Relationships between Biomarkers of Vascular Dysfunction and Peripheral Arterial Disease

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Abstract- *Introduction.* According to data in recent literature, adipocytokines are involved in endothelial dysfunction, pro- and anti-inflammatory protein factors. Inflammation appears to be involved at all stages of atherosclerosis. Peripheral arterial occlusive disease (PAD) can be considered as less studied manifestation of systemic atherosclerosis.

Matherial and Method. The study is performed on group of 75 patients with atherosclerotic PAD hospitalized at the Surgical Clinic No. II of Cluj-Napoca, Romania and a control group including an equal number of healthy patients. We examined markers: adiponectin and tumor necrosis factor-α (TNF-α), which are useful in an assessment of atherogenesis in PAD patients. Also, we studied single nucleotide polymorphism (SNP) of plasma adiponectin gene.

Results and Discussion. The results of our study showed that hipoadiponectinemia may be associated with atherosclerotic PAD. The SNP of plasma adiponectin was associated with decreased risk of atherosclerotic PAD. Linking data of our study, we can concluded that some of this parameters can be considerated biomarkers of vascular dysfunction.. If this is confirmed, these factors can be possibly considered as potential predictive factors, which open new fields of research.

Key words. Adiponectin, tumor necrosis factor-α, endothelial dysfunction.

I. INTRODUCTION

Atherosclerosis is the main pathological process that affects the arteries, being a degenerative disease characterized by the accumulation of cells and lipids in a conjunctive matrix at the level of the arterial intima. Lower limb peripheral arterial disease (PAD), one of the preferred localizations for atherosclerosis, affects 12% of the general population and 20% of individuals over 70 years of age. PAD has proven to be an independent risk factor for general mortality and especially for cardiovascular mortality.

II. RESEARCH HYPOTHESIS

The presence of inflammation-generating signs is an important risk factor in the initiation and evolution of atherosclerotic lesions in PAD. The inflammatory response is modulated by pro- and anti-inflammatory cytokines that play a critical role in the adjustment of the balance and in the occurence of PAD-related complications.

Adiponectin has an antiatherogenic effect on endothelial cells, inhibits the proliferation of vascular smooth muscle cells, and suppresses the conversion of macrophages to foamy cells. There is a general consensus about a putative protective role of adiponectin from an inflammatory state, at least at an endothelial-vascular level, although not uniquely (1,2).

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine released in large quantities in the case of obese or diabetic patients, also playing a role in the initiation and propagation of atherosclerotic lesions.

The human adiponectin gene, also known as: ACDC; ADPN; APM1;APM-1; GBP28; ACRP30; adiponectin; ADIPOQ, is located on the 3q27 chromosome, at a locus that is susceptible to the metabolic syndrome and type 2 diabetes. It is encoded by 3 exons and it stretches over a section of about 13 kb.(4,5) It displays a structure similar to the C_{1q} complement protein and to the TNF family, and it plays an important role in inflammation, the immune system, and atherosclerosis.

The +45 polymorphisms in exon 2 and +276 in intron 2 have often been associated with type 2 diabetes and obesity in the studies conducted on populations in Japan and the Caucasus. (6,7) The fragment studied by the author includes these polymorphisms (which have not been studied so far in the case of patients having PAD), with the purpose of establishing a possible correlation between their presence and the atherosclerotic peripheral arterial disease in the case of the studied individuals.

III. MATERIAL and METHOD

The study is an analytic observational case-control study, performed on group of 75 patients with atherosclerotic lower limb peripheral arterial disease (PAD) hospitalized at the Surgical Clinic No. II of Cluj-Napoca and a control group including an equal number of healthy patients.

The criteria for selection and inclusion in the study were in accordance with international consensuses and were based on clinical and biochemical data and imaging.

A clinical trial protocol was developed, in accordance with the ethical principles regarding research involving humans and approved by the Ethics Board of the institution where the research was conducted. The clinical trial protocol was applied identically to all patients. Participation in the trial was voluntary and confidentiality was absolute. All patients had tests performed in order to evaluate disease severity and possible dysfunctions of other organs.

Serum levels of adiponectin and TNF-α were measured by using the ELISA - sandwich test, using Quantikine® reagents (R&D Systems, USA): Human Adiponectin and Human HS TNF-α/TNFSF1A.

For the genetics study, the biological material consisted of blood samples collected from the batches of selected patients. The method by which genomic DNA is extracted from blood was represented by an automated protocol based on the use of the MagNA Pure LC DNA Isolation Kit I (Roche) in combination with the MagNA Pure LC (Roche) platform. The DNA quantification method was represented by the spectrophotometric method, using the NanoDrop UV VIS ND-1000 apparatus.

The PCR-RFLP analysis method involves a first stage of amplification via the PCR technique, followed by an amplicon digestion stage, specific to the RFLP reaction.

