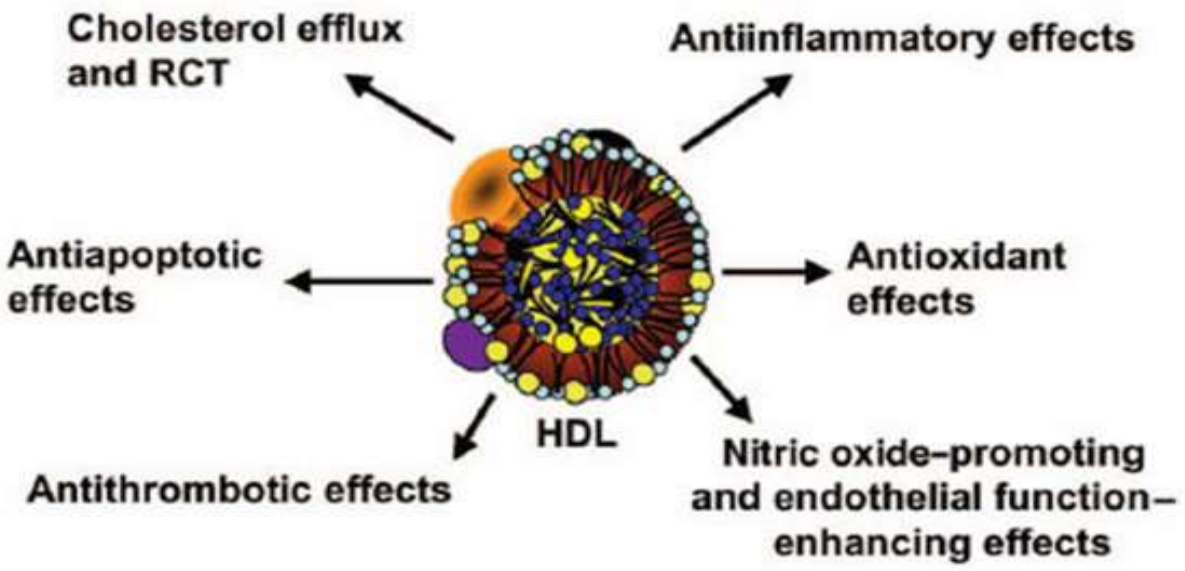


Table 3: Major components of the HDL proteome

Protein	Major Function	M_r, KDa
Apolipoproteins		
ApoA-I	Major structural and functional apolipoprotein, Interacts with cellular receptors, activates lecithin–cholesterol acyltransferase (LCAT) and exhibits antiatherogenic activity.	28
ApoA-II	Structural and functional apolipoprotein	17
ApoA-IV	Structural and functional apolipoprotein	46
ApoC-I	Modulator of CETP activity, LCAT activator.	6.6
ApoC-II	Activator of LPL.	8.8
ApoC-III	LPL and hepatic lipase inhibitor.	8.8
ApoC-IV	Regulates TG metabolism	11
ApoD	Responsible for the binding and transport of small hydrophobic molecules.	19
ApoE	Structural and functional apolipoprotein, ligand for LDL-R and LRP.	34
ApoF	Inhibitor of CETP	29
ApoJ	Binding of hydrophobic molecules, interaction with cell receptors.	70
ApoM	Binds small hydrophobic molecules, primarily sphingosine-1-phosphate (S1P), as well as oxidized phospholipids.	25
Enzymes		
LCAT	Esterification of cholesterol to cholesteryl esters.	63
PON1	Calcium-dependent lactonase. Antioxidant role	43

PAF-AH(LpPLA2)	Hydrolyzes short chain oxidized phospholipids. Macrophages are the most important source of the circulating enzyme	53
GSPx-3 (glutathione selenoperoxidase 3)	A component of the system of protection against the oxidative damage of molecules. Catalyzes the redox reaction of peroxides (hydrogen peroxide to water or lipid peroxides to the corresponding alcohols) with glutathione.	22
Lipid transfer proteins		
PLTP	Conversion of HDL into larger and smaller particles, transport of LPS.	78
CETP	Hetexchange of CE and TG and homoexchange of PL between HDL and apoB-containing lipoproteins.	74
Acute-phase protein		
SAA1	Major acute-phase reactant	12
SAA4	Minor acute-phase reactant	15
Alpha-2-HSglycoprotein	Negative acute-phase reactant	39
Fibrinogen alpha chain	Precursor of fibrin, cofactor in platelet aggregation.	95
Complement components		
C3	Complement activation	187
Proteinase inhibitors		
Alpha-1- antitrypsin	Inhibitor of serine proteinases.	
Other proteins		
Transthyretin	Thyroid hormone binding and transport	55
Serotransferrin	Iron binding and transport.	75
Vitamin D-binding protein	Vitamin D binding and transport.	58
Hemopexin	Heme binding and transport.	52

Apolipoproteins

ApoA-I; Circulating apoA-I represents an amphipathic protein and is synthesised and secreted by liver and small intestine. Apolipoprotein A-I is the major structural and functional HDL protein [45]. ApoA-I exerts different roles such as interaction with cellular receptors, activation of lecithin/cholesterol acyltransferase (LCAT), anti-oxidative effects.

ApoA-II; ApoA-II is the second major HDL apolipoprotein (approximately 15–20 % of total HDL protein). About a half of HDL particles may contain apoA-II [46]. ApoA-II is more hydrophobic than apoA-I and circulates as a homodimer composed of two identical polypeptide chains and is predominantly synthesised in the liver but also in the intestine.

ApoA-IV; ApoA-IV is an O-linked glycoprotein. It is the most hydrophilic apolipoprotein which readily exchanges between lipoproteins and circulates in a free form. ApoA-IV contains thirteen 22-amino acid tandem repeats, nine of which are highly alpha-helical; many of these helices are amphipathic and may serve as lipid-binding domains. apoA-IV is synthesised in the intestine and is secreted into the circulation with chylomicrons.

ApoCs; ApoCs are a family of small exchangeable apolipoproteins primarily synthesised in the liver. ApoC-I is the smallest apolipoprotein which associates with both HDL and VLDL and readily exchange between them. ApoC-I is involved in activation of LCAT and inhibits hepatic lipase and cholesteryl ester transfer protein (CETP) [48,49].

ApoD; ApoD is a glycoprotein which is mainly associated with HDL [50,51]. The protein is expressed in many tissues including liver, kidney, breast, lung, intestine. It does not possess a typical apolipoprotein structure and belongs to the lipocalin family which also includes retinol-binding protein such as lactoglobulin and uteroglobulin. Lipocalins are small lipid transfer proteins of amino acid sequence identity but it has a common tertiary structure. ApoD transports small hydrophobic ligands. In plasma, apoD compose disulfide-linked homodimers and heterodimers with apoA-II.

ApoE; ApoE is a key structural and functional glycoprotein component of HDL [52]. ApoE acts as ligand for apoB/apoE receptors and ensures lipoprotein binding to cell-surface glycosaminoglycans. ApoE is synthesised in many tissues and cell types including liver, endocrine tissues, central nervous system, and macrophages.

ApoF; ApoF is a sialoglycoprotein of human HDL and low-density lipoprotein (LDL) **[**53], also known as lipid transfer inhibitor protein (LTIP) due to its ability to inhibit CETP [54].

ApoJ; It is called clusterin and complement-associated protein SP-40. The distinct structure of apoJ allows binding of both a wide spectrum of hydrophobic molecules and specific cell-surface receptors [55,56].

ApoM; It is an apolipoprotein mainly found in HDL [57]. It possesses an eight stranded antiparallel beta-barrel lipocalin fold and a hydrophobic pocket of binding of small hydrophobic molecules, primarily sphingosine-1-phosphate (S1P). ApoM reveals 19 % homology with apoD, another apolipoprotein member of the lipocalin family and is synthesised in the liver and kidney. The binding of apoM to lipoproteins is assured by its hydrophobic N-terminal signal peptide, which is retained on secreted apoM, a phenomenon atypical for plasma apolipoproteins [57-58].

2. Enzymes and lipid transfer proteins associated to HDL surface

Among proteins associated to HDL, some exert catalytic activities and are involved in the pleiotropic roles exerted by HDL. Some roles of the main enzymes associated to HDL surface are described in this paragraph (**Table 3**).

Phospholipid transfer protein (PLTP)

PLTP is synthesised in different tissues and mediates the transfer of phospholipids from apoB-containing triglyceride-rich lipoproteins to HDLs. Therefore, it participates in phospholipid exchanges between lipoproteins and contributes to HDL conversion and remodelling [59].

Lipoprotein-associated Phospholipase A2 (Lp-PLA2)

Platelet-activating factor acetyl hydrolase (PAF-AH) is also known as lipoprotein-associated phospholipase A2 (LpPLA2). It is a calcium-independent, N-glycosylated enzyme which degrades PAF by hydrolysing the sn-2 ester bond to yield biologically inactive lyso-PAF [60]. Macrophages are the most important source of the circulating enzyme [60]. Plasma PAF-AH circulates in association with LDL and HDL particles, with most of the enzyme bound to small, dense LDL and to lipoprotein(a) [61].It has been linked to oxidative stress and it has been demonstrated that can release atherogenic isoprostanes from esterified phospholipids and that may be involved in inflammation and atherosclerosis [61].Several studies have recently suggested that it may be a potential biomarker for cancer due to its role promoting cancer cell migration and invasion [62]. Moreover, abnormal expression and increased secretion plasma levels of Lp-PLA2 have been found in patients with colon cancer [63]and prostate cancer [64].

