Original Article

APOPROTEIN(a) ISOFORMS RISK FACTORS FOR ATHEROGENESIS IN ESRD PATIENTS ON HAEMODIALYSIS

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Abstract

Introduction. End-stage renal disease (ESRD) patients undergoing hemodialysis are at an increased risk of arteriosclerotic vascular disease (ASVD). The increased risk is commonly attributed to the traditional risk factors related to ESRD. However, interest for more recent risk factors for ASVD, such as the level of lipoprotein(a) and its specific apoprotein(a) has been promoted.

The aim of this paper is to determine whether apo(a) phenotype is a risk factor for arteriosclerotic vascular disease in ESRD patients who are on hemodialysis.

Methods. Apo(a) phenotypisation was performed by using the Western Blot Technique of blood samples from 96 end-stage renal disease patients who were undergoing hemodialysis, and from 100 healthy individuals.

Results. Frequency distribution of the basic apo(a) isoforms calculated by means of the χ^2 -test has shown that there was no significant statistical difference in distribution among patients on hemodialysis, and healthy carriers (χ^2 -0.36, $p < 0.548$ -for carriers of single apo(a) isoforms, χ^2 -0.10, $p < 0.7545$ -for carriers of double apo(a) isoforms). The calculated relative risks have demonstrated that apo(a) phenotype was not a risk factor for ASVD in HD patients.

Conclusion. Based on the results obtained, it can be concluded that the apo(a) phenotype is not a risk factor for arteriosclerotic vascular disease in patients undergoing hemodialysis.

Key words: apo(a) phenotype, ESRD hemodialysis

Introduction

End-stage renal disease (ESRD) patients undergoing hemodialysis are at an increased risk of arteriosclerotic vascular disease (ASVD). The increased risk is commonly attributed to the traditional risk factors related to ESRD [1]. However, interest for more recent ASVD risk factors, such as the level of lipoprotein(a) and its specific apoprotein(a), has been promoted [2].

Lipoprotein(a) is composed of an LDL particle to which apoprotein(a) has a covalent bonding with S-S bridges. Apoprotein(a) [apo(a)] is a glycoprotein with the so-called Kringle structure (in German kringle means "pretzel"). There is a different number of Kringle IV-type 2 units in various individuals, which are coded by a high polymorph APO(a) gene [3]. The repetition number affects the size of the apo(a) molecule, and the size of this molecule is in an inverse correlation with the Lp(a) concentration in the people's blood [4].

The apo(a) polymorphism molecule size is detected by using SDS-PAGE and a follow-up of the Western Blot Technique. On a polyacrilamide gel, based on the mobility in relation to Apo B100, 6 apo(a) isomorphs have been separated in humans [5].

The aim of this paper is to determine whether apo(a) phenotype is a risk factor for arteriosclerotic vascular disease in ESRD patients who undergo hemodialysis.

Material and methods

Examinees. A total of 96 end-stage renal disease (ESRD) patients undergoing dialysis at the Dialysis Ward of the University Clinic of Nephrology in Skopje were included and compared to the control group of 100 healthy individuals. The sole inclusion element for the study was the dialysis treatment, regardless of possible previous kidney disorders which had led to the patients' ending up with hemodialysis.

The two groups, patients and healthy individuals, were first asked to sign an Inform Consent Document for participating in the study.

K₃EDTA plasma blood samples and healthy individuals' serum were taken after a 12 hour (overnight) deprivation of food. The blood samples of the patients were taken from the arteriovenous fistula before the dialysis treatment. The patients were undergoing a regular dialysis regime (3-5 hours three times a week).

Following the low velocity blood centrifugation, the plasma samples were divided in small portions of 1.5 ml plastic capped Ependorpha and frozen at -80°C until the actual analysis. The fresh serum was used to define the lipid status of the examinees.

