Title: Early and global estimation of microvascular target organ damage in hypertension by use of innovative software

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Disclosure: none

Conflicts of interest: none

Key Words: hypertension, target organ damage, microcirculation, vessels, retinopathy, aldosterone, cardiovascular risk

Abstract

In the cardiovascular disease arena, the central role of microcirculation has been verified, and research towards global cardiovascular risk assessment and early

identification of hypertensive target organ damage is continuously expanding. In this concept, quantitative microcirculation measures have been developed, applied to the easily accessible arterioles and venules of the retina, the skin and the kidney. Indeed, subtle retinal vascular alterations, capillary rarefaction and microalbuminuria have been each associated with increased risk of cardiovascular mortality. However, data regarding the concomitant presence of microvascular lesions in the above target organs in the early stages of hypertension, association of the number of affected organs with cardiovascular risk, and the effect of aldosterone on multiple target organ damage are lacking. We therefore studied consecutive naïve, never-treated patients attending the Hypertenion Unit of our Department with recent duration of hypertension (<1 year), confirmed with 24-hour ambulatory blood pressure monitoring, and healthy volunteers. Innovative, semi-automated software was specifically developed by our Hypertension Unit and the Institute of Computer Science, Foundation for Research and Technology–Hellas (FORTH) to estimate retinal vascular diameters obtained by retinal photography and capillary density obtained by nailfold capillaroscopy photography. The Framingham Risk Score was used to determine future cardiovascular risk. Biochemical parameters including serum aldosterone were also derived. A total of 118 subjects, 77 hypertensives and 41 normotensives, were included. Hypertensive patients exhibited a significantly greater number of affected target organs compared to normotensives ($p=0.018$), with retinopathy representing the most common target organ among hypertensives. The number of microcirculation target organ damage was linearly correlated with increased Framingham score ($r=0.276$, $p=0.015$). Aldosterone levels linearly correlated ($r=0.398$, $p<0.001$) and significantly predicted ($p=0.03$) the number of microcirculation target organ damage even after adjustment for other variables. Physicians dealing with the hypertensive patient should be aware of the possibility of diffuse microvascular impairment and seek for multiple target organ damage even in the early stages of hypertension, in order to apply appropriate treatment and decelerate its progression towards cardiovascular disease.

Introduction

Hypertension and its cardiovascular complications represents the leading cause of morbidity and mortality worldwide and has been acknowledged as the most important reversible risk factor for cardiovascular diseases (1). In this concept, assessment of global cardiovascular risk and early identification of hypertensive target organ damage emerges as extremely important in terms of life prolongation, quality-of-life improvement and health-care resources sparing.

A novel concept for assessing global cardiovascular risk in hypertension is implementation of quantitative microcirculation measures in everyday clinical practice. Of the whole framework of small vessels spread through all internal and external organs in the human body, the retina, the skin and the kidney represent an open and easily accessible window for the in-vivo study of microcirculation.

In experimental data derived from both animals and humans, hypertension was found to coexist with changes in small vessels of all the above organs. Capillary rarefaction was observed in the skin; several qualitative and quantitative alterations have been described in retinal arterioles and venules, while increased excretion of albumin in urine is traditionally used to identify early kidney damage in hypertension. The predictive value of the above microcirculation measures in terms of cardiovascular morbidity and mortality has been validated in several studies.

Recently, we showed for the first time that quantitative, more detailed signs of retinal vascular alterations are present even in untreated, otherwise healthy, recently diagnosed hypertensives as well as in two very important pre-hypertension phenotypes such as masked and white coat hypertensives, compared to normotensive individuals (2;2). Of great significance, hypertensive retinopathy alterations are associated not only with blood pressure but also with other markers of hypertensive target-organ damage, such as microalbuminuria, renal impairment and left ventricular hypertrophy (3), as well as with established cardiovascular complications, such as stroke(4) and myocardial infarction (5). Preliminary results of our work also correlate subtle retinal alterations with increased arterial stiffness, another independent marker of macrovascular damage (6).