For the digestion of the amplified fragment via the specific primers and then the highlighting of the SNP +45 T/G, the conditions characteristic of the used enzime were taken into account, and namely AvaI (Fermentas). Similarly, for the detection of the SNP +276 G/T, the DNA fragments that resulted from the amplification via the specific primers were digested with the HinfI (Fermentas) enzime.

After testing all continuous variables for normality of data using Kolmogorov-Smirnov Test, data analyses were performed using Student T test or Mann-Whitney U Test, according to distribution. Normally distributed variables were expressed as mean± standard deviation, while non-normally distributed variables were expressed as median (minimum- maximum). Qualitative data was tested using Chi square/ Fisher Exact Test, based on standard application criteria. Results were considered significant for $p<0.05$. Statistical package SPSS 17.0 was use for all data analyses.

IV. RESULTS

Description of the descriptive variables:

1. Quantitative variables

The average age of the patients included in the first batch was 61 +/- 10,32 (standard deviation-SD), the minimum extreme being 41 and the maximum extreme – 87, while in the case of the second batch patients, the average age was 63+/-11,20 (SD), with the minimum extreme at 39 years of age and the maximum extreme at 80.

2. Qualitative variables

a. The distribution according to sex was as follows: 9 patients of the feminine sex (12%) and 66 patients of the masculine sex (88%), with a M:F sex ratio of 7.33/1 in the case of the patients of the PAD batch, and 11 patients of the feminine sex (14.66%) and 64 patients of the masculine sex (85.33%), with a sex ratio of 5.82/1, in the case of the patients of the witness batch, respectively. This data is consistent with that found in specialty literature, according to which men display lower limb atherosclerotic PAD more frequently than women.

Evaluation of risk factors: smoking, diabetes mellitus (DM), cardiovascular-associated diseases, coronary artery disease (CAD), arterial hypertension (AHT), hypercholesterolemia and obesity, by using anamnestic methods and paraclinical examinations which were then statistically processed, was the primary goal of our study. A percentage of 73.8% of the patients had at least one risk factor, and particularly: 30.78% DM; 56.39% AHT; 52.67% CAD, with a very high percentage (73.02%) of smokers.

Biochemical characteristics, including variations of adiponectin and TNF-α in the patients of the studied groups are presented in Table 1.

Table 1. Biochemical data for PAD patients and control study.

	Quantifier	PAD patients (n=75)	Controls (n=75)	P value
Total cholesterol (mg/dL)	mean±SD	186.2 ± 49.8	173.7± 50.8	0.275
HDL-cholesterol (mg/dL)	mean± SD	49.2 ± 19.54	47.7 ± 21.2	0.078
Triglycerides (mg/dL)	mean± SD	115.9 ± 45.8	129.0 ± 89.3	0.849
Fasting glucose (mg/dL)	mean± SD	109. 7± 56.8	118.2 ± 58.4	**0.051**
Adiponectin (pg/ml)	median (range)	1170.0 (17-3957)	1267.0 (69-4510)	**0.041**
TNF-α (ng/ml)	median (range)	2,184 (0.487-3,490)	0.896 (0.337-2,589)	0.010

For TNF-α, the normal in serum values were considered those between 0.550–2,816 pg/ml, and for adiponectin 865-21,424 ng/ml. The higher mean value of TNF-α in the case of the patients included in the PAD group (2,184 pg/ml) as compared to that of the control group (0.896 pg/ml) led to distinctly significant differences (p=0.01).

The median value of adiponectin in patients included in the PAD group was of 1170.0 (17-3957)pg/ml, as compared to 1267.0 (69-4510) pg/ml in the control group ($p<0.05$).(Fig.1)

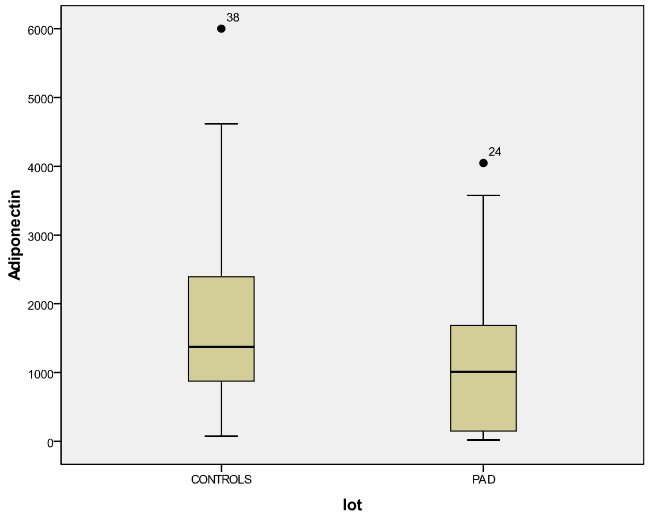

Fig. 1. Diagram of variation (boxplot) of adiponectin values compared in patients with PAD and control subjects.

