

Association between skin lymphangiogenesis parameters and arterial hypertension status in patients: An observational study

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Abstract

Background. Recent studies have indicated that the skin lymphatic system and interstitium may play a role in the pathophysiology of arterial hypertension (AH).

Objectives. We aimed to determine whether the set of pathway parameters described previously in rodents would allow for the distinction between hypertensive and normotensive patients.

Materials and methods. Molecular and histopathological parameters from the skin and blood of patients with AH (AH group, n = 53), resistant AH (RAH group, n = 32) and control (C group, n = 45) were used, and a statistical multivariate bootstrap methodology combining partial least squares–discriminant analysis (PLS–DA) and selectivity ratio (SR) were applied.

Results. The C vs RAH model presented the best prediction performance (AUC test = 0.90) and had a sensitivity and specificity of 73.68% and 83.33%, respectively. However, the parameters selected for the C vs AH group model were the most important for the pathway described in the rodent model, i.e., greater density of the skin lymphatic vessels (D2–40 expression) and greater number of macrophages (CD68 expression), higher expression of the messenger ribonucleic acid (mRNA) of nuclear factor of activated T cells 5 (NFAT5), vascular endothelial growth factor C (VEGF–C) and podoplanin (PDPN) in the skin, greater concentration of hyaluronic acid (HA) in the skin, and lower serum concentration of VEGF–C.

Conclusions. Our study suggests that the NFAT5/VEGF–C/lymphangiogenesis pathway, previously described in rodent studies, may also be present in human HA. Further experiments are needed to confirm our findings.

Key words: lymphangiogenesis, chemometrics, salt-sensitivity, nuclear factor of activated T cells 5 (NFAT5), vascular endothelial growth factor C (VEGF–C)

Background

The lymphatic system is complementary to the circulatory system, and its principal function is to maintain fluid homeostasis and prevent tissue edema.¹ There is growing evidence that the lymphatic system of the skin interstitium may also play an essential role in the pathophysiology of arterial hypertension (AH).^{2–5}

Previous rodent studies revealed that sodium ions (Na^+) accumulate in the skin in an osmotically inactive form, binding to glycosaminoglycans (GAGs).^{6–9} The ^{23}Na magnetic resonance imaging (^{23}Na -MRI) studies confirmed that Na^+ is also stored in human skin and (in smaller quantities) in muscles.^{10,11} The increased skin Na^+ content in rodents resulted in more intensive GAGs skin synthesis,² polymerization and degree of sulfation.^{9,12,13} A higher concentration of Na^+ in the skin of rodents resulted in hypertonic stress and subsequent stimulation of a pathway that started with infiltration of the skin by macrophages¹⁴ and activation of nuclear factor of activated T cells 5 (NFAT5).¹⁵ The next step was macrophage secretion of vascular endothelial growth factor C (VEGF-C)¹⁴ and activation of lymphangiogenesis in the skin.² Blocking the macrophages – VEGF-C – lymphangiogenesis axis in the skin of rodents fed a high-sodium diet by genetic or pharmacological interventions resulted in salt-sensitive AH.^{3,4}

The regulatory pathway proposed in the experimental rodent model may be protective in AH. Vascular endothelial growth factor C, as the main stimulator of lymphangiogenesis, is also a regulator of the lymphatic vessels' pump activity¹⁶ and a known inducer of the endothelial nitric oxide synthase (eNOS) expression, associating with the vasodilation of blood vessels and, potentially, with lowering blood pressure via NO elevation.^{2,13,17} Moreover, newly formed lymphatic vessels might enable the removal of Na^+ and water from the skin interstitium.^{2–5,18,19}

Our previous studies demonstrated that the lymphatic system and the skin interstitium may also play a role in the pathophysiology of AH in humans. In our 1st study, we demonstrated that the hypertensive and control groups did not differ in the skin concentration of Na^+ , that a higher skin concentration of Na^+ was associated with increased water content, and that the patients with resistant hypertension had a higher number of macrophages in the skin and lower concentration of serum VEGF-C than the control group.²⁰ Our 2nd study showed that the patients with hypertension and a higher concentration of Na^+ in the skin had a significantly greater density of skin lymphatic vessels, and that skin water content correlated with factors associated with lymphangiogenesis in the skin, i.e., messenger ribonucleic acid (mRNA) expression of NFAT5, VEGF-C, and podoplanin (PDPN), and number of macrophages (expression of CD68).²¹ We managed to separate a group of patients with AH that most closely matched the experimental rodent model of salt-sensitive hypertension in the histological and molecular assessment (it was the group of patients

with AH and a high Na^+ concentration in the skin), and there were some significant relationships among the parameters important in the pathway described in rodents. However, the direct differences in the individual parameters important for this pathway between hypertensive and normotensive groups were mainly insignificant.²¹

In the present study, we addressed whether it is possible to determine the sets of parameters important for the NFAT5-VEGF-C-lymphangiogenesis pathway to distinguish hypertensive from normotensive patients. Mathematically, this can be done using a multivariate discriminant analysis.^{22,23} Therefore, we applied a multivariate bootstrap method that combined partial least squares-discriminant analysis (PLS-DA) and the selectivity ratio (SR).