Regarding the capillary network, of the several abnormalities known to occur in essential hypertension, capillary rarefaction appears as the most important (7).

Capillary rarefraction in essential hypertension may be present in the early stages of hypertension, often preceding its development (8;9). Last but not least, presence of microalbuminuria as another form of target organ damage in hypertensive patients increased their cardiovascular risk, when compared to patients with similar blood pressure and no albuminuria(10).

Although the above microcirculation alterations have been identified as different forms of target organ damage in hypertensive patients, little is known on the co-existence of these lesions in the early stages of hypertension compared to normotensives individuals, or their association with cardiovascular risk proposed by the Framingham risk score model. In addition, there is a lack of data regarding their association with other factors implicated in the pathogenesis hypertensive vascular disease and the development of target organ damage, including activation of the renin-angiotensin-aldosterone system. Of the utmost significance, investigation of the clinical meaning of microvascular target organ damage largely lies on the development of the essential technology that will allow the clinician to visualize retinal and skin vessels rapidly and non-invasively and obtain robust microcirculation quantitative measures.

Aim

Therefore, the aim of this study was:

1) to determine **the relationship of high blood pressure with the whole spectrum of microcirculation alterations**, through the simultaneous investigation of the status of small vessels of the kidney, the eye and the skin

2) to examine if combined microcirculation damage represents **a stronger predictor of increased cardiovascular risk** estimated with the Framingham risk score

3) to investigate a possible **association between presence of different forms of target organ damage and activation of renin-angiotensin-aldosterone system,**

in a group of meticulously selected, naïve, never-treated hypertensive patients and normotensives individuals, confirmed by 24-hour ambulatory blood pressure monitoring (ABPM).

Methods

-Participant characteristics

Consecutive patients attending the Hypertension Unit of the 2nd Propedeutic Department of Internal Medicine, Aristotle University, Thessaloniki, were included in the study. All subjects were Caucasian and gave written informed consent. The study was approved by the Ethics Committee of our University and was conducted in accordance with the principles of the Helsinki declaration. Participants had never been treated with anti-hypertensive agents and had no other known health problems. Only patients with recent appearance of hypertension (home BP measurements within normal limits during the previous year) were included. The group of normotensives was recruited from subjects admitted for regular check-up. Patients with secondary causes of hypertension and other comorbities, diagnosed through medical examination and laboratory tests, were excluded.

-Blood pressure measurements

After 10 minutes in rest, office BP was measured using standard methodology, and was determined as the mean of the second and third value of three consecutive BP recordings taken at a 2-minute interval. Hypertension was defined as office BP (oBP) higher than 140/90 mmHg according to guidelines (Seventh Report of the Joint National Committee (11) and European society of Hypertension(ESH)-European Society of Cardiology(ESC)) (12). ABPM was then performed using a SpaceLabs 90207 device according to a standard protocol. Blood pressure was measured at 15-minute intervals during a usual working day and 30-minute intervals during the night. Only ABPM assessments that achieved a minimum of 70% of successful readings were regarded as technically sufficient. Hypertensive patients according to the ABPM were those with a daytime ABPM≥135/85 mmHg. Only patients with true hypertension (oBP≥140/90 mmHg and ABPM≥135/85 mmHg) or confirmed normotension (oBP<140/90 mmHg and ABPM<135/85 mmHg) participated in the protocol (12).

