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ORIGINAL ARTICLE

Long-term effect of pioglitazone vs glimepiride on lipoprotein oxidation in patients with type 2 diabetes: a prospective randomized study

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Abstract

Aims Type 2 diabetes (DM2) is associated to oxidative modifications of high-density lipoproteins (HDL), which can interfere with their function. Pioglitazone has proved effective in raising HDL cholesterol (HDL-C) and lowering small dense low-density lipoprotein (LDL), but no clinical studies have examined its effect on lipoprotein oxidation in patients with DM2.

Methods We assessed the effect of pioglitazone vs glimepiride after 1 year on HDL oxidation, expressed as relative abundance of peptides containing $Met^{112}O$ in ApoA-I (oxApoA-I) estimated by mass spectrometry (MALDI/TOF/TOF), in 95 patients with DM2. The oxLDL and AGE were quantified by ELISA.

Results Patients receiving pioglitazone showed a significant increase in the concentration of ApoA-I ($\Delta = 7.2 \pm 14.8$ mg/dL, $p < 0.02$) and a reduction in oxApoA-I ($\Delta = -1.0 \pm 2.6\%$, $p < 0.02$); this reduction was not significantly different from glimepiride. oxLDL showed a slight, but not significant increase in both treatment groups. Regression analysis showed a correlation between ΔoxApoA-I and ΔAGE ($r = 0.30$; $p = 0.007$) in all patients, while both of these parameters were unrelated to changes in HbA1c, HDL-C, duration of illness, or use of statins.

Conclusions Long-term treatment with pioglitazone was effective in reducing the oxidation of HDL, but not LDL in patients with DM2, while glimepiride didn't. This finding seems to be associated to the change of glyco-oxidation status, not to any improvement in glycemic control or lipid profile.

Trial registration NCT00700856, ClinicalTrials.gov Registered June 18, 2008

Keywords Clinical trials · Diabetes · Mass spectrometry · Oxidized lipids · Lipoproteins

Introduction

High-density lipoprotein cholesterol (HDL-C) concentrations are known to correlate inversely with the incidence of cardiovascular events in patients with type 2 diabetes (DM2) [1]. Irrespective of a patient's HDL-C levels, HDL have anti-inflammatory and anti-oxidative properties [2, 3] that may be impaired in certain pathological conditions, especially DM2, in which a state of subclinical inflammation and accumulation of reactive oxygen species (ROS) is associated with both hyperglycemia and whole-body insulin resistance [4–6]. In vitro studies have suggested that apolipoprotein A-I (ApoA-I) functionality is reduced in DM2 due to downregulation of its expression or post-translational modifications of its structure [7].

A greater formation of methionine sulfoxides (MetO) on methionine residues of ApoA-I (Met86, Met112, Met148) has been observed in patients with type 1 diabetes (DM1) by comparison with healthy subjects [8]: these residues preserve biochemical functions related to cholesterol outflow and activation of the lecithin–cholesterol acyltransferase (LCAT) enzyme [9], acting as endogenous antioxidants.

In a previous cross-sectional study we showed for the first time that non-diabetic subjects experiencing a premature

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myocardial infarction and DM2 patients with no cardiovascular disease have a significantly higher proportion of MetO in ApoA-I than healthy controls [10]. Interestingly, this increased HDL oxidation in patients highly susceptible to coronary artery disease (CAD) was uninfluenced by the individuals' HDL-C levels.

Pioglitazone is a PPAR-γ receptor agonist with a demonstrated efficacy in lowering triglycerides (TG) and raising HDL-C levels, superior to that of metformin or sulfonylurea, in patients with DM2 [11]. Moreover, despite its neutral effect on low-density lipoprotein cholesterol (LDL-C), treatment with pioglitazone reduces the number of small dense LDL [12]. These quantitative effects on the lipid profile were confirmed by long-term studies [13], regardless of an individual's glycemic control or any concomitant use of statins.

Pioglitazone has also been associated with an anti-inflammatory and antioxidant activity in patients with DM2 [14], particularly as regards advanced glycation end products (AGE), a marker of glyco-oxidation [15]. AGE tend to accumulate in the cells and tissues of patients with DM2 and are believed to have a role in the pathogenesis of microvascular and macrovascular complications of DM2 [16, 17].