Paraoxonase-1 (PON1)

Human paraoxonases (PON1, PON2 and PON3) belongs to multitask calcium-dependent lactonases. All enzymes exert antioxidant roles. PON1 activity has been extensively studied in many diseases since 1986 [65-67]. Mainly, PON1 and PON3 are localized in the plasma. PON2 is localized in the plasma membrane, endoplasmic reticulum, nuclear envelop, and inner mitochondrial membrane. These enzymes protect biological membranes and lipoproteins against potentially harmful ROS which contribute to lipid peroxidation [67]. PON1 exerts a role in metabolism of homocysteine thiolactone [68].PON1 is also able to hydrolyse the organophosphate substrate paraoxon including aromatic carboxylic acid esters. Using PON1 purified from human plasma, it has been shown that enzyme is able to hydrolyze lipid hydroperoxides and it can delay or inhibit lipid peroxidation induced by metal ions on LDLs [65-67]. Previous studies have demonstrated that HDL-PON1 also exerts a protective role against lipid peroxidation of biological membranes [66].Lipid

oxidation of LDL is critical for the initiation and propagation of atherosclerosis. Therefore, PON1 is believed to play a central role in the antioxidant activity of HDL [65-67]. PON1 interaction with apoA-I is critical for enzyme stability. A PAF-AH/PON1-coupled protective function of apoA-I could effectively divert proatherogenic LOOH to less harmful products. Therefore, a lower PON1 activity is an important determinant of HDL dysfunctionality [69]. In addition, there is growing evidence that PON1 may exert part of its atheroprotective effect through regulating cholesterol efflux from macrophages and maintaining cholesterol homeostasis [70].

Many studies have investigated the relationship between PON enzymes and various diseases associated with oxidative stress as cardiovascular diseases [65-69]. Alterations of PON1 activity have also been observed in serum and plasma of patients affected by several human cancers [71,72]. PON1 paraoxonase and arylesterase activities are significantly decreased in the serum of BC subjects with respect to controls [73].As far as concerns the physiological relevance of the observed alterations, Okuturlar et al [74] have shown that serum PON and ARE levels were lower in patients who needed neoadjuvant chemotherapy than in patients who did not need the therapy. Previous studies have also shown that the decrease of serum paraoxonase-1 concentration demonstrated in patients with urinary bladder cancer was associated with higher levels of chemokine (C-C motif) ligand 2 C-reactive protein than the control individuals [74]. Furthermore, a relationship between PON1 and clinical data was reported with lower PON1 concentration in patients with tumour recurrence [75]. This latter study also showed an inverse relationship between serum PON1 activity and serum amyloid A concentrations. Both these proteins are carried in the circulation bound to HDL particles, and they tend to change in opposite directions in case of inflammation [75]. PON1 is also considered a part of the innate immune system due to its antioxidant, anti-inflammatory properties [76, 77]. The relationship between PON1 gene polymorphism and BC has demonstrated that RR genotype was more common in bladder tumours [78]. Finally, PON1 is a glycoprotein, and alterations of protein glycosylation patterns have been described during cancer progression with tumour-specific proteins that actively involved in cancer cell growth [79].

In addition to alterations of activity and concentration of PON1, modifications of PON2 have been demonstrated in BC [80]. The comparison of expression levels of PON2 in paired tumour and normal bladder tissue samples from patients affected with BC, most of which underwent radical cystectomy for the treatment of advanced disease (pT3-4), has shown that PON2 expression levels were significantly higher (2.01-fold) in BC compared with those detected in normal tissue. Furthermore, PON2 expression in urinary exfoliated cells obtained in BC patients was significantly higher compared to that in patients affected with tumours invading subepithelial connective tissue or extending outside the bladder (pT1-3). PON2 overexpression on

bladder tumour cells (T24) was associated with higher proliferation and lower susceptibility to oxidative stress by tert-butyl hydroperoxide [80].

Lecithin-Cholesterol Acyltransferase (LCAT)
LCAT catalyses the esterification of free cholesterol to cholesteryl esters and contributes to HDL maturation. Almost 75 % of plasma LCAT activity is associated with HDL. It has been suggested that LCAT activity could also contribute to the antioxidant properties of HDL [81].

Glutathione Peroxidase GPx-1; GPx-1, another enzyme detected in HDL, can reduce lipid hydroperoxides to corresponding hydroxides and thereby detoxify them.
Multiple clinical studies suggest an atheroprotective role for GPx-1. It seems that the role of GPx-1 in the development of atherosclerosis is particularly prominent under conditions of significant oxidative stress.

Cholesterol ester transfer protein (CETP)
CETP is primarily expressed by the liver and adipose tissue. In the circulation, CETP shuttles between HDL and apoB-containing lipoproteins and facilitates the bidirectional transfer of cholesteryl esters and triglycerides. The structure of CETP includes a hydrophobic tunnel filled with two cholesteryl ester molecules and plugged by an amphiphilic phosphatidylcholine (PC) molecule at each end [82].

3. Role of HDL-mediated cholesterol efflux and influx pathway in normal and cancer cells

Reverse Cholesterol Transport (RCT) RCT, the transport of excess cholesterol from the peripheral tissues to the liver for utilization and excretion with the bile [33], is considered a classical function of HDL. The dysregulation of cholesterol metabolism in cancer, is the object of extensive discussion [10-12, 83]. Epidemiological studies have reported an association between cancer, serum cholesterol levels and HDL-C. A positive association between elevated serum cholesterol levels and increased cancer risk has been found in melanoma, prostate cancer, non-Hodgkin's lymphoma, endometrial and breast cancer [8,9,83]. Interestingly, cancer development and progression are closely related to cholesterol metabolism at the cellular level. As aforementioned, intracellular cholesterol levels and its compartmentalization are modulated by complex mechanisms and cholesterol efflux depends on membrane proteins such as ATP-binding cassette A1 transporter protein (ABCA1) and ATP-binding cassette G1 transporter protein (ABCG1) [84-87]. Regulation of cholesterol levels is essential for cell homeostasis and HDL are actively involved in intracellular cholesterol metabolism**.** The first and critical step rate-limiting of reverse cholesterol transport is efflux of free cholesterol from the cell plasma membrane to HDL and efflux of cellular phospholipid and free cholesterol to apolipoprotein A-I.

There are four different routes by which cholesterol can be transported or effluxed from the cell namely:
1) passive diffusion of cholesterol to mature HDL particles,
2) SR-B1 mediated facilitated diffusion,
3) Active efflux to apo A1 via ABCA1
4) ABCG1 mediated efflux to mature HDL.

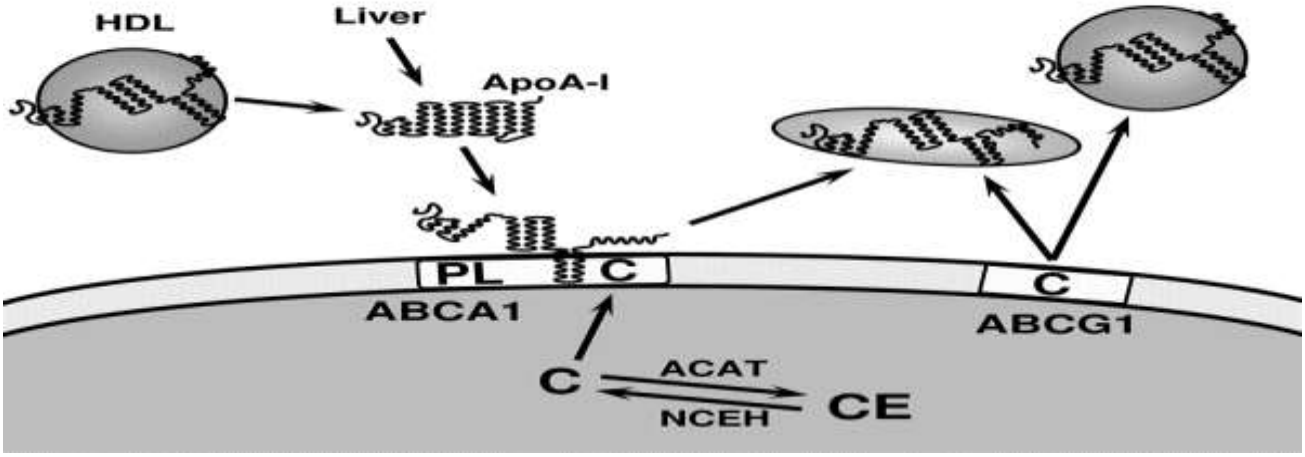
The role of ABCA1, ABCG1 and SR-B1 in cell cholesterol metabolism are in described.

ATP-binding cassette A1 transporter protein (ABCA1)

ABCA1 belongs to the ABC family of transporters protein, a protein encoded by the ABCA1 gene in humans (Abca1 in rodents). ABCA1 mediates the transfer of cellular cholesterol across the plasma membrane to apoA-I and is expressed in the liver, macrophages, brain, and various other tissues [84-87]. ABCA1 also promotes efflux of cellular phospholipids to lipid-free or minimally lipidated apoA-I and other apolipoproteins [84-87] **(Figure 3).** As cancer is a highly cooperative process of oncogenic mutations that causes multiple metabolic changes including changes in gene expression patterns, ABCA1 has been identified as one of the cooperation response genes, nonmutant genes synergistically downregulated by multiple cancer gene mutations in the processes of malignant cell transformation. Several studies

showed alterations of expression of ABCA1 in cancer cells. Chou et al. [88] showed that *ABCA1* was upregulated by TGF-β and was hypermethylated in a subset of ovarian cancer cell lines. Expression of ABCA transporters was also associated with poor outcome in serious ovarian cancer, implicating lipid trafficking as a potentially important process in OC [89]. Other authors have shown that expression of ABCA1 is significantly higher in Triple Negative breast cancer (TNBC) tissues, compared with the non-cancerous mammary tissues [90]. In addition, ABCA1 is highly expressed in M14 melanoma cell lines [91], and in LDL1 colon cancer cell lines and colon cancer tissues [92]. Lee et al. [93] have demonstrated ABCA1 promoter hypermethylation in prostate cancer but not in benign prostatic tissue. The epigenetic alteration had a higher prevalence in intermediate and high-grade cancers when compared with low grade cancers. An impairment of cellular cholesterol homeostasis in pancreatic ductal adenocarcinoma (PDAC) has also been related to ABCA1 upregulation [94].