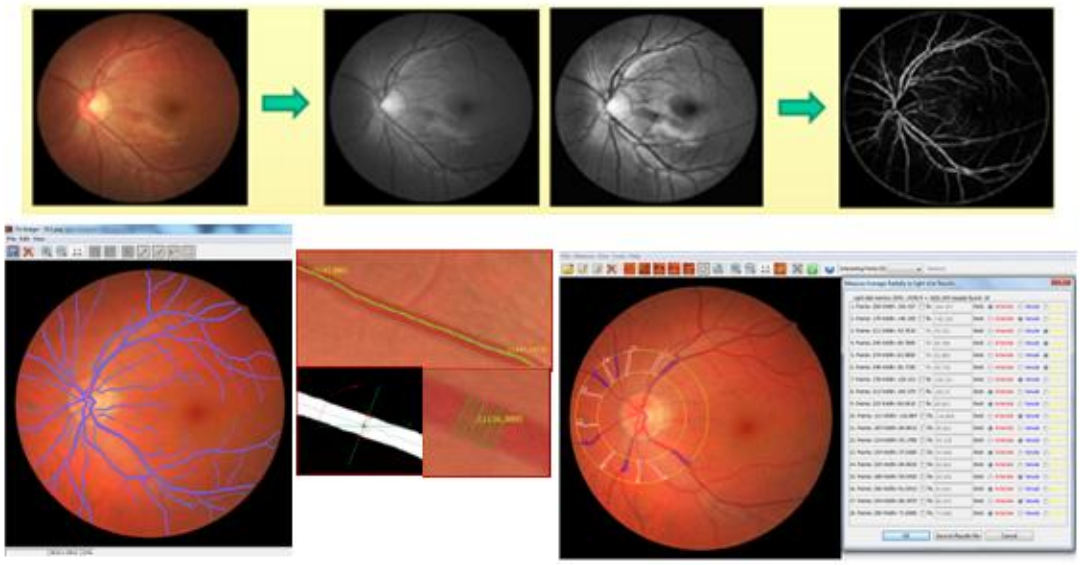
Microcirculation assessment

-Retinal photography and analysis

All patients underwent a bilateral, non-mydriatic digital fundus photography using a NIDEK AFC-230/210 camera. Two photographs were obtained from each eye and the best one was examined by a trained grader masked to the subjects' identity and BP group assignment.

To achieve retinal vessels measurement and analysis a semi-automated computer software was developed by our Hypertension Unit and the Institute of Computer Science, Foundation for Research and Technology–Hellas (FORTH)(13;14), to assess retinal photographs according to a standard protocol, Figure 1. The software was comprised of a measurement module, which estimates vessel diameter in the input images, and a Graphical User Interface module, which facilitates user intervention at points of interest. Employing image processing methods, retinal vessels are segmented and distances of the vessel boundaries from the medial axes at any given point are estimated. After estimation of the mean diameter of each vessel, the software allows vessel representation editing, in order to correct segmentation errors. The measurement area was defined as the area from one half to one disc diameter from the optic disc margin. Parr and Hubbard formulas, as modified in the Atherosclerosis Risk in Communities (ARIC) protocol (15) were calculated automatically to summarize indices of the average retinal arterioral and vascular diameters, referred to as the central retinal artery (CRAE) and central retinal vein (CRVE) equivalent respectively(15;16). Arteriovenus ratio (AVR) was calculated as the ratio of these (CRAE/CRVE). The accuracy (0.937-0.932), the sensitivity (0.741-0.712) and the specificity (0.967-0.966) of this method are very satisfactory and have been previously described in detail(14). Intra- and inter- rater variability (from the analysis of 20 retinal photographs) was 0.823 and 0.798, respectively (2).