Objectives

To determine whether the pathway parameters previously described in rodents would allow for distinguishing hypertensive and normotensive patients.

Materials and methods

Study design

Molecular and histopathological parameters from the skin and blood of patients with AH and without AH (control group) were used, and a statistical multivariate bootstrap methodology combining PLS-DA and SR was applied.

Participants

Patients hospitalized in the Department of Surgery in the 4th Military Hospital in Wrocław (Poland), with and without AH, who had undergone elective surgery with an abdominal skin incision, were enrolled in the study. The participants represent a continuous population of those with planned surgical intervention of abdominal skin incisions. Among the most common indications for abdominal surgery were abdominal aortic aneurysm, Leriche's syndrome, gallstones, and abdominal hernia.

The exclusion criteria were secondary hypertension, diabetes mellitus, kidney failure, and a body mass index (BMI) $>40 \text{ kg/m}^2$.

To ensure that the groups did not differ in age, we removed patients under or equal to 50 years from the control group. We also removed 1 patient with a BMI of 42.9 kg/m^2 because this patient was enrolled in our study incorrectly (BMI $> 40 \text{ kg/m}^2$ was an exclusion criterion). Additionally, we removed 1 patient with a BMI of 38.1 kg/m^2 from the AH group because this BMI value was significantly higher than the BMI values in all other patients. The study groups did not differ in sex. The measurements of 1 control patient (patient No. 46 from the control group) were

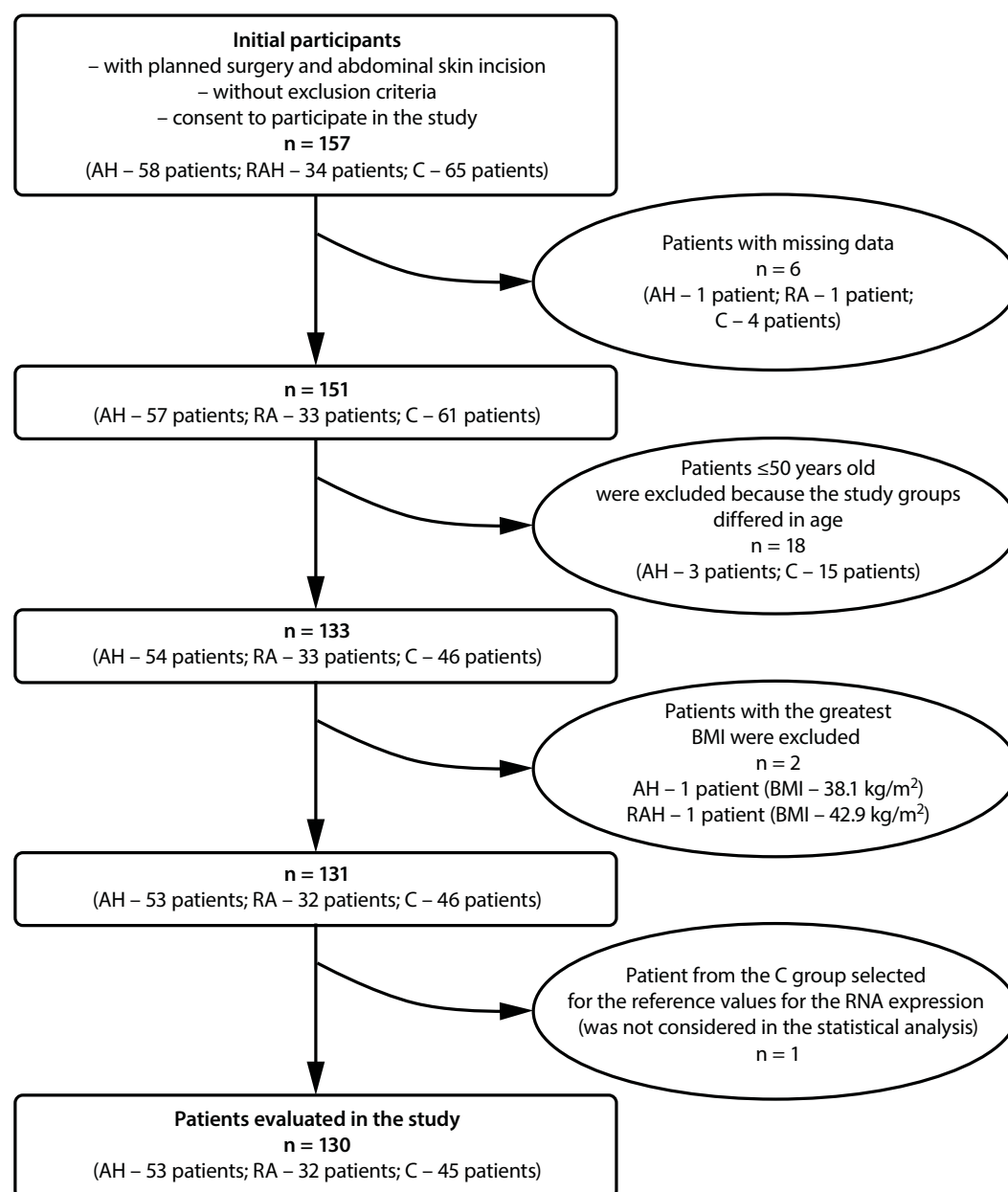


Fig. 1. Patient selection flowchart

AH – group with arterial hypertension; RAH – group with resistant arterial hypertension; C – control group; BMI – body mass index.

selected as the reference values for estimating the RNA expression parameters. This patient was not considered in the statistical analysis in this study. A flowchart of patient selection is presented in Fig. 1.

