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Synergistic effects of serum albumin and HDL cholesterol concentrations on serum oxidized LDL cholesterol concentration in obese individuals with normal glycoregulation and patients with type 2 diabetes mellitus

Sinergistički efekti koncentracije serumskog albumina i HDL holesterola na koncentraciju oksidovanog LDL holesterola u serumu gojaznih osoba sa normalnom glikoregulacijom i obolelih od dijabetesa melitusa tipa 2

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Abstract

Background/Aim. Serum albumin and high-density lipoprotein (HDL) cholesterol molecules have multiple physiological functions, including an antioxidant role in neutralizing the harmful effects of oxidized low-density lipoprotein (oxLDL). In obese individuals, albumin and HDL cholesterol molecules are unable to counteract the unfavorable effects of oxLDL cholesterol adequately. The aim of the study was to examine the functional relationships between oxLDL cholesterol, HDL cholesterol, and serum albumin. **Methods.** The study included 30 obese individuals with newly diagnosed type 2 diabetes mellitus (before and after a three-month treatment with metformin), 30 obese individuals with normal glucose tolerance, and 30 normal-weight subjects (control group). The groups were age- and sex-matched. **Results.** Both qualitative and quantitative changes in the levels of HDL cholesterol and albumin were detected among the groups. Statistically significant changes were found in the linear correlations between albumin and ox-LDL cholesterol among the study groups. Furthermore, by forming a synergistic influence of independent variables (HDL cholesterol and albumin), expressed through a complex polynomial of the dependent variable (oxLDL) of the quadratic type, statistically significant qualitative and quantitative changes in maximal oxLDL values were observed in all examined groups. **Conclusion**. The results of our study indicate a potential synergistic effect of albumin and HDL cholesterol in the prevention of oxidative damage, as well as a possible alteration in the quality of the ratio of these parameters in relation to oxLDL cholesterol molecules under conditions characterized by increased oxidative stress.

Key words:
albumins; diabetes mellitus, type 2; lipoproteins, hdl; lipoproteins, ldl; obesity; oxidative stress.

Apstrakt

Uvod/Cilj. Serumski albumin i molekuli *high-density lipoprotein* (HDL) holesterola imaju višestruke fiziološke funkcije, uključujući antioksidativnu ulogu u neutralisanju štetnih efekata oksidovanog lipoproteina niske gustine (*oxidized low-density lipoprotein* – oxLDL). Kod gojaznih osoba, molekuli albumina i HDL holesterola nisu u stanju da se adekvatno suprotstave nepovoljnim efektima oxLDL holesterola. Cilj rada bio je da se ispitaju funkcionalni odnosi između oxLDL holesterola, HDL holesterola i albumina u serumu. **Metode.** Studijom je obuhvaćeno 30 gojaznih osoba sa novodijagnostikovanim dijabetesom melitusom tipa 2 (pre i posle tromesečnog lečenja metforminom), 30 gojaznih osoba sa normalnom tolerancijom glukoze i 30 ispitanika normalne težine (kontrolna grupa). Grupe su bile podudarne po starosti i polu. **Rezultati**. Utvrđene su kvalitativne i kvantitativne razlike u nivoima HDL holesterola i albumina među grupama. Pokazane su statistički značajne promene u linearnim korelacijama između albumina i oxLDL holesterola među ispitivanim grupama. Takođe, formiranjem sinergističkog uticaja nezavisnih varijabli (HDL holesterola i albumina), izraženih kroz složeni polinom zavisne varijable (oxLDL) kvadratnog tipa, uočene su statistički značajne kvalitativne i kvantitativne

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promene u maksimalnim vrednostima oxLDL u svim ispitivanim grupama **Zaključak.** Rezultati našeg istraživanja ukazuju na mogući sinergistički efekat albumina i HDL holesterola u prevenciji oksidativnog oštećenja, kao i na mogućnost promene kvaliteta odnosa tih parametara u odnosu na molekule oxLDL holesterola u uslovima koje karakteriše povećan oksidativni stres.

Ključne reči:
albumini; dijabetes mellitus, insulin nezavisni; lipoproteini, hdl holesterol; lipoproteini, ldl holesterol; gojaznost; stres, oksidativni.

Introduction

Serum albumin has multiple physiological functions, including an antioxidant role in plasma and extracellular fluid. Conditions characterized by increased oxidative stress (OS) can reduce the capacity of albumin, resulting in modification of its structure. Increased levels of glycated albumin in diabetes mellitus (DM) may contribute to the development of chronic complications [1].

A decrease in high-density lipoprotein (HDL) cholesterol levels can be observed in different inflammatory disorders. The cause of this phenomenon is most likely the oxidative modification of HDL cholesterol during OS, with a consequent structural change of its molecule [2]. Although there is evidence of the existence of "dysfunctional HDL cholesterol", biomarkers for its monitoring in routine clinical practice have not yet been fully established [3, 4]. Both molecules participate in the reduction of oxidative and inflammatory damage by oxidized low-density lipoprotein (oxLDL) particles [5], which is well documented, but to our knowledge, their joint action has not been described in the previous literature.

This study aims to examine the functional relationships between oxLDL, HDL cholesterol, and serum albumin in obese individuals with newly diagnosed type 2 DM (T2DM) (before and after a three-month treatment with metformin), obese individuals with normal glucose tolerance (NGT), and normal-weight (NW) subjects.

Methods

Participants

The study was conducted at the Clinic for Endocrinology, Diabetes, and Metabolic Disorders of the University Clinical Center of Vojvodina, Serbia, and included 30 obese individuals with newly diagnosed T2DM before treatment (T2DMBT) and after a three-month treatment with metformin (T2DMAT) [mean values (MV) and standard deviation (SD) of body mass index (BMI) at the baseline was 34.41 ± 4.68 kg/m^2], 30 obese individuals with NGT (group ONGT) (37.37 ± 6.11 kg/m^2), and 30 NW subjects (control group) (23.34 ± 3.12 kg/m^2). The groups were age- and sex-matched. A detailed medical history was taken from all patients, and a physical examination and other detailed laboratory analyses were performed.

Inclusion criteria for all study groups except the NW group were as follows: a BMI above 30 kg/m^2; patients who were not suffering from diseases that could influence the oxidative status (liver and kidney diseases, arterial hypertension, hyperlipidemia, etc.); non-smokers; patients who were not taking vitamin supplements or drugs with established effects on OS (statins, fibrates, angiotensin-converting enzyme inhibitors, calcium channel blockers, etc.). Exclusion criteria for all groups were the following: patients suffering from diseases that could influence the oxidative status (liver and kidney diseases, arterial hypertension, hyperlipidemia, etc.); smokers; patients taking vitamin supplements or drugs with established effects on OS (statins, fibrates, angiotensin-converting enzyme inhibitors, calcium channel blockers, etc.). T2DM patients who took therapy irregularly, occasionally smoked, or took drugs that affect the oxidative status of the organism were excluded from the study.