Figure 3 -Model for ABCA1- and ABCG1-mediated lipid transport from macrophages.. C indicates free cholesterol; CE, cholesteryl esters; PL, phospholipids; ACAT, acyl-CoA:cholesterol acyltransferase; NCEH, neutral cholesteryl ester hydrolase.



ATP-binding cassette G1 transporter Protein (ABCG1)

ABCG1, a 110kDa protein, is a member of the ABC family expressed in several types of cells and it mediates cholesterol transport through its ability to translocate cholesterol and oxysterols across membranes **(figure 3)** [95,96]. Recently, an investigation of genome-wide association studies (GWASs) have identified two single nucleotide polymorphisms (SNPs), rs225388 G > A and rs225390 A > G [97]. Overexpression of ABCG1 has been implicated in HKULC4 lung cancer cell line with enhanced proliferation rate and reduction in apoptosis [98]. A recent study has shown ABCG1 overexpression in aggregative metastatic colon cancer cells. Expression of ABCG1 is also enhanced in cancer cell lines as well as in prostate cancer patients. ABCG1 has also been reported in tumour growth and sustenance by endoplasmic reticulum (ER) homeostasis. In mesenchymal glioblastoma, knockdown of ABCG1 has increased expression and activation of CCAAT-enhancer-binding protein homologous protein (CHOP) in response to ER stress and enhanced

apoptosis [99]. HDL obtained from CETP deficient subjects or patients treated with the CETP inhibitors torcetrapib or anacetrapib was shown to have enhanced ability to promote ABCG1-dependent cholesterol efflux from macrophages [100]. In addition, ABCG1 was shown to promote efflux of 7 oxysterols from macrophages and endothelial cells to HDL, thus protecting cells from apoptosis [101,102].

Scavenger receptor of the B1 class transporter protein (SRB1)

SR-BI is an 82 kDa membrane glycoprotein, also known as CLA-1. The protein consists of a large extracellular domain, two transmembrane domains, and two cytoplasmic amino and carboxyterminal domains. The main cellular function of SR-BI is to mediate the selective uptake of HDL cholesterol by cells, primarily in the form of cholesteryl esters **(figure 4).** This involves transfer of the cholesteryl esters from the hydrophobic core of the HDL particle to the cell without transfer of the apolipoprotein at the surface of the particle. SR-BI also mediates the bidirectional flux of unesterified cholesterol and phospholipids between HDL and cells. Since SR-BI facilitates the cellular uptake of cholesterol from LDL and HDL , it has been proposed that the receptor modulates cell growth and proliferation [103-105].In fact, in addition to transport of cholesterol to cells, SR-BI is described as a key factor involved in intracellular signalling [103-105] **(Figure 4)**. In fact, it modulates mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signalling pathway activation. The activation of these pathways subsequent to esterified cholesterol (CE) and lipid transfer to cells favours cancer cell survival [106].SR-BI is mainly expressed in the liver, steroidogenic tissues, and endothelial cells but is also found in other tissues [107]. Previous studies have identified SR-BI upregulation in Leydig cell tumours, nasopharyngeal carcinoma, and prostate and breast cancer [108-110]. Studies in vitro have also demonstrated that SR-BI downregulation reflects in alterations of cholesterol availability necessary for the androgen synthesis pathways in prostate cancer cell lines and significantly decreases cellular viability and prostate specific antigen (PSA) secretion **[**110,111].

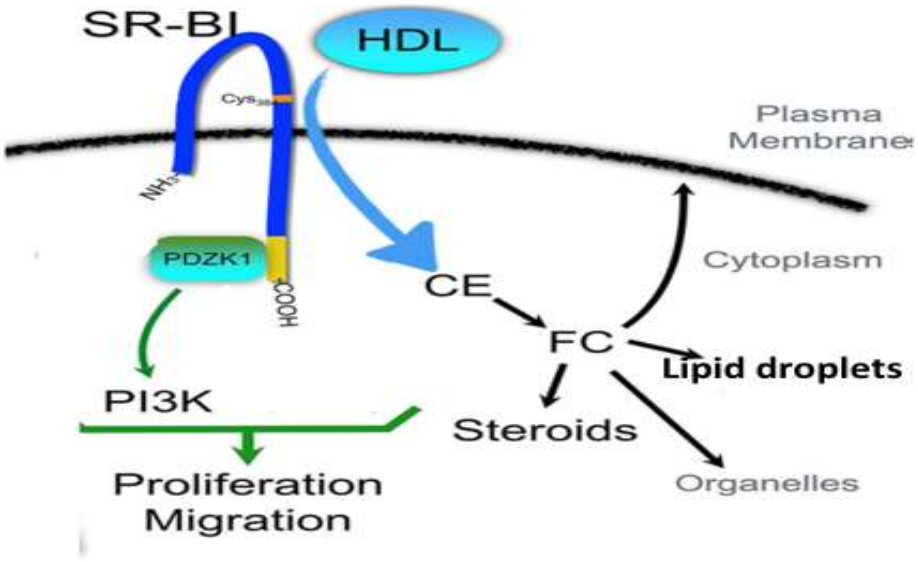
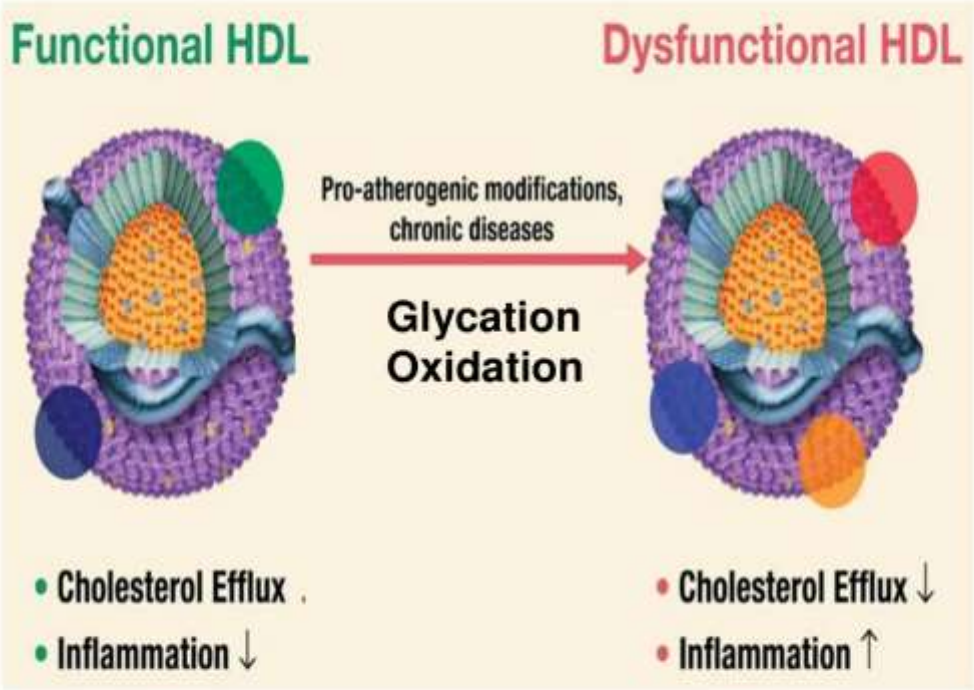


Figure 4: Role of SR-BI in the regulation of cellular signaling pathways.SR-BI contributes to the uptake of cholesteryl ester (CE) from HDL. A relevant residue in SR-BI for this interaction is Cys384 (denoted with an orange label). CE may be converted into free cholesterol (FC) which can be incorporated in the plasma membrane or organelles (such as mitochondria), stored in lipid droplets (LD), or used as substrate for steroids and other cholesterol metabolites. The C-terminal domain of SR-BI (denoted in yellow) activates PI3-kinase (PI3K) pathways to promote proliferation and migration

4. Dysfunctional HDL in human diseases

Nowadays, it is widely accepted that the role of HDL in both physiological and pathophysiological conditions goes far beyond its cholesterol content. As summarized previously, the term HDL functionality refers to a plethora of known HDL-mediated effects [24,40,112]. In addition to RCT, HDL have the ability to increase endothelial nitric oxide (NO) bioavailability, a capacity to reduce oxidative stress and inflammation, and an ability to reduce the expression of endothelial adhesion markers and transendothelial monocyte migration. Numerous structural and functional changes in the HDL particle may result in its transformation from an anti-atherogenic particle to a pro-inflammatory equivalent **(Figure 5).**As aforementioned, structural components of HDL, i.e. proteins and complex lipids associated with HDL particles exert a role in HDL functions. Literature data suggests that High-density lipoproteins (HDL) are susceptible to oxidation, glycation and homocysteinylation [112-114]. Modifications of HDL can occur due to general inflammatory processes, but also due to disease-specific processes [112]. In addition, genetic factors can also cause modifications of the HDL particles. Here the alterations of functions observed in glycated HDL and oxidized HDL are described.

Figure 5 From normal HDL to dysfunctional HDL .