The Bioethical Commission of Wrocław Medical University approved the study protocol (approval No. KB-578/2012). All of the participants were informed about the purpose of the study, and written informed consent was signed by every patient before their inclusion. The study was conducted following the principles outlined in the Declaration of Helsinki.

Study groups

We evaluated the following study groups: 1) with AH (patients with AH who required no more than 3 antihypertensive drugs to control their blood pressure, n = 53;

among them, there were 9 patients with AH recognized as de novo); 2) with resistant AH (RAH) (RAH patients with AH who required more than 3 different antihypertensive drugs to control their blood pressure, n = 32); 3) control (C) group (patients without AH, n = 45).

Diagnosis of AH was defined as systolic blood pressure (SBP) ≥ 140 mm Hg and/or diastolic blood pressure (DBP) ≥ 90 mm Hg.²⁴ Blood pressure measurements were repeated using the Riva–Rocci method in a seated rest period during daily hospitalization, also in the control group. The final AH diagnosis, or lack of such diagnosis, was based on carefully analyzing each patient's medical history and clinical evaluation, including repeated blood pressure measurements. Most patients qualified to the AH group had the AH diagnosis stated in the past and had been taking antihypertensive medications before admission to the hospital. Blood pressure measurements were

the basis for qualifying the patients with AH to the group with resistant AH (the RAH group) or controlled AH with less than 3 drugs (the AH group). Moreover, among patients without diagnosis of AH at admission to the hospital (initially the control patients), those with repeated blood pressure values above 140/90 mm Hg were diagnosed with AH recognized as *de novo*, and they started to take the antihypertensive treatment. They were finally qualified for the group with AH (the AH group).

Variables

Skin samples were taken from abdominal skin at the beginning of surgery at the site of the planned surgical incision. They were then cut into smaller parts and placed in appropriate test tubes (for histological examination, Na⁺ concentration, water content, hyaluronic acid (HA), real-time polymerase chain reaction (PCR), and protein assessment with western blot), as previously described.²¹

We took the following parameters into account in the statistical analysis: skin Na⁺, water content in the skin, HA in the skin (HA, as a significant component of GAGs in the skin,²⁵ which can leave the skin interstitium through the lymphatic vessels and is present in the bloodstream²⁶), skin mRNA expression of NFAT5, VEGF-C, and PDPN, number of macrophages (as CD68 expression) in the skin, and the density of lymphatic vessels in the skin (as the PDPN/D2-40 expression). In addition, we also considered the expression of CD31 in the skin (regarded as a blood endothelium marker; however, in light of recent findings, CD31 is a pan-endothelial marker and may also be detected on several immune cells and lymphatic endothelium cells),^{27–30} VEGF-D mRNA (another known stimulator of the process of lymphangiogenesis), skin tumor necrosis factor alpha (TNF- α) mRNA (as a marker of inflammation in the skin),³¹ plasma concentrations of HA, Na⁺, K⁺, and creatinine, and the parameters which have been suggested to be the markers of salt-sensitivity, i.e., plasma renin activity (PRA),^{32,33} plasma concentration of N-terminal pro atrial natriuretic peptide (NT-proANP)^{34,35} and serum concentration of circulating VEGF-C.³⁶

Detailed descriptions of the study samples and laboratory procedures were published elsewhere,²¹ except the method used to evaluate the expression of CD31 in the skin, which was described below.

Immunohistochemistry of CD31 expression in the skin

Immunohistochemical (IHC) reactions were performed on 4- μ m thick paraffin sections, as previously described. As primary antibodies (20 min incubation), mouse monoclonal against CD31 (clone JC70A, ready-to-use, IR610; Dako, Glostrup, Denmark) was used. Expression of CD31 was assessed according to the Weidner method.³⁷ The IHC reactions were evaluated with a BX-41 light microscope

(Olympus Corp., Tokyo, Japan). First, the slides were examined under low magnification ($\times 100$) to identify areas with the highest vascular density (hotspots). Then, under $\times 400$ magnification, stained vessels were counted in 3 hotspots. The final score for each slide was presented as a mean number of vessels per mm². Any stained cells were counted as a single microvessel, even in the absence of a vessel lumen.³⁷

Statistical univariate and multivariate chemometric analysis

Univariate and multiple analyses used a classic and non-parametric analysis of variance (ANOVA) for the initial data categorization. The Kolmogorov–Smirnov test was also used to assess whether each sample was drawn from a normally distributed population. As a nonparametric variant of classic ANOVA, the Kruskal–Wallis test was used, followed by a post hoc Dunn's test with the Sidak adjustment formula.³⁸ Any values missing completely at random (ca. 7.6%) were not considered in the comparisons. A χ^2 test assessed whether the hypertension diagnosis and family history of hypertension were independent and if AH diagnosis and gender were independent.