Phenotype analysis of apoprotein(a) isomorphs was done by means of the Western Blot Test: first the separation with 3-15% gradient denaturation SDS-PAGE of Mi-

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ni-Protean II Bio-Rad Vertical System (Bio-Rad Laboratories, Hercules, CA, USA) was done and second, the transfer of proteins onto a nitrocellulose (NC) membrane by electro-blotting with Hoefer TE 22 Mini Tank Transfer Unit which was connected to a circulating bath set up at $+10^{\circ}\text{C}$. The plasma samples were treated with SDS, glycerol, bromophenol-blue and β -mercaptofenol, and then applied in wells of mini 3-15% gradient gels. Simultaneously, we applied the commercial standard (Immuno-AG, Vienna, Austria) in two wells of one mini gel. The Apo(a) standard contained 6 apo(a) isomorphs: B, F, S1, S3, S4, >S4. Electrophoresis was leaking into a TRIS-glycerin puffer until the blue front left the gel. With the electro-blotting in the TRIS-glycerin-methanol puffer the proteins were transferred to the NC membrane (S&S NC; BA 83; 0.2 mm) with the above mentioned instruments.

On the third day, the apo(a) isomorphs were immune-chemically visualized by means of Lp(a) phenol-typifying. The details of the complete procedure have already been published [6].

Based on their relative electrophoretic SDS-PAGE mobility, compared to ApoB100, and by applying the Utermann nomenclature, the apo(a) isomorphs were separated into F, B, S1, S3, S4 and >S4 single-band isomorphs and appropriate apo(a) double-banded isomorphs. In case a blob was not visualized at the application place, the phenotype was defined as "null" phenotype [7] (Figure 1).

Fig. 1. NC membrane with samples of individuals who carry a single apo(a) isoform (1), double apo(a) isoform (2 and 3), null phenotype (4); St- apo(a) standard (Immuno-AG)

The molecule mass (MM) of each apo(a) isoform of the samples was calculated by comparing it to apo(a) isoform MM present in the standard by means of a laser scanner and Image Master Software (Pharmacia Biotechnology, Uppsala Sweden).

According to Utermann *et al.* >S4, S4 and S3 are high molecular apo(a) isoforms which contribute to the low Lp(a) concentration in the blood, whereas F, B, S1 are low molecular apo(a) isoforms which are responsible for the high Lp(a) values in the carriers' blood [6].

Determination of the concentration of lipoprotein(a), apolipoproteins and lipid parameters

The Lp(a), ApoA-1 and ApoB concentration was determined in a plasma sample with immunonephelometric kit (DADE-Behring, Germany of Behring Nephelometer Analyzer). Triacylglycerols and the total cholesterol were determined in a fresh serum with enzymatic CHOD-PAP test kits (Randox Laboratories-UK). HDL-cholesterol was determined by means of an enzymatic test kit in the supernatant that was received after depositing the other lipoproteins with a mixture of phosphowolframat and MgCl_2 (Randox Laboratories-UK). LDL-cholesterol was mathematically calculated by means of the Friedewald formula. Glucose was determined by means of the Beckman Glucose Analyzer.

Statistical analysis

The results obtained are presented as a medium arithmetical value $\pm$ standard deviation (SD); the lipoprotein (a) [Lp(a)] concentration is also presented as a median. The Student's t-test was used for comparing the age, the concentration of Lp(a), apoproteins and the parameters of the lipid status between the ESRD patients undergoing hemodialysis and the healthy individuals (control group). χ^2 -test was used for comparison between the single apo(a) isoforms of the ESRD patients undergoing hemodialysis and the healthy individuals. The statistical analysis was conducted with STATWIN statistical package (Statsoft Inc, Tulsa Oklahoma USA). The $p < 0.05$ value was considered to be statistically significant.

Results

The clinical characteristics and the data acquired with the laboratory research for HD patients and the control group are presented in Table 1. Increased blood pressure was measured in 27.08% of the patients, but in none of the control examinees. The positive family anamnesis of kidney diseases was found in 20.83% of the patients. The duration of the hemodialysis was from 1-36 years (9.33 ± 7.32 years). Due to the fact that no significant statistical difference in the Lp(a) concentration was determined between the two genders and the two groups of examinees ($p < 0.12$ -patients; $p < 0.16$ -control group), the results are given as a sum for both genders and for the groups of patients and the controls.