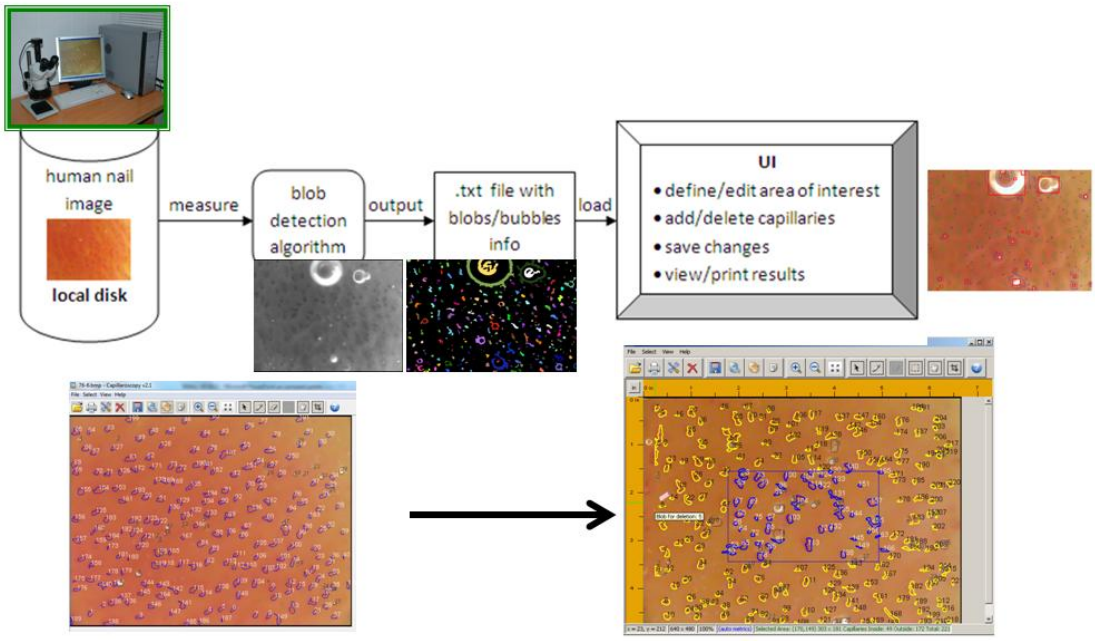
Figure 1. *Retinal vessels caliber measurement software*



-Capillaroscopy photography and analysis

All participants were examined with nailfold capillaroscopy (DS Medica, Milan Italy – 200 x magnification). A semi automated software, for the detection and measurement of the capillaries in each image, was developed by our Hypertension Unit and FORTH(17) (figure 2).

Figure 2. *Capillary density measurement software*



The software was comprised by a measurement module and a Graphical User Interface module. The measurement module segments capillaries in the input image. The user interface provides methods for the automatic and interactive measurement of capillaries at regions of interest. In addition, it provides methods for editing the obtained capillary representation, in order to correct segmentation errors.

For the specific protocol the best two microscopic images were chosen and examined by a grader masked to the subjects' identity and BP group assignment. The numbers of the capillaries were recorded both in an entire visual field as well as in the area with the greater density per quadrant, which was automatically detected by the software. At least two measurements from each participant analyzed both automatically by the software and semi-automatically by a trained operator, were used in the analysis.

-Biochemical measurements

The determination of microalbuminuria was made by immuno-turbidimetric method in samples of 24-hour urine collection, which is considered the most reliable method of microalbuminuria estimation (18). Microalbuminuria was defined as an albumin excretion rate between 30-300 mg/24 h.

Plasma renin activity (PRA) (ng/mL/h) and serum aldosterone (ng/dl) levels were estimated by radioimmunoassay method. Patients lied in the supine position for two hours before blood sampling, which was performed in the morning between 0800 and 1000h.

-Framingham risk score

The Framingham Risk Score is a scoring system used to determine an individual's 10-year cardiovascular risk. In this study we used the Medical college of Wisconsin Calculator based on age, sex, smoking, diabetes, blood pressure and cholesterol level (19). Information was applicable only for the ages 30 -74. All participants were categorized in individuals with normal or increased cardiovascular risk, by comparison to the lowest 10 year risk of their relevant age group.

Statistical analysis

Analysis was performed using the Statistical Package for Social Sciences (SPSS) 19. Student t or Mann Whitney test was used to estimate differences between mean values between two groups and Anova, with Bonferroni multiple comparisons test when was necessary, between more than 2 groups. Analysis of qualitative variables was made by Chi Square or Kendals tau b test when the categories were more than 2. Correlation coefficients were calculated with Pearson and Spearman rank tests. To explore the relationship between the number of microcirculation target organ damage forms and aldosterone, while controlling for other covariates, we applied multivariate linear regression analysis. For retinal and capillary parameters, the first 2 quartiles of a population consisting of 250 otherwise healthy, except for the high BP (data not yet published), individuals were considered as normal values. Where needed to transform a non to normal distribution we used the logarithmic mean of the parameter. A probability value of $p \leq 0.05$ was considered statistically significant.