The strategy for a multivariate discriminant analysis combined a PLS-DA with the SR method for selecting the most important parameters for distinguishing 2 respective groups. A bootstrapped method, with a replacement, was used to estimate the quality of the models with the selected parameters, and the prediction was evaluated for the test set. The sensitivity, specificity and efficiency were presented as figures of merit to evaluate any model's prediction ability. To obtain a representative model set that contained samples that described all of the variability that was characteristic for each group (e.g., C, AH or RAH) and was balanced to avoid the recalculation of the cutoff value in the PLS discriminant model,³⁹ the Kennard and Stone algorithm⁴⁰ was used. Thus, 26 samples were considered in the model set for each of the 3 pair-group comparisons, while the remaining samples ($n_C = 19$, $n_{AH} = 27$ and $n_{RAH} = 6$) were placed in the respective test set. The complexity of every PLS-DA model for each bootstrapped sample (out of 1,000) was selected automatically after a leave-one-sample-out cross-validation procedure. Since the data contained missing values, the Kennard and Stone algorithm was modified to estimate the distances using the partial distance strategy,⁴¹ with which the squared Euclidean distance was only calculated for the observed elements, and the result was proportionally rescaled to account for missing values. An expectation–maximization approach in PLS-DA^{42,43} was adopted to manage the incomplete model sets and to perform the prediction for the incomplete test sets. The strategy may be described using, e.g., the C vs RAH model. First, a bootstrap sample (26 C and 26 RAH samples) for the model set was created by randomly withdrawing samples from each group with

a replacement. Then, a PLS-DA model of a given complexity was constructed for the autoscaled parameters, and the respective SR values for all of the parameters (1 SR value for each parameter), or the area under the receiver operating curve (AUC) value for the entire model, were estimated. This calculation was repeated for 1,000 different bootstrap samples, and the respective 1,000 SR values for each variable were used to obtain the estimates of the mean and standard deviation ($M \pm SD$) or a histogram of the AUC values. A cutoff value for the average SR values was then selected, considering the relationship between the average discriminative power of any parameter and the selectivity ratio, which was 0.1 in this case. A bootstrap procedure was used to evaluate the average AUC values and the uncertainty of the model with a reduced number of parameters and, finally, to obtain the prediction for the test set. The scheme of the entire procedure was presented elsewhere.⁴⁴

All calculations employed Statistical Toolbox 8.0 with MATLAB 7.0 (R14) (<https://www.mathworks.com>), and in-house tested and validated algorithms were performed on a personal computer (Intel(R) Pentium(R) M, 1.60GHz with 2GB RAM) using the Microsoft Windows XP (service pack 2) operating system (Microsoft Corp., Redmond, USA).

Results

Univariate analysis

The study groups did not differ in the mean age or sex values. The hypertensive groups had significantly higher BMI than the control group ($p = 0.003$, Kruskal–Wallis test). The basic demographic and medical data of all studied groups are listed in Table 1.

A comparison of the primary groups using ANOVA and Kruskal–Wallis tests showed that the concentrations of Na^+ in the skin were similar in the hypertensive and control groups. Only the mean and median values of creatinine and renin concentration in plasma in the RAH group and NEAT5, VEGF-C and PDPN mRNA expressions in the skin in the AH group were found to be significantly higher than in the C group.

Multivariate models using a partial least squares-discriminant analysis

The next hypothesis was whether the changes in the combination of the studied parameters were responsible for distinguishing any 2 defined groups. To describe the relationship among all of the parameters and determine which of them might be responsible for distinguishing the normotensive individuals from the non-resistant hypertensive patients (C vs AH), the normotensive individuals from patients with resistant hypertension (C vs RAH) and the non-resistant and resistant hypertensive patients (AH vs RAH), multivariate discriminant models used a PLS-DA was constructed under the assumption that a linear combination of parameters (not a single parameter) might be responsible for describing the differences between any 2 groups. This is a straightforward assumption to account for any covariance/correlations among all of the parameters and the impact of these relationships in the discriminant analysis, which is impossible to conduct using univariate analysis.

First, the quality of the 3 pairwise multivariate discriminant PLS-DA models (C vs AH, C vs RAH and AH vs RAH) was evaluated and described by the average and

Table 1. Selected demographic and medical parameters of the studied groups

Parameter		C group (n = 45)	AH group (n = 53)	RAH group (n = 32)	p-value
Age, mean \pm SD (min–max)		65 \pm 7 (56–81)	68 \pm 8 (51–89)	67 \pm 8 (56–81)	0.092*
BMI [kg/m ²], mean \pm SD		25.4 \pm 3.6	27.0 \pm 3.2	28.6 \pm 4.0 [#]	0.003*
Male gender [%]		68.9	73.6	68.8	0.842**
SBP [mm Hg], mean \pm SD		124.23 \pm 8.76	140.16 \pm 15.42 [#]	148.14 \pm 19.66 [#]	<0.001*
DBP [mm Hg], mean \pm SD		77.63 \pm 5.28	83.47 \pm 8.13 [#]	84.90 \pm 13.31 [#]	0.003*
Number of antihypertensive medications used by the patients [%]	1 medication	–	30.6	–	–
	2 medications	–	44.9	–	
	3 medications	–	24.5	59.4	
	4 medications	–	–	28.1	
	5 medications	–	–	12.5	
Mean time from diagnosis of hypertension [years]		–	9.6	12.5	–
Family history of hypertension [%]		14.3	44.0	51.7	0.008**