A putative role for AGE in lipoprotein oxidation has been suggested. In an in vivo study, exposure to high concentrations of AGE correlated with a reduced cholesterol efflux capacity from macrophages to ApoA-I, through interference with the ATP-binding cassette A-1 (ABCA1), a pivotal carrier for reverse cholesterol transport efficiency [18]. The increased glyco-oxidative stress typical of DM2 probably contributes to causing a reduced HDL function in these patients, and their consequent residual cardiovascular risk.

To our knowledge, no clinical trials have examined the long-term effects of pioglitazone treatment on the oxidative changes in HDL in patients with DM2. The main purpose of this study was thus to assess the long-term effect of pioglitazone, by comparison with glimepiride, on HDL oxidation, measured by means of relative abundance of peptides containing $Met^{112}O$ in ApoA-I (oxApoA-I), in patients with DM2 treated with metformin. A second aim was to ascertain whether any pioglitazone-induced change in oxApoA-I related to glyco-oxidative stress, expressed as the concentration of serum AGE, and its impact on LDL oxidation (oxLDL).

Patients and methods

Subjects

The study was conducted within the framework of the TOSCA.IT trial (thiazolidinediones or sulphonylureas and cardiovascular accidents. Intervention Trial; NCT00700856, ClinicalTrials.gov), a randomized clinical trial (RCT) designed to assess the cardiovascular effects of two different hypoglycemic drug regimens (sulfonylurea or pioglitazone). Details of the study protocol, and the inclusion and exclusion criteria have been published elsewhere [19]. The protocol was approved by the Ethics Review Committee/Institutional Review Board of the Coordinating Center and each participating center. The study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines. Written informed consent was obtained from participants before beginning any protocol-specific procedure and participants were told that they had the right to withdraw from the study at any time.

Allocation to treatment was assigned centrally by telephone after checking for all the eligibility criteria. The treatment allocation schedule was computer-generated in blocks and stratified by clinical status and previous cardiovascular events. The participating centers were masked to the randomization sequences, which were generated at the Epidemiology Unit.

Data reported in this study were collected from the 95 patients recruited for the TOSCA.IT trial at the Diabetology and Dietetics Unit in Padua, Italy. Patients were randomized to take pioglitazone 15 mg or glimepiride 2 mg, in addition to the maximum tolerated dose of metformin. A blood test was performed at the time of their randomization and after 1 year, in accordance with the RCT procedures, to obtain biochemical parameters of glyco-oxidation and lipid profiles.

Basic blood chemistry parameters and markers of glyco-oxidation

HbA1c was measured by high-performance liquid chromatography (HPLC, Menarini Akray ADAM A1c HA-8180v), in line with IFCC standards (International Federation of Clinical Chemistry) [19]. Total cholesterol, LDL-C and HDL-C were measured using an enzymatic colorimetric method (COBAS 8000; Roche, Milan) [20], as were TG (GPO-PAP colorimetric enzyme tests; Roche Diagnostic System) [21].

The albumin-creatinine ratio (ACR) was established from the relationship between albuminuria and creatinine concentrations on random urine samples. The glomerular filtration rate (GFR) was calculated using the CKD-EPI formula, which takes serum creatinine concentrations, and patients' weight, age and sex into account. Although it does not overcome the risk of underestimating the real GFR, this formula is currently the most accurate for the purpose of following up renal function, particularly in patients with diabetes [22].

An ELISA method was used to assay oxLDL, with 2 monoclonal antibodies specific for antigens on apolipoprotein B (Mercodia Oxidized LDL ELISA, Uppsala, Sweden), obtaining an intra-assay coefficient of variation < 5% and an interassay coefficient of variation < 10% [23].

Serum AGE were estimated by means of an ELISA assay, using a polyclonal antibody [24].