Results

In total, 118 subjects with a mean age of 42.9±11.6 years were included in the study. According to their office and ABPM, 77 participants were classified as hypertensives and 41 comprised the normotensive-control group. Baseline demographic and clinical characteristics of the study population are depicted in Table 1.

Table 1. Baseline characteristics of the study population.				
	Overall (n=118)	**Hypertensives** (n= 77)	**Normotensives** (n=41)	**p value**
Age, years	42.9±11.6	43.3±11.4	42.4±11.9	0.434
BMI, Kg/m^2	27.2±4.3	27.7±4.1	26.1±4.7	0.112
Sex (MALE%)	64.4	76.6	41.5	<0.001
Smoking (% yes)	39.5	41.6	35.1	0.511
SBP, mmHg	138.8±19.4	149.9±13.8	118.1±9.4	<0.001
DBP, mmHg	88.0±13.7	94.8±10.8	75.4±8.8	<0.001
24hSBP, mmHg	131.2±14.9	139.7±10.3	115.5±7.7	<0.001
24hDBP, mmHg	83.0±11.1	88.5±9.3	72.8±5.3	<0.001
Day SBP, mmHg	136.7±15.5	145.6±10.4	120.2±7.7	<0.001
Day DBP, mmHg	87.4±11.1	92.9±9.3	77.2±5.4	<0.001
Night SBP, mmHg	118.8±15.6	126.8±12.6	104.3±8.5	<0.001
Night DBP, mmHg	73.2±11.8	78.2±11.1*	64.6±6.5	<0.001
Plasma renin activity (ng/mL/h)	0.51(0.01-4.96)	0.93(0.01-4.96)	0.82(0.01-2.90)	0.661
Aldosterone (ng/dl)	10.4(2-34)	12.5(2-34)	7.5(2.2-27.4)	0.004
SBP: Systolic blood pressure, DBP: Diastolic blood pressure, BMI: Body Mass Index				

1) Prevalence of different microcirculation target organ damage in hypertensive and normotensive individuals

Prevalence of target organ damage per blood pressure status (hypertension, normotension) is depicted in Figures 3 and 4. Hypertensive patients exhibited a significantly greater number of affected target organs compared to normotensives (p=0.018). Only 19.3% of hypertensive patients were free from target organ damage, compared to 35.3% of normotensives (Figure 4). Retinopathy was the most common target organ damage in hypertensive patients (52.6%), whereas capillary rarefraction was the most frequent target organ damage in participants with normal blood pressure (44%).

Figure 3. *Concomitant presence of different forms of target organ damage per hypertension status*