* Kruskal–Wallis test (Kolmogorov–Smirnov normality test for all parameters $p < 0.001$); * χ^2 test. [#] statistically significant differences vs C group in the multiple comparison Dunn–Sidak post hoc testing; C – control group; AH – group with arterial hypertension; RAH – group with resistant arterial hypertension; SD – standard deviation; BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure.

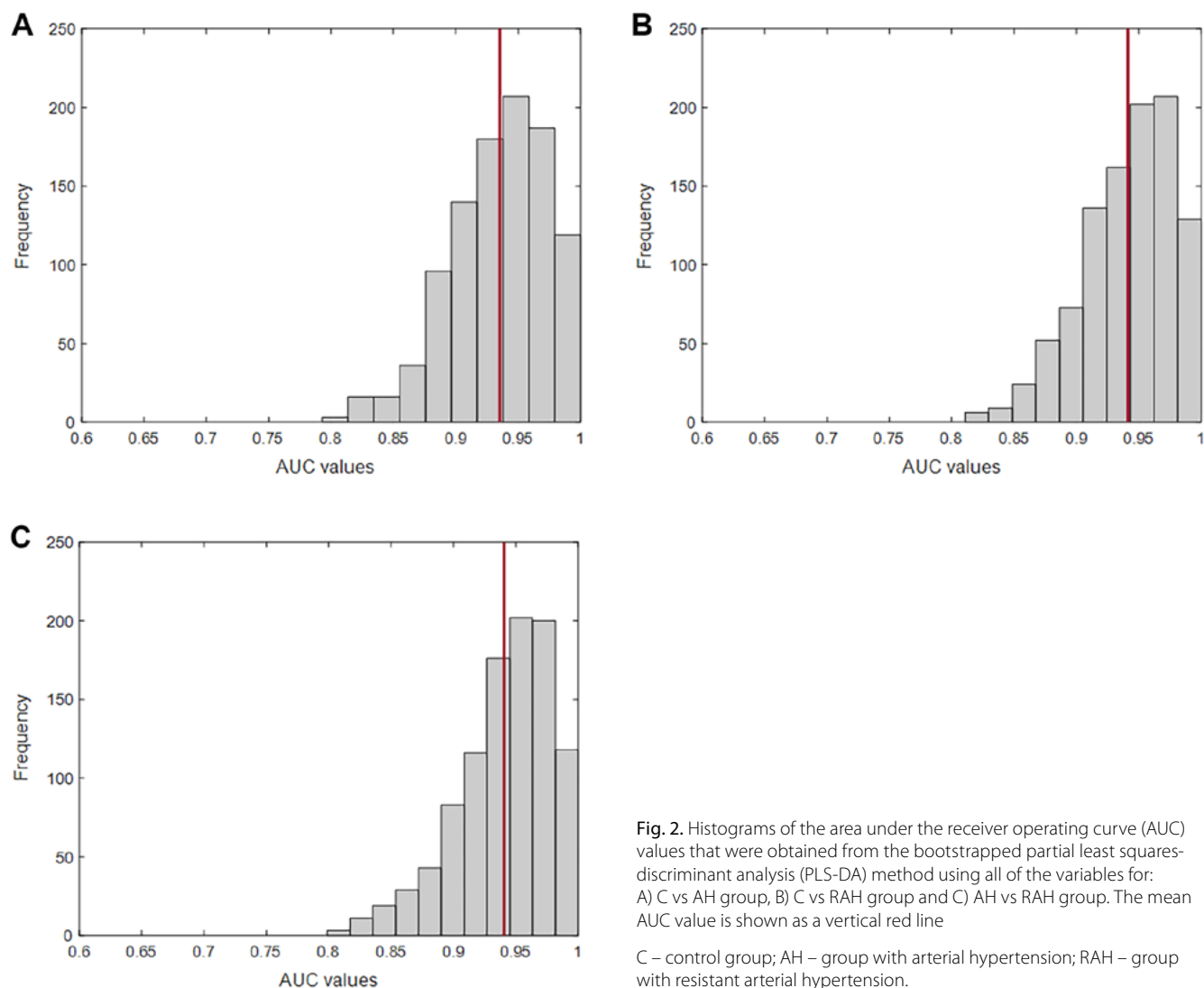


Fig. 2. Histograms of the area under the receiver operating curve (AUC) values that were obtained from the bootstrapped partial least squares-discriminant analysis (PLS-DA) method using all of the variables for: A) C vs AH group, B) C vs RAH group and C) AH vs RAH group. The mean AUC value is shown as a vertical red line

C – control group; AH – group with arterial hypertension; RAH – group with resistant arterial hypertension.

