

Profiles of Fatty Acids and the Main Lipid Peroxidation Products of Human Atherogenic Low Density Lipoproteins

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Cardiovascular diseases (CVD), a worldwide leading cause of mortality and morbidity, are generated mainly by atherosclerosis. The serum fatty acids (FA) and some of their peroxidation products, such as 4-hydroxy-trans-2-nonenal (4-HNE) and malondyaldehyde (MDA), are known risk factors for CVD. Our aim was to determine in atherogenically-modified low-density lipoproteins (LDL), either oxidized or glycated, the FA composition, quantify the levels of 4-HNE, MDA and of cholesterol's main oxidation product, 7-ketocholesterol (7-KC). Profiles of FA, conjugated dienes, 4-HNE, MDA and 7-KC were determined in native LDL (nLDL) isolated from blood donors from the Hematology Center, Bucharest, copper-oxidized LDL (oxLDL) and non-enzymatically glycated LDL (gLDL). The FA composition was determined by Gas Chromatography with a flame ionization detector and the conjugated dienes by absorption in UV at 234 nm. Free 4-HNE and 7-KC levels were determined by Gas-Chromatography-Triple Quadrupole Mass Spectrometry in Electron Ionization mode and total MDA by reversed-phase Ultra High Performance Liquid Chromatography. Results showed that the polyunsaturated fatty acids (PUFAs) were totally reduced in oxLDL (except linoleic acid) and decreased in gLDL, while the monounsaturated fatty acids remained almost unchanged in both oxLDL and gLDL. Lipid peroxidation products increased as follows: conjugated dienes were significantly increased both in gLDL (29%, $p < 0.05$) and oxLDL (1.5 fold higher, $p < 0.01$) compared to nLDL; free 4-HNE level in oxLDL (2.75 ± 1.01 nmoles/mg protein) was ~100 times the level in nLDL (0.02 ± 0.01 nmoles/mg protein), while gLDL (0.05 ± 0.02 nmoles/mg protein) had only twice the nLDL level; MDA levels in oxLDL were statistically increased (1.05 ± 0.44 nmoles/mg protein) compared to nLDL (0.05 ± 0.02 nmoles/mg protein) and to gLDL (0.19 ± 0.05 nmoles/mg protein); 7-KC level was significantly increased in oxLDL (674.39 ± 207.70 nmoles/mg protein) and gLDL (1.21 ± 0.32 nmoles/mg protein) compared to nLDL (0.13 ± 0.04 nmoles/mg protein). Our data demonstrate that the alteration of the FA composition of modified LDL has two negative consequences: (i) the reduction of PUFA levels, most importantly the essential ω -3 and ω -6 fatty acids, and (ii) the increase of the main peroxidation products of FA and cholesterol such as conjugated dienes, free 4-HNE, MDA and 7-KC, transforming LDL into atherogenic lipoproteins.

Keywords: Fatty acids, 4-HNE, oxidized LDL, glycated LDL, GC/MS/MS

Cardiovascular diseases (CVD) caused by atherosclerosis and their associated adverse complications are major causes of morbidity and mortality in western societies [1]. The non-enzymatic glycation of plasma proteins, such as in diabetes, can increase their atherogenic potential. During the last years a considerable amount of research was focused on the susceptibility of low-density lipoproteins (LDL) to oxidation. Modified LDL that cause cellular lipid loading were proven to be atherogenic and involved in the development of CVD [2]. Glycation of LDL is significantly increased in diabetic patients even in the case of good glycemic control [3]. LDL, either oxidized and/or glycated, are present in the plasma and in the affected vasculature of obese, diabetic and CVD patients [4].

Fatty acids (FA) profiling of biological samples (plasma) has a great importance for the understanding of the relation between dietary lipids and the development of diabetes and CVD [5]. Conventional methods for the profiling of FA consist of several steps: lipids extraction with organic solvents, hydrolysis, methylation/derivatization, and finally, analysis and quantification by gas chromatography (GC). Linoleic acid and arachidonic acid are the major polyunsaturated fatty acids (PUFAs) in LDL, the former being

present mostly in the cholesteryl esters (CE) core of the lipoproteins. The FA composition varies between various sources of LDL (different subjects) and depends on the diet [2].