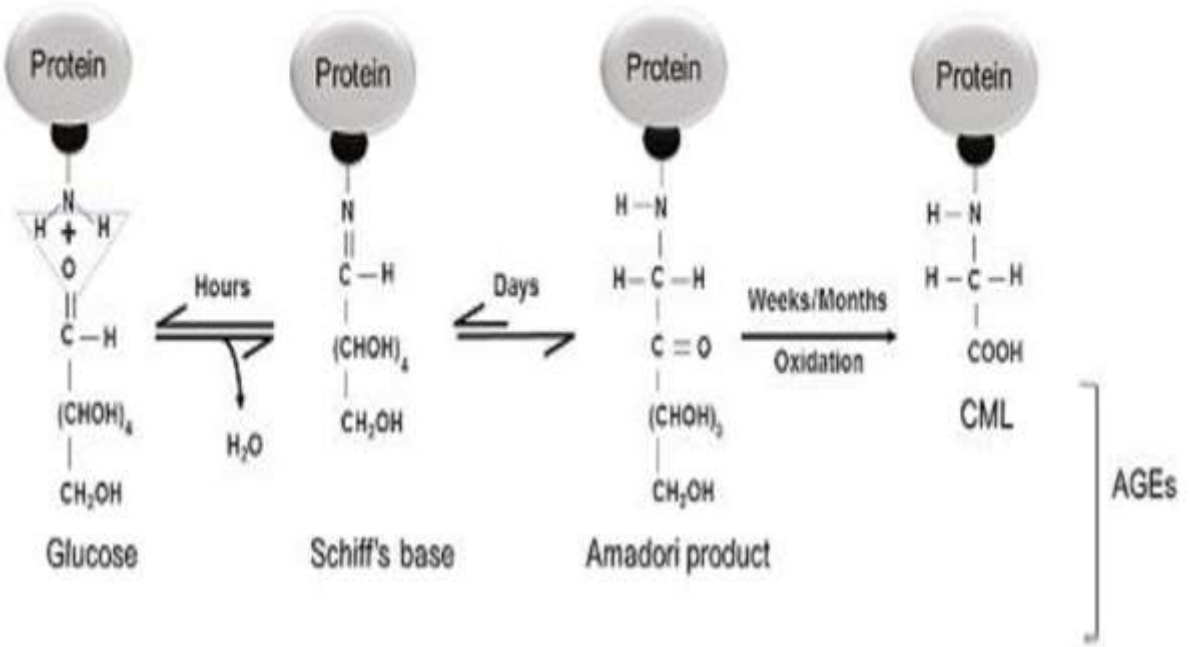


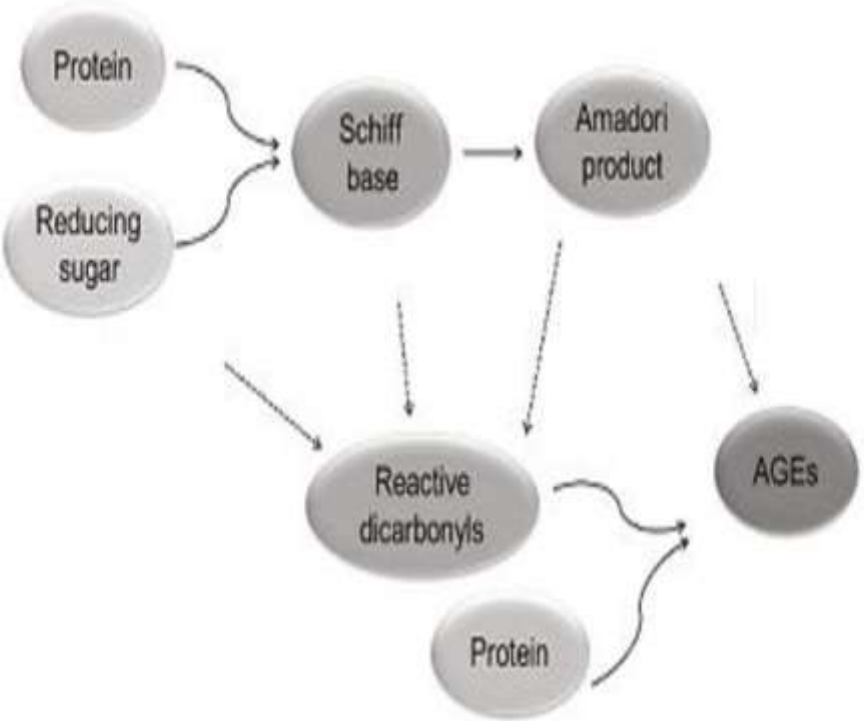
4.1 Non-enzymatic glycation

Diabetes mellitus is a common endocrine disorder characterised by hyperglycaemia and predisposes to chronic complications. Hyperglycaemia has an important role in the pathogenesis of diabetic complications by increasing protein glycation and the gradual build-up of advanced glycation end-products (AGEs) in body tissues. Glycation is linked to multiple oxidative and nonoxidative reactions, collectively termed the Maillard reaction with generation of advanced glycation end products (AGEs) **(figure 6) [**114]. Nonenzymatic glycation may occur under conditions of chronic hyperglycaemia, leading to alterations of HDL composition (lipids, apoproteins and enzymes) and functionality [112]. It has been demonstrated that nonenzymatic glycosylation process contributes to alterations of apoproteins due to reactions between reducing sugars, MGO and aminoacid residues such as lysine, arginine [112-114].

The glycation of apoA-I alters the conformation of the apoprotein in regions that are critical for LCAT activation [115], reduces its ability to promote cholesterol efflux from macrophages and to inhibit the expression of adhesion molecules and ROS formation [116]. Bacchetti et al. [116] have demonstrated that 3 days incubation of HDL with glucose (50 and 100 mM) significantly increases levels of TBARS and conjugated dienes with respect to control HDL. HDL incubated with glucose also show modifications of their physico-chemical properties as well as alterations of HDL associated enzyme activity such as PON1, CEPT, LCAT [112, 116]. *In vitro,* the exposure of endothelial cells to glycated HDL induces apoptosis by triggering mitochondrial dysfunction [117] and increases oxidative stress [118,119]. In addition, glycated HDL are less effective in counteracting the inhibitory effect of OxLDL on endothelium-dependent vasorelaxation and trigger vascular smooth muscle cell proliferation and migration [117]. An increase of levels of intracellular markers of glycoxidation contributes also to cellular lipid accumulation due to the reduced cellular cholesterol removal by apoA-I in an ABCA1 dependent pathway [119]. Several studies have been observed that glycation of lipoproteins as observed in diabetes mellitus, increases risk of cancer progression [120]. For instance, HDL isolated from Diabetic patients have a stronger capability to promote cell proliferation, migration, and invasion of breast cancer cells through the Akt, ERK, and p38 mitogen-activated protein kinase (MAPK) pathways [121]. These observations were also found using HDL glycated in vitro, compared with normal HDL [121]. Pre-treatment with diabetic, glycated, and oxidized HDL also promoted the metastasis capacity of breast cancer cells in animal models and it increased their capacity of adhesion to human umbilical vein endothelial cells (HUVECs) and attachment to the extracellular matrix in vitro, compared with normal HDL. These effects mainly were due to elevated PCK activity, which, in turn, stimulate secretion of integrins, which are important in promoting breast cancer metastasis [121,122].

Figure 6 - Glycation is linked to multiple oxidative and nonoxidative reactions, collectively termed the Maillard reaction with generation of advanced glycation end products (AGEs)





4.2 Oxidation of HDL

Oxidation of HDL can be triggered by incubation with metal ions (copper ions), hypochlorous acid and other agents [112]. Under pathological conditions associated with oxidative stress, HDL can become dysfunctional, independently of their plasma levels, due to compositional and functional changes of lipoprotein. For example, in patients with cardiovascular diseases, diabetes mellitus or chronic renal disease, HDL function is impaired [112,113], as they exhibit significantly reduced protective functions, and rather acquire pro-atherogenic features. Oxidative stress of HDL lead to lipid peroxidation and protein modification and may significantly affect the functions of HDL. Using different experimental models of oxidized HDL, it has been demonstrated that oxidation involves both surface (protein, phospholipid, and cholesterol) and core (cholesteryl ester) components and structural and compositional modifications in lipids and apolipoproteins of HDL. As a result, Oxidation of HDL increases the levels of lipid peroxidation markers including conjugated dienes, thiobarbituric acid reactive substances (TBARs). Moreover, alterations of physico-chemical properties of HDL such as fluidity, molecular order are described in OX-HDL. It has been reported that the modification of oxidized HDL can impair its atheroprotective functions [112]. Myeloperoxidase and superoxide anion radical (O2 −) and H_2O_2 are potential candidates for generation of oxidized HDL in vivo. Hypochlorite is an important ROS, which can be released by activated white blood cells in different diseases during pro-inflammatory states [122]. Zheng et al. showed the clinical relevance of such modifications and demonstrated that apoA-I is a target of MPO-catalyzed modifications [122]. These modifications are associated with the loss of lipid binding properties of apoA-I and also impairs ABCA1 dependent efflux of cholesterol from macrophages [122]

Oxidized HDL exert also cytotoxic effect on cultured cells such as HAECs, macrophages. The alterations of Ox-HDL have been also related to modifications of HDL enzymes such as paraoxonase (PON) [112, 123,125], LCAT (lecithin cholesterol acyltransferase) [124]. The interactions between ox-HDL and tumour cells has been previously studied using HDL oxidized in vitro. Hypochlorite-oxidized HDL stimulate cell proliferation, migration, invasion, and adhesion in vitro of breast cancer cells [125]. The molecular mechanisms involve the activation of protein kinase C (PKC) pathway, which regulates numerous cellular responses including cell proliferation and the inflammatory response. This modified HDL promoted breast cancer cell pulmonary and hepatic metastasis compared with normal HDL *in vivo*. [125,126].

