ApoD and ApoA-IV, novel proteomic components in HDL of diabetic kidney disease without dialysis

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Research

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Abstract

Background and aims: Diabetic kidney disease (DKD) is associated with lipid derangements worsening kidney function and enhancing cardiovascular (**CVD**) risk. The management of dyslipidemia, hypertension and other traditional risk factors does not completely prevent **CVD** complications bringing up the participation of untraditional risk factors such as advanced glycation end products (AGEs), carbamoylation and changes in HDL proteome and functionality. We analyzed HDL composition, proteome, chemical modification and functionality in non-dialytic DKD subjects categorized according to estimated glomerular filtration rate (eGFR) and urinary albumin excretion rate (AER).

Methods: DKD individuals were divided in eGFR>60 mL/min/1.73 m^2 plus AER stages A1 and A2 (n=10) and eGFR<60 plus A3 (n=25) and matched by age with control subjects (eGFR>60; n=8).

Results: Targeted proteomic analyses quantified 28 proteins associated with HDL in all groups, although only 2 were more expressed in eGFR<60+A3 group in comparison to controls: apolipoprotein D (**apoD**) and **apoA-IV**. HDL from eGFR<60+A3 presented higher levels of total AGEs (20%), pentosidine (6.3%) and carbamoylation (4.2 x) and a reduced ability in removing ^{14}C-cholesterol from macrophages (33%) in comparison to controls. The antioxidant role of HDL (lag time for LDL oxidation) was similar among groups but HDL from eGFR<60+A3 presented a higher ability in inhibiting the secretion of IL6 and TNF alpha (95%) in LPS-elicited macrophages in comparison to control group.

Conclusion: The increment in **ApoD and ApoA-IV** seems to counteract the HDL chemical modification by AGE and carbamoylation that contributes for HDL loss of function in well-established DKD.

Background

In diabetes mellitus (DM), abnormal kidney function is one of the most frequent complications being the leading cause of end-stage kidney disease. Besides, kidney function impairment increases the risk of cardiovascular disease (CVD) that is the major cause of mortality in both type 1 (DM1) and type 2 DM (DM2) [1]. Apart from traditional risk factors that are commonly observed in DM, including dyslipidemia, hypertension and other components of the metabolic syndrome, untraditional risk factors - advanced glycation, carbamoylation and oxidation - contribute to macrovascular disease in diabetic kidney disease (DKD) [2-4].

In DKD, the reduction in kidney function represented by the diminished estimated glomerular filtration rate (eGFR) is not unvariable accompanied in the same extension by elevation in the urinary albumin excretion rate (AER) [5]. In fact, many individuals with DKD with a marked reduction in eGFR can still present normal (A1 stage) **or** slightly reduced AER (A2 stage) and in some cases people in the A2 stage can revert to A1 [6]. The incidence of CVD is positively related to the reduction in eGFR as well as AER, and both parameters have additive effects on **CVD** risk in any stage of abnormal kidney function [7].

Advanced glycation end products (AGEs) are prevalent in DM and in chronic kidney disease (CKD) due to hyperglycemia, oxidative stress, **inflammation** and detoxyfication failure of intermediate compounds of the glycation reaction. AGEs independent predictors of **CVD** risk by impairing the reverse cholesterol transport (RCT) and lipid metabolism, inducing inflammation and altering vasodilation [8-10]. In addition, the reaction of isocyanic acid - derivated from urea or from the **myeloperoxidase activity -** with proteins, leads to protein carbamoylation that is also related to atherogenesis [4]. **Diet and tobacco contribute as unpredictable sources of thiocyanate that favors carbamoylation and AGEs, increasing the body pool of carbamoylated and glycated macromolecules.**

The reduction of high- density lipoprotein (HDL) cholesterol in plasma is a hallmark in DM although HDL dysfunction is also considered as having an important role in **CVD** morbidity and mortality. This is especially reputable when analyzing clinical trials where the increment in HDL cholesterol did not contribute for **CVD** risk improvement. HDL are antiatherogenic particles that mediate the removal of excess cholesterol from the arterial wall macrophages allowing its traffic to liver and excretion in feces by the RCT. Besides, HDL exert several others atheroprotective actions including antioxidant, antiinflammatory, vasodilation and anti-aggregant and improving glucose tolerance and insulin sensitivity. HDL is a cargo lipoprotein transporting many proteins, **microRNAs and other molecules** that are able to control metabolism in different tissues and in the arterial wall [11].

The HDL proteome that follows its complexity and functionality has been analyzed in CKD associated or not with DM, but in most of the studies, the individuals were on dyalisis that may have influenced the results. In addition, the stratification of CKD subjects by eGFR or AER alone may not reflect changes in HDL proteome and function that takes place in an unusual evolution of chronic disease typically in DKD. **Our hypothesis is that the HDL proteome and its chemical modification may influence the functionality of this lipoprotein and its role in CVD in DKD without dialysis.** We then analyzed the HDL proteome, composition and modification by advanced glycation and carbamoylation, together with its functionality in non- dyalisis DKD subjects categorized according to the eGFR and AER in comparison to age-matched control subjects. We found that the HDL proteome was enriched in **ApoD** and **ApoA-IV** and that HDL was modified by advanced glycation and carbamoylation, according to the reduction in eGFR and increased AER. The ability of HDL in removing cellular cholesterol was reduced in DKD with eGFR < 60 mL/min/1.73 m^2 plus A3 although its antioxidant activity was preserved and its capacity to prevent inflammation in macrophages was even increased. This may be related to the increment of HDL in **ApoA-IV** that exerts antiatherogenic actions and **ApoD** counteracting HDL loss of function that is reported in CKD.

Material And Methods

DM2 subjects with DKD were recruited at the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. **The inclusion criteria was based on the diagnosis of DM2 for at least 10 years, and in the classification of chronic kidney disease according to the loss in the glomerular filtration**

rate (eGFR; < 60 mL/min/1,73m^2) and enhanced urinary albumin excretion rate [AER; A1: < 30 mg/g creatinine; A2: 30 - 300 mg/g creatinine and A3: > 300 mg/g creatinine] for at least 3 months [12]. HbA1c values were between 7 and 10% avoiding greater variations in DM control and carbonyl stress due tue hyperglycemia.

Healthy control individuals matched by age were selected at Faculdade de Medicina da Universidade de São Paulo. All participants were properly informed about the procedures and the study and signed an informed written consent form that was previously approved by The Ethical Committee for Human Research Protocols of the Clinical Hospital (#15024), in accordance with the Declaration of Helsinki. Subjects on dyalisis, with other chronic diseases, rapid loss in eGFR (> 3 mL / min / year), refractory hypertension, BMI < 18.5 Kg / m^2, current smoke or alcohol abuse were not included. **Eighty percent of DKD subjects were in use of insulin and 70 % were on statins and beta-blockers. Angiotensin-converting-enzyme inhibitors (ACEi) and metformin were used for A1 and A2 subjects only and angiotensin II receptor blockers (ARBs) by groups A1 (39 %), A2 (50 %) and A3 (36%). Eritropoietin was utilized by A2 (7 %) and A3 (12 %) groups.**

Blood was drawn after overnight fasting and HbA1c was determined by high performance liquid chromatography (HPLC). Plasma was immediately isolated from the same sample in a refrigerated centrifuge (4°C). Glycemia, triglycerides (TG), total cholesterol (TC), HDL cholesterol (HDLc), fructosamine, TSH, creatinine and urea were determined in plasma by enzymatic techniques after overnight fasting and albumin in 24h-urine. DKD individuals were categoried according to the eGFR above 60 mL/min/1.73 m^2 plus AER stages A1 (< 30 mg/g creatinine) and A2 (30 - 300 mg/g creatinine) and eGFR below 60 mL/min/1.73 m^2 plus stage A3 (> 300 mg/g creatinine). Control subjects presented eGFR above 60 mL/min/1.73 m^2 plus A1.