Preparation of the HDL fraction

Blood samples were collected in vacutainers after 12–14 h of overnight fasting. To protect the methionine residues from oxidation, serum was prepared immediately by low-speed centrifugation at 2500 rpm for 15 min at 10 °C, adding Na2-EDTA (0.04% w/v) and HDL were isolated from the serum by sequential ultracentrifugation on the day when the blood was collected, using the Beckman 50 Ti fixed-angle rotor in an Optima XL90 ultracentrifuge (Beckman Instruments, Palo Alto, California, USA) [25]. The density of 6 mL of serum was adjusted to 1.21 kg/L by adding solid KBr (Carlo Erba reagents, Milan, Italy). The resulting solution was placed in polyallomer quick-seal centrifuge tubes (Beckman Instruments, Palo Alto, California, USA) and over-layered with a density solution of KBr, $d = 1.21$ kg/L, pH 7.4. Densities were adjusted and checked on a Densito 30P density meter (Mettler Toledo, Switzerland). Centrifugation was done at 40,000 rpm, at 6 °C for 30 h. All lipoproteins were removed in 4 mL of supernatant by tube slicing. The density of 4 mL of supernatant was adjusted to 1.063 (kg/L). Centrifugation was repeated as described above, at 40,000 rpm and a temperature of 6 °C, for 24 h. HDL ($d = 1.063$–1.210 kg/L) were removed in 4 mL of the bottom fraction and used in the subsequent analysis. Recovery of the 3 fractions of cholesterol was 90%. The HDL fraction was exhaustively dialyzed and concentrated with an Amicon Ultracel 10K centrifugal filter (Millipore Corporation, Billerica USA). Apo-AI and cholesterol concentrations were measured in serum and in the fractions isolated by ultracentrifugation using an automatic Cobas Mira plus analyzer (Horiba ABX, Montpellier, France). ApoA-I was measured with the immune turbidimetric method (Horiba ABX, Montpellier, France). HDL cholesterol was measured after precipitation of the apo-B-containing lipoproteins with phosphotungstate precipitant (Roche Diagnostics, Mannheim, Germany) [26] using the same enzymatic-colorimetric method (Cholesterol CHOD-PAP, Roche-Diagnostics, Mannheim, Germany) [19] as for the total cholesterol.

Tryptic digestion of HDL fractions

The HDL fractions were digested using trypsin according to the following procedure: 1 mg of the ApoA-I fraction was dissolved in 1 mL of 50 mM NH4HCO3 buffer solution (pH 8.5) and 100 mL of trypsin solution (100 ng/mL) were added (substrate to enzyme ratio = 40:1 w/w). The final solutions were left to react at 37 °C overnight. The reaction was stopped by adding 50 mL of 10% trifluoroacetic acid in aqueous solution. The digestion mixture was desalted and purified with ZipTip-C18 pipette tips (Millipore, Bedford, USA), following the procedure described in the ZipTip user's guide.

MALDI/MS and characterization of Met112 and Met112-O containing peptides

MALDI/time of flight (TOF) and MALDI/TOF/TOF measurements were obtained using a MALDI/TOF/TOF UltrafleXtreme instrument (Bruker Daltonics, Bremen, Germany), equipped with a 1 kHz smartbeam II laser ($l = 355$ nm) and operating in the positive reflectron ion mode. The instrumental conditions were: IS1 = 25 kV; IS2 = 21.65 kV; reflectron potential = 26.3 kV; delay time Z 0 nsec. The matrix was a-cyano-4-hydroxycinnamic acid (HCCA) (saturated solution in H2O/acetonitrile [50:50 v/v] containing 0.1% TFA). Five mL of purified tryptic digest and 5 mL of matrix solution were mixed together, then 1 mL of the resulting mixture was deposited on the stainless steel sample holder and allowed to dry before placing it in the mass spectrometer. External mass calibration (Peptide Calibration Standard) was based on monoisotopic values of $[M+H^+]$ of angiotensin II, angiotensin I, substance P, bombesin, ACTH clip [1–17], ACTH clip [18–39], somatostatin 28 at m/z 1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714. TOF/TOF experiments were performed using the LIFT device under the following experimental conditions: IS1: 7.5 kV; IS2: 6.75 kV; Lift1: 19 kV; Lift2: 3.7 kV; Reflector1: 29.5 kV; delay time: 70 ns.