In all groups without T2DM, glucose levels were measured both fasting and 2 hrs after starting the oral glucose tolerance test, along with hemoglobin A1c (HbA1c) levels. Obese patients were included in the study based on their BMI values, while T2DM patients were included according to the American Diabetes Association criteria for the diagnosis of DM. Ethical approval for this study was granted by the Ethics Committee of the University Clinical Center of Vojvodina (approval No. 00-08-10).

Biochemical analysis

The HDL cholesterol levels were measured using a direct enzymatic colorimetric test with the Ultra HDL reagent kit (Abbot, USA) on the automated Architect ci4100 analyzer (Abbot, USA). Cholesterol concentration in oxLDL particles was determined manually using the oxLDL/MDA Adduct enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Germany), following the ELISA method with an RT-2100C microplate reader and RT-2600C microplate washer (Rayto, China). HbA1c levels were determined using the latex agglutination-inhibition method with the HbA1c reagent kit (Abbot, USA), also on the automated Architect ci4100 analyzer (Abbot, USA). Serum albumin levels were determined during serum protein electrophoresis using the spectrophotometric bromine cresol green method on the Siemens Advia 1800 chemistry analyzer.

Statistical analysis

The basic parameters of MV and SD were calculated, and a distribution was established for each statistical group, verified using the Chi-squared test (Table 1). For the parameter HbA1c, the same procedure was applied: MV and SD were calculated, and the distri-bution was verified using the Chi-squared test (Table 2). Statistical analysis included parameter sets and corresponding tests of dispersive analysis using analysis of variance (ANOVA). A linear correlation

test was performed using Pearson's product-moment correlation coefficient (Tables 3 and 4). For the analytical expression of the relationship between parameters in obese individuals with T2DM before and after metformin treatment, regression analysis was applied. Changes in the values of correlation coefficients indicate the effects of metformin therapy. The significance of these changes was verified by Pearson's correlation coefficient test (Table 4).

Table 1

The basic parametric and non-parametric characteristics with ANOVA of HDL cholesterol, albumin, and oxLDL

Parameter/group	Mean value (ANOVA test)	Standard deviation	Distribution	*p*-values (Chi-square test)
HDL cholesterol (mmol/L)				
NW	1.5109[A]	0.3077	normal	0.4258
ONGT	1.1400[B]	0.2186	log-normal	0.1205
T2DMBT	**1.0475[C]**	0.2252	log-normal	0.3117
T2DMAT	**1.0669[C]**	0.2180	log-normal	0.1225
Albumin (g/L)				
NW	**47.6967[D]**	2.3946	uniform	0.6238
ONGT	**45.8540[D]**	3.3474	log-normal	0.0920
T2DMBT	45.4593[E]	2.8407	log-normal	0.2133
T2DMAT	**46.0807[D]**	3.0443	uniform	0.2933
oxLDL (ng/mL)				
NW	57.4194[F]	59.9692	exponential	0.1101
ONGT	111.7000[G]	75.9165	exponential	0.0689
T2DMBT	183.4594[H]	227.5207	exponential	0.1034
T2DMAT	129.6192[I]	117.1498	exponential	0.0708

ANOVA – analysis of variance; HDL – high-density lipoprotein; oxLDL – oxidized low-density lipoprotein; NW – normal-weight individuals; ONGT – obese individuals with normal glucose tolerance; T2DMBT – obese individuals with newly diagnosed type 2 diabetes mellitus (T2DM) before metformin treatment initiation; T2DMAT – obese individuals with newly diagnosed T2DM after a three-month metformin treatment.

***Note*: Between the values marked with identical capital letters, there is no significant difference according to the ANOVA test for the significance threshold of $p < 0.05$. There are no significant differences between the T2DMBT and T2DMAT groups in HDL values (marked with the capital letter C), while other differences are significant. There are no significant differences in the NW, ONGT, and T2DMAT groups in serum albumin values (marked with the capital letter D), while other differences are significant. All oxLDL values are significantly different between study groups.**

Table 2

The basic parametric and non-parametric characteristics of hemoglobin A1c (HbA1c)

Groups	Mean value of HbA1c (%) (ANOVA test)	Standard deviation	Distribution	*p*-values (Chi-square test)
NW	**5.1967[A]**	0.296439	normal	0.4493
ONGT	**5.5200[A]**	0.334781	normal	0.2102
T2DMBT	8.2696[B]	2.186131	normal	0.0526
T2DMAT	7.4176[C]	1.509281	normal	0.0382

For abbreviations, see Table 1.

***Note*: Between the values marked with identical capital letters, there is no significant difference according to the ANOVA test for the significance threshold of $p < 0.05$. There are no significant differences between the NW and ONGT groups in HbA1c values (marked with the capital letter A), while other differences are significant.**

Table 3

Correlations between HDL cholesterol and oxLDL with Pearson tests

		HDL cholesterol			
		NW	ONGT	T2DMBT	T2DMAT
oxLDL	NW	***r* = +0.2900**[Fig 1A]	$p = 0.1394$	$p = 0.0905$	$p = 0.1235$
	ONGT		***r* = +0.0037**[Fig 1B]	$p = 0.4115$	$p = 0.4518$
	T2DMBT			***r* = -0.0628**[Fig 1C]	$p = 0.4573$
	T2DMAT				***r* = -0.0308**[Fig 1D]

Fig – figure. For other abbreviations, see Table 1.

***Note*: On the diagonal of Table 3, the values of linear correlations (marked with the letter *r*) of HDL and oxLDL (bold and underlined values) are given. The correlation graphs are shown in Figure 1 (A for the NW group, B for the ONGT group, C for the T2DMBT group, D for the T2DMATx group). Outside the diagonal are the values of the results of the Pearson correlation test (*p*-value) differences are significant for $p < 0.05$. There are no significant differences between correlations.**

Table 4

Correlations between serum albumin and oxLDL with Pearson tests

		Serum albumin			
		NW	ONGT	T2DMBT	T2DMAT
oxLDL	NW	***r* = -0.1840**$_{\text{Fig 2B}}$	p = 0.0642	***p = 0.0186***	p = 0.3122
	ONGT		***r* = +0.2310**$_{\text{Fig 2B}}$	p = 0.2978	***p = 0.0281***
	T2DMBT			***r* = +0.3656**$_{\text{Fig 2C}}$	***p = 0.0073***
	T2DMAT				***r* = -0.3096**$_{\text{Fig 2D}}$