Isolation of lipoproteins

Venous blood samples were drawn after overnight fasting and plasma immediately isolated in a refrigerated centrifuge. Preservatives were added to the plasma and density adjusted with bromide potassium to 1.21 g/mL. Low- density (LDL; d = 1.019-1.063 g/mL) and high- density lipoprotein (HDL; d = 1.063-1.21 g/mL) were isolated from plasma by discontinous density gradient ultracentrifugation (100 000g, 24 h, 4 °C, Sw40 rotor; Beckman ultracentrifuge). Samples were dialyzed against phosphate buffer saline containing EDTA (PBS).

HDL composition in lipids and ApoA- I

The amount of lipids and **ApoA-I** in HDL were determined, respectively, by enzymatic techniques [TC and TG; Labtest diagnóstica S. A., Minas Gerais, Brazil; phospholipids (PL); Randox Laboratorier LTD. Crumlin, Co. Antrem, United Kingdom] and immunoturbidimetry.

Determination of total AGE and pentosidine in HDL

The contents of total AGE and pentosidine were determined in isolated HDL by fluorescence measurement (Synergy HT Multi-Mode Microplate Reader, SpectraMax M5). Samples were excited at a wavelength of 370nm and the fluorescence emitted at 440 nm and 378 nm, respectively, for total AGE and pentosidine [13].

Determination of HDL carbamoylation

HDL carbamoylation was determined in the isolated lipoprotein by ELISA (- STA-877 Protein Carbamylation Sandwich ELISA;Cell Biolabs Inc., San Diego, CA, USA).

Determination of carboxymethyllysine in plasma

Carboxymethyllysine (CML) was determined in plasma by ELISA (Circulex CML, Woburn, MA, USA).

Proteolytic digestion of HDL

The HDL protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Ten micrograms of HDL-protein was solubilized in 100 mM ammonium bicarbonate, dithiothreitol, and iodoacetamide, following digestion with trypsin (1:40, w/w Promega, Madison, WI, USA) for 4 h at 37 °C. Trypsin was further added to samples (1:50, w/w HDL) and the incubation was done overnight at 37 °C. Samples were desalted using solid phase extraction (Oasis PRIME HLB SPE column; Waters) after acidic hydrolysis with 2% trifluoroacetic acid, **dried** and kept frozen at - 80°C until MS analyses. Prior to MS analysis, samples were resuspended in 0.1% formic acid (final protein concentration of 25 ng/µL).

Angiotensin peptide (DRVYIHPFHL, 0.2 pmol/µL) spiked in each sample was used as a global internal standard to control the robustness of PRM methodology. Variability in the integrated peptide area was monitored across 87 injections, and low variance was obtained with CV of 13%.

Targeted proteomic analyses

Digested HDL proteins (50 ng protein) were quantified by parallel reaction monitoring (PRM), as previously described [14]. Briefly, an Easy-nLC 1200 UHPLC (Thermo Scientific, Bremen, Germany) was used for peptide separation. **Each sample was loaded onto a trap column (nanoViper C18, 3 µm, 75 µm × 2cm, Thermo Scientific), and after, the trapped peptides were eluted onto a C18 column (nanoViper C18, 2 µm, 75 µm × 15 cm, Thermo Scientific).** Acquisition of the data was performed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, Bremen, Germany) using a nanospray Flex NG ion source (Thermo Scientific, Bremen, Germany). A scheduled (3-min window) inclusion list containing m/z of precursor peptides of interest and corresponding retention times was generated using Skyline software [15].

Selection of HDL peptides for targeted quantification

PRM methodology was assembled **using data derived** from shotgun proteomics analyses as previously described [14]. Ninety-one proteins were identified, but this number was reduced to 47 proteins eliminating proteins **that could be potential contaminants or were in low abundance** (keratin, proteins with <2 unique peptides and peptides with high interfering signal). Peptides susceptible to ex-vivo modification (e.g., methionine-containing peptides) were also avoided, and only peptides satisfactorily detected **(with a good chromatographic peak, containing at least 4 co-eluted transitions, and with mass error <10 ppm)** were included in the final analysis. After our exclusion criteria, 28 proteins remained. **For each protein quantification, a surrogate peptide was chosen by first selecting a peptide pair with best Pearson's correlation coefficient, followed by empirically selecting the final peptide based on a good chromatographic peak. Quantification was performed using the sum of peak areas obtained for each transition of each surrogate peptide, and at least 4 transitions per peptide were used. The 28 surrogate peptides chosen for HDL proteins are highlighted in Figure 1.**

Acetylation of LDL

LDL was acetylated as previously described by Basu et al [16]. Samples were extensively dialyzed before incubation with macrophages.

Measurement of ^{14}C-cholesterol efflux

This study was approved by the Institutional Animal Care and Research Advisory Committee (#1015/2018) and was performed following the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J mice were housed in a conventional animal facility at 22±2°C under a 12-h light/dark cycle with free access to commercial chow (Nuvilab CR1, São Paulo, Brazil) and drinking water. Bone marrow-derived cells were isolated from male, 6 week-old mice and macrophages were differentiated [17]. Briefly, tissue from the femur and tibias were cleaned and isolated at the knee joint. A needle size 26 and ½ and a 20 mL syringe filled with bone marrow medium (low glucose DMEM with 0.8% penicillin/streptomycin, 10% heat-inactivated fetal calf serum, and 10% L929 cell-conditioned medium was utilized to cut the end of each bone and to expel the bone marrow from both ends of the bones. Bone marrow was aspirated and expelled by utilizing a needle size 18 and ½ attached to a 20 mL syringe. Cells were centrifuged (6 min, 1,000 rpm in room temperature), resuspended in bone marrow medium, plated in culture dishes, and incubated for 5 days at 37°C under a 5% (v/v) CO2. Then, the medium was changed to low glucose DMEM containing 1% penicillin/streptomycin + 10% heat-inactivated fetal calf serum.

Bone marrow-derived macrophages (BMDM) were overloaded with acetylated LDL (50 µg/mL DMEM) and ^{14}C-cholesterol (0.3 µCi/mL) for 48 h. HDL from controls and DKD subjects (50 µg/mL) were utilized as cholesterol acceptors in 6- h incubations and the % of cholesterol efflux calculated as: **^{14}C-cholesterol in the media/ ^{14}C-cholesterol in the medium + ^{14}C-cholesterol in cells x 100. Control incubations were performed in the presence of the DMEM containing freee fatty albumin (FAFA) in the absence of HDL and the results subtracted from those obtained in the presence of HDL, as previous described**[8].

Measurement of the HDL antioxidant activity

The ability of HDL from controls and DKD individuals in inhibiting LDL oxidation was determined by incubation of LDL (40 µg/mL) isolated from a unique healthy plasma donor with $CuSO_4$ solution (1 mL; final **concentration 10 µmol/L**) in the presence of HDL (80 µg/mL). Lipoproteins were dialyzed against PBS without EDTA prior incubations. The absorbance at 234 nm was continuously monitored every 3 min during 4 h and the lag time phase for LDL oxidation (min) and the maximum ratio of conjugated dienes formation calculated [18].