The mean value of the Lp(a) concentration was moderately increased compared to the control group, but it was statistically insignificant. Table 1 illustrates an increased level of triacylglycerols and hypocholesterolemia in the ESRD patients compared to the control group [7]. The sum distribution of the basic apo(a) isoforms carriers in the patients and the control group, separated by 3-15% gradient SDS-PAGE isoforms, expressed in percent, is presented in Figure 2.

Table 1. Clinical characteristics and concentration of lipid status parameters in ESRD HD patients and the control group (healthy individuals)

Variables	ESRD patients undergoing HD n =96	Control group n = 100	p-value ESRD pat./ control group
Age (years)	51.59±13.26	50.69±12.89	n.s.
Smokers (%)	14.00	16.00	n.s.
NTA (%)	27.08	-	-
Duration of hemodialysis (years)	9.33±7.32	-	-
Positive family history of kidney disease (%)	20.83	-	-
Lp(a) mg/dl	17.33±17.44	14.14±9.58	n.s.
(median)	(9.62)	(9.62)	
Lp(a) ≥ 30 mg/dl (%)	12.63	7.69	-
Lp(a) ≥ 30 mg/dl	57.79±21.28	55.49±18.35	n.s.
(median)	(53.75)	(51.6)	
ApoA1 (g/L)	0.98±0.20	1.19±0.19	0.001
ApoB (g/L)	0.871±0.29	0.81±0.25	n.s.
Triacilglycerols (mmo/L)	1.93±0.95	1.32±0.71	0.01
Total cholesterol (mmol/L)	3.91±1.04	4.45±0.89	0.001
HDL- chol. (mmol/L)	0.822±0.29	1.22±0.30	0.001
LDL -chol. (mmol/L)	2.22±0.94	2.88±0.99	0.001
Glycise (mmol/L)	5.60±2.37	5.32±0.77	n.s.

ApoA1-apoprotein A1; ApoB-apoprotein B; Total chol.- total cholesterol; HDL- chol.- HDL- cholesterol; LDL-chol.-= LDL cholesterol.; n.s.-statistically insignificant differences

The analysis shows that the distribution of the basic apo(a) isoforms carriers between the HD patients and the control group is rather similar. Most frequent are carriers of single apo(a) isoforms (60%), and then come the carriers of the double apo(a) isoforms (35%).

The percent of the "null" phenotype carriers in ESRD patients undergoing hemodialysis (3.13%) is lower compared to the control group (4.95%), but the difference is statistically insignificant. χ^2 -test demonstrated that there is no significant statistical difference in the basic

Fig. 2. Frequency distribution of basic apo(a) isoforms (single and double and null phenotypes) in HD patients and control group

apo(a) isoform distribution frequency between patients on hemodialysis and the control group (χ^2 -0.36, $p < 0.548$ -for the single apo(a) isoform carriers, χ^2 -0.10, $p < 0.7545$ -for the double apo(a) isoform carriers).

Figure 3 (A and B) shows the separated single (A) and double apo(a) isoforms and the carriers of the "null"

phenotype for the patients undergoing hemodialysis and the control group.

The Figure shows that high molecular single >S4, S4, apo(a) isoforms were more present in patients undergoing hemodialysis compared to the control group, whereas S3 apo(a) isoforms were less present compared to the control

Fig. 3a.

Fig. 3b.