Table 2. The average area under the receiver operating curve (AUC) values (uncertainty in the AUC evaluation) for the model and the AUC values for the test set that were obtained from partial least squares-discriminant analysis (PLS-DA). Sensitivity, specificity and efficiency for the test sets with all of the variables

Model	Average AUC values for model set	AUC for test set	PLS-DA (complexity)	Sensitivity [%]	Specificity [%]	Efficiency [%]
C vs AH	0.94 ± 0.04	0.70	3	94.74	44.44	65.22
C vs RAH	0.94 ± 0.04	0.95	3	84.21	100.00	88.00
AH vs RAH	0.94 ± 0.04	0.70	3	37.04	100.00	48.48

C – control group; AH – group with arterial hypertension; RAH – group with resistant arterial hypertension.

standard error values of the AUC, which was estimated for 1,000 bootstrapped samples, with a replacement. The AUC values measure the model's ability to distinguish between the groups, with a higher AUC indicating a better model. The statistical analysis section describes the selection of a balanced model and test sets. The histograms of the AUC values (the mean AUC value is presented by a vertical red line) are presented in Fig. 2. The sensitivity, specificity and efficiency of the prediction are listed in Table 2.

From the results presented in Table 2, the best prediction was obtained from the model built to distinguish the resistant hypertensive group from the control group ($AUC_{test} = 0.95$). The best prediction expression means that the sensitivity, specificity and efficiency estimated for the independent tests set were as close as possible to 100%. The test samples were not used to build the model. Three normotensive individuals were incorrectly recognized as resistant hypertensive patients, and this resulted in a sensitivity of 84.21%. The model had the highest specificity

Table 3. Univariate, multivariate and descriptive statistics for the parameters that were found to be the most important using the selectivity ratio – partial least squares-discriminant analysis (SR-PLS-DA). Bootstrapping was used to estimate the mean selectivity ratio (SR) for each parameter. A cutoff value of 0.10 for average SR was used to select the important parameters (*)

Parameters	Mean values of parameters \pm SD			Kruskal–Wallis test p-value	Parameters selected (*) by SR-PLS-DA in all models (average SR > 0.10)		
	C group (n = 45)	AH group (n = 53)	RAH group (n = 32)		C vs AH	C vs RAH	AH vs RAH
Plasma Na ⁺ [mmol/L ⁻¹]	140.67 \pm 3.23	140.85 \pm 3.11	141.21 \pm 2.70	0.632	*	–	*
Plasma K ⁺ [mmol/L ⁻¹]	4.20 \pm 0.40	4.28 \pm 0.48	4.47 \pm 0.54	0.117	*	*	*
Plasma creatinine [mg/dL ⁻¹]	0.82 \pm 0.18	0.91 \pm 0.26	1.06 \pm 0.40 [#]	0.022	*	*	*
eGFR [mL/min ⁻¹]	92.16 \pm 27.34	86.93 \pm 28.63	90.44 \pm 32.48	0.624	*	*	*
Plasma NT-proANP [nmol/L ⁻¹]	2.69 \pm 1.64	3.15 \pm 2.17	3.69 \pm 2.89	0.500	*	*	*
Plasma renin activity [uIU/mL ⁻¹]	75.69 \pm 52.15	186.76 \pm 332.96	291.05 \pm 428.40 [#]	0.018	*	*	–
Skin lymphatic vessels, D2-40 [μ m ²]	7,889.59 \pm 4,684.28	9,392.61 \pm 5,090.00	8,193.45 \pm 4,525.78	0.301	*	–	*
Skin CD68 [number of macrophages/mm ⁻²]	4.33 \pm 3.69	7.05 \pm 11.58	4.06 \pm 5.85	0.179	*	–	*
Skin log (NFAT5 mRNA)	0.66 \pm 0.50	0.97 \pm 0.40 [#]	0.90 \pm 0.41	0.003	*	*	–
Skin log (VEGF-C mRNA)	0.47 \pm 0.33	0.65 \pm 0.30 [#]	0.60 \pm 0.34	0.029	*	*	–
Skin log (VEGF-D mRNA)	0.52 \pm 0.51	0.60 \pm 0.45	0.48 \pm 0.53	0.299	–	–	*
Skin log (PDPN mRNA)	0.50 \pm 0.60	0.82 \pm 0.51 [#]	0.74 \pm 0.54	0.008	*	*	–
Serum VEGF-C [pg/mL ⁻¹]	4,784.11 \pm 1,800.16	4,483.74 \pm 1,668.33	4,084.76 \pm 1,667.89	0.122	*	*	–
Serum VEGF-D [pg/mL ⁻¹]	343.78 \pm 150.98	338.60 \pm 139.29	317.00 \pm 156.66	0.466	–	–	*
Skin water [% of dry weight]	53.13 \pm 39.92	52.7 \pm 40.81	35.36 \pm 18.47	0.063	–	*	*
Skin hyaluronic acid [mg/g ⁻¹]	0.56 \pm 0.25	0.74 \pm 0.81	0.54 \pm 0.24	0.714	*	*	*
Skin blood vessels, CD31 [μ m ²]	12.45 \pm 5.79	13.32 \pm 7.44	12.67 \pm 8.23	0.443	–	–	–
Skin log (TNF- α mRNA)	–0.10 \pm 0.29	–0.13 \pm 0.33	–0.12 \pm 0.29	0.880	–	–	–
Serum hyaluronic acid [ng/mL ⁻¹]	40.51 \pm 40.04	45.15 \pm 38.07	42.38 \pm 36.55	0.316	–	–	–
Skin Na ⁺ [mg/kg ⁻¹]	624.97 \pm 485.04	567.79 \pm 409.64	583.45 \pm 353.69	0.939	–	–	–