Determination of oxApoA-I was performed accordingly to procedures described and validated in our previously published paper [10]. Briefly, MS/MS experiments performed on the two ions at m/z 1283.6 and 2645.4 showed that the sequences of the corresponding peptides are W^{108}QEEM^{112}ELYR and V^{97}QPYLDDFQKKWQEEM^{112}ELYR, both of which contain the methionine residue in position 112 (Met112). The percentages of OxApoA-I were calculated dividing the sum of the relative abundances of the peaks at m/z 1299 and 2661—originating from oxidation of Met^{112} and differing from the above-described species by 16 Da—to the sum of the abundances of the four peaks of interest [10].

Statistical analysis

Continuous values are given as means ± standard deviations (SD). The comparison between continuous variables at baseline and after 1 year of treatment was calculated as the pre–post difference: negative delta values (Δ), therefore, indicate the actual decrease in the parameter depending on the treatment administered. The statistical significance of these differences (pre-post) in each treatment group was analyzed using Student's t test for paired data, after checking the

normality of the distribution of the parameters according to the skewness and kurtosis parameters, and after normalizing differences at the baseline.

The two treatment groups (pioglitazone vs glimepiride) at the baseline and after 1 year of treatment were compared with Student's *t* test for unpaired data.

For the nominal variables, the differences between the two groups (pioglitazone vs glimepiride) were estimated with Fisher's exact test. All differences were considered statistically significant with a p value < 0.05.

The possible associations between continuous variables was investigated by conducting linear correlation tests and ascertaining the Pearson coefficient. To prevent any interference of anthropometric and clinical characteristics in the associations between variables, a stepwise forward multiple regression analysis was performed, where appropriate.

For a more in-depth analysis of the variables of interest, the non-parametric multivariate method of principal component analysis (PCA) was also used. PCA enables the dimensions of the dataset to be reduced, thus helping to identify new, meaningful underlying variables. The reduced set contains what are called 'principal factors', i.e., linear combinations of the original variables. The first principal component accounts for as much of the variability in the data as possible, with each successive component accounting for the remaining variability. Biplot software was used as an Excel add-in. The first three components were considered for data classification purposes. A biplot graphic display was used to present the behavior of the variables to examine their correlation. The most useful variable is the cosine of the eigenvectors suggesting correlations between different variables. When the angle between eigenvectors nears 0°, the variables are positively correlated, while the angle for negative correlations approaches 180°, and angles of 90° indicate no correlation.

Results

The data for all 95 patients assessed at the baseline and after 1 year were considered in the statistical analysis. There were no dropouts from the study, and no serious adverse events (particularly cardiovascular events, revascularization procedures, tumors, fractures) were recorded in either treatment group.

Clinical characteristics

The patients randomly assigned to the two treatments were similar as regards the following variables at the baseline (Table 1): male/female ratio, duration of diabetes, smoking habits, blood pressure, body mass index (BMI) and waist circumference (WC), renal function (expressed as the calculated GFR), and ACR. The two groups were also comparable in terms of glycemic control (HbA1c = 7.7 ± 0.4 glimepiride vs 7.6 ± 0.4 pioglitazone), lipid profiles, markers of lipid oxidation, and there were no differences in their treatment with statins and/or fibrates, which did not change significantly during the study.

Table 1 Baseline anthropometric and clinical parameters of the two treatment groups. Continuous data are expressed as mean ± SD

	Glimepiride ($n = 47$)	Pioglitazone ($n = 48$)
Gender (male/female)	22/25	32/16
Age (year)	64.4 ± 6.6	64.0 ± 7.0
Smoking (yes/no)	8/39	7/41
Disease duration (year)	8.0 ± 5.0	8.0 ± 4.7
Statin/fibrates (yes/no)	25/22	27/20
BMI (kg/m^2)	29.4 ± 4.0	29.1 ± 4.1
HbA1c (%)	7.7 ± 0.4	7.6 ± 0.4
Apo A1 (mg/dL)	140.2 ± 23.8	134.3 ± 21.0
Apo A1ox (%)	3.7 ± 3.3	3.8 ± 2.6
AGE (μg/mL)	25.78 ± 12.48	27.42 ± 14.10
LDLox (U/L)	41.07 ± 8.00	41.94 ± 9.25

Differences between groups were assessed with Student's *t* test for continuous variables, and Fisher's exact test for nominal variables