Measurement of the HDL antiinflammatory activity

BMDM were isolated and cultured as described above; then overloaded with acetylated LDL (50 µg/mL DMEM) and treated for 24 h with HDL **(50 µg/mL DMEM)** from controls and DKD subjects. After washing, macrophages were incubated with lipopolyssacharide (LPS; 1 µg/mL DMEM) for 24 h. Medium was collected and the amount of TNF alpha and interleukin 6 (IL-6) determined by ELISA (R&D System-Duo Set, Minneapolis, EUA) [9].

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 program (GraphPad Software, Inc. 2007). Comparisons were made by the Kruskal-Wallis test with Holm-Sidak post test, Mann- Whitney or Student t test and Sperman linear correlation as appropriated. The value of $p < 0.05$ was considered statistically significant.

Results

Anthropmetric and biochemical data of control and DKD subjects are depicted in table 1. In the group eGFR <60 + A3 there was a greater predominance of male individuals (72%) compared to the control (50%) and eGFR> 60 + A1 and A2 (30%) groups. Age was similar among groups as well as the time of DM comparing both DKD grups. **As expected, eGFR was lower in eGFR <60 + A3 as compared to eGFR > 60 + A1 and A2 and control groups and AER was greater in eGFR <60 + A3 as compared to eGFR > 60 + A1 and A2.** BMI and CVD history were higher in the group eGFR > 60 + A1 and A2. **The measuring of traditional variables of glycemic control showed that fructosamine and HbA1c levels were higher in DM groups as compared to controls. On the other hand, the major circulating AGE specie in plasma**, CML did not reached statistically difference among groups. Total cholesterol (TC) was lower in the group eGFR > 60 + A1 and A2 as compared to controls.

Proteomic analysis was performed in order to analyze the protein cargo of HDL and its possible implication in HDL functionality. Twenty-eight proteins associated with HDL were quantified by PRM using nanoscale liquid chromatography coupled with mass spectrometry (Nano-LC / MS / MS) **(figure 1, box)**. Of these, 2 were more expressed in the **eGFR** < 60 + A3 group as compared to the control group: **ApoA-IV(figure 1 panel A) andApoD(figure 1 panel B)**.

The HDL composition **(figure 2) was addressed to verify possible changes in lipids and ApoA-I content that may modulate the HDL ability in removing cell cholesterol and inhibiting oxidation and inflammation**. The HDL content in TC **(figure 2 panel A)** and TG **(figure 2 panel B)** was similar between all groups. There was a decrease in PL **(figure 2 panel C)** and ApoA-I **(figure 2 panel D)** in the HDL of the **eGRF** group <60 + A3 compared to the control group. A positive correlation was observed between HDL-PL **(Figure 2 panel E)**, and ApoA-I **(Figure 3 panel F)** with the eGFR.

Chemical modification by glycation and carbamoylation was determined in isolated HDL. Total AGE **(figure 3 panel A)** and pentosidine **(figure 3 panel B)** were higher in HDL isolated from individuals with eGFR < 60 + A3 **(20% and 6.3 %, respectively),** as compared to the control group. Although the values of HbA1c and fructosamine were similar between groups, carbonyl stress as a function of albuminuria and eGFR < 60 can be attributed to the renal changes that accompany macroalbumininuria and are reflected in the lower detoxification of glycation reaction precursors and greater oxidative stress. The modification of HDL by isocyanic acid that reflects uremic stress and **induces HDL carbamoylation** was greater in the group with eGFR < 60 + A1 compared to the control group **(figure 3 panel C).** A positive correlation was observed between total AGE **(Figure 3, panel D)** and pentosidine in HDL **(Figure 3 panel E)** with AER.

HDL was utilized as acceptors of ^{14}C-cholesterol from **BMDM as a tool to determine the HDL intrinsic capacity to mediate cholesterol efflux.** As shown in the **figure 4 (panel A)**, ^{14}C-cholesterol efflux mediated by HDL isolated from the **eGFR** < 60 + A3 group was lower as compared to that mediated by the HDL from the control group. The antioxidant activity of HDL **that minimizes LDL oxidation** was determined by mesuring LDL oxidation with $CuSO_4$ along time. The lag phase for LDL oxidation determined by the presence of HDL was similar among all groups **(figure 4 panel B)** as well as the maximum ratio of conjugated dienes formation in LDL **(figure 4 panel C)**. **Additionally, we measured the ability of HDL from DKD and controls in inhibiting the secretion of inflammatory cytokines by LPS-challenged cells.** In the **figure 4 (panel D and E)**, it is demonstrated the secretion of inflammatory cytokines, IL6 (panel D) and TNF alpha (panel E) in macrophages treated with HDL from controls and DKD patients with eGFR < 60 + A3 groups and after challenged with LPS. For both cytokines, it was observed a very lower secretion when macrophages were exposed to DKD HDL in comparison to C-HDL.

ApoA-IV in HDL proteome was positively correlated with urea (figure 5 panel A), creatinine (figure 5 panel B), and AER (figure 5 panel D) and inversely correlated with eGFR (figure 5 panel C). No correlation was observed between apoA-IV with all measured parameters of HDL functionality or HDL chemical modification (data not shown). ApoD was also positively correlated with urea urea (figure 6 panel A) and creatinine urea (figure 6 panel B), and inversely correlated with eGFR (figure 6 panel C), although no correlation was observed with AER (data not shown). A positive correlation was observed between ApoD with pentosidine and total AGE in HDL (figure 6 panels D and E). Besides, ApoD was negatively correlated with the secretion of IL-6 (figure 6 panel F) but not associated with TNF alpha (data not shown).

Discussion

The prevalence of kidney complications in DM is high and the AER together with the reduced eGFR independently predict **CVD** morbidity and mortality [19]. Changes in the HDL proteome and functionality may modulate the antiatherogenic actions of this lipoprotein and consequently the development of atherosclerosis [20]. In this study, we evaluated in DM2 patients with DKD, the composition, chemical modification and proteomics of the HDL particles and their ability to remove cholesterol from macrophages, inhibit LDL oxidation and macrophage inflammation.

Targeted proteomics quantified 28 proteins in HDL of all experimental groups, although only 2 – ApoD and ApoA-IV - were differently expressed in DKD with eGFR < 60 plus A3. **ApoD is an atypical apolipoprotein mainly expressed in the central nervous system, testes, adrenal glands and kidneys[21]. Its expression is elevated by aging[22] and is implicated with neurological [23] and psychiatric[24] disorders. In mice ApoD protects against oxidative stress, which is related to its ability in scavenging free radicals[25].** In HDL, this apolipoprotein contributes for the HDL hydrophobic nucleus remodeling by facilitating the lecithin-cholesterol acyltransferase (LCAT) anchoring to the lipoprotein structure and carrying lysophosphatidylcholine, although it is not clear whether it has the potential to activate or inhibit the enzyme [26]. Some studies show an increase of ApoD in the HDL proteome of individuals with coronary artery disease and in areas of human atherosclerotic lesion as well as in apolipoprotein E knockout mice plasma [26,27,28]. However, it is not clear yet whether this increase in ApoD in these conditions refers to its role in inducing atherosclerosis or whether it represents a compensatory adaptive mechanism to changes observed in cardiovascular disease [26]. **In the present investigation, ApoD was related by the first time with markers of kidney failure (including urea, creatinine, and eGFR). Besides, apo D was positively correlated with HDL advanced glycation, although negatively related to the secretion of IL-6 by LPS-challenged macrophages that were treated with HDL. Although there is no direct evidence of a cause-effect relationship, which is a limitation of this study our findings point for apoD as a marker of DKD that may contribute to HDL function, despite lipoprotein chemical modification, which deserves future investigation.**