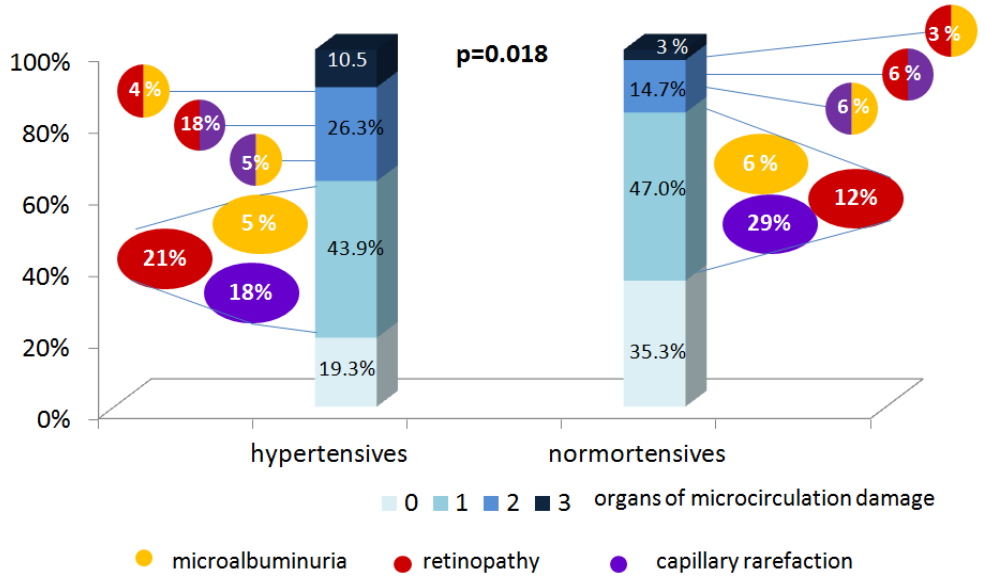
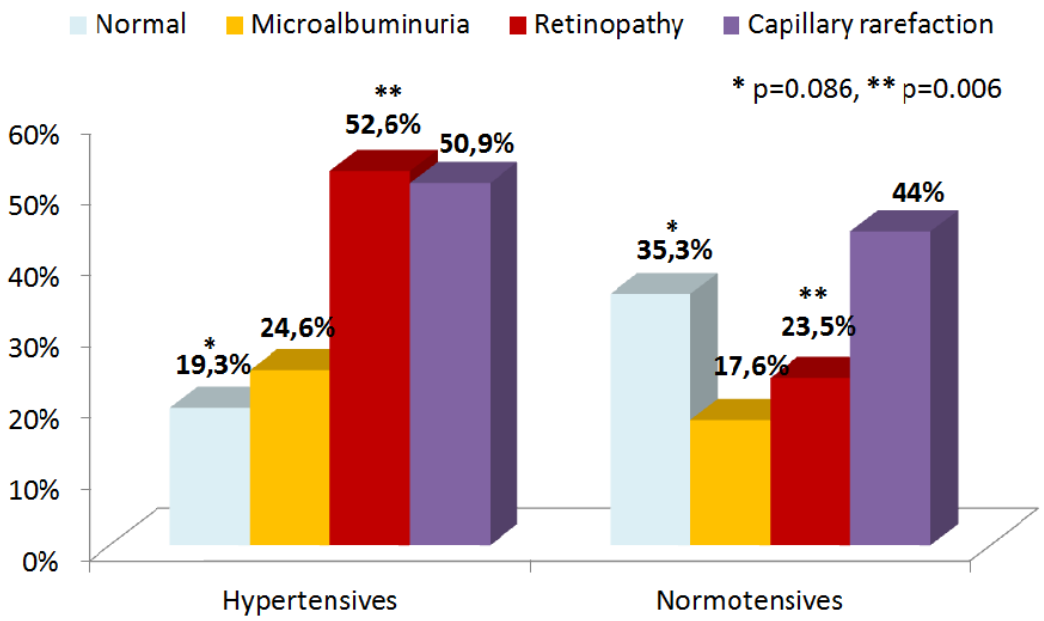


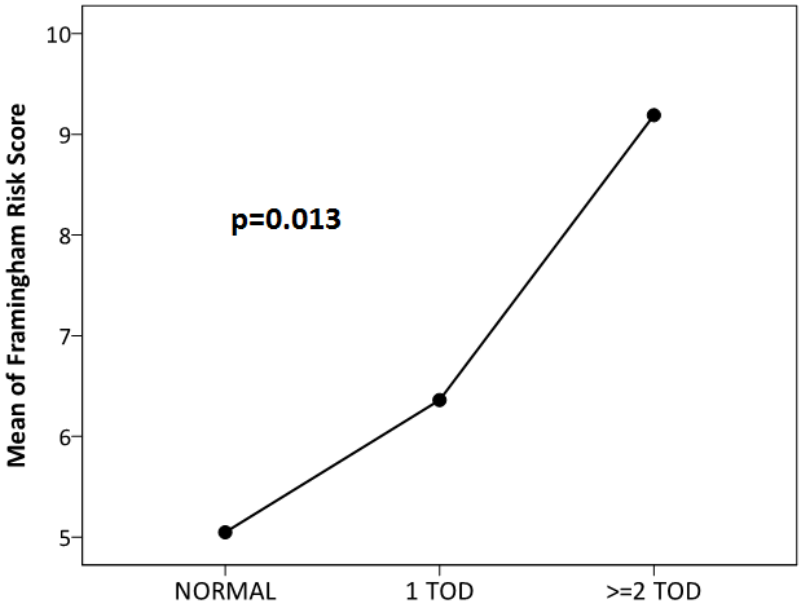
Figure 4. *Prevalence of different forms of microcirculation target organ damage in hypertensive and normotensives participants*