Fig. 3a and 3b. Separated single (A) and double (B) apo(a) isoforms and carriers of null phenotype in HD patients and the control group (in %)

Table 2. Relative risks of individual apo(a) isoforms in the atherogenesis of HD patients compared to the control group

Apo(a) isoforms	>S4	S4	S3	S1	B
HD(n)	20	47	19	4	3
Control (n)	23	48	23	3	3
OR	1.82	0.69	0.94	2.59	1.92
95% CI	0.90-3.69	0.41-1.16	0.48-1.80	0.48-14.95	0.30-12.21
RR	1.65	0.84	0.95	2.53	1.90
95% CI	0.96-2.85	0.66-1.71	0.58-1.55	0.58-11.06	0.39-9.21
p	0.072	0.139	0.831	0.200	0.342

OR-odds ratio; RR-relative risk; 95% CI-confidence interval; p-statistical significance for the assessed apo(a) relative risks between patients and control group

group. However, while scanning the NC membrane on the LKB Image Scanner, which was used to measure the apo(a) molecular isoform mass, we found that the mean arithmetic value of the S3 apo(a) isoform molecular mass (611.24 ± 46.29) in HD patients was statistically significantly higher compared to the healthy carriers of the same S3 apo(a) isoform (586.18 ± 20.85) ($p > 0.0065$). In

carriers of the double high molecular >S4S4 apo(a) isoform was found in higher percent (11.46%) in patients undergoing hemodialysis compared to the carriers of the same phenotype in the control group (6.72%). We also found low molecular S1 phenotype carriers both among the patients and among the healthy examinees, whereas low molecular B phenotype was found only in

the healthy individuals. The χ^2 -test analysis showed no statistically significant difference in the apo(a) isoform frequency between the two examined groups.

The role of the apoprotein (a) isoforms in the development of atherogenesis in patients undergoing hemodialysis is presented in Table 2 by estimating the relative risks. The values of certain apo(a) isoforms are a result of the sum of single apo(a) isoforms and the predominance of the double apo(a) isoforms.

From the assessed relative risks it can be concluded that none of the apo(a) isoforms is a risk factor in the development and progress of atherogenesis in patients undergoing hemodialysis compared to the relevant control examinees.

Discussion

The kidneys play an active role in the Lp(a) catabolism. This is why it is considered that the increased Lp(a) concentration in patients undergoing hemodialysis is primarily a result of the decreased clearance Lp(a), rather than the increased Lp(a) synthesis, regardless of the apo(a) phenotype [8]. The HD patients have an increased risk for atherosclerotic disease [1,9].

The frequency distribution of apo(a) isoforms for ESRD patients undergoing hemodialysis was very similar to the distribution in the healthy group which was confirmed with the χ^2 -test and which is in line with the finding of Parsons *et al.* [10].

In our study we have not found that apo(a) isoforms are a risk factor for atherosclerotic vascular diseases, and in particular, we have not found a relation between the low molecular apo(a) isoforms and CAD, which may be due to the small number of patients on hemodialysis, and low molecular apo(a) isoform carriers. The reference data are contradictory. According to the Choice Study conducted on 871 examinees on HD, there was no connection between apo(a) isoforms and CAD, particularly between the small apo(a) isoforms and atherosclerotic changes in these patients. However, in the same study, by applying the Cox Hazard Population Analysis it was found that patients of both races (white and black race representatives were included), the carriers of the low molecular apo(a) isoforms had a significantly shorter life [2,11]. Kronenberg *et al.* in a study including 404 randomly selected HD patients found that CAD was associated with low molecular apo(a) phenotype (RR-2.3, $p=0.0008$), but not with the high Lp(a) values in the patient's blood. According to them, the size of the apo(a) molecule and Lp(a) concentration have a synergetic role in the progress of atherosclerosis in its more advanced stage. In addition, they believe that apo(a) phenotype, which precisely reflects the Lp(a) level, may be considered to be a marker for those patients who have a more damaged vascular endothelium before the beginning of ESRD;

these patients may develop a "galloping" atherosclerosis to follow the beginning of ESRD, thus they need to be more aggressively treated with statins and other medicine which can decrease the Lp(a) level for at least a little in order to prevent undesired vascular changes [12].

Conclusions

Based on the results obtained we can say that apo(a) phenotype is not a risk factor for atherosclerotic vascular diseases in patients undergoing hemodialysis. It is most likely that the conventional risk factors (lipid parameters, increased blood pressure, smoking) are still the most responsible factors for ASVD in HD patients.

Conflict of interest statement. None declared.

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