Fig – figure. For other abbreviations, see Table 1.
***Note*: On the diagonal of Table 4, the values of linear correlations (marked with the letter *r*) of albumin and oxLDL (bold and underlined values) are given. Correlation graphs are shown in Figure 2 (A for NW, B for ONGT, C for T2DMBT, D for T2DMAT). Outside the diagonal are the values of the results of the Pearson correlation test (*p*-value); differences are significant for $p < 0.05$, italic bold values.**

Based on empirical data, theoretical two-dimensional approximation functions were established, with HDL cholesterol and albumin as independent variables and oxLDL as the dependent variable. The reliability of the approximation was established by correlation. Based on the application of double integrals, the volume of the dependent variable defined by approximate functions was calculated over the domain D, representing the real intervals of the empirical independent variables, as well as over the maximum domain D_{max}. This double integral model was applied to investigate the synergistic effect of HDL cholesterol and albumin on oxLDL. The significance was set at 0.05 for all parametric and non-parametric verifications, and at 0.10 for the Pearson test. The results were calculated using classical mathematical analysis methods, specifically double integrals, without reliance on commercial software.

Results

The basic parametric and non-parametric characteristics, along with ANOVA test results for HDL cholesterol, albumin, and oxLDL, are presented in Table 1, and for HbA1c in Table 2. Table 1 illustrates the distribution dynamics and differences in MVs of the examined parameters. The HDL cholesterol group primarily followed a log-normal distribution, with the lowest levels observed in newly diagnosed T2DM patients and those undergoing treatment. Serum albumin levels tended toward a uniform distribution, with the lowest values found in newly diagnosed T2DM patients. Quantitative statistical significance was given between MVs, precisely indicated by different superscript capital letters. Values marked with the same capital letter showed no statistically significant difference (ANOVA test and significance threshold set at $p = 0.05$ were emphasized). Qualitative differences between study groups were expressed in the types of distributions. The significance threshold was set at $p = 0.05$, and all distributions were verified using the Chi-squared test, with confirmed verification in all cases ($p > 0.05$). If different verified distributions were identified among groups, a significant qualitative difference was established. Qualitative changes in distribution types were observed for HDL cholesterol (Normal and Log-normal) and serum albumin [Even (Uniform) and Log-normal] between groups, whereas no qualitative changes were found in oxLDL distributions (exponential distribution of all groups) (Table 1). For the parameter HbA1c, quantitative differences were observed between groups, but there were no qualitative changes as all cluster distributions were verified as normal ($p > 0.05$) (Table 2).

The linear correlation coefficients between HDL cholesterol and oxLDL across the different studied groups are presented in Table 3 and Figure 1. It can be seen that there was a change in the direction of the linear regression, especially in individuals with newly diagnosed T2DM before metformin treatment initiation. At a significance threshold of $p = 0.10$, the Pearson test revealed a statistically significant change in correlation between the T2DMBT group and the NW group. Table 3 displays the correlation coefficients along the main diagonal, representing relationships within the same group, while the values symmetrical to the diagonal represent the Pearson test results indicating changes in correlations between groups. These findings are illustrated graphically, with group indices as follows: 01 – NW; 02 – obese normal glucose tolerance; 03 – T2DM before therapy; 04 – T2DM during metformin therapy.

The linear correlation coefficients between serum albumin and oxLDL in the different studied groups are given in Table 4 and Figure 2. Figure 2 shows the exceptional correlation dynamics in serum albumin. From the NW group to ONGT and the T2DMBT, there was a significant positive change in the angle of linear regression, which returned to the initial value after therapy with metformin. Changes in correlation coefficients were significant. In Table 4, the main diagonal displays correlation coefficients within each group, while the other values represent Pearson test results that indicate the dynamics of changes between groups. The indices on the graphs are highlighted (01 – NW, 02 – obese normal glucose tolerance, 03 – T2DM before therapy, 04 – T2DM during metformin therapy).

Differences in parametric and non-parametric characteristics (Table 1), as well as variations in correlations (Tables 3 and 4), provided the foundation for further investigation.

In this research, a two-dimensional function was applied, with oxLDL as the dependent variable and HDL cholesterol and serum albumin as independent variables. Figures 3–6 illustrate both empirical data and approximated theoretical distributions of the two-dimensional functions, with oxLDL as the dependent variable and HDL cholesterol and alb-