2) Association between number of affected target organs and cardiovascular risk prediction estimated by the Framingham risk score

Increase in the number of microcirculation target organ damage was linearly correlated with increased Framingham score in our sample (spearman test, r=0.276, p=0.015). Moreover, individuals with multiple TOD had higher Framingham Risk Score (Anova with Bonferroni correction post hoc tests, p=0, 013), as depicted in Figure 5.

Figure 5. *Cardiovascular risk score according to microcirculation TOD*

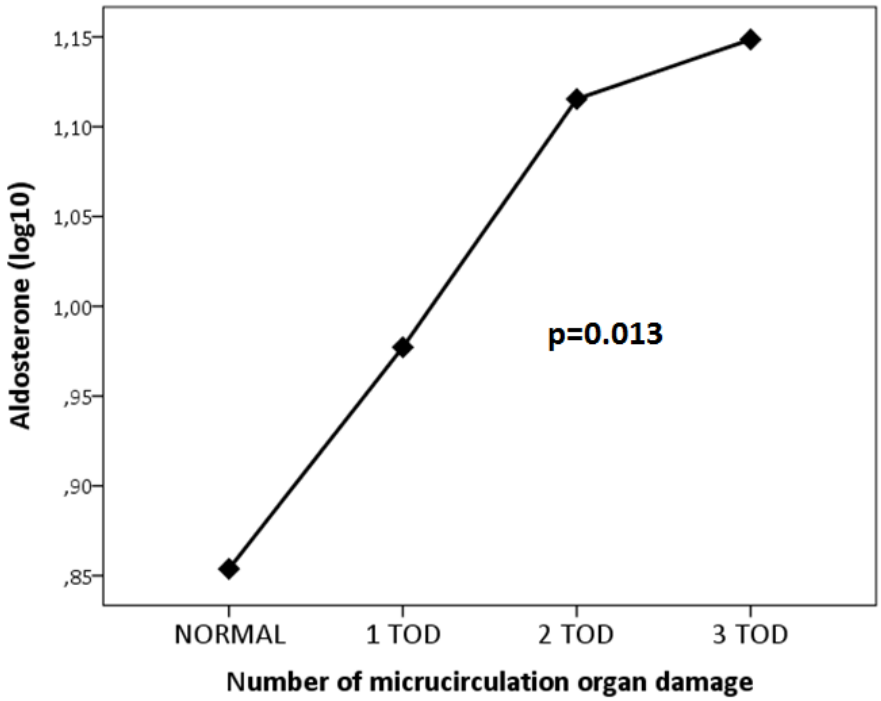


3) Interaction between microcirculation damage and the RAAS

Plasma renin activity was not significantly associated with the number of affected organs in our population.

On the contrary, aldosterone levels were increasing (figure 6), and linearly correlated (r=0.398, p<0.001), in accordance with the number of microcirculation target organ damage.

Figure 6. *Aldosterone levels according to microcirculation TOD*



Furthermore, multiple linear regression analysis revealed that aldosterone remained a significant predictive factor of the number of microcirculation TOD after the adjustment for age, smoking, BMI, office and ambulatory BP (table 2).