Normality test (Kolmogorov–Smirnov): failed for all parameters ($p < 0.001$); [#]statistically significant differences vs C group in the multi-comparison Dunn–Sidak post hoc testing; SD – standard deviation; C – control group; AH – group with arterial hypertension; RAH – group with resistant arterial hypertension; Na⁺ – sodium, K⁺ – potassium; eGFR – estimated glomerular filtration rate; NT-proANP – N-terminal proatrial natriuretic peptide; NFAT5 – nuclear factor of activated T cells 5; VEGF-C – vascular endothelial growth factor C; VEGF-D – vascular endothelial growth factor D; PDPN – podoplanin; TNF- α – tumor necrosis factor alpha.

(100%), indicating that all resistant hypertensive patients were correctly recognized. A specificity of 100% was also obtained for the model built to distinguish between non-resistant and resistant hypertensive patients (AH vs RAH in Table 2). However, this model had very poor sensitivity (37.04%), and the probability of correctly identifying a non-resistant hypertensive patient (AH patient) was very low. In contrast, the probability of an incorrect identification of an AH patient as an RAH patient was relatively high. Indeed, the model that described the differences between the normotensive individuals and the non-resistant hypertensive patients (C vs AH) showed a relatively high sensitivity of 94.74%, but a low specificity (44.44%). This suggests that the probability of incorrectly recognizing a normotensive patient as non-resistant hypertensive was very low. In contrast, the probability of incorrectly identifying a non-resistant hypertensive patient as normotensive was relatively high. Thus, all of the parameters that were usually considered to describe the mechanism of Na⁺ regulation

in the skin are best explained by the differences between normotensive and resistant hypertensive individuals.

An important question was whether reducing the number of parameters would also provide a good description of the models regarding the sensitivity, specificity and prediction efficiency. The parameters were selected using the SR approach in PLS-DA, and the method is abbreviated as SR-PLS-DA.^{45–47} The SR is a figure of merit that describes the importance (in terms of the explained variance) of each parameter in the model construction. The larger the SR value for a parameter, the more important it is in explaining the differences between the groups in the PLS-DA model. The SR values for each parameter were estimated during the bootstrapping procedure (1,000 bootstrapped samples), and parameters with average SR values (e.g., the average value of 1,000 SR values obtained for each parameter) larger than 0.10 were selected as important. The descriptive statistics for the parameters found to be the most important in each model are shown in Table 3.

Table 4. The average area under the receiver operating curve (AUC) values (uncertainty in the AUC estimation) for the model and test sets with the selected variables from the selectivity ratio – partial least squares-discriminant analysis (SR-PLS-DA). Sensitivity, specificity and efficiency for the test sets with selected variables are marked by an asterisk (*). in Table 3. The cutoff value for selectivity ratio (SR) in all of the models was 0.10

Model	Average AUC values for model set	AUC for test set	PLS-DA (complexity)	Sensitivity [%]	Specificity [%]	Efficiency [%]
C vs AH	0.90 ± 0.04	0.68	2	89.47	33.33	56.52
C vs RAH	0.89 ± 0.04	0.90	2	73.68	83.33	76.00
AH vs RAH	0.87 ± 0.05	0.73	2	22.22	100.00	36.36

C – control group; AH – group with arterial hypertension; RAH – group with resistant arterial hypertension.

The C vs AH and AH vs RAH models used 11 of the 20 parameters, but only 6 selected parameters (54%) were common. The parameters important for differentiating C and RAH were unimportant for distinguishing the AH and RAH patients, and it was renin, log (NFAT5 mRNA), log (VEGF-C mRNA), log (PDPN mRNA), and serum VEGF-C. Conversely, the parameters important for distinguishing AH and RAH groups that were unimportant for distinguishing the C and RAH groups were plasma Na⁺, D2-40, CD68, log (VEGF-D mRNA), and serum VEGF-D. The C vs AH model required a larger number of parameters (13 of the 20) and had a larger number of parameters that were common with the other 2 models (ten common parameters concerning the C vs RAH model and 8 common parameters compared to those that had been used with the AH vs RAH model). Specifically, skin water, log (VEGF-D mRNA) and serum VEGF-D were unimportant for distinguishing the normotensive and non-resistant hypertensive patients. Still, they were found to be important in the other 2 models.