5. Experimental Part

Materials and Methods

Chemicals and Antibodies

Methanol, n-butanol, ethanol, n-isopropanol,sodium dodecyl sulphate (SDS), Tween-20, H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate), 2-Mercaptoethanol, PageRuler™ Plus Prestained Protein Ladder were purchased from Thermo Fisher Scientific (Cedarlane, Toronto, Canada).D-(+)-Glucose, Butylated hydroxytoluene, Cupric sulphate pentahydrate, Tris/HCl, potassium bromide, sodium chloride,Hydrochloric acid, sodium hydroxide, glycine, glycerol, L-valine, 2,4,6-Trinitrobenzene sulfonic acid (TNBS), 1,1,3,3-Tetramethoxypropane (Malonaldehyde bis (dimethyl acetal),2-Thiobarbituric acid, Trichloro acetic acid, Thiazolyl blue Tetrazolium Bromide (MTT), N-ACETYL-L-CYSTEINE (NAC),Triton X-100,RIPA lysis buffer, protease inhibitor cocktail, Tert-butyl hydroperoxide (TBHP),Dimethyl sulfoxide, diethyl p-nitro-phenyl phosphate (paraoxon), Ponceau S, 100 mM PBS powder purchased from Sigma chemicals (St. Louis, MO, USA).Bis-acrylamide 40 % was from Calbiochem (Billerica, MA, USA). Laemmli buffer (4X), TEMED and ammonium persulfate were obtained from Bio-Rad (Saint-Laurent, Canada).Tritium radiolabelled cholesterol (3H-cholesterol) was purchased from Perkin Elmer (Boston, MA,USA), and Western Lightning® Plus-ECL were from Perkin Elmer (Waltham, MA, USA). J82 cell lines (Bladder carcinoma), Eagle's Minimum Essential Medium (EMEM), Trypsin were purchased from ATCC (Manassas, VA, USA).Bovine serum albumin (BSA), Fetal bovine serum (FBS),phosphate-buffered saline (PBS), antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin) were purchased from Wisent Inc. (Saint-Jean-Baptiste, Quebec, Canada). Antibodies were obtained from anti β-actin antibody, Anti-scavenging receptor SB-B1, Anti-ABCA1, Anti-ABCG1 and Goat Anti-Rabbit IgG H&L (HRP) and Rabbit Anti-Mouse IgG H&L (HRP) from Abcam Inc. (Toronto, ON, Canada).

Preparation of Human HDL

Blood was drawn from 12 healthy normolipidemic volunteers using heparin as anticoagulant. Plasma was collected by centrifugation at 3,000 rpm for 10 minutes and stored at -80^{o}C until isolation of lipoproteins. HDL (d=1.063-1.210 g/ml) was isolated from human plasma by using precipitation of non HDL particles with polyethylene glycol and dialysed at 4^{o}C for 24 h against 10 mM phosphate-buffered saline (PBS) solution (pH 7.4) and further sterilised by filtration through 0.22µm

filter. HDL protein concentration was measured by commercial assay (Bio-rad, Mississauga, Canada).

In vitro Glycation of HDL

HDL (100 µg/ml) was re-suspended in 10 mM PBS (pH 7.4) containing 25 µM BHT and increasing concentrations of glucose (30-100 mM). Samples were incubated for 7 days at 37 ^{o}C. At the end of the incubation, Normal-HDL (N-HDL) or glycated HDL (G-HDL) were dialysed against 0.9% NaCl solution at 4^{o}C for overnight to remove glucose and BHT. Levels of free amine groups were evaluated in N-HDL and G-HDL using 2,4,6-Trinitrobenzene sulfonic acid (TNBS) assay using L-valine as standard [126]. Levels of malondialdehyde (MDA) in N-HDL and G-HDL were evaluated by Thiobarbituric acid reactive substance (TBARS) assay as previously described [127]. Levels of conjugated dienes were evaluated using OD at 233 nm.

Cell culture

The human urinary bladder cell line J82 was purchased from American type culture collection (ATCC® HTB-1™, Manassas, VA, USA). J82 cells were grown in Eagles's Minimum Essential Medium (EMEM) containing 10% Fetal Bovine Serum (FBS)and antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin) in an incubator kept at 37^{o}C with 5% CO_2.

Cell proliferation Assay (MTT)

Cell proliferation was assayed using a colorimetric method with 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl tetrazolium bromide (MTT). J82 cells (5x10^{3}) were seeded into a 96-well microtiter plate. Cells were incubated in the medium containing pre-incubated N-HDL and G-HDL (100 µg/ml). Cell proliferation was evaluated at different times (24, 48 and 72 hours). Briefly, 10ul of MTT working solution (5 mg/ml in PBS) was added to the cells and incubated for 4.30 hour at 37^{o} C. The medium was discarded and 200 ul DMSO (stock concentration) were added. The number of living cells measured correlate with the amount of formazan crystals formed. The absorbance at 570 nm was measured using a microplate reader (Victor X5, Perkin Elmer, USA) as described [80]. Results were expressed as percentage of the control and all experiments were repeated three times.

Western blot analysis

J82 cells (5 x 105) were grown on 6-well plates until confluency (80-90%). Cells were treated in cAMP (0.3mM) (as positive control), N-HDL, G-HDL for 24 hours and 48 hours for the determination of cell membrane transporter protein expression (ABCA1, SR-B1, ABCG1). After that cells were washed three times with PBS, and RIPA buffer containing protease inhibitor cocktails (150 µl/well) was put on 4^{o}C for 30 minutes. Cell lysates were collected onto Eppendorf tubes on ice for sonication for 15 seconds and centrifuged at 13,000xg for 10 minutes. Equal amounts of proteins (30-40µg/lane) containing Laemmli buffer (4X) were heated on 95^{o}C for 5 minutes and were electrophoresed on (10%) SDA-PAGE and transferred onto PVDF (polyvinylidene fluoride) regarding pre-stained protein ladder. Membrane of protein migration was detected by ponceau S and membrane was washed with TBS-T until removing of colour. After that membranes were blocked in 5% non-fat milk and 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Primary antibodies were blocked in 5% non-fat milk in TBS-T (ABCG1 & SRB1) and 5% BSA in TBS-T (β-actin & ABCA1) according to the manufacturer's instructions and incubated for overnight at 4^{o}C dark place. Membranes were washed with TBS-T (4 times for 10 minutes) and the horseradish peroxidase-conjugated secondary antibody (Goat Anti-Rabbit IgG & Rabbit Anti-Mouse IgG) were blocked with 5% non-fat milk in TBS-T and 5% BSA in TBS-T for 1 hour at room temperature according to the manufacturer's instructions. Finally, membranes were washed with TBS-T (3 times for 10 minutes) and visualized using Western Lighting Plus-ECL solutions (1:1) ratio by Li-cor (Perkin Elmer, USA). Gel bands were quantified by ImageJ software.

HDL-mediated cholesterol efflux measurement

Cells J82 (1x105) were plated on a 24-well plate and incubated overnight in the medium containing 10% FBS. Cells were washed with PBS twice and incubated with complete medium and were labelled with[3H]- cholesterol (1µCi/ml) for 24 h. Cells were then washed with PBS, and were incubated with medium containing 1% BSA in the presence or absence of G-HDL or N-HDL for 18 h . After 18 h, medium was collected, centrifuged at 8,000 rpm for 10 minutes, and counted for radioactivity. The residual radioactivity in the cell fraction was measured after an extraction with 1 M NaOH. Cholesterol efflux was determined by liquid scintillation counting and the percentage of cholesterol efflux was calculated by dividing radioactivity (cpm) in culture medium by the sum of the radioactivity in the medium and cells [(medium)/ (medium + cells) ×100%].

Measurement of Cholesterol Influx

Cholesterol influx was measured by radiolabelling J82 cells with 3H-cholesterol (incubation of J82 cells with 1 µCi/ml 3H-cholesterol for 24 h). Cells were then washed with PBS twice, and were incubated in medium containing 1% BSA in the presence or absence of G-HDL or N-HDL for 18 h. The medium was then removed from the culture plates and the cells were washed with PBS, lysed with 1 M NaOH and then sonicated. The cholesterol influx cholesterol content was determined by liquid scintillation counting and the percentage of cholesterol influx was calculated by using the following formula: [(cpm in the cell/cpm in the cell + medium) ×100].

Measurement of intracellular ROS oxidative stress

Intracellular ROS and oxidative stress levels were measured by using 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA) probe. J82 cells were cultured on T-25 flask and incubator at 37^{o}C confluency (80-90%). Cells were then treated in the presence of G-HDL or N-HDL for 48 h at 37^{o}C and medium was washed before incubated with fresh medium containing DCFH2-DA (50 µM) in the dark for 45 min at 37^{o}C. Cells (30×103)were after washed to remove the extracellular DCFH2-DA and were placed on 96-well fluorometric black plates under clear bottom and then treated for 3 hours in the absence or presence of tert-butyl hydroperoxide (TBHP) (50-200 µM). The fluorescence was measured on a fluorescence plate reader at Ex/Em= 485/535 nm (Victor X5, Perkin Elmer, USA) [80].

Measurement of cell lipid peroxidation

J82 cells (50^{x}103) were grown on 24-well plates until reach on confluency (80-100 %). Cells were treated in N-HDL or G-HDL at 37^{o}C and then washed with PBS, the cells were scraped and sonicated at 15 seconds for 2 times and protein content was quantified by BCA. The remaining samples was complemented with 5 µL Triton-X100, vortexed for 10 seconds and incubated 5 minutes at room temperature and then added with TBA solution in each samples and heated tubes at 99^{o}C for 30 minutes. Samples was centrifuged at 13,000g for 30 seconds at room temperature (to pellet cell debris). Samples was transferred into 96-wells plate (200 µL/each well). The extent of TBA derivatives substance was assayed by monitoring absorbance at 532 nm (Victor X5, Perkin Elmer, USA). Lipid peroxidation of cells was expressed as TBARS (nmol/mg of proteins).