Table 2. Multiple linear regression models of microcirculation TOD			
Microcirculation TOD-Adjusted R Square=0.203, R Square=0.279, p=0.004			
	Unst. C.	**Stand. C**	**p**
Age(years)	-0.004	-0.062	0.697
Smoking	0.124	0.070	0.549
BMI (Kg/m^2)	-0.004	-0.019	0.878
Office BP (DBP) mmHg	-0.002	-0.033	0.812
Ambulatory BP (night SBP) mmHg	0.021	0.380	0.006
Aldosterone (ng/dl)	1.002	0.284	0.030
BMI: Body Mass Index, BP: Blood Pressure, DBP: Diastolic BP, SBP: Systolic BP			

Discussion

To our knowledge, this is the first study investigating the concomitant presence of different forms of microvascular target organ damage (capillary rarefaction, impaired retinal diameter calibers and microalbuminuria) in a series of “naïve”, never-treated, true hypertensive patients with only recently established hypertension, compared to their normotensive healthy individuals. Even in the very early stages of arterial hypertension (within just one year of elevated BP), microcirculation damage is more prevalent (80.7% versus 64.7%) and present in a greater number of organs (10.5% versus 3% in all of the examined organs) compared to their normotensives counterparts. The clinical interpretation of this finding is of paramount importance. Physicians dealing with the hypertensive patient should be aware of the possibility of diffuse microvascular impairment and seek for multiple target organ damage even in the early stages of hypertension, to decelerate its progression.

Of equal or even greater importance, we showed that the number of the affected organs is linearly correlated with increased Framingham risk score (Figure 5). In other words, identification of multiple target organ damage with our software denotes risk assessment of subsequent development of cardiovascular disease. Whether aggressive hypertension treatment aiming at the reversal of the examined microvascular damage would lead to cardiovascular risk reduction, remains to be investigated in future studies.

Of the examined target organs, the retina appears to be mostly affected by high blood pressure. The deleterious effects of hypertension on the retinal microcirculation are overt in more than half of hypertensive population compared to only 23.5% of normotensives ($p=0.006$). Capillary rarefaction comes next in hypertensive patients, affecting 50.9% of hypertensives, while it represents the most frequently encountered form of microvascular impairment in normotensive individuals. The increased frequency of capillary rarefraction in hypertensive patients may be at least partially explained by the fact that capillary rarefaction often precedes the development of hypertension (8;9), as implied by the increased portion

of normotensives exhibiting the same target organ damage (Figure 4). Both capillary rarefaction and microalbuminuria followed the same pattern of increased prevalence among hypertensive patients compared to individuals with normal blood pressure, but differences failed to reach statistical significance maybe due to the sample size.

While hypertensive vascular disease has been traditionally conceived as a result of altered hemodynamics, several factors are now implicated in the development of adverse structural and functional changes within the vessel wall in the continuous effect of high blood pressure. Of them, aldosterone has been shown to exert a direct effect on the cardiovascular system, mediating myocardial fibrosis and the accumulation of collagen fibers and growth factors in the arterial wall, eventually leading to remodeling of hypertensive vessels, vascular injury and target organ damage (20). We showed for the first time that aldosterone levels significantly and linearly correlate with the number of the affected organs. In addition, aldosterone was identified as a significant predictor of the number of affected organs even after adjustment for other factors. This is a hypothesis-generating result, suggesting a common pathophysiological pathway for the simultaneous development of multiple target organ damage in the early stages of essential hypertension, in which aldosterone-mediated effects play a prime role.

There are significant strengths in the present study. First, the meticulous selection of our study population (naïve, otherwise healthy, never-treated hypertensive patients with hypertension onset within a year confirmed by 24-hour ABPM and healthy volunteers) allows the assessment of net blood pressure effect on target organs, independently of other cardiovascular risk factors and diseases. Most importantly, innovative software for microcirculation assessment was developed and applied. Easy and prompt applicability of such non-invasive methods in the everyday clinical practice is a prerequisite for the conduction of large studies investigating the impact of microvascular alterations on the cardiovascular system and their subsequent wide use within the clinical setting. Our software was specifically designed to serve this purpose. Retinal (obtained by fundus camera) and nailfold

capillaroscopy (obtained by a simple capillaroscope) image analysis with our software offers a novel non-invasive measurement of early changes in the vasculature, not detectable on routine clinical examination until now, that may allow the identification of individuals at risk for subsequent cardiovascular complications.

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