Table 2 shows clinical and serum parameters in the two groups of patients, before and after treatment with the two drugs. For a more accurate evaluation of data, and to take into account patient's individual variability, the variations in the biochemical parameters have been evaluated as net differences between after-minus-before each respective treatment, as indicated in Table 3. Both treatments led to a significant improvement in HbA1c values ($\Delta_{gli} = -0.5 \pm 0.9\%$ vs $\Delta_{pio} = -0.2 \pm 0.9\%$; p between groups n.s.). Only patients treated with pioglitazone experienced an increase in HDL-C ($\Delta_{pio} = +5.7 \pm 8.2$ mg/dL, p pre/post < 0.001), with a non statistically significant reduction in TG and LDL-C. On the other hand, patients treated with glimepiride had a slight but statistically significant increase in LDL-C ($\Delta = +2.3 \pm 25.3$ mg/dL, p pre/post < 0.05).

Qualitative changes in lipoproteins and glyco-oxidation

Markers of glycol-oxidation—namely serum AGE, oxApoA-I, oxLDL—were not different at baseline among the two treatment groups (Table 1).

As summarized in Table 3, patients treated with pioglitazone showed a significant increase in ApoA-I concentrations ($\Delta_{pio} = +7.2 \pm 14.8$ mg/dL, p pre/post < 0.05), and a reduction in oxApoA-I ($\Delta_{pio} = -1.0 \pm 2.6\%$, p pre/post < 0.05). Treatment with glimepiride was associated with no significant changes in any of the above parameters. As for LDL, the

Table 2 Clinical and serum parameters in the two groups of patients, before (PRE) and after (POST) treatment with the two drugs (*SULF* glimepiride 2 mg, $n=47$; *PIO* pioglitazone 15 mg, $n=48$)

Parameter	PRE		POST	
	SULF	PIO	SULF	PIO
Gender (male/female)	22/25	32/16	–	–
Age (year)	64.4 ± 6.6	64.0 ± 7.0	–	–
Smoke (yes/no)	8/39	7/41	–	–
Statin use (yes/no)	25/22	27/20	24/23	28/19
BMI (kg/m^2)	29.4 ± 4.0	29.1 ± 4.1	29.8 ± 4.0	29.5 ± 4.7
HbA1c (%)	7.7 ± 0.4	7.6 ± 0.4	7.2 ± 0.8	7.4 ± 0.7
PAS (mmHg)	137.9 ± 12.3	132.2 ± 11.8*	135.4 ± 14.0	134.1 ± 17.3
PAD (mmHg)	81.5 ± 7.8	80.9 ± 7.3	78.9 ± 7.3	81.0 ± 8.3
CV (cm)	100.5 ± 9.6	100.9 ± 11.5	101.0 ± 9.8	99.7 ± 18.5
ACR (mg/g)	36.6 ± 143.2	39.7 ± 128.8	14.4 ± 25.6	48.9 ± 133.3
GFR (mL/min)	99.0 ± 25.4	104.6 ± 31.5	97.5 ± 26.0	99.0 ± 28.8
CT (mg/dL)	181.8 ± 33.7	178.9 ± 35.8	187.2 ± 38.3	182.6 ± 33.1
C-HDL (mg/dL)	48.6 ± 12.9	46.4 ± 11.0	49.2 ± 13.2	52.1 ± 13.3
C-LDL (mg/dL)	110.8 ± 29.2	110.2 ± 29.6	113.1 ± 34.9	108.5 ± 32.0
TG (mg/dL)	138.1 ± 63.8	136.8 ± 55.5	147.8 ± 66.6	124.5 ± 80.0
Apo A1 (mg/dL)	140.2 ± 23.8	134.3 ± 21.0	139.6 ± 22.4	141.5 ± 22.2
Apo A1ox (%)	3.7 ± 3.3	3.8 ± 2.6	3.4 ± 2.4	2.9 ± 2.7
LDL ox (U/L)	41.07 ± 8.00	41.94 ± 9.25	44.64 ± 10.62	44.00 ± 9.32
AGE (μg/mL)	25.78 ± 12.48	27.42 ± 14.10	25.62 ± 11.34	23.03 ± 11.08