Measurement of PON1 paraoxonase activity

PON1 activity in N-HDL and G-HDL was measured using paraoxon (O, O-diethyl-O-p-nitrophenyl phosphate; Sigma) as the substrate as previously described. [125]. Briefly, 50 µL of samples and 1 mL 100 mmol/L Tris-HCL buffer (pH8.0) containing 2 mmol/L CaCl2 and 5.5 mmol/L paraoxon was resuspended and the enzymatic reaction was monitored in absorbance at 412 nm (UH5300 spectrophotometer, Hitachi, Japan) and enzymatic activity was calculated using molar extinction coefficient 17100 M-1cm-1. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay condition.

Statistical Analysis

Values were represented as mean ± standard deviation (S.D). Differences between groups were determined using ANOVA with (Dunnett's & Tukey's) multiple comparison test and unpaired T-test. Statistical analysis was performed using GraphPad Prism 8.1.2 (332) (GraphPad prism software, USA). Values were considered statistically significant at a p value less than 0.05.

6. Results

Effects of Glycation on HDL composition and paraoxonase (PON1) activity

In HDL incubated 7 days at 37°C with increasing concentrations of glucose30-100mM, the levels of amino groups decreased with respect to N-NDL **(Figure 7).**The decrease realized at a higher extent using glucose 100 mM therefore this concentration was used to investigate the effect of glycated HDL on J82 cells.

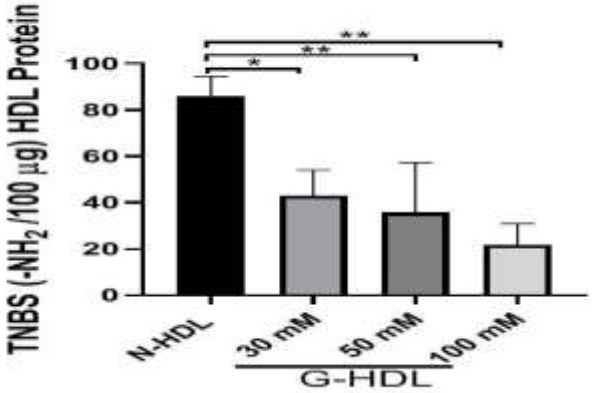


Figure 7 : Effect of incubation of HDL with glucose on levels of free amino groups Human HDL (100 µg/ml) was incubated in the presence or absence of 30-100 mM glucose at 37°C for 7 days. Values were expressed as mean ± SD (n=4) (*p<0.01 vs N-HDL, **p<0.005, vs N-HDL).

Levels of TBARS and conjugated dienes were higher in G-HDL compared with N-HDL and the differences were statistically significant **(figure 8, figure 9a).** These results demonstrate that lipid peroxidation occurs during glycation treatment. The enzyme activity of paraoxonase (HDL-PON1) was significantly decreased in G-HDL with respect to N-HDL **(Figure 9b).**

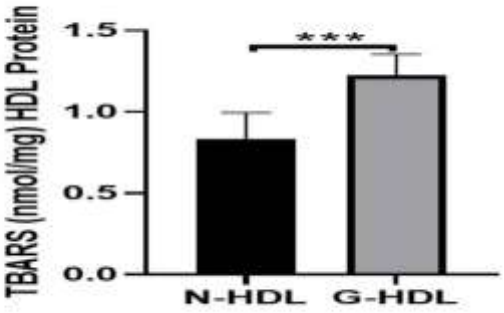


Figure 8 : Effect of incubation of HDL with glucose 100 mM on levels of TBARS Human HDL (100 µg/ml) was incubated in the presence or absence of 100 mM glucose at 37°C for 7 days.
(***p<0.001 G-HDL vs N-HDL).

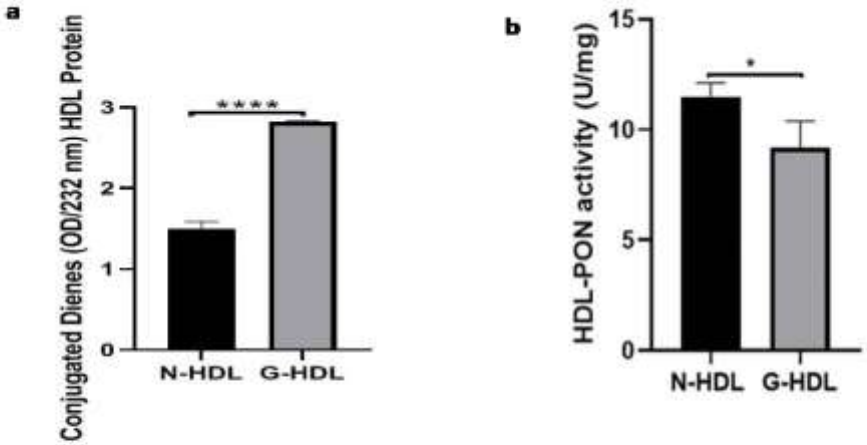


Figure 9 : Effect of incubation of HDL with glucose 100 mM on levels of conjugated dienes and PON1 activity.
a. Conjugated dienes (****p<0.001 G-HDL vs N-HDL).
b. HDL-PON1 activity (*p<0.04 G-HDL vs N-HDL)

Effect of normal HDL and glycated HDL on J82 cell proliferation

Incubation of cells with untreated HDL (N-HDL) had no effects on J82 cell proliferation. On the contrary after incubation of 48 hours, G-HDL promoted bladder cell proliferation. The effect of G-HDL was time-dependent and was statistically significant after 72 hours compared to cells incubated with N-HDL **(figure 10).**

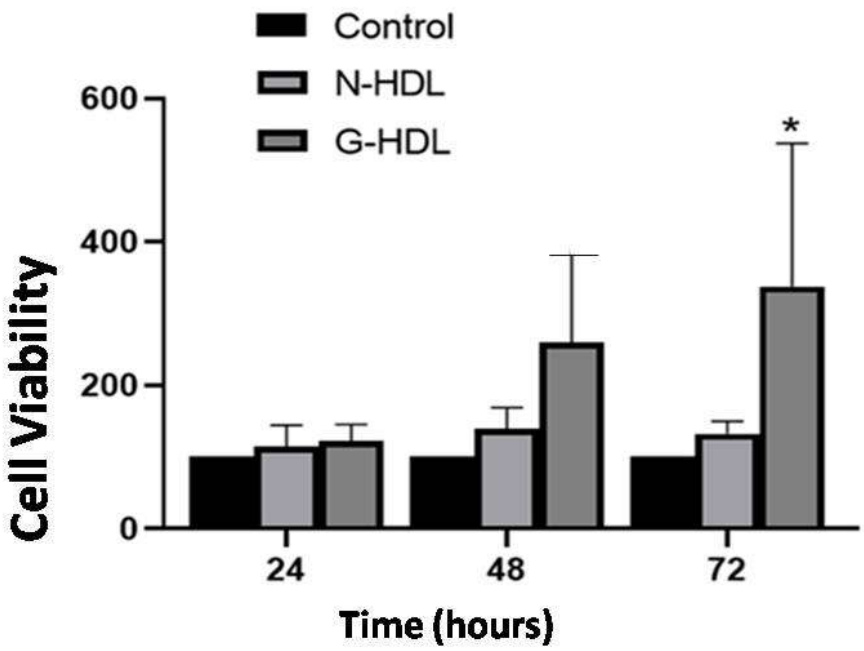


Figure 10 : Effects of normal HDL (N-HDL) or glycated HDL (G-HDL) on cell proliferation. Cells were treated for different times of incubation (24 h, 48 h, 72 h) with N-HDL or G-HDL (*p< 0.05 vs N-HDL)

Effects of normal HDL and glycated HDL on levels of intracellular ROS in J82 cells

The levels of intracellular ROS in J82 cells was evaluated in cells incubated with G-HDL or N-HDL in the presence of increasing concentrations of tert-butyl hydroperoxide (TBHP). As shown in **Figure 11a**, an increase of ROS was observed in J82 cells incubated with TBHP when compared to cells incubated alone. The increase of ROS was dependent on the concentration of TBHP in the range (50-200 uM). Incubation with N-HDL or G-HDL significantly increased the ROS levels. The increase realized at a higher extent in the presence of G-HDL compared to N-HDL (Figure 11a). Preincubation of J82 cells with the antioxidant NAC (10 mmol/L) for 30

minutes, before treatment with N-HDL or G-HDL significantly decreased the levels of ROS **(figure 11b).**

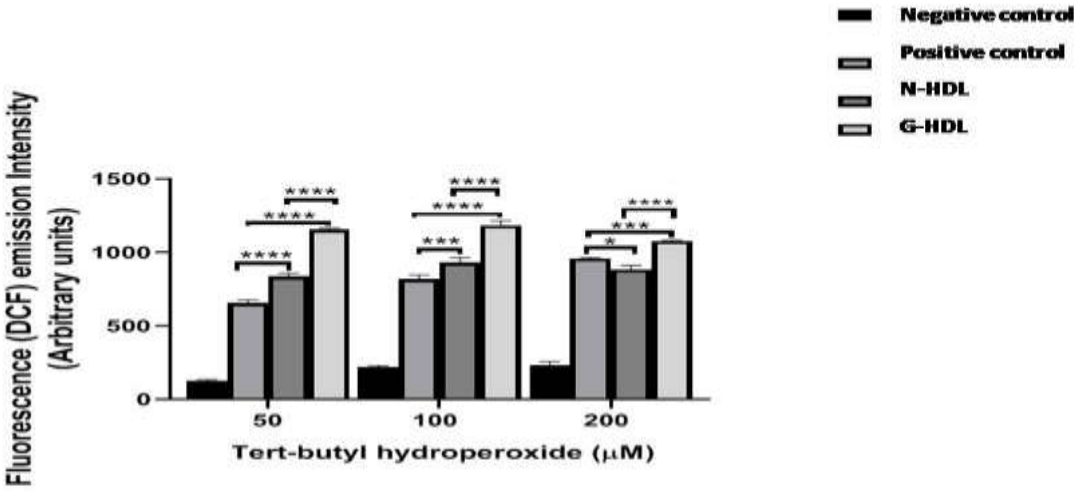


Figure 11a : Effect of N-HDL and G-HDL on intracellular levels of ROS in J82 cells. J82 cells were treated in the presence or absence of G-HDL (100 µg/ml) or N-HDL (100 µg/ml), and after 48 h treatment, tert-butyl hydroperoxide (TBHP) were incubated for 3 hours. Positive control (cells treated only with TBHP), Negative control (cells incubated alone). (***p<0.0001 vs positive control; ****p<0.0001 G-HDL vs N-HDL