Several studies have demonstrated that ApoA-IV can be used as an early marker of kidney failure in individuals with CKD and in the general population, although further studies are needed to understand the pathophysiological basis of this association [29]. Besides, in individuals on hemodialysis, the increment in ApoA-IV was associated with an increased risk of all causes mortality [30]. Our data agree with these findings and an increased ApoA-IV expression was observed in the HDL proteome of individuals with higher AER and reduced eGFR as compared to controls. **Also, ApoA-IV was positively correlated with urea, creatinine, and AER, and inversely correlated with eGFR.** This apolipoprotein is synthesized in the intestine and secreted in the mesenteric lymph, being transported by chylomicrons but mainly by HDL, being the third most abundant apolipoprotein in this lipoprotein [29,30]. ApoA-IV presents many anti-atherogenic functions, acting in the removal of cellular cholesterol [31] and promoting activation of lipoprotein lipase [32], LCAT and cholesteryl ester tranfer protein (CETP) [33]. In addition, its anti-atherogenic activity is complemented by its anti-inflammatory and antioxidant properties [34,35]. **Nonetheless, in the present**

investigation ApoA-IV was not correlated with citokynes secretion by macrophages treated with HDL neither to other HDL functions (cholesterol efflux and inhibition of LDL oxidation). Future experiments with apoA-IV enrichment or deletion in HDL particle would help to clarify its involvement in CVD protection in DKD.

Similarly to other HDL-apolipoproteins, ApoA-IV is modified by advanced glycation in DM and CKD. Recently, our group demonstrated that E. coli recombinat **ApoA-IV** submitted to advanced glycation in vitro mantains its ability in removing macrophage excess cholesterol, despite its large amount pyrraline, CML and argypyrimidine [36]. This may explain the fact that no major reductions in cholesterol efflux were observed in the present study, even with a significant increase in total AGE and pentosidine in the HDL of the group eGFR < 60 + A3 as compared to controls. In addition, the in vitro glycation of **ApoA-IV** only partially impaired its ability to inhibit the inflammatory response promoted by LPS in macrophages [37]. On the contrary, ApoA-I has its ability in removing cell cholesterol and antioxidant and anti-inflammatory properties severely impaired by advanced glycation [29].

The HDL proteome has been analyzed in CKD, but the vast majority of studies were performed in individuals undergoing hemodialysis, which can interfere with the observed results. Also, many studies included CKD together with DKD indistinctively. Mangé et al [38] found 40 differently expressed proteins in HDL from CKD subjects. ApoC-II and ApoC-III were higher while transtirretin and haptoglobin-related protein (HPTR) were lower in HDL from CKD subjects on hemodialysis compared to healthy subjects. In another study, individuals on hemodialysis, with or without DM, an increase in SAA1, albumin, phospholipase A2 and ApoCIII was observed in HDL proteome. Along with the reduction in the content of PL and an increase in TG and lysophospholipids, these modifications were linked to the reduction in the HDL-mediated cholesterol efflux [39]. In agreement HDL dysfunction was also related to changes in HDL proteome by Florens et al [40]. Shao et al [41] found proteins related to renal injury (beta 2 microglobulin, complement factor D, cystatin C, prostaglandin-H2 D-isomerase, retinol-binding protein 4 and AMBP) increased in HDL of individuals with CKD.Others were more present in the control group, among them: ApoA-I, ApoA-II, ApoL-I, ApoM and paraoxonase 1 (PON-1), confering greater damage to the anti-atherogenic activities of HDL in CKD.

Recently, the HDL proteome was analyzed in 191 individuals with DM1, in the Diabetes Control and Complications Trial (DCCT) study. Eight proteins were associated with proteinuria, although only **PON-1** was simultaneously associated with AER and coronary calcium content [42]. Wang et al [43] demonstrated enrichment in 8 proteins related to inflammation and lipid metabolism (serum amyloid A1, A2, and A4; hemoglobin beta, HPTR, CETP, PLTP and ApoE) in HDL isolated from individuals in short-term as compared to long-term dyalisis therapy.

ApoA-I determined by immunoturbidimetry was reduced in HDL of the **eGFR** group < 60 + A3 with a negative correlation with AER. Therefore, it is likely that in vivo, cellular cholesterol removal may be even more compromised than that observed in the present study due to the lower content of ApoA-I in

individuals with established DKD. In our efflux assay HDL concentration was matched by the protein component that is mainly represented by ApoA-I, making more difficult to observed differences.

A decrease in the HDL-PL was also observed in the eGFR group < 60 + A3 with a positive correlation with eGFR. Notably, PLs are positive modulators of cell cholesterol removal, as they facilitate the interaction of HDL with plasma membrane domains that guarantee cholesterol exportation by diffusion and / or mediated by specific receptors.

Reduced ability in removing cell cholesterol was previously reported in CKD individuals stage 3 and 4 as compared to healthy controls although not independently of age [44]. In this sense, there are studies showing the role of aging on the ability of HDL in mediating cholesterol efflux that may is a bias for many studies dealing with controls and CKD subjects at different ages [45,46]. In dialytic individuals, Yamamoto et al [47] reported a reduced cholesterol efflux mediated by HDL from DM with or without DKD in comparison to controls.

Diferently from our work, there are many studies that measured the cholesterol efflux mediated by the ApoB- depleted serum. Although, HDL is the only lipoprotein in that serum, there are many other components including albumin, cytokines, haptoglobin and insulin that could affect cholesterol removal. **In addition, measurement of cholesterol removal mediated by ApoB- depleted serum hides the variations in HDL cholesterol levels that are frequently altered in DM and DKD**[48,49].

Ganda et al [50] did not find any alteration in the cholesterol efflux mediated by the ApoB-**depleted serum** isolated from CKD subjects (stages 4 and 5), although the expression of *Abca1* in monocytes isolated from those subjects was reduced compromising the cholesterol efflux to ApoA-I. Chemical modification of HDL by advanced glycation and carbamoylation that was observed in the present study was related to the stage of AER and eGFR. In individuals in hemodialysis or peritoneal dialysis a greater amount of pentosidine was found in plasma that correlated with the progression of kidney disease. Besides, pentosidine levels were higher in CKD stage 5 subjects as compared to stage 1 [51]. **Pentosidine and other AGEs found elevated in HDL from subjects included in the present investigation are consequences of the hyperglycemia that is inerent to DM as well as failure in glycation intermediate destoxyfication due to CKD. Those conditions simultaneously contribute to AGEs generation that can be further aggravated by inflammation and exogenous sources.** In CKD subjects undergoing hemodialysis it was demonstrated elevated concentration of carbamoylated HDL [52,53]. **Both glycation and carbamoylation have negative impact in atherogenesis by favoring the uptake of LDL in detriment of the HDL-mediated cholesterol efflux that is impaired by these modifications [54,55].**

The antioxidant capacity of HDL assessed by the lag time for LDL oxidation was similar among groups, and as for the other HDL functions the impact of ApoD and ApoA-IV in HDL proteome should be investigated by using appropriated experimental protocols. For instance, the HDL antioxidant role can be assessed by using cell-based models that sometimes show divergent results as compared to the measurement of the lag phase utilized by us in the present investigation.

Interestingly,the HDL anti-inflammatory ability was greatly increased in DKD-HDL as compared do controls. This was demonstrated by the reduced secretion of inflammatory cytokines, IL6 and TNF alpha by macrophages challenged by LPS.