Continuous data are expressed as mean ± SD

*$p<0.05$, SULF vs PIO, Student's t test for unpaired data. Differences for nominal variables was evaluated using Fisher's exact test; no significant difference was detected between the two groups

Table 3 Variations in clinical and serum parameters in the two groups of patients, calculated as after-minus-before treatment with the two drugs

Parameter	Glimepiride ($n=47$)	Pioglitazone ($n=48$)
BMI (kg/m^2)	0.4 ± 1.9	0.4 ± 1.2
HbA1c (%)	− 0.5 ± 0.9**	− 0.2 ± 0.8*
SBP (mmHg)	− 2.5 ± 15.4	2.0 ± 15.9
DBP (mmHg)	− 2.6 ± 9.7	0.1 ± 8.8
WC (cm)	0.5 ± 2.8	− 1.3 ± 15.5
ACR (mg/g)	− 23.5 ± 127.3	10.1 ± 89.8
GFR (mL/min)	− 1.4 ± 21.9	− 5.2 ± 16.8*
TC (mg/dL)	5.5 ± 26.5	3.7 ± 30.3
HDL-C (mg/dL)	0.6 ± 6.1	5.7 ± 8.2**
LDL-C (mg/dL)	2.3 ± 25.3*	− 1.8 ± 26.2
TG (mg/dL)	9.6 ± 45.6	− 12.3 ± 62.2
Apo A1 (mg/dL)	− 0.6 ± 13.7	7.2 ± 14.8*
oxApoA-I (%)	− 0.3 ± 2.2	− 1.0 ± 2.6*
oxLDL (U/L)	3.33 ± 9.23	2.31 ± 8.47
AGE (μg/mL)	− 0.33 ± 9.62	-4.62 ± 11.04*

Data are expressed as mean ± SD

**$p<0.001$; *$p<0.05$, Student's t test for paired data. A negative value indicates a decrease in the parameter following the treatment

concentration of oxLDL rose slightly, but not significantly in both groups.

The concentration of AGE was significantly lower after treatment with pioglitazone ($\Delta = -4.6 \pm 11.04$ mg/dL, p pre/post < 0.01), while there were no significant changes in the group treated with glimepiride.

Correlations between markers of lipoprotein oxidation, glyco-oxidation, clinical parameters and glycemic control

To investigate the association between changes in oxApoA-I, oxLDL and other clinical/ biochemical parameters, we first conducted a non-parametric multivariate analysis (PCA). This analysis was performed on all 95 patients enrolled and, in addition to disease duration, pre-post changes (Δ) in the following parameters were included as covariates: HDL-C, AGE, oxLDL, oxApoA-I and HbA1c.

The biplot obtained as a graphical representation of the PCA (Fig. 1) suggested a close association between ΔoxApoA-I and ΔAGE, while both of these parameters were unrelated to ΔHbA1c, ΔHDL-C or duration of disease. A more modest correlation was seen between ΔoxLDL and ΔAGE.

Based on the biplot, a linear regression analysis was run between ΔoxApoA-I and ΔAGE, considering the data for all patients (Fig. 2a), and this confirmed a statistically significant relation between said variables ($r=0.30$; $p=0.007$).

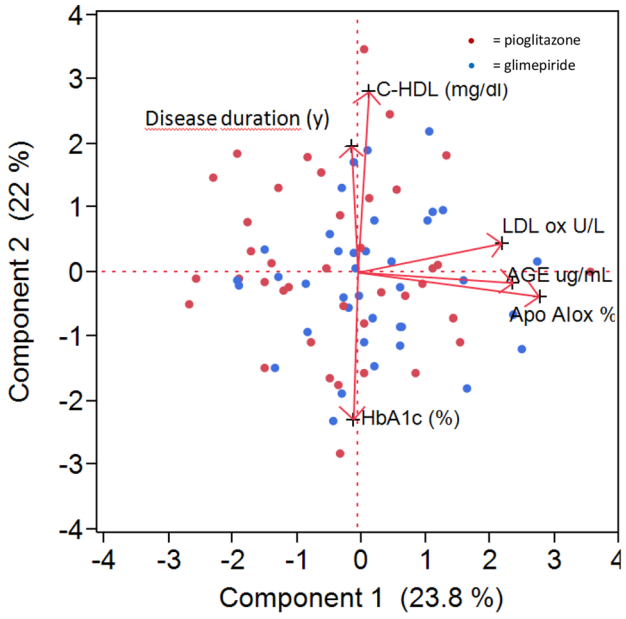

Fig. 1 Biplot obtained by PCA conducted on 95 patients with correlations between ΔoxApoA-I, ΔoxLDL, ΔAGE, ΔHDL-C, ΔHbA1c and disease duration