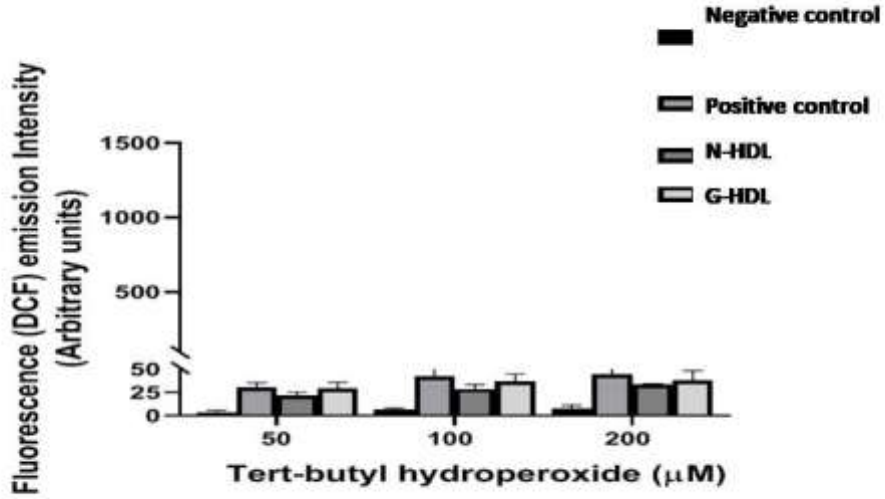


Figure 11 b : Effect of N-HDL and G-HDL on intracellular levels of ROS in J82 cells incubated with NAC
Positive control (cells treated with TBHP)
Negative control (cells incubated alone)

Effects of normal HDL and glycated HDL on lipid peroxidation in J82 cell

As shown **in figure 12**, TBARS levels in J82 cells incubated with N-HDL or G-HDL were higher with respect to cells incubated alone. A four-fold increase was observed after incubation with G-HDL compared with cells incubated alone and the difference was statistically significant**.**

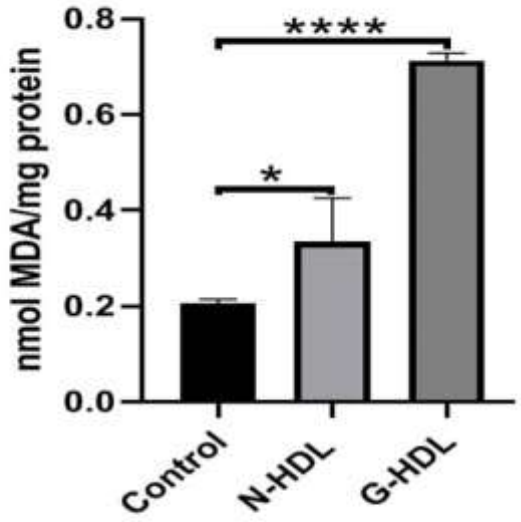


Figure 12: Effect of N-HDL or G-HDL on Levels of TBARS in J82 cells (*p<0.02 N-HDL vs untreated cells; ****p<0.0001, G-HDL vs N-HDL).

Effects of normal HDL and G-HDL on Cholesterol efflux and influx in J82 cells

Cholesterol efflux from J82 cells incubated with normal HDL was higher with respect to cells incubated alone, The efflux was higher during incubation with G-HDL compared to cells incubated with N-HDL **(Figure 13a).**

On the contrary the effect of glycated HDL on cholesterol influx was significantly decreased with respect to N-HDL **(Figure 13b).**

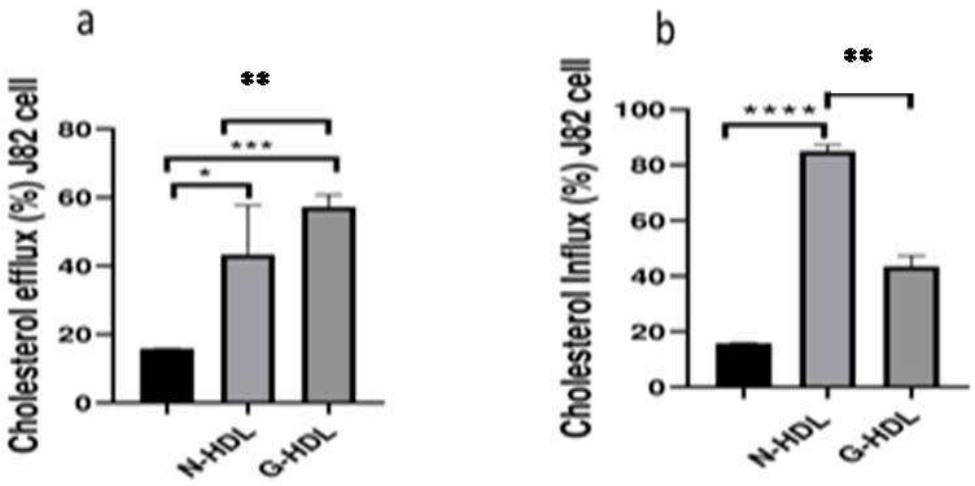


Figure 13 : a, Effect of incubation of N-HDL or G-HDL on cholesterol efflux of J82 cells and Cholesterol influx (b)

a) *p<0.01, N-HDL vs untreated cells, **p<0.001 G-HDL vs N-HDL; ****p<0.0001 vs G-HDL control cells

b) **** N-HDL vs control cells, ** p< 0.01 G-HDL vs N-HDL

Effects of N-HDL or glycated HDL on the expression of ABCA1, ABCG1 and SRB1, in J82 cells

The expression of ABCA1 protein in J82 cells was lower in cells incubated for 24 hours with N-HDL or G-HDL compared with untreated cells. More precisely, the expression of ABCA1 was lower compared to cAMP treated cells (positive control) **(Figure14).**

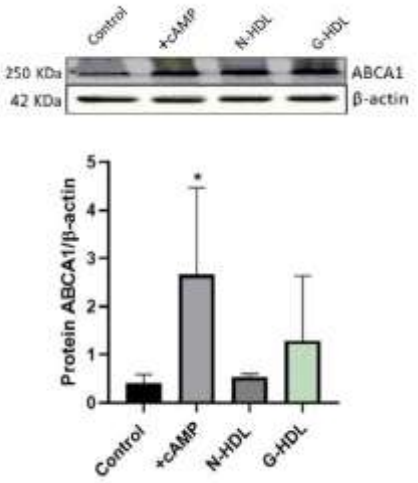


Figure 14 :Effect of N-HDL and glycated HDL on expression of ABCA1 J82 bladder cancer cells were treated with cAMP (0.3 mM) (positive control), N-HDL or G-HDL.
*p<0.001 vs Control

Moreover, the expression of ABCG1 protein in J82 cells was lower in cells incubated with N-HDL **(figure 15)**

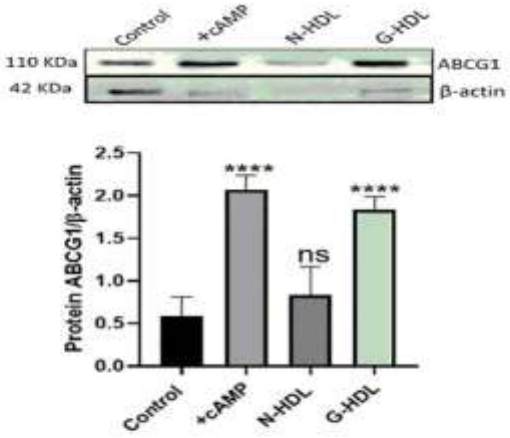


Figure 15 :Effect of glycated HDL on expression of ABCG1
****p<0.001, vs control cells

As shown in **Figure 16,** the expression of SRB1 protein was upregulated in cells incubated with G-HDL for 48-hour compared to N-HDL.