Within the genotypical study of PAD patients, after the extraction of genomic DNA from the blood, the quantification of the DNA, the design of specific primers (the results being analyzed with the aid of a computer), in order to define the experimental strategy with the purpose of identifying the SNP in the ADIPOQ, the human adiponectin gene was studied, namely the +45 polymorphisms in exon 2 and +276 in intron 2.

SNP +45 T/G

The amplified fragment had a length of 305 pb, while the primers used (3) were:

- The sense primer (F) 5'- TGT GTG TGT GGG GTC TGT CT-3' and the antisense primer (R) 5'-TGT GAT GAA AGA GGC CAG AA-3

SNP +276 G/T

The amplified fragment had a length of 110 pb, while the primers used (3) were:

- The sense primer (F) 5'- CTA CAC TGA TAT AAA CTA TAT GGA G -3' and the antisense primer (R) 5'-CCC CAA ATC ACT TCA GGT TG -3'

The BLASTN analyses have confirmed the fact that the sequence of the PCR product corresponds to the DNA sequence identified in the NCBI gene bank. In the analyzed population, in the case of loci rs59775053, rs1501299, rs62625693, rs13061862, and rs62622816, only one allele was identified, while in the case of loci rs2241767 and rs2241766, two alleles were identified.

V. DISCUSSION

Serum levels of adiponectin in the our PAD patients are significant lower, and the level of TNF-α significant higher, than the levels in the control subjects, which suggests the role of hypoadiponectinemia and TNF-α in the development of PAD.

In order to genotype the patients with atherosclerotic PAD, alongside the method based on the sequencing of the DNA of interest, the PCR-RFLP method was also used, the latter costing less and being easier to realize, but having a lower confidence value. The frangments amplified with the specific primers had 305 pb in the case of SNP +45 T/G and 110 pb in the case of SNP + 276 G/T.

The analyses that were conducted allowed the identification of the SNPs of interest at the level of the human adiponectin gene and, at the same time, the highlighting with greater precision of the modifications present at the nucleotidic sequence level. By comparing the data obtained as a result of the PCR-RFLP analyses with the data obtained as a result of sequencing, it has been proven that the methods were employed correctly.

Thus, one may conclude that the recent progress in the field of genomics is extremely promising as far as the transformation of the medical practice in cardiovascular pathology is concerned – arguments that have prompted the author to undertake the present study. Despite the important therapeutic progress achieved in the last few years, the clinical consequences of PAD justify the constant efforts in the field of research.

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REFERENCES

1. Gualillo O, González-Juanatey JR, Lago F. The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives.Trends Cardiovasc Med. 2007 Nov;17(8):275-83.
2. Lago F, Diéguez C, Gómez-Reino J, Gualillo O: The emerging role of adipokines as mediators of inflammation and immune responses. Cytokine Growth Factor Rev 2007; 18:313–325.
3. Mackevics, V., Heid, I.M., Wagner, S.A., Cip, P., Doppelmayr, H., Lejnieks, A., Gohlke, H., Ladurner, G., Illig, T., Iglseder, B., Kronenberg, F., Paulweber, B. The adiponectin gene is associated with adiponectin levels but not with characteristics of the insulin resistance syndrome in healthy Caucasians. Eur. J. Hum. Genet., 2006, 14, 349-356.
4. Takahashi, M., Arita, Y., Yamagata, K., Matsukawa, Y., Okutomi, K., Horie, M., Shimomura, I., Hotta, K., Kuriyama, H., Kihara. S., Nakamura, T., Yamashita, S., Funahashi, T., Matsuzawa, Y., Genomic structure and mutations in adipose-specific gene, adiponectin, Int J Obes Relat Metab Disord. 2000, 24(7):861-8.
5. Vionnet, N., Hani, E.H., Dupont, S., Gallina, S., Francke, S., Dotte, S., De Matos, F., Durand, E., Leprêtre, F., Lecoeur, C., Gallina, P., Zekiri, L., Dina, C., Froguel, P., Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24, Am J Hum Genet 2000, 67(6):1470-80.
6. Hara, K., Boutin, P., Mori, Y,. Tobe, K., Dina, C., Yasuda, K., Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population, Diabetes 2002, 51:536-540.
7. Vasseur, F., Helbecque, N., Dina, C., Lobbens, S., Delannoy, V., Gaget, S., Single-nucleotide polymorphism haplotypes in the both proximal promoter and exon 3 of the APM1 gene modulate adipocyte-secreted adiponectin hormone levels and contribute to the genetic for the type 2 diabetes in French Caucasians, Hum Mol Genet 2002, 11, 2607-2614.