During the formation of hydroperoxides from unsaturated FA, conjugated dienes are typically produced, due to the rearrangement of the double bonds. The resulting conjugated dienes exhibit a characteristic absorption at 234 nm. An increase in UV absorption theoretically reflects the formation of primary oxidation products in lipids [6, 7].

The oxidation of PUFAs generates highly reactive α,β -unsaturated hydroxyalkenals, such as 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE) and 4-hydroxydodecadienal (4-HDDE) [6]. Oxidation of ω -6 PUFAs (linoleic and arachidonic acids) leads to the formation of 4-HNE [8], whereas oxidation of ω -3 PUFAs (docosahexaenoic acid, eicosapentaenoic acid and linolenic acid) generates 4-HHE [9]. 4-HNE can react with histidine (His), cysteine (Cys) or lysine (Lys) residues of proteins, leading to the formation of stable Michael adducts with a hemiacetal structure [10], modifying the proteins' epitopes that interact with specific receptors. The hydroxy-alkenals levels have been measured with high performance liquid chromatography (HPLC) using the UV absorption detector.

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However, this method's sensitivity is limited. Another method far more sensitive for the determination of 4-HNE and 4-HHE is the GC coupled to a mass spectrometer (MS) [6].

MDA is one of the most abundant aldehydes, resulting from the peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acids [11]. MDA reacts with the Lys residues of proteins to form Schiff bases [8] and plays a major role in the LDL oxidative modifications [12-13]. The oxidative hypothesis of atherosclerosis proposed by Steinberg postulates that 4-HNE- and MDA-modified LDL are taken up by macrophages in the artery wall, which become foam cells and form fatty streaks, an early step of the atherogenic process [14]. MDA can be measured in biological fluids as well as in isolated cells by the thiobarbituric acid (TBA) reaction and the resulting absorbance at 532 nm or fluorescence can be determined by HPLC [6].

Cholesterol may be oxidized by enzymatic or non-enzymatic pathways [15]. Cholesterol oxidation products have received increasing attention as diagnostic markers of oxidative stress, intermediates in cholesterol transport and messengers for cell signaling or intermediates in bile acid biosynthesis [15]. 7-Ketocholesterol (7-KC), a major cholesterol oxidation product (oxysterols), is found in high concentrations in atherosclerotic plaques and contributes to its development [16].

Experimental part

Materials and methods

4-HNE was purchased from Cayman Chemicals, 1,1,3,3-tetramethoxypropan (malonaldehyde bis(dimethyl acetal) 99% (TMP), 5-cholesten-3 β -ol-7-one (7-ketocholesterol), 5 α -cholestane, hexane, sodium hydroxide, methanol (HPLC grade), perchloric acid 70%, hydrochloric acid 37% were purchased from Sigma-Aldrich. FAME MIX 37 and hexamethyldisilazane:trimethylchlorosilane:pyridine 3:1:9 (Sylon HTP) were purchased from Supelco. *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine PFBHA HCl, *N,O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) and 2,4-dinitrophenylhydrazine (DNPH) 50%, were purchased from Fluka. Acetonitrile (HPLC grade), methanol (HPLC grade) and toluene were purchased from Merck and acetic acid (HPLC grade) was purchased from Panreac Quimica Spain.

LDL isolation, in vitro oxidation and glycation

LDL was isolated from human plasma of healthy donors from the Blood Hematology Center, Bucharest by density gradient ultracentrifugation in Optima LE-80 and XP-80 ultracentrifuges (Beckman Coulter International SA, Nyon, Switzerland).

Oxidized LDL (oxLDL) was prepared by incubation for 24 h with 10 μ M CuSO₄ as previously described [17].

Non-enzymatically irreversibly glycosylated LDL (gLDL) was prepared by incubation of LDL with 0.2 M D(+) glucose for 4 weeks at 37°C, in the presence of antioxidants (1 mg/mL EDTA and 1 M BHT) under sterile conditions and extensively dialyzed before use [18].

Determination of Protein

Protein concentration of LDL was measured by an adapted Lowry method as modified for lipoproteins employing bovine serum albumin as a standard [19].