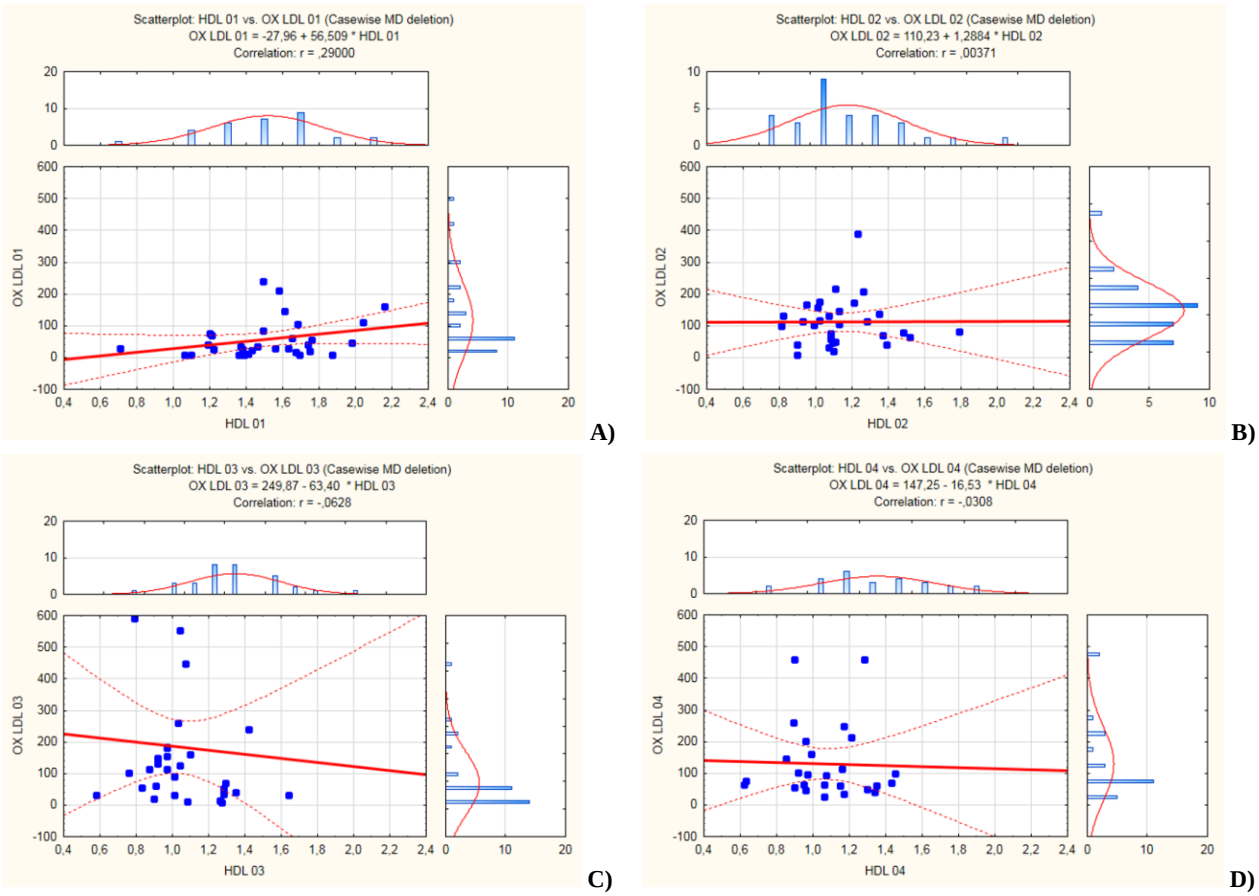


Fig. 1 – The linear correlations of HDL cholesterol and oxLDL in the examined groups [A – 01 – normal weight, B – 02 – obese normal glucose tolerance, C – 03 – type 2 diabetes mellitus (T2DM) before therapy, C – 04 – T2DM during metformin therapy].
MD – missed data. For other abbreviations, see Table 1.
***Note*: Changes in the slope (coefficient) of the linear regression express the dynamics of the HDL and oxLDL relationship between the groups. The changes in the correlations of these groups were not statistically significant, with a note that they approach a statistically significant difference, especially in the T2DM group before therapy and the control group ($p = 0.0905$).**

umin as independent variables. Approximate functions, where x stands for HDL cholesterol and y for serum albumin, are shown in all the Figures. In the theoretical function, the independent empirical variables (HDL cholesterol as variable x and serum albumin as variable y) are analytically expressed. The theoretical oxLDL data were obtained when the empirical values of HDL cholesterol (as variable x) and serum albumin (as variable y) were included in the theoretical function. The theoretical distribution function of oxLDL for each group is given in Figures 3–6. Based on empirical data, it was not possible to perform an analysis of the migration of oxLDL maximum between groups. That is why it was necessary to approximate empirical data with theoretical data, i.e., with a function that can be applied as in an integral calculation.

In the NW group, there was no pronounced maximum of oxLDL values, with a high agreement between empirical and theoretical data. In the remaining groups, there is an obvious migration of the maximum levels of oxLDL (highlighted in red on the abscissas of the independent variables HDL and serum albumin in Figures 4–6).

In the NW group, the correlation between empirical and theoretical data was $r = +0.4551$, while in the ONGT group, the correlation was $r = +0.4241$. The maximum oxLDL levels were observed at higher values of HDL cholesterol, $\in[1.2, 1.8]$, and higher values of serum albumin, $\in[46, 52]$. In this zone, the oxLDL MV was 129.32, and in the complementary zone, it was 97.51. A statistically significant difference between these values was established using the ANOVA test ($p = 0.0193$).

In the T2DMBT group, the correlation between empirical and theoretical data was $r = +0.5291$. The maximum oxLDL levels were observed at lower values of HDL$\in[0.8, 1.2]$, and higher values of serum albumin$\in[46, 52]$. In this zone, the oxLDL MV was 302.56 and 182.62 in the complementary zone. A statistically significant difference between the stated values was established by the ANOVA test ($p = 0.0439$).

In the T2DMAT group, the correlation between empirical and theoretical data was $r = +0.5193$. The maximum oxLDL levels were found at higher values of HDL$\in[1.2, 1.8]$, and lower values of serum albumin$\in[38,44]$. In this zone, the