Conclusion

We demonstrated an enhanced ApoD and ApoA-IV content in the HDL proteome in DKD subjects without dialysis that may modulate antiatherogenic functions of this lipoprotein. The increment in ApoD and ApoA-IV seems to counteract the HDL chemical modification by AGE and carbamoylation that contributes for HDL loss of function in well-established DKD and should be further investigated.

Declarations

Ethics approval and consent to participate: All participants were properly informed about the procedures and the study and signed an informed written consent form that was previously approved by The Ethical Committee for Human Research Protocols of the Clinical Hospital (#15024), in accordance with the Declaration of Helsinki.

Consent for publication: Not applicable

Availability of data and material: The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request. The authors declare that all data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Autors contribution

MFMS: performed all experiments and helped in manuscript preparation; ALAL: selected patients and controls and helped in clinical data processing; RSP: helped in experiments performance; CAM: helped in patients recruitment and clinical data; ARMS: performed proteome analysis and data processing; MIBACS: helped in statistics; ERN: helped in data interpretation; MLCCG: helped in data interpretation;

MSQ: supervised patients recruitment and helped in data interpretation; GER: supervised the proteome analysis and data interpretation; MP: conceived the study and experiments and wrote the manuscript.

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Abbreviations

ApoA-I – apolipoprotein A-I

ApoA-IV – apolipoprotein A-IV

ApoC-IV – apolipoprotein C-IV

ApoD – apolipoprotein D

AER – albumin excretion rate

AGEs – advanced glycated end products

BMDM – bone marrow derived-macrophages

CKD – chronic kidney disease

CVD – cardiovascular disease

DKD – diabetic kidney disease

eGFR – estimated glomerular filtration rate

HDL – high density lipoprotein

LDL – low density lipoprotein

RCT – reverse cholesterol transport

SAA4 – serum amyloid protein A-4

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Table

	Control eGFR > 60	DM eGFR > 60 A1 + A2	DM eGFR < 60 A3
n (F/M)	8 (4/4)	10 (7/3)	25 (7/18)
Age (years)	68.5 (58-84)	68 (53-75)	69 (55-87)
eGFR (mL/min/1.73m^2)	84.5 (63-102)	82.0 (61-131)	25 (10-46)$^{\#}$
AER (mg/g creatinine)	-	9.5 (2.4-114)	1128 (317-6430)$^{@}$
Time of DM (years)	-	14 (4-26)	18 (6-30)
CVD history (%)	-	2 (20%)	11 (44%)
BMI (kg/m^2)	24.9 (21-29)	33 (25-45) *	27 (21-38)
HbA1c (%)	5.6 (5.0-6.0)	8.4 (7.0-8.0)$^{\#}$	8.3 (6.0-10.0) $^{\#}$
Fructosamine (µmol/L)	219 (205-262)	308 (158-428)$^{\&}$	331 (329-506) $^{\&}$
TC (mg/dL)	208 (155-290)	144 (114-186)$^{\$}$	154 (92-313)
TG (mg/dL)	111 (71-362)	88 (64-151)	148 (78-329)
HDLc (mg/dL)	49 (33-87)	46 (35-92)	40 (21-140)
CML (µg/mL)	0.5 (0.3 - 1.0)	1.7 (0.5 - 3.4)	1.0 (0.7 - 7.3)

Table 1. Anthropometric and clinical data of control subjects and individuals with DKD categorized according to the estimated glomerular filtration rate (eGFR; mL/min/1.73 m^2) and albumin excretion rate (AER; A1 = normoalbuminuria, A2 = microalbuminuria; A3 = macroalbuminuria). BMI = body index mass; HbA1c = glycated hemoglobin; TC = total cholesterol; TG = triglycerides; HDLc = HDL cholesterol (mean ± SD); CML = carboxymethyl-lysine (median - range)**. Results were compared by the Kruskal-Wallis test with Holm-Sidak post test; * p < 0.004; $^{\#}$ p < 0.0001; $^{\&}$ p < 0.001 and $^{\$}$ p < 0.006 in comparison to ControleGFR > 60, and by Mann- Whitney test; @ p < 0.001 in comparison to eGFR > 60 A1 + A2.**

Figures

Figure 1

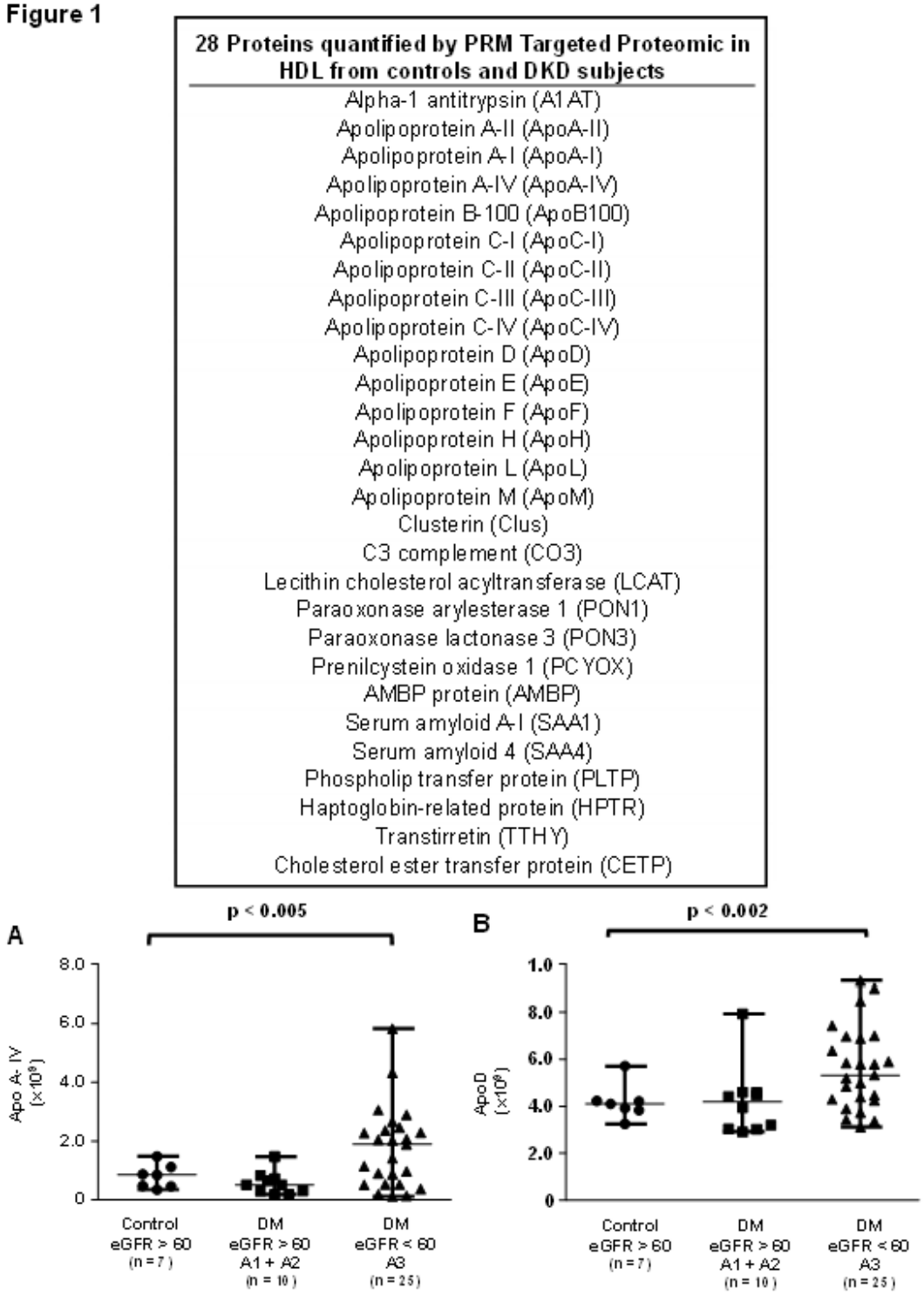

Figure 1

HDL proteome. Twenty-eight proteins were quantified in HDL using a targeted proteomic analysis (box). Significant proteins found in the CKD-HDL proteome, ApoA-IV and ApoD (panels A to B). Comparisons were made by the the Kruskal-Wallis test with Holm-Sidak post test.