Analysis of fatty acids distribution by GC-FID

Lipids were extracted by a modified Bligh and Dyer method [20] from LDL samples. The fatty acids methyl esters (FAMES) were obtained by mild methanolysis with

8% HCl methanolic solution, according to the procedure suggested by Ichihara and Fukubayashi [21].

GC-FID analyses were performed on an Agilent Technologies gas chromatograph GC 7890A coupled with FID detector. A fused-silica capillary column DB-225 ms (60 m \times 0.25 mm i.d., 0.25 μ m film thickness) from Agilent J&W was used for separation FAMES. Aliquots (each 1 μ L) were injected in the split mode (1:100) with an auto injector. Helium was used as the carrier gas, at a flow rate of 1 mL/min. The initial temperature of 120°C was maintained for 1 min and then increased to 220°C at 4°C/min. The temperature of the injector was 260°C and the FID detector was set at 280°C. Identification of fatty acids methyl esters was made with a standard mixture of 37 esters of fatty acids (FAME MIX 37) from SUPELCO.

Determination of conjugated dienes

The formation of conjugated dienes was detected in UV at λ =234 nm in native and modified LDL [6, 7].

Determination of free 4-HNE by GC/MS/MS

According to the procedure suggested by van Kuijk and Rauli [22, 23], LDL sample was incubated for 30 min with 0.05 M PFBHA HCl in water with continuous stirring. The pentafluorobenzyl-oxime (PFBO)-derivatives were extracted with hexane and evaporated under nitrogen. The sample was derivatised with 50 μ L of *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) at 80°C for 1 h and were transformed in PFBO-TMS of 4-HNE derivatives (**2**, scheme 2).

GC/MS/MS analyses were performed on an Agilent Technologies 7000A GC/MS Triple Quad directly interfaced with a gas chromatograph GC 7890A. A fused-silica capillary column HP-5 ms (30 m \times 0.25 mm i.d., 0.25 μ m film thickness) from Agilent J&W was used for separation of 4-HNE derivatives. Aliquots (each 2 μ L) were injected in the split less mode with an auto injector. Helium was used as the carrier gas, at a flow rate of 1 mL/min. The initial temperature of 85°C was maintained for 1 min and then increased to 145°C at 20°C/min (3min), and to 300°C at 8°C/min (10 min). The temperature of the injector was 260°C and the transfer line was set at 280°C.

Mass spectrometry detection was performed in Electron Ionization mode (EI) with a 70 eV electron energy, at 230°C source temperature and 150°C MS1 and MS2 temperature. The optimization of the collision energy voltage and the selection of precursor ions to generate product ions will be presented in the section Results and discussions.

The calibration curve was constructed with known amounts of standard 4-HNE solution. The highest level of target analyte was 2 nmoles/mL. Six calibration solutions were prepared by 1:2:2:2:2:2 dilution ratios. The standard solutions were derivatized in the same mode as samples.

Determination of total MDA by UHPLC

For the alkaline hydrolysis of protein bound MDA, a volume of 6 M NaOH was added to the LDL sample and incubated in a 60°C water bath for 45 min [24]. After protein precipitation with 35% perchloric acid and centrifugation, 250 μ L supernatant were derivatized with 25 μ L DNPH solution 5 mM in 2 M HCl and incubated for 10 min at room temperature. The samples were filtered through a 0.22 μ m filter. Aliquots of 3 μ L were injected in an Ultra High Performance Liquid Chromatography (UHPLC) system (Agilent Technologies 1290 Infinity) equipped with binary solvent delivery pump (with four channels), auto sampler, thermostatic column compartment, a Diode Array Detector and Agilent ChemStation software for data acquisition and processing.

The chromatographic separation was performed on a ZORBAX Eclipse Plus C18 column narrow bore RR (150 mm x 2.1 mm, 3.5 μ m) with the mobile phase consisting of a 0.2% aqueous acetic acid and acetonitrile (62:38) v/v at a flow rate 0.25 mL/min. The detector wavelength was set at 310 nm and the column was maintained at 25°C.

MDA standards were prepared from 1 mM stock solution of TMP. The calibration curve was constructed in the range 0.625-10 nmol/mL TMP. 250 μ L of standards were mixed with 25 μ L DNPH in the same condition as the samples.