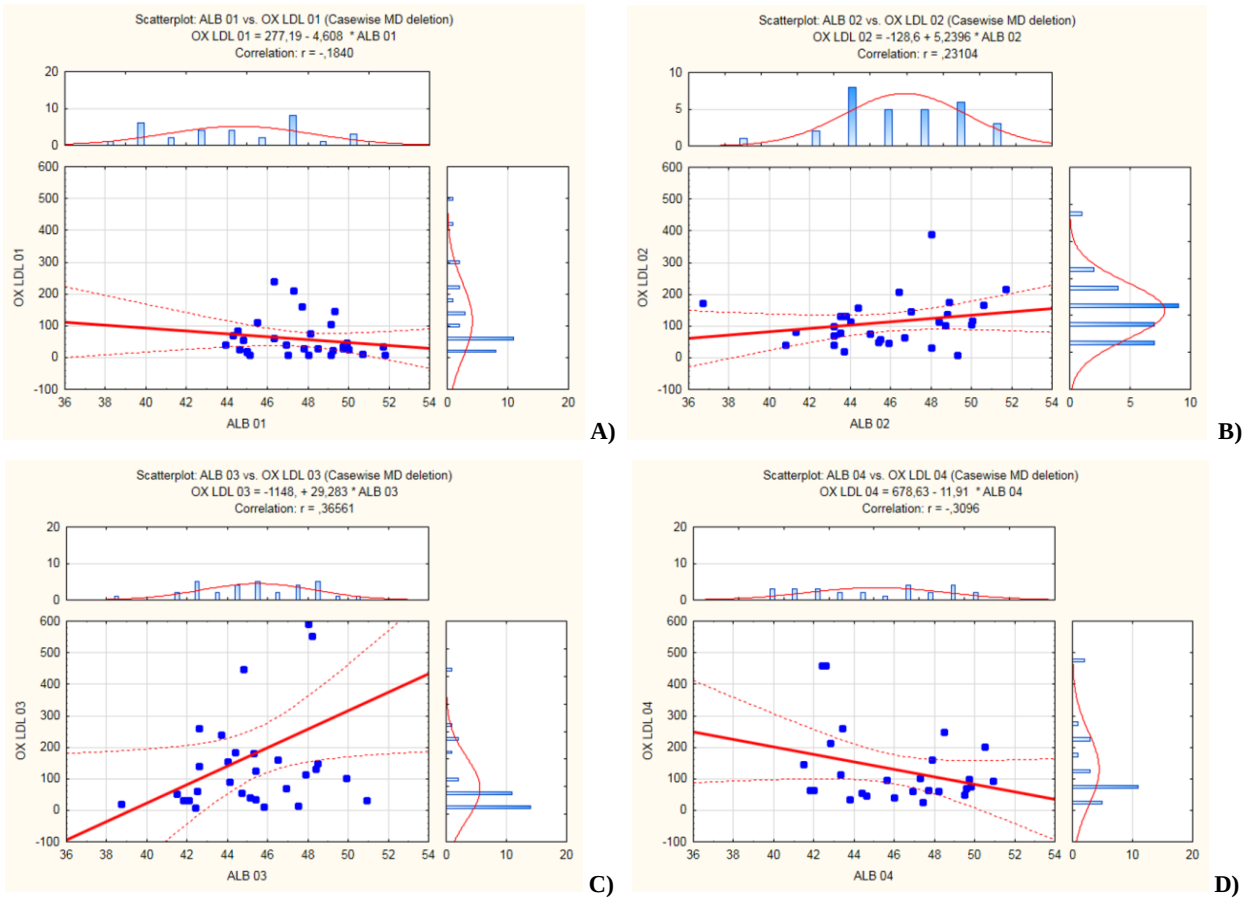


Fig. 2 – The linear correlations of albumin and oxLDL in the examined groups [A – 01 – normal weight, B – 02 – obese normal glucose tolerance, C – 03 – type 2 diabetes mellitus (T2DM) before therapy, C – 04 – T2DM during metformin therapy].
MD – missed data. For other abbreviations, see Table 1.

***Note*: Changes in the slope (coefficient) of the linear regression express the dynamics of the serum albumin and oxLDL relationship between the groups. Metformin therapy reduces the relationship between these two parameters to a relationship that is statistically in agreement with the control group. Changes in correlations were significant. Correlations of obese patients without diabetes and T2DM patients before therapy are significantly different compared to the control group and T2DM patients during metformin therapy (see Table 4).**

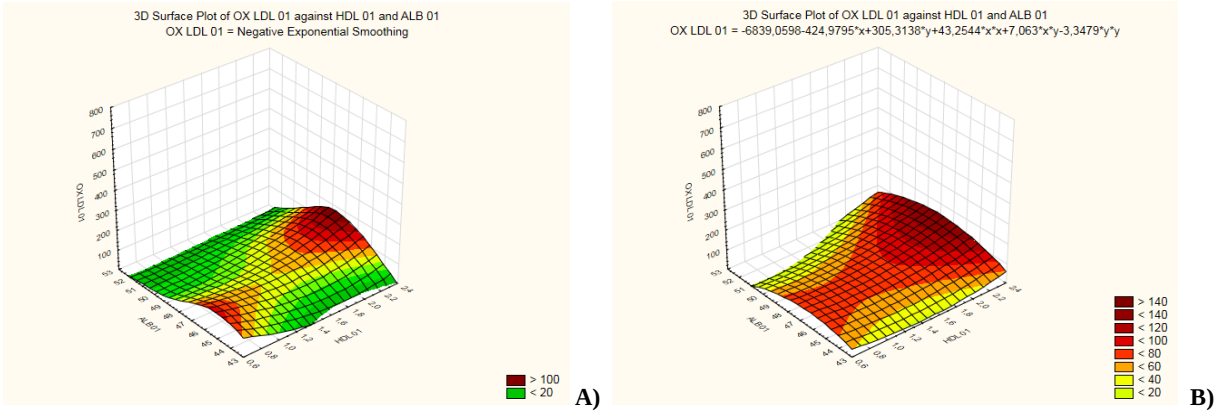
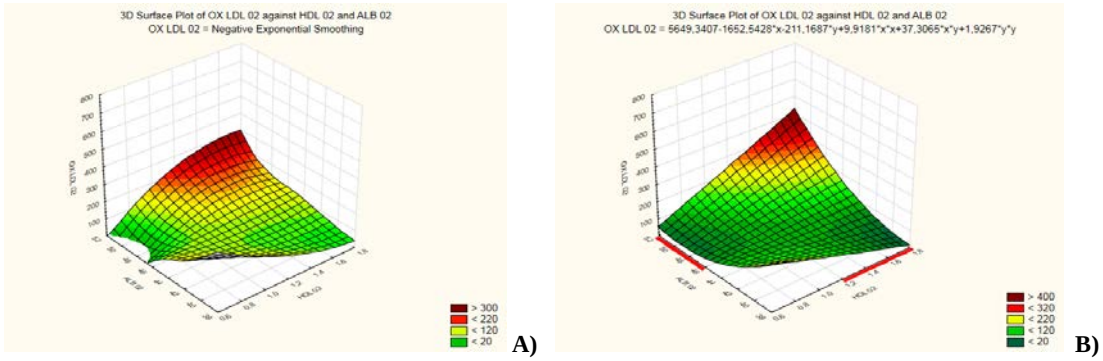


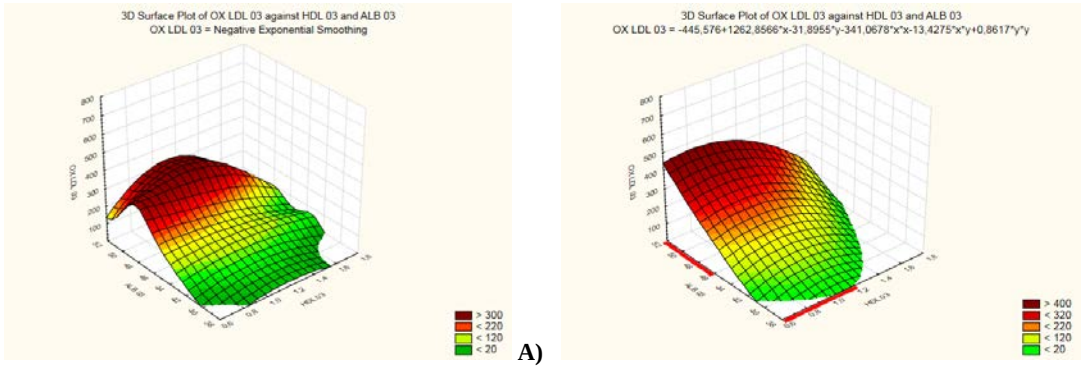
Fig. 3 – Distribution of A) empirical and B) theoretical data in the group of normal weight subjects, two-dimensional function of oxLDL as dependent variable and HDL cholesterol and albumin as independent variables.
ALB – albumin. For other abbreviations, see Table 1.



A) B)

Fig. 4 – Distribution of A) empirical and B) theoretical data in the group of obese individuals with NGT, two-dimensional function of oxLDL as dependent variable (HDL cholesterol and albumin as independent variables). ALB – albumin; NGT – normal glucose tolerance. For other abbreviations, see Table 1.