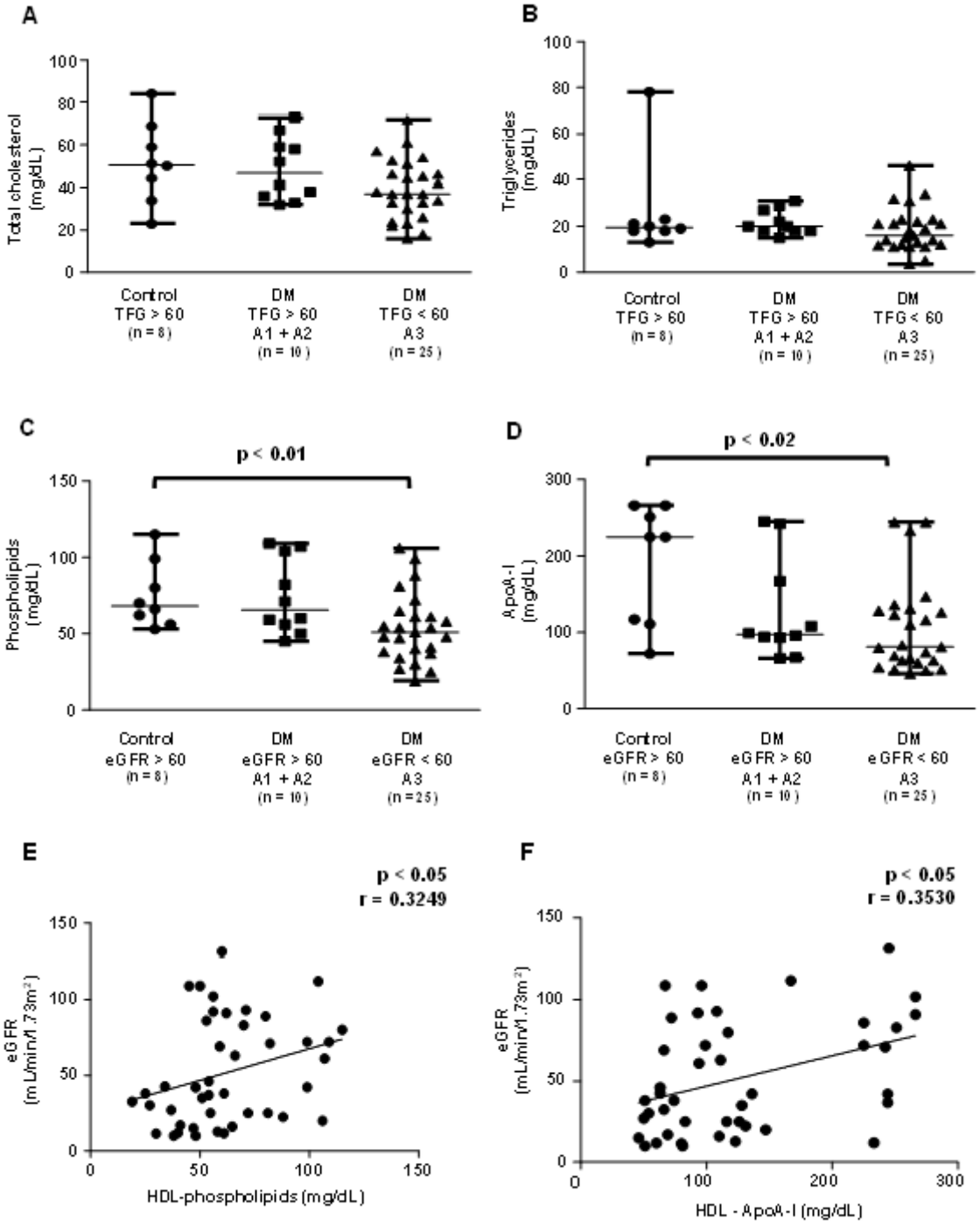

Figure 2

HDL composition. The HDL content of TC, TG and PL was determined by colorimetric enzymatic methods and ApoA-I, by immunoturbidimetry (panel A to D). The results were compared by the Kruskal-Wallis test with Holm-Sidak post test. Associations between HDL components with eGFR (panels E to F) were performed by Spearman´s correlation.