Determination of 7-ketocholesterol by GC/MS/MS

The sample preparation method was an adapted method after Matysik [25]. Briefly, to LDL sample/standard were added 2000 ng of 5 α -cholestane internal standard (IS) and freshly prepared 1 M potassium hydroxide in ethanol to cleave the ester bonds for 60 min at 25°C under magnetic agitation. Afterwards, the reaction solution was adjusted to pH 7 and sterols were extracted with hexane. The solvent was evaporated to dryness under nitrogen and the residue was dissolved in 100 μ L of the reagent Sylon HTP for derivatization, 1 h at 60°C. The derivatized samples were transferred to GC vials for direct injections.

For separation of the trimethylsilyl ether derivatives of 7-ketocholesterol (TMS derivatives of 7-KC) (1, scheme 1) was used the same technology and the same column as for determination of 4-HNE. Aliquots (each 2 μ L) were injected in the splitless mode with an auto injector. Helium was used as the carrier gas, at a flow rate of 1.2 mL/min. The initial temperature of 180°C was maintained for 1 min and then increased to 250 at 20°C/min, and to 295°C at 5°C/min and to 300°C at 1°C/min (10 min). The temperature of the injector was 275°C and the transfer line was set at 280°C.

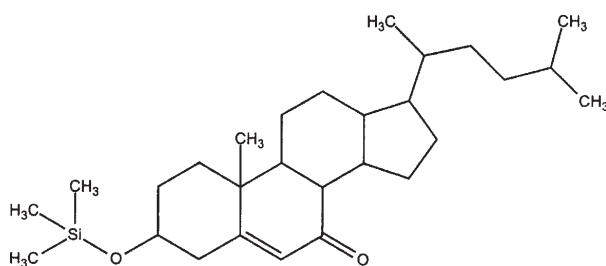
Mass spectrometry detection was performed in EI mode with a 70 eV electron energy, at 230°C source temperature and 150°C MS1 and MS2 temperature. The collision energy voltage was optimized to fragment the precursor ion m/z 472.5 by collision-induced dissociation (CID) to generate the product ions selected as quantification and qualifier ions in the multiple reactions monitoring (MRM) method for analyte. The quantification ion was m/z 455.4 and qualifier ion m/z 382.6.

Precursor ion of 5 α -cholestane (IS) was m/z 357.4. The quantification ion was m/z 189.2 and qualifier ion m/z 203.1. The calibration curve was constructed in the range 50-5000 ng/mL in the same mode as samples.

Results and discussions

It is well known that the high saturated fat intake generates high serum lipids levels and the development of atherosclerosis. The analysis of serum and tissue FA distribution was included in population studies on CVD and their risk factors [26].

It has been determined the content of linoleic acid (C18:2) (34.22 \pm 1.57, expressed as % (w/w) and



Scheme 1. Chemical structure of TMS derivatives of 7-ketocholesterol (1)

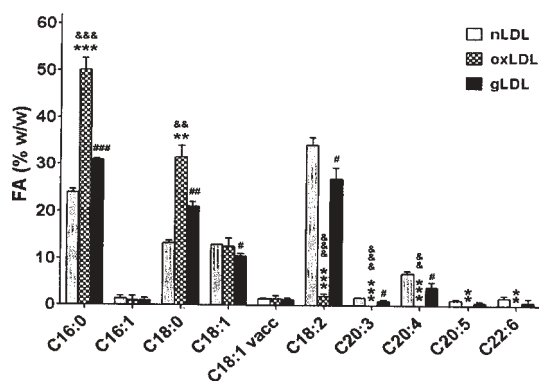


Fig. 1. Composition of fatty acids (FA) in oxidized low density lipoproteins (oxLDL) and glycated low density lipoproteins (gLDL) compared to native low density lipoproteins (nLDL). The data are expressed as mean \pm SD; n=3 samples for each histogram, ** p <0.01 oxLDL versus nLDL, *** p <0.001 oxLDL versus nLDL, # p <0.05 gLDL versus nLDL, ## p <0.01 gLDL versus nLDL, ### p <0.001 gLDL versus nLDL, && p <0.01 oxLDL versus gLDL, &&& p <0.01 oxLDL versus gLDL.

arachidonic acid (C20:4) (6.97 \pm 0.54), known as the most abundant ω -6 PUFAs present in nLDL, and ω -3 PUFAs, such eicosapentaenoic acid, EPA (C20:5) and docosapentaenoic, DHA (C22:6), which occur only in minor amounts (fig. 1).