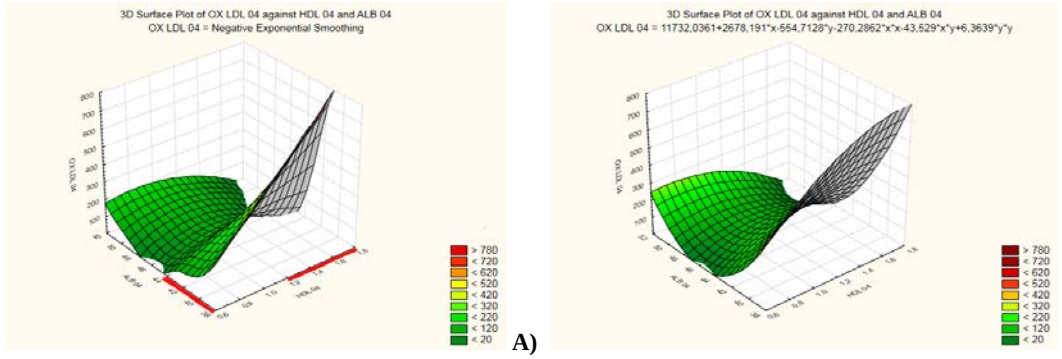
***Note*: The P_{max} area (maximum oxLDL values) in the ONGT group was found at high HDL cholesterol and high albumin values (the red line on the ordinates indicates the maximum oxLDL values). The volume under the graph of the function and above the surface P_{max} is 2.30 times greater than the volume over the complementary domain in which the oxLDL values are lower (see Table 5).**



A) B)

Fig. 5 – Distribution of A) empirical and B) theoretical data in the group of obese individuals with T2DM prior to metformin treatment initiation, two-dimensional function of oxLDL as dependent variable (HDL cholesterol and albumin as independent variables). ALB – albumin. For other abbreviations, see Table 1.

***Note*: The P_{max} area (maximum oxLDL values) in the T2DMBT group is found at low HDL cholesterol values and high albumin values (the red line on the ordinates indicates the maximum oxLDL values). The volume under the graph of the function and above the surface P_{max} is 3.49 times greater than the volume over the complementary domain in which the oxLDL values are lower (see Table 5).**



A) B)

Fig. 6 – Distribution of A) empirical and B) theoretical data in the group of obese individuals with T2DM after a three-month metformin treatment, two-dimensional function of oxLDL as dependent variable (HDL cholesterol and albumin as independent variables). ALB – albumin. For other abbreviations, see Table 1.

***Note*: The P_{max} area (maximum ox LDL values) in the T2DMAT group is found at high HDL cholesterol values and low albumin values (the red line on the ordinates indicates the maximum oxLDL values). The volume between the plot of the function and surface P_{max} is 3.19 times greater than the volume over the complementary domain where the oxLDL values are lower (see Table 5). Observe that the applied metformin therapy completely inverted the maximum values of oxLDL in relation to the values of HDL cholesterol (low values before the therapy and high values after the therapy), as well as serum albumin (high values before the therapy and low values after the therapy with metformin).**

oxLDL MV was 336.85, and in the complementary zone, it was 112.35. A statistically significant difference between the stated values was established using the ANOVA test ($p = 0.0065$).

This procedure proved the qualitative characteristic of oxLDL peak migration as a function of HDL cholesterol and serum albumin. This two-dimensional dependence can therefore be declared as synergistic.

Further on, we can calculate the quantity of this same relationship with double integrals, calculating the volume under the theoretical distribution of the two-dimensional function dependent variable oxLDL from independent variables HDL cholesterol and serum albumin. All volumes are calculated for the total domain D, which is standardized for all groups: HDL∈[0.6, 1.8] and low values of albumin∈[38,52]. The maximum domain is already specified for each group individually.

For the NW group (with no pronounced maximum of oxLDL), the total volume under the two-dimensional function was:

$$D_{NWH} = \int_{0,6}^{1.8}\int_{38}^{52}(-6{,}830.05 - 424.97x + 305.31y + 43.25x^2 + 7.06xy - 3.34y^2)dxdy = 528.58$$

For the ONGT group, the total volume under the two-dimensional function and declared maximum (red highlight on abscissas) was:

$$D_{ONGT} = \int_{0,6}^{1.8}\int_{38}^{52}(5{,}649.34 - 1{,}652.54x - 211.16y + 9.91x^2 + 37.30xy + 1.92y^2)dxdy = 1{,}900.27$$

$$D^{max}_{ONGT} = \int_{1,2}^{1.8}\int_{46}^{52}(5{,}649.34 - 1{,}652.54x - 211.16y + 9.91x^2 + 37.30xy + 1.92y^2)dxdy = 732.65$$

For the T2DMBT group, the total volume under the two-dimensional function and the maximum (red highlight on abscissas) was:

$$D_{T2DMBT} = \int_{0.6}^{1.8}\int_{38}^{52}(-445.57 + 1{,}262.85x - 3.89y - 341.06x^2 - 13.42xy + 0.8617y^2)dxdy = 14{,}878.77$$

$$D^{max}_{T2DMBT} = \int_{0.6}^{1.2}\int_{46}^{52}(-445.57 + 1{,}262.85x - 3.89y - 341.06x^2 - 13.42xy + 0.8617y^2)dxdy = 7{,}261.26$$

For the T2DMAT group, the total volume under the two-dimensional function and the maximum (red highlight on abscissas) was:

$$D_{T2DMAT} = \int_{0.6}^{1.8}\int_{38}^{52}(11{,}732.03 + 2{,}678.19x - 554.71y - 270.28x^2 - 43.52xy + 6.36y^2)dxdy = 3{,}277.19$$

$$D^{max}_{T2DMAT} = \int_{1,2}^{1.8}\int_{38}^{44}(11{,}732.03x - 554.71y - 270.28x^2 - 43.52xy + 6.36y^2)dxdy = 1{,}525.41$$

The total area of the domain D in all groups is equal to P = (1.8–0.6) × (52–38) = 16.8. The area of the maximum oxLDL in the groups ONGT, T2DMBT, and T2DMAT is standardized and equal to $P_{max} = 3.6$, as shown in Figures 4, 5, and 6, where the red-marked zones on the HDL cholesterol and serum albumin axes indicate the regions of maximum intensity. From the previous results, we can introduce the resultant of the intensity of the synergic effect as a quotient of the total volumes of oxLDL levels, which we denote by D on the reference surface P, i.e., $\rho = D/P$ (Table 5). The interpretation of this value is somewhat analogous to the MVs (129.32, 302.56, and 336.85, respectively, previous ANOVA tests). The basic problem of dimensional alignment is conditioned by the synergistic effect in complex metabolic relationships (HDL cholesterol expressed with mmol/L, serum albumin expressed with g/L, and oxLDL expressed with ng/mL). Nevertheless, the numerical expression of the synergistic effect is obvious – the quotient of these relations for each group and relation to the group with the highest intensity (T2DMBT group) is:

$\rho_{NW} = 528.58/16.8 = 31.46$ ($R_{NW} = 28.14$)
$\rho_{ONGT} = 1{,}900.27/16.8 = 113.11$ ($R_{ONGT} = 7.82$)
$\rho_{T2DMBT} = 14{,}878.77/16.8 = 885.64$ ($R_{T2DMBT} = 1.00$)
$\rho_{T2DMAT} = 3{,}277.19/16.8 = 195.07$ ($R_{T2DMAT} = 4.54$)

The T2DMBT group had an R_{NW} 28.14 times higher intensity of synergistic effect than the NW group, R_{ONGT} had 7.82 times higher intensity of synergistic effect than the ONGT group, and R_{T2DMAT} had 4.54 times higher intensity of synergistic effect than the T2DMAT group.