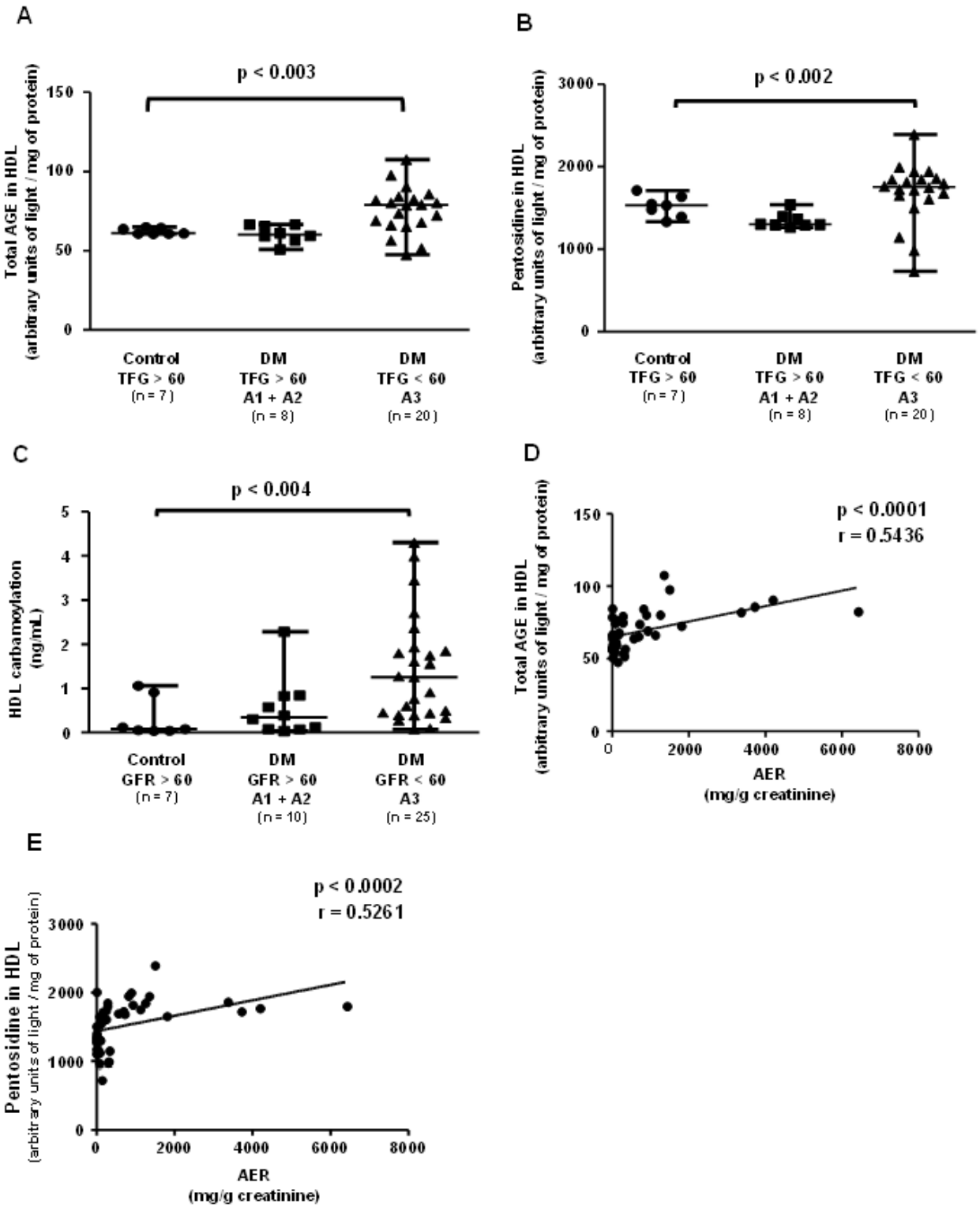

Figure 3

HDL modification by advanced glycation and carbamoylation. The amount of total AGE (panel A), and pentosidine (panel B) was determined in HDL by measuring the absorbance in the fluorescence range at 440 nm (total AGE) and 378 nm (pentosidine) and carbamoylation (panel C), by ELISA. The results were compared by the Kruskal-Wallis test with Holm-Sidak post test. Associations between HDL chemical modification with AER (panels D and E) were performed by Spearman´s correlation.

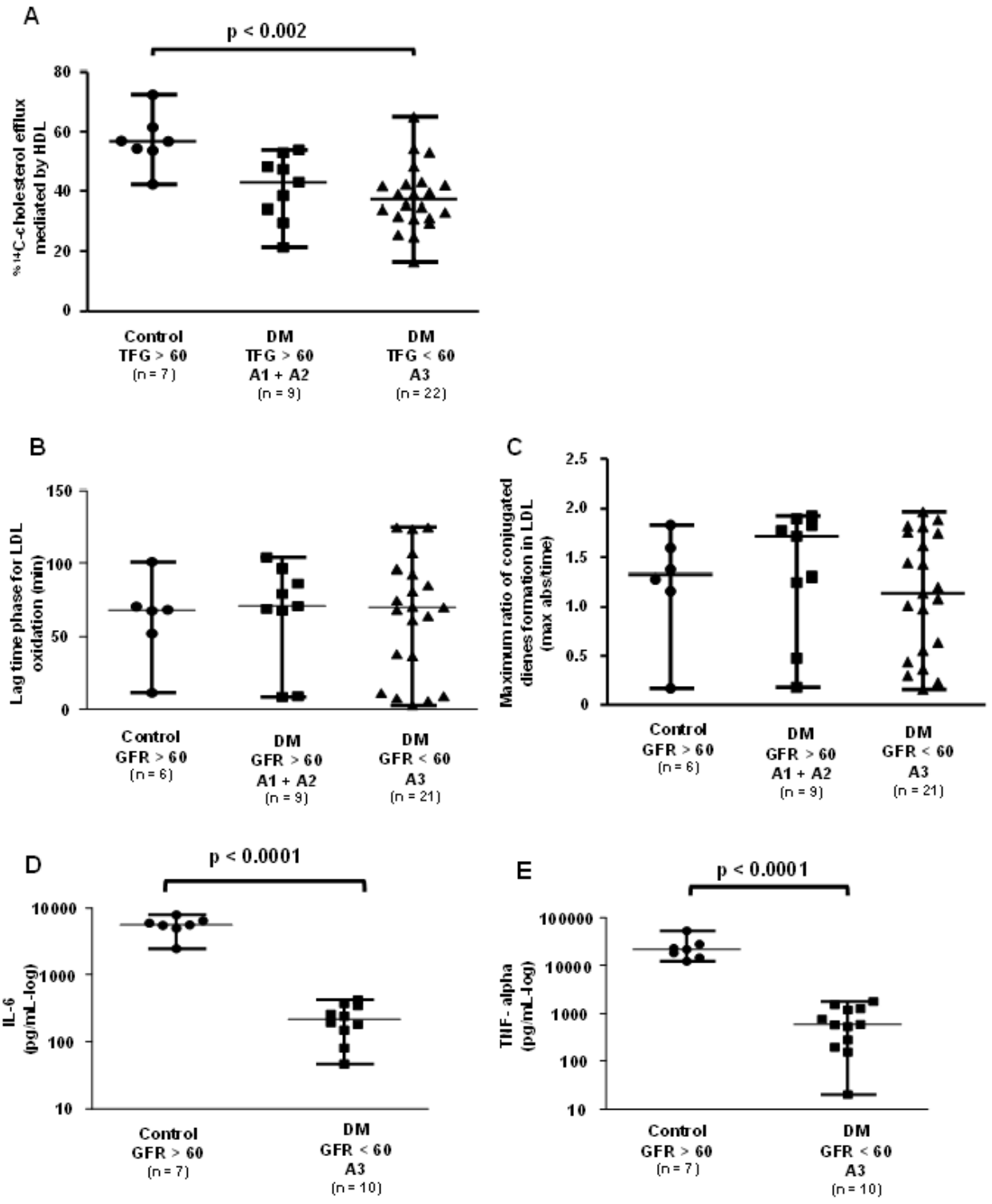

Figure 4

HDL functionality. (panel A) cholesterol efflux: HDL was isolated from DKD and control subjects and utilized as acceptor of cellular cholesterol. Bone marrow-derived macrophages (BMDM) overloaded with acetylated LDL and 14C-cholesterol were incubated with 50 µg of HDL / mL of medium for 6 h. Cholesterol efflux was determined after measuring the radioactivity in the culture medium and that remaining in cells, being calculated as: 14C-cholesterol in the medium / 14C-cholesterol in the medium +

14C-cholesterol in cell x 100. Control incubations were performed in the presence of the DMEM/FAFA in the absence of HDL and the results subtracted from those obtained in the presence of HDL. (panels B and C) antioxidant activity: the lag time (panel B) and the maximum rate of LDL oxidation (panel C) was determined in incubations with LDL (40 µg of protein) isolated from a healthy donor with CuSO4 solution and HDL from DKD or controls (80 µg of protein). (panels D and E) antiinflammatory activity: BMDM overaloaded with acetylated LDL (50 µg / mL) were treated with HDL (50 µg / mL), for 24 h. After washing, cells were treated with LPS (1 µg / mL), for 24 h, and interleukin 6 (IL-6, panel D) and TNF-alfa (panel E) determined in the medium by ELISA. Results were compared by the the Kruskal-Wallis test with Holm-Sidak post test or Student t test.