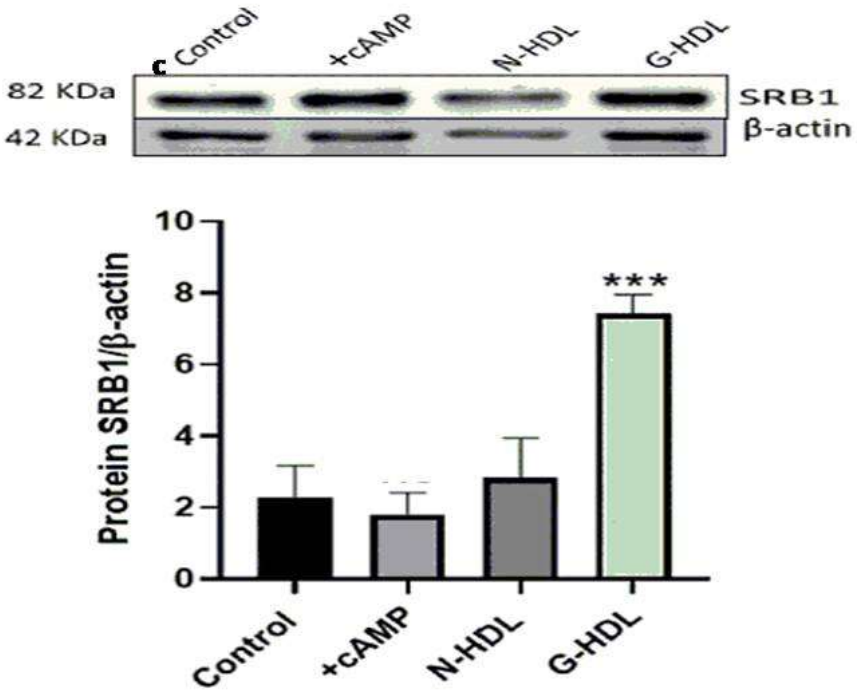


Figure 16:Effect of glycated HDL on expression of receptor protein SR-B1 on J82 cell. *** p<0.01 G-HDL vs control cells

7. Discussion

Recent studies have shown an increase in certain cancers such as breast cancer and bladder cancer in Diabetic patients [16]. Limited studies have investigated the molecular mechanisms. HDL exert several physiological functions (reverse cholesterol transport, antioxidant, anti-inflammatory and anti-apoptosis [17]. Dysfunctional HDL as those observed in human diseases associated with oxidative stress show compositional changes and alterations of their functions [40,112]. For instance, glycated HDL or oxidized HDL exert a lower ability to mediate cholesterol efflux and exhibit lower antioxidant properties [112]. The functional alterations have been related to modifications of lipoprotein lipids and apoproteins. We confirmed that glycation treatment of HDL in our experimental conditions, is associated with alterations of apoprotein with a significant decrease of free lysine amino groups as shown by TNBS assay. Glycated HDL also showed an increase of markers of lipid peroxidation and a significant decrease of PON1 activity. These results, in good agreement with previous studies [115,116] confirm that glycation treatment reflects in a higher susceptibility to oxidative stress with formation of glyco-oxidized HDL. The results suggest that HDL glycated in our experimental functions could behave as dysfunctional lipoproteins.

Previous studies have demonstrated that HDL can behave as growth factor and increase cell proliferation [118,119,128-130]. The effects of normal or dysfunctional HDL on bladder cancer cells has not been previously investigated. In this study, we found that glycated HDL (G-HDL) promoted proliferation of J82 cells with respect to cells incubated with N-HDL. In agreement with our results, previous studies in other cells models in culture revealed that G-HDL can enhance the capacity of cells for proliferation and migration [118,119]. An increased level of intracellular reactive oxygen species (ROS) was observed in J82 cells incubated with tert-butyl-hydroperoxide. The increase in intracellular ROS realized at a higher extent in cells incubated in the presence of tert-butyl-hydroperoxide and G-HDL compared to cells incubated with N-HDL. Preincubation of J82 cells with the antioxidant NAC (10 mmol/L) before treatment with G-HDL (100 µg/ml) and tert-butyl hydroperoxide (TBHP) completely suppressed the increase in ROS. The increase of intracellular ROS was associated with higher levels of TBARS in cells incubated with G-HDL with respect to cells incubated with N-HDL. Our results suggest that dysfunctional HDL promote lipid peroxidation in J82 cells. A relationship between compositional changes of dysfunctional HDL and increased cell oxidative stress has been previously

demonstrated. In fact, previous studies have shown that interaction of glycated HDL or ox-HDL trigger an increase of ROS and oxidative stress in cells in culture. In addition, these alterations promote the proliferation and migration [119,131,132]. Some hypotheses can be advanced to explain the effect of glyco-oxidized HDL on cell oxidative stress and proliferation. At molecular levels, oxidative stress plays a pivotal role in the initiation and progression of cancer cells. For instance, oxidized HDL (ox-HDL) induces ROS production due to NADPH oxidase activation. Several studies have demonstrated that ROS are a heterogeneous group of highly reactive molecules and can modulate several signaling pathways mediating cell proliferation and differentiation. Among signaling pathways activated by ROS there are MAPK and PI3K/Akt pathways [129].

The alterations of J82 after incubation with G-HDL could also be related to modifications of the interactions between G-HDL and cell receptors involved in cholesterol transport and metabolism. In fact, literature data suggest that the behaviour of HDL as potential growth factor for cells in culture could be related to the interactions between HDL and cell receptors involved in cholesterol efflux and influx such as ABCG1, ABCA1 and the SR-BI scavenger receptor [95,106]. The interactions between HDL and membrane transporters/receptors are key steps in HDL-mediated modulation of cell cholesterol content and its involvement in functional activities and cell signalling [133].The interactions between SR-B1 and HDL mediate the selective transfer of cholesteryl ester from HDL molecules to cells. The entry of cholesteryl ester entry via HDL-SR-BI and Akt signaling is considered to relevant in the regulation of cellular proliferation as demonstrated in other cell models [134,135]. Selective cellular influx and efflux of lipid molecules (cholesteryl esters (CEs), lipid-soluble vitamins, such as vitamin E and carotenoids [105, 106], is also mediated by SR-B1. Depending on the type of cancer, high or low SR-BI expression may promote poor survival. In our experimental conditions, expression of receptor protein SR-B1 and ABCG1 was increased in cells incubated with G-HDL. No significant modifications of expression of ABCA1 on J82 was observed. The mechanisms involved in the regulation of expression of receptor protein SR-B1, ABCA1 and ABCG1 are not completely dilucidated. Other authors have shown that glycated proteins isolated from poorly controlled diabetes mellitus serum reduce protein content of ABCA-1 and ABCG-1 in macrophages and impair cholesterol efflux and induce intracellular lipid accumulation. The higher expression of SR-B1 in cells incubated with G-HDL suggests that dysfunctional HDL affect cholesterol homeostasis of J82 cells. These modifications could affect cell proliferation. In fact,

as aforementioned, cholesterol is a key factor which modulates physico-chemical properties of biological membranes and cellular functions. An increased need of cholesterol in tumour cell proliferation has been previously described [8-10]. Alterations of cholesterol homeostasis in J82 cells incubated with G-HDL are supported also by the modifications of Cholesterol efflux and of cholesterol influx in cells incubated with G-HDL compared to cells incubated with N-HDL. We suggest that also alterations of HDL-PON1 in G-HDL could be involved and deserve of further studies. This hypothesis is supported by previous studies on macrophage and to the contribute of HDL-associated PON1 in HDL binding and HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter [70,136].

The role of oxidative stress, dysfunctional HDL and interactions with cell receptors have been mainly investigated in cardiovascular diseases [137,138]. More recent studies on dysfunctional HDL support their modulatory role in the rapid progression and aggressiveness of certain types of cancer [131, 134]. In the present study, we demonstrated that G-HDL trigger oxidative stress and lipid peroxidation in bladder cancer cells. Moreover, alterations of cholesterol homeostasis have been observed. Cancer cells show alterations in different aspects of cholesterol metabolism, which can affect the availability of structural lipids for the synthesis of membranes, contribution of lipids to energy homeostasis, and lipid signaling functions, including the activation of inflammation-related pathways. All these changes are related to important cellular processes, including cell growth, proliferation. A potential role of cholesterol in bladder cancer (BC) cells and signaling pathways is supported by recent studies by Wang G, et al. [12]. Moreover, high dietary cholesterol intake increases the risk of bladder cancer [4].

8. conclusions

High density lipoprotein and their role in prevention of human diseases associated with oxidative stress have been widely studied. Results of some large clinical studies indicate an inverse association for HDL-C and cancer risk; however, these findings have not been reproduced in all epidemiological studies and are still debated. Potential molecular protective mechanisms include HDL role in reverse cholesterol transport, antioxidant, and anti-inflammatory properties. Oxidants or oxidizing agents contribute to cancer risk attacking by biomolecules such as lipids, proteins, and DNA. A persistent proinflammatory state can sustain cancer cell growth and survival and has also been found to contribute to other chronic diseases; Proteins associated to HDL surface may modulate inflammation.

Basic research studies have determined the important role of cholesterol and its transporters in cancer development. Dysfunctional HDL such as oxidized and glycated HDL may promote different cancer via several mechanisms. In fact, an interplay among modified lipoproteins, oxidative stress, proinflammatory signaling pathways and alterations of intracellular cholesterol levels can promote cell proliferation. In this study, we found that glycation treatment of HDL in vitro is associated with an increase of lipid peroxidation markers of HDL and a decrease of the activity of the antioxidant enzyme HDL-paraoxonase. Glyco-oxidized HDL remarkably promote proliferation and oxidative stress in J82 cells as models of bladder cancer cells. The increase of intracellular ROS was associated to higher levels of lipid peroxidation products and to alterations of cholesterol homeostasis. In detail cholesterol efflux was increased. On the contrary, cholesterol influx was significantly decreased in cells incubated with G-HDL. Furthermore, expression of receptor protein SR-B1 and ABCG1 was increased. Both diabetes and cancer are prevalent diseases whose incidence is increasing globally. Diabetes Mellitus is associated with elevated bladder cancer or cancer mortality risk. Our results could have a physio-pathological significance in particular in Diabetes. In fact, dysfunctional HDL including glycated and oxidized HDL are widely described in patients with type 2 diabetes mellitus. Further studies are needed to identify the details which HDL components are responsible for the observed effects in J82 cells. Our results suggest that the study of HDL cholesterol levels and HDL functionality could be used to help advise patients about lifestyle modifications. HDL-based treatments should be considered for treatment of bladder cancer.

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