Table 5

Calculation of the ratios of the domain of the maximum and the complementary domain in groups

Groups	D_{max}	D	P_{max}	$D–D_{max}$	$P–P_{max}$	ρ_{max}	ρ_{com}	ρ_{max}/ρ_{com}
ONGT	732.65	1,900.27	3.6	1,167.62	13.2	203.5139	88.45606	2.30
T2DMBT	7,261.26	14,878.77	3.6	7,617.51	13.2	2017.017	577.0841	3.49
T2DMAT	1,525.41	3,277.19	3.6	1,751.78	13.2	423.725	132.7106	3.19

D – the volume of the double integral over the entire surface domain; D_{max} – the volume of the double integral on the domain of maximal oxLDL values; P_{max} area of the domain of maximum (red highlight area); $\rho_{max} = D_{max}/P_{max}$, synergistic resultant of serum albumin and HDL cholesterol over the maximal domain; $\rho_{com} = (D–D_{max})/(P–P_{max})$, synergistic resultant of serum albumin and HDL cholesterol over the complementary domain; ONGT – obese individuals with normal glucose tolerance; T2DMBT – obese individuals with newly diagnosed type 2 diabetes mellitus (T2DM) before metformin treatment initiation; T2DMAT – obese individuals with newly diagnosed T2DM after a three-month metformin treatment. For other abbreviations, see Table 1.

***Note*: The standardized area (domain) P_{max}, which represents the maximum values of oxLDL, changes position between the tested groups. In the ONGT group, the maximum values of oxLDL were found at high HDL cholesterol and high albumin values (Figure 4). In the T2DMBT group, the maximum values of oxLDL were found at low HDL cholesterol and high albumin values (Figure 5). In the T2DMAT group, the maximum values of oxLDL were found at high HDL cholesterol and low albumin values (Figure 6). Two-dimensional linear correlations (Figures 1 and 2) contain indications of P_{max} migrations. The three-dimensional representation in the Figures above clearly highlights the migrations of P_{max}. A more detailed description is given in the following graphics.**

Comparison of the intensity and MV of the synergistic effect of the independent variables HDL cholesterol and serum albumin on oxLDL in the maximum zones and complementary zones can be obtained when the volume under the function and the maximum zone are divided by the corresponding surfaces. The results are presented in Table 5, excluding the NW group, as it lacks a pronounced peak zone. In the zones of maximum, the intensity of oxLDL was formed by the dominant synergistic effect of HDL cholesterol and serum albumin.

In the ONGT group, the intensity in the maximum domain was 2.30 times higher than the complement zone (maximum domain participation 69.7%); in the T2DMBT group, it was 3.49 times higher than the complement zone (maximum domain participation 78.4%), and in the T2DMAT group, it was 3.19 times higher than the complement zone (maximum domain participation 78.7%) (Table 5).

It should be noted that metformin therapy reduced the intensity of the synergistic effect of independent variables by 3.54 times. However, in the maximum zone of oxLDL, there was a minimal reduction from 3.49 to 3.19, which in total participation represents only a few percent success, i.e., although this is indisputably proven, statistically significant (Table 1, ANOVA test) reduction of oxLDL with the use of therapy. Nevertheless, the application of therapy had a dominant effect on one group of patients, but in the maximum zones of oxLDL, this undeniable influence did not produce the expected results. In other words, there was a group of patients who reacted more inertly to therapy and the reduction of oxLDL levels.

Discussion

The results of our study revealed the existence of the following: a) both qualitative (different verified groups distributions) and quantitative (statistical differences in MVs) changes in HDL cholesterol in the examined groups and the incapability of metformin treatment to restore those changes to the pattern observed among NW individuals; b) both qualitative and quantitative changes in albumin in the examined groups and the capability of metformin treatment to restore only the quality (therapy restores the distribution of serum albumin to uniform one as in the control group) but not the quantity to the pattern observed among NW healthy individuals; c) only quantitative changes in oxLDL in the examined groups and the incapability of metformin treatment to restore those values to the levels observed among NW individuals

The most likely cause of the quantitative decrease and qualitative change of HDL cholesterol particles is their transformation from an antiatherogenic to a proatherogenic form under the influence of systemic OS and chronic inflammation [6]. The dysfunctionality of HDL cholesterol particles, already present in obesity, becomes exacerbated with the onset of hyperglycemia, which causes additional oxidative damage through glycation processes. It has been hypothesized that the reduced values of HDL cholesterol in conditions of elevated OS represent a purposeful response [7]. Moreover, previous studies have shown that HDL cholesterol particles undergo oxidative modification much faster than LDL cholesterol particles [8]. Interestingly, a recent epidemiological study demonstrated that high HDL cholesterol levels may also represent a risk factor for cardiovascular disease (CVD), despite the fact that HDL cholesterol is recognized as an antiatherogenic lipoprotein [9, 10].

Additionally, no statistically significant changes in the correlations between HDL cholesterol and oxLDL were observed across the examined groups. However, there was a tendency towards statistically significant change in correlations among obese individuals with T2DM prior to metformin treatment initiation and NW subjects. For the significance threshold of $p = 0.10$, a significant change in correlation compared to the NW group was observed in the T2DM group prior to therapy based on the Pearson test.

On the other hand, significant changes in the correlations between serum albumin and oxLDL were observed across the examined groups. A significant change in the ratio of linear correlations of these two parameters indicates the existence of biochemical dynamics and underscores the effect of metformin treatment on the improvement of the albumin oxidative status in the setting of T2DM.

Obese individuals with NGT and those with T2DM prior to metformin treatment initiation had an exactly proportional ratio of oxLDL and albumin, which did not differ significantly. However, metformin treatment reduced the linear correlation ratio of oxLDL and albumin to the linear correlation level observed among NW subjects. The observed shift in these two parameters indicates a change in the nature of the ratio of oxLDL and albumin in conditions characterized by increased OS (T2DM before metformin treatment) and after treatment initiation.