The FA distribution in oxLDL (expressed as %w/w) was altered after LDL oxidation with copper ions as compared to the FA distribution in nLDL. The percent of saturated FA in oxLDL increased compared to nLDL: the palmitic acid (C16:0) (50.06 \pm 2.45 versus 23.99 \pm 0.69, p <0.001) and the stearic acid (C18:0) (31.60 \pm 2.39 versus 13.39 \pm 0.46, p <0.01). The abundance of monounsaturated FA, such as the palmitoleic acid (C16:1) (1.47 \pm 0.52 versus 1.51 \pm 0.59), oleic acid (C18:1) (13.05 \pm 0.06 versus 12.66 \pm 1.79) and (C18:1 *cis* vaccenic) (1.41 \pm 0.17 versus 1.51 \pm 0.59) remained statistically unchanged. The linoleic acid was statistically decreased in oxLDL (1.94 \pm 0.56 versus 34.22 \pm 1.57, p <0.001) compared to nLDL, while the ω -6 dihomogammalinolenic acid (C20:3) (1.72 \pm 0.04, p <0.001) and arachidonic acid (C20:4) (6.97 \pm 0.54, p <0.001) were completely oxidized in oxLDL (fig. 1).

The ω -3 FA, EPA (1.16 \pm 0.23, p <0.001) and DHA (1.71 \pm 0.54, p <0.001) were totally reduced, being transformed in peroxidation products (fig. 1).

In gLDL compared to nLDL, the decrease of linoleic acid (27.03 \pm 2.31 versus 34.22 \pm 1.57, p <0.05), of dihomogammalinolenic acid (0.97 \pm 0.29 versus 1.55 \pm 0.29, p <0.05) and arachidonic acid (3.98 \pm 1.03 versus 6.97 \pm 0.54, p <0.05), was statistically significant.

There is a tendency for ω -3 FA abundance to decrease, as they were reduced in half in gLDL compared to nLDL, EPA (0.58 \pm 0.50 versus 1.16 \pm 0.23 and DHA (0.58 \pm 0.83 versus 1.71 \pm 0.54) (fig. 1).

In oxLDL compared to gLDL, the linoleic acid (1.94 \pm 0.56 versus 27.03 \pm 2.31, p <0.01) was significantly decreased.

Conjugated dienes, detected in UV at λ =234 nm, were significantly increased in both gLDL and oxLDL with 29%, p <0.05 and \sim 1.5 fold higher, p <0.01, respectively, compared to nLDL (fig. 2). Conjugated dienes detection by UV is easy, but the method is neither very specific, nor very sensitive.

Like other aldehydes, 4-HNE was derivatized into pentafluorobenzyl oxime directly in the LDL sample as previously described [22, 23]. Their derivatives were extracted with hexane, and the hydroxyl group was converted into trimethylsilyl ether with *N,O*-bis(trimethylsilyl)-trifluoroacetamide, and the penta-

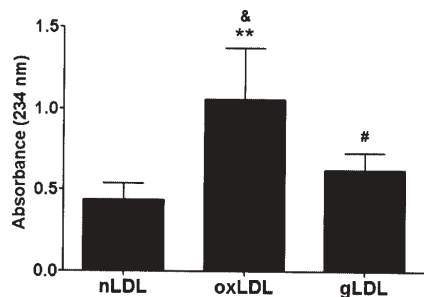
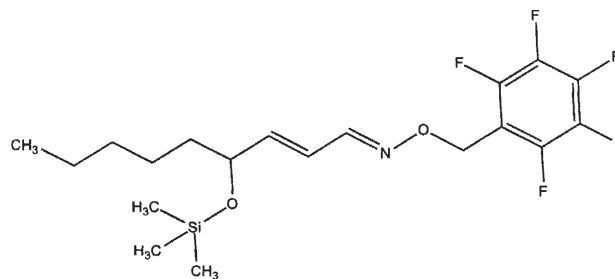


Fig. 2. Conjugated dienes in native low density lipoproteins (nLDL), oxidized low density lipoproteins (oxLDL) and glycated low density lipoproteins (gLDL). The data are expressed as mean \pm SD; n=6 samples for each histogram, ** p <0.01 oxLDL versus nLDL, # p <0.05 gLDL versus nLDL, & p <0.05 oxLDL versus gLDL.