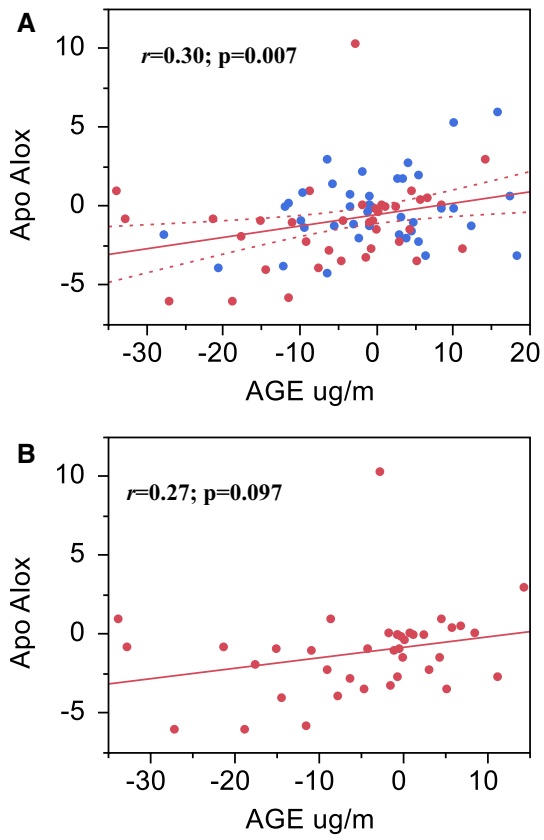

Fig. 2 Linear regression between ΔoxApoA-I and ΔAGE pre-post treatment in all patients (**a**), and in patients treated with pioglitazone (**b**). *AGE* advanced glycation end products, *ApoAIox* percentage of methionine sulfoxide of apolipoprotein A–I, *r* Pearson's coefficient

The sub-analysis conducted for 48 patients treated with pioglitazone (Fig. 2b) confirmed a slight correlation, that failed to reach statistical significance ($r = 0{:}27$; $p = 0.098$).

To exclude the possible influence of clinical parameters on the correlation between anthropometric ΔoxApoA-I and ΔAGE, a stepwise forward multivariate regression analysis was run with ΔoxApoA-I as the independent variable, while the other parameters (sex, duration of disease, use of statins and/or fibrates, ΔHDL-C, ΔBMI, ΔACR, ΔHbA1c, ΔAGE) were included as covariates. This analysis confirmed the significance of the correlation between ΔoxApoA-I and ΔAGE ($r = 0{:}31$; $p = 0.008$), while none of the parameters entered in the model were found to influence this correlation (Supplementary table S1).

Discussion

This study showed that long-term treatment with pioglitazone in addition to metformin is more effective than glimepiride in improving the qualitative characteristics of HDL in patients with DM2. In particular, pioglitazone significantly reduced HDL oxidation, expressed as relative abundance of peptides containing $Met^{112}O$ in ApoA-I (oxApoA-I).

It has been suggested that HDL function may be more important than its content in cholesterol (HDL-C), especially in pathological conditions—and DM2 in particular—on the grounds of in vitro studies demonstrating a lack of anti-inflammatory and antioxidant potential of HDL in such cases [7]. Clinical data concerning the assessment of HDL function parameters in patients with DM2 are still fragmentary, however, and their possible modulation by pharmacological strategies has only been thoroughly investigated using ex vivo techniques [27].

In a previous study by our group, oxApoA-I levels were significantly higher in non-diabetic subjects with premature CAD (< 55 years) than in healthy controls, but they were also higher in DM2 patients without CAD than in non-diabetics with premature CAD [10]. These findings suggest that an increased Apo-AI oxidation may be due to a decline in antioxidant capacity, typical of DM2 patients with long-standing disease.