The most significant finding relates to the maximum values of oxLDL, which were established in obese individuals with NGT and continuously appeared among obese T2DM individuals, both prior to and after metformin treatment initiation. The basic quality was determined by the different locations of the oxLDL maximal values, which highlighted the unique dynamics of the synergistic influence of HDL cholesterol and albumin on oxLDL values. This synergistic influence is also significantly different from their individual influences established by linear correlations. The unique dynamic refers to the intense migration of maximum oxLDL values in the groups, where we observed different relationships and values of the investigated parameters of oxLDL cholesterol, HDL cholesterol, and albumin.

Namely, among NW subjects, the highest values of oxLDL were observed at high HDL cholesterol values and MVs of albumin, which shows greater engagement of HDL cholesterol compared to albumin in states characterized by lower OS levels, as well as the existence of functional preservation of both HDL cholesterol and albumin. On top of that, the absolute value of oxLDL was significantly lower among NW subjects compared to all other examined groups.

On the other hand, in obese individuals with NGT (Figure 4), the highest oxLDL values (the red line on the ordinates indicates the maximum oxLDL values in each group) were observed with high values of both HDL cho-

lesterol and albumin, which shows greater involvement of albumin in states of increased OS. The decrease in absolute HDL cholesterol values in this group may represent a useful adaptive mechanism due to the potential transformation of antiatherogenic into proatherogenic HDL cholesterol particles under the influence of systemic OS and chronic inflammation in obesity. Hypothetically, at this point, albumin molecules may already show dysfunction due to their oxidative modification, as significantly elevated levels of oxLDL are associated with high albumin values. Additionally, the absolute oxLDL value in obese individuals with NGT was four times higher compared to NW subjects.

Furthermore, among obese individuals with T2DM prior to the initiation of metformin treatment, the highest values of oxLDL were observed alongside lower values of HDL cholesterol and high values of serum albumin, demonstrating a greater involvement of albumin, but also the existence of additional oxidative and inflammatory stress caused by T2DM onset, which further deepens the compensatory decrease in the HDL cholesterol level. As observed in obese individuals in general, the absolute value of oxLDL was higher among obese individuals with T2DM prior to metformin treatment initiation than in NW subjects.

Finally, among obese individuals with T2DM after metformin treatment initiation, significantly higher oxLDL values were observed at high values of HDL cholesterol and low values of albumin. This finding indicates a complete inversion of the ratio of these parameters caused by the initiation of treatment. The unfavorably high value of HDL cholesterol points out the persistence of systemic inflammation but also indicates a partial recovery, returning its quantitative levels closer to the ones observed among obese individuals with NGT. The presence of low albumin values underscores a partial recovery and lower levels of OS (significantly higher levels of serum albumin in this group than in group T2DMBT) due to a quicker recovery than HDL molecules. Low albumin values may also occur due to consumption and degradation during antioxidant neutralization of oxLDL. Additionally, this suggests a functional association between albumin and the neutralization of oxLDL, as lower albumin values were associated with higher levels of oxLDL.

These findings demonstrated the existence of functional dependence on the examined parameters and a significant change in the value of linear correlations between the examined groups, thus suggesting different relationships between HDL cholesterol, albumin, and oxLDL depending on the level of oxidative damage. Additionally, albumin showed a more significant functional recovery compared to HDL cholesterol in relation to oxidative damage after metformin treatment initiation in the setting of T2DM. This model undeniably proved the special dynamics of oxLDL's maximum migration due to the synergistic influence of HDL cholesterol and serum albumin, as a special qualitative feature, which according to our knowledge, was not previously observed or investigated.

The potential therapeutic application of albumin in states of increased OS should be limited to those conditions in which high albumin levels would not favor further oxidative damage. Based on the findings of our study, this could follow the metformin treatment lasting for at least three months. Additionally, therapeutic strategies aimed at increasing HDL cholesterol levels should not be implemented before achieving adequate glycemic control, due to the potentially unfavorable effects of high levels of oxidatively modified HDL cholesterol, which may lead to an increase in oxLDL values.

Albumin is involved in redox reactions non-specifically, owing to the fact that its concentration in the extracellular compartment is very high, while renewal occurs in about twenty days. Due to its flexible structure, albumin is also easily modulated. Undoubtedly, all these properties of albumin should be considered in the development of treatments for illnesses and disorders associated with OS [1]. Glycated and oxidatively modified albumin significantly contributes to the pathogenesis of DM and other diseases. Recent data indicate that albumin is a major blood plasma protein that represents a molecular "core" and a link between various tissues and organs, essential for the health of the entire organism. The ratio of oxidized albumin to total albumin can be increased in liver disease, DM, and CVD [1, 11]. Among individuals with T2DM, deleterious vascular effects could originate from a complementary mechanism of action, including higher levels of OS biomarkers alongside the loss of antioxidant capacity of albumin. This underscores the importance of considering albumin quality in maintaining homeostasis between glycoxidation and the antioxidant system [12].

As far as compensatory reduction of dysfunctional HDL cholesterol is concerned, it has been demonstrated that acrolein modification of HDL cholesterol produces a dysfunctional particle that may promote atherogenesis by impairing its cholesterol transport function [13]. Recent studies measuring other indices of HDL cholesterol, such as the functionality, size, or number of its particles, revealed that HDL-lipid hydroperoxides (LOOH) (HDL-LOOH) and HDL-triglyceride (HDL-TG) represent clinically available markers for predicting approximate risks of CVDs [14].

Although both HDL cholesterol and albumin have a protective effect in preventing oxLDL modification and consequent progression of atherosclerosis and other complications, it turns into its opposite during HDL cholesterol and albumin oxidative damage in obesity and DM.

The strength of this study shows the synergistic effect of HDL cholesterol molecules and albumin in neutralizing the negative effects of oxLDL. In the presented study, these relationships change in the study groups according to the levels of OS, inflammation, and the longevity of the investigated parameters.

Limitations of the study

The limitations of this study are the small study group and the relatively short follow-up time of the subjects. Qualitative determination of glycosylated and oxidized fractions of HDL cholesterol and albumin molecules could contribute to further investigations in this field.

Conclusion

The results of our study indicate a potential synergistic effect of albumin and high-density lipoprotein cholesterol in the prevention of oxidative damage, as well as a possible alteration in the quality of the ratio of these parameters in relation to oxidized low-density lipoprotein cholesterol molecules under conditions characterized by an increased oxidative stress. In this context, the focus should be moved from albumin and high-density lipoprotein cholesterol quantity to their quality, particularly in terms of their oxidative modifications, provided that further studies are required to elucidate their relationship with cardiometabolic disorders. Future prospective studies enrolling a large number of participants are needed to confirm these assumptions.

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