Scheme 2. Chemical structure of PFBO-TMS derivatives of 4-HNE (2)

Fig. 3. Mass spectra of Q1 scan after positive electron ionization (EI) of the PFBO-TMS-derivatives of 4-HNE standards.

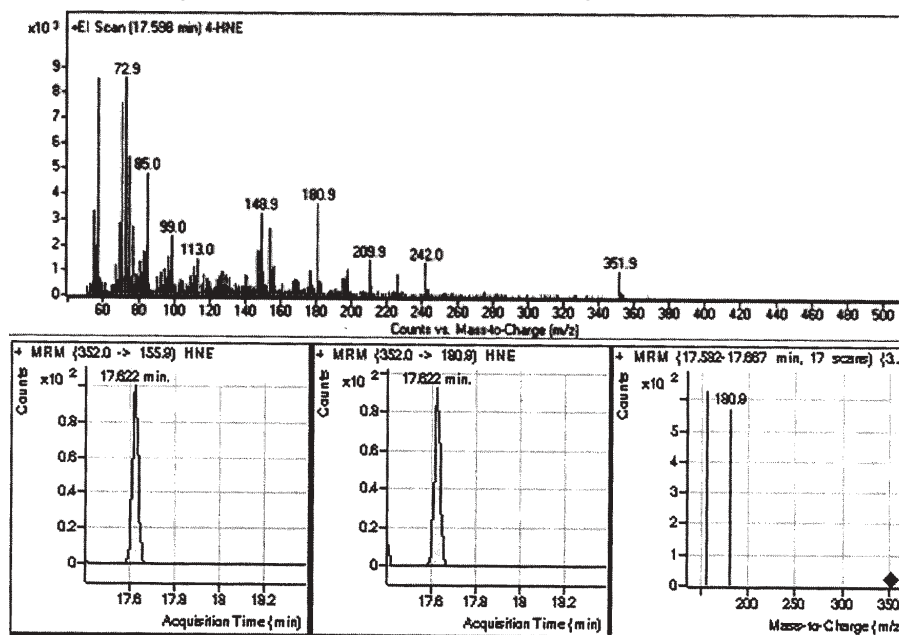


Fig. 4. MRM chromatograms of quantifier, qualifier and peak spectrum of PFBO-TMS derivatives of 4-HNE standards.

fluorbenzyl oxime trimethylsilyl ether derivatives (PFBO-TMS) of 4-HNE (2, scheme 2) were analyzed by negative ion chemical ionization (NICI) GC-MS [22, 23].

A GC/MS/MS Triple Quad in EI mode method for quantification of 4-HNE has been developed. First was performed scan a quadrupole 1 (Q1) to produce a MS pattern in EI mode for PFBO-TMS derivatives of 4-HNE from standard solutions. The characteristic fragment ions (fig. 3) corresponded well with those given in the literature [27].

Two precursor ions (m/z 242 and m/z 352) were chosen for product scans and the most abundant product ions were chosen to define specific multiple reaction monitoring (MRM). The collision energy voltage was optimized in the range 1-10 eV to fragment the most abundant precursor ion m/z 352 by collision-induced dissociation (CID) to generate the product ions selected as quantification and qualifier ions in the multiple reaction monitoring (MRM) method for analyte. The optimum collision energy voltage was 10 eV and the dwell time was fixed at 150 ms for MRM transition. The quantification ion was m/z 155.9 and the qualifier ion, m/z 180.9 (fig. 4).

The 4-HNE levels were significantly increased in oxLDL compared to both nLDL and gLDL (2.75 ± 1.01 versus 0.02 ± 0.01 nmoles/mg protein, p <0.001, and 0.05 ± 0.02 nmoles/mg protein, p <0.05, respectively) (fig. 4).