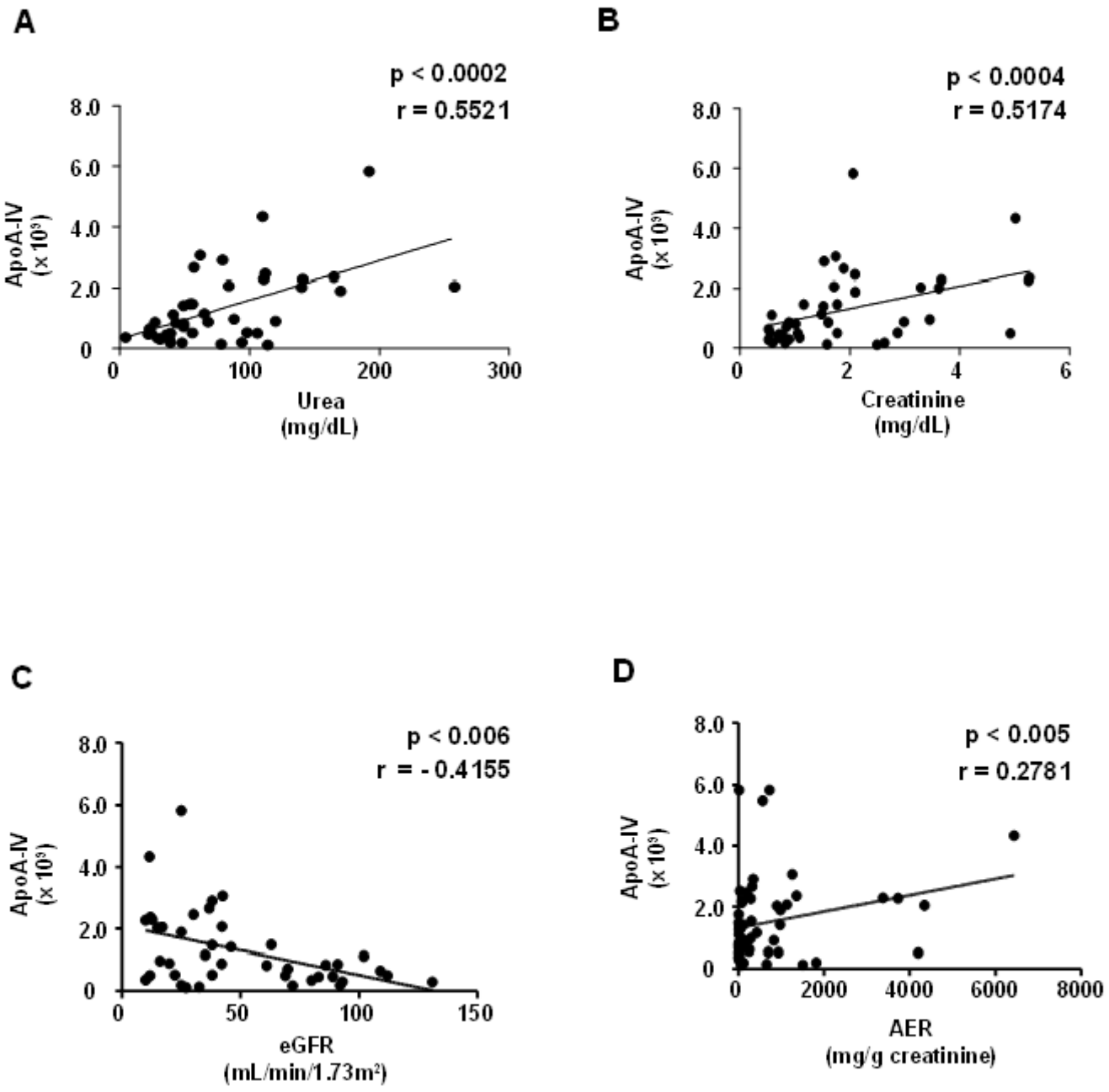

Figure 5

Correlations between ApoA-IV with parameters of CKD. Correlations were performed using Spearman correlation.

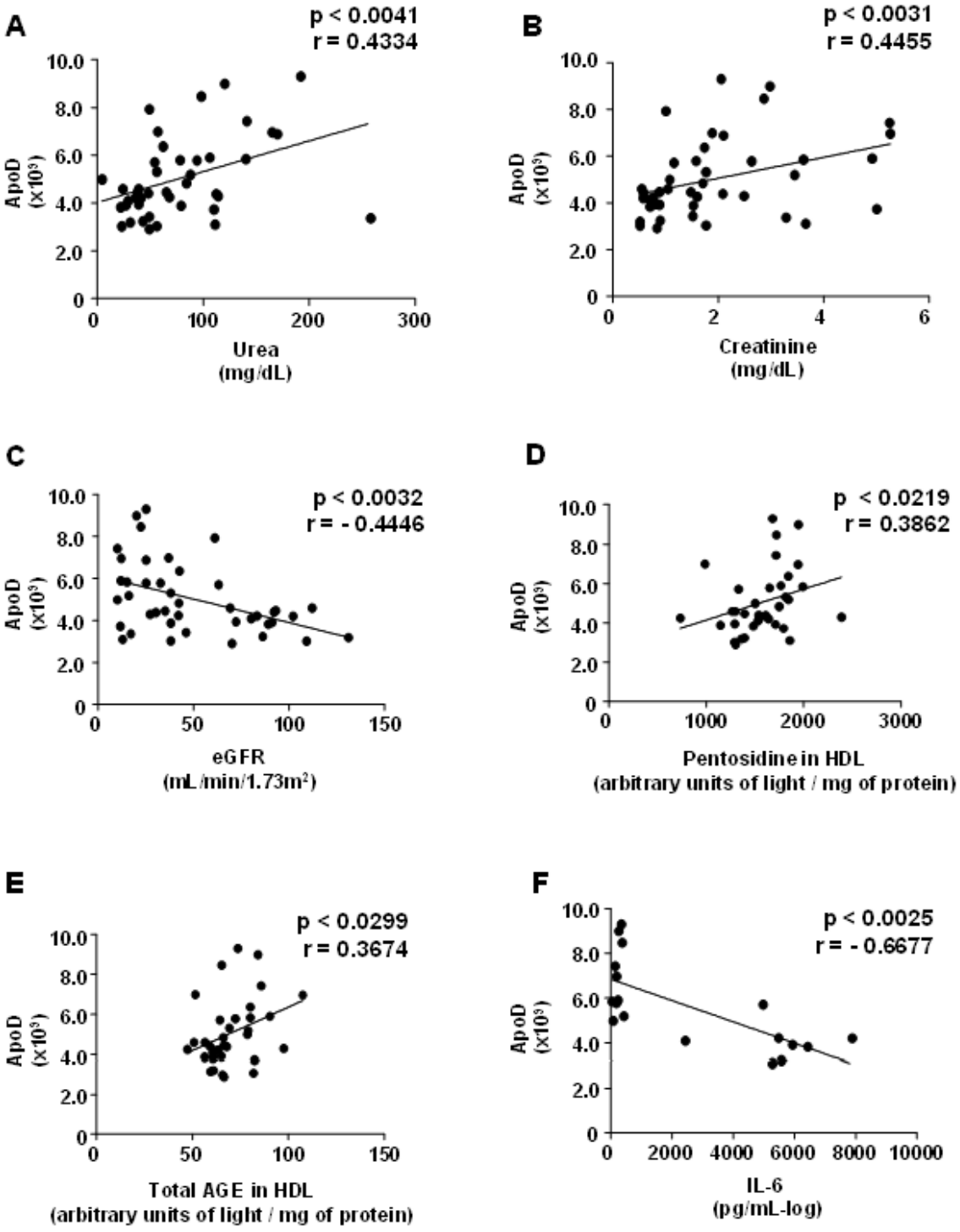

Figure 6

Correlations between ApoD with parameters of CKD, HDL chemical modification and functionality. Correlations were performed using Spearman correlation.

Supplementary Files

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