Another interesting study found a higher percentage of oxApoA-I in patients with acute coronary syndrome [28]. In addition, oxApoA-I was associated with an increase in biochemical markers of thrombosis and vascular dysfunction [29], pointing to a pathogenic link between oxidative changes in HDL and CAD, while an in vitro study confirmed that MetO—measured in our study too—has a negative impact on reverse cholesterol transport [30].

Pioglitazone has been demonstrated positive CV outcomes in the long-term treatment of patients with DM2 [31], as recently confirmed for cerebrovascular events in

patients without diabetes [32]. Treatment with pioglitazone has also demonstrated positive effects on the lipid profiles, oxidative stress and inflammation of patients with DM2, irrespective of their glycemic control [33, 34], even at low dose administrations [35]. Moreover, treatment with pioglitazone has been shown to enhance cholesterol efflux capacity from macrophages, a relevant marker of atheroprotection, albeit representing a limited fraction of overall flux through the reverse cholesterol transport pathway [36].

Furthermore, treatment with pioglitazone results in a downregulation of the AGE-RAGE system in patients with DM2, probably due to the activation of antioxidant enzymes (especially glutathione peroxidase) as a consequence of PPAR-γ modulation [15].

In our study, oxApoA-I was found positively correlated with variations in serum AGE, which were significantly reduced by treatment with pioglitazone, but not with glimepiride. This correlation was uninfluenced by any of the main clinical and anthropometric parameters, suggesting that serum AGE may independently contribute to HDL oxidation.

AGE-mediated glycoxidation of HDL has been shown to decrease several key functions of the particle mainly through a marked reduction in paraoxonase (PON) enzymatic activity of ApoA-I, which has a pivotal role in preventing LDL oxidation [37]. Our data suggest that the reduction of serum AGEs with pioglitazone results in a qualitative improvement in HDLs, likely to be independent of the PON enzymatic system, as demonstrated by the absence of effect on oxLDL levels. On the other hand, the observed reduction of oxApoA-I may have repercussions on HDL reverse cholesterol transport, although we did not confirm this hypothesis by an in vitro functional test.

As further suggestion of the specific role of pioglitazone on HDL metabolism, we saw no significant changes in oxLDL levels during treatment with pioglitazone in our study. The hypothesis that this specificity may involve AGE is reinforced by an in vivo study conducted by Passarelli et al., which demonstrated that glyoxal (one of the most common AGE) has a negative effect on cholesterol uptake by HDL in monocytes, while not affecting LDL metabolism [17]. On the other hand, since LDL have a shorter half-life and a faster turnover, oxLDL may be a less reliable marker of systemic oxidative stress in patients with DM2.

This study has some methodological limitations. First, the sample size was too small to demonstrate a statistically significant correlation between changes in oxApoA-I and AGE in the group treated with pioglitazone (though this was not the primary endpoint of this trial). Second, though mass spectrometry with MALDI/TOF/TOF is accurate, highly sensitive and specific, this method is currently not suitable for use in routine clinical assessments.

On the other hand, this was the first RCT to examine the effect of pioglitazone, as compared with a sulfonylurea, on HDL and LDL oxidation in patients with DM2. The correlation between oxApoA-I and serum AGE, now confirmed in a clinical study, also offers ways to further investigate the pathophysiological link between the glycation and oxidation of lipoproteins in diabetic patients.

In conclusion, the present study extends the evidence of a positive effect of pioglitazone on the qualitative features of HDL. OxApoA-I may represent an interesting biomarker of HDL functionality in patients with DM2, since the putative link between HDL oxidation and impaired reverse cholesterol transport could become increasingly important in the near future, although it needs to be confirmed by further studies.

Author contributions NCC wrote the manuscript. GS contributed to study design. NCC, SB, GS researched and analyzed data. AL edited and reviewed manuscript. OV contributed to the discussion. ER contributed to data access and statistical support. RS, RM, MR, CC contributed to data collection and performed the relevant laboratory measurements. All authors read and approved the final version to be published.

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Compliance with ethical standards

Conflict of interest The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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