The mean MDA level in oxLDL was significantly increased compared to nLDL (1.05 ± 0.44 versus 0.05 ± 0.02 nmoles/mg protein, p <0.001) and to gLDL (0.19 ± 0.05 nmoles/mg protein, p <0.001). The increase of MDA in gLDL compared to nLDL was statistically significant

(0.19 ± 0.05 versus 0.05 ± 0.02 nmoles/mg protein, p <0.001) (fig. 5).

The mean MDA level was ~3 fold lower in oxLDL (1.05 ± 0.44 nmoles/mg protein) than 4-HNE (2.75 ± 1.01 nmoles/mg protein), while in gLDL, the MDA level (0.19 ± 0.05 nmoles/mg protein) was ~ 4-fold higher than 4-HNE level (0.05 ± 0.02 nmoles/mg protein).

These differences of MDA levels versus 4-HNE in gLDL could be explained by the fact that our GC/MS/MS method for quantification 4-HNE has a high degree of specificity while the determination of MDA by HPLC with DNPH

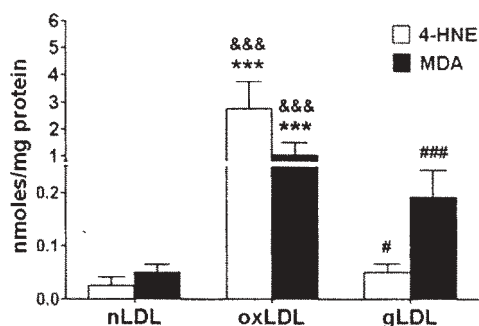


Fig. 5. Free 4-HNE and total MDA in native low density lipoproteins (nLDL), oxidized low density lipoproteins (oxLDL) and glycated low density lipoproteins (gLDL). The data are expressed as mean \pm SD; n=6 samples for each histogram, *** p <0.001 oxLDL versus nLDL, # p <0.05 gLDL versus nLDL, ### p <0.001 gLDL versus nLDL, &&& p <0.001 oxLDL versus gLDL.

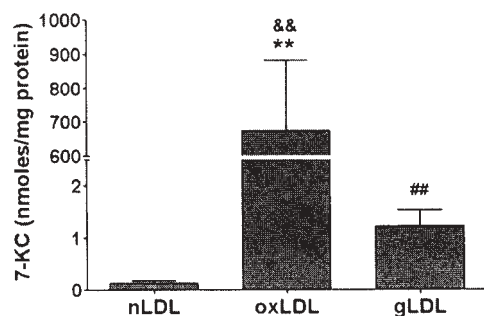


Fig. 6. 7-Ketocholesterol (7-KC) in native low density lipoproteins (nLDL), oxidized low density lipoproteins (oxLDL) and glycated low density lipoproteins (gLDL). The data are expressed as mean \pm SD; n=3 samples for each histogram, ** p <0.01 oxLDL versus nLDL, ## p <0.01 gLDL versus nLDL, && p <0.01 oxLDL versus gLDL. derivatization is specific for all carbonyl groups and gLDL have a high number of carbonyl groups.

Next to 27-hydroxycholesterol, 7-ketocholesterol is the next most abundant oxysterol in advanced human atherosclerotic lesion [29]. During the *in vitro* oxidation of LDL, a substantial loss of free and esterified cholesterol occurs as both these molecules are oxidized [15].

The mean 7-KC level was significantly increased in oxLDL compared to both nLDL and gLDL (674.39 ± 207.70 versus 0.13 ± 0.04 nmoles/mg protein, $p < 0.01$, 1.21 ± 0.32 nmoles/mg protein, $p < 0.01$, respectively) (fig. 6). The mean 7-KC level was ~ 550 -fold higher in oxLDL (674.39 ± 207.70 nmoles/mg protein) than in gLDL (1.21 ± 0.32 nmoles/mg protein).

Conclusions

The experimental results demonstrate that the glycation and mostly the oxidation of LDL have negative consequences on the particles biochemical composition involving the following processes: (i) the reduction of PUFAs levels, most importantly the essential ω -3 and ω -6 fatty acids and (ii) the increase of the main peroxidation products of FA and cholesterol, such as conjugated dienes, free 4-HNE, MDA and 7-KC, which taken together transform LDL into atherogenic lipoproteins.

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