The performance of the models with the selected parameters was re-evaluated using the bootstrapping procedure; the results are presented in Table 4. Once again, the best performance for the test set ($AUC_{test} = 0.90$) was obtained when differentiating the normotensive individuals from the resistant hypertensive patients. Compared to the model with all parameters, this model had a lower sensitivity, specificity and efficiency for the test set. However, the C vs AH model also had a very poor specificity (33.33%), while the AH vs RAH model had a very low sensitivity (22.22%).

Discussion

The group in our study that most corresponded to the sequence of events documented in the rodents' skin was the AH group. The parameters that distinguished AH group from C group were also important for the mechanism presented in the studies of Machnik et al.,^{2,3} Wiig et al.⁴ and Nikpey et al.,⁵ i.e., a greater density of lymphatic vessels (expressed as D2-40), a greater number of macrophages in the skin (expressed as CD68), a higher expression of NFAT5, VEGF-C and PDPN mRNA, a higher concentration of HA in the skin, and a lower concentration of serum

VEGF-C. As in the studies of Machnik et al.,^{2,3} the expression of TNF- α mRNA was not important for differentiating the studied groups.

Although more than a dozen years have passed since the NFAT5-VEGF-C lymphangiogenesis pathway was described in experimental AH rodent studies,^{2–5} our studies (this and recently published) are the first human studies that directly translate the rodent model of the involvement of the lymphatic system in the pathogenesis of AH to the patients. Studying this mechanism in humans is challenging since the pathogenesis of human AH is heterogeneous, and salt sensitivity is only one of the causes. That may be the reason why the groups in our study did not differ significantly in the majority of parameters important for studying the pathway using univariate analysis (except for the higher NFAT5, VEGF-C and PDPN mRNA expression in the skin in the AH compared to the C group). By utilizing a multivariate discriminant analysis method, such as PLS-DA, we could provide additional evidence that the mechanisms observed in the skin of rodents may also be applicable to humans.

The dominant pathophysiological mechanisms that led to resistant hypertension were probably various in the RAH group, which we primarily hypothesized would be the most salt-sensitive.⁴⁸ The lack of a tendency to lower PRA values in the RAH group might suggest mechanisms other than salt sensitivity,^{32,33} which led to resistant hypertension in this group. Another explanation is that there was an insufficient activation of the NFAT5-VEGF-C pathway and, therefore, no effect on the increase in the number of macrophages and the density of lymphatic vessels in the skin in the RAH group.

Interestingly, the lower concentration of skin water in the RAH group was one of the parameters that differentiated the RAH group from the C or AH group. This observation might have resulted from increased vascular contraction and arterial remodeling, characteristic of long-lasting resistant AH.⁴⁹ The greater vascular resistance might be associated with a decreased filtration from the capillaries at the microcirculation level and, therefore, there was a lower skin water content. It might be an important observation concerning the pathophysiology of the NFAT5-VEGF-C-lymphangiogenesis pathway because we showed in our previous study that the skin water content was strongly associated with lymphangiogenesis

in the skin, i.e., it was associated with greater mRNA expression of NFAT5, VEGF-C and PDPN, and a number of macrophages.²¹

Assuming that the skin water content triggers skin lymphangiogenesis, a smaller amount of water passing through the skin microcirculatory system through vessels hardened by long-lasting hypertension might result in inadequately activated NFAT5-VEGF-C-lymphangiogenesis in patients with RAH. However, this suspicion requires further elucidation.

In this study, the concentration of Na⁺ in the skin did not differ in hypertensive and the control groups, as we had previously reported.²⁰ Moreover, the amount of skin Na⁺ was not responsible for the differentiation between pairs of studied groups in the multivariate analysis. However, we would expect a difference in the skin Na⁺ concentrations if the healthy individuals in an experiment consumed excessive amounts of salt in their diets and the results of measuring Na⁺ in their skin were compared with those of healthy subjects. In our study, we did not control the intake of dietary salt. Therefore, some normotensive individuals could have had higher levels of skin Na⁺ than usual. In comparison, the hypertensive patients could have had lower levels of skin Na⁺ than the control due to the administration of antihypertensive medications or because of following a low-sodium diet, which is usually prescribed for patients with AH.

In our study, the patients in the RAH group had a higher BMI than the C group. However, we do not consider the difference in BMI as a limiting factor. The relationship between the higher body weight (BMI approx. 30 kg/m²) and AH has been well documented,^{50,51} as patients with primary hypertension in the same age group generally present a higher BMI.

Limitations

The main limitations of the study were previously described in detail.^{20,21} Additionally, there is the marginal possibility of patients' incorrect classification to hypertensive groups based on medical history and non-standardized BP measurement without ambulatory blood pressure monitoring (ABPM), especially since the patients were pre-surgical. The patients from the control group did not have AH because neither the medical history nor measurements during hospitalization indicated it. The group with hypertension treated with <3 antihypertensive drugs mainly consisted of patients with a previously diagnosed and treated AH (before hospitalization), and only 9 patients from this group had AH diagnosed de novo, based on blood pressure measurements during hospitalization. Even if we assume that some of the patients with AH recognized de novo were included in the AH group (the AH group) instead of the control group (elevated blood pressure might be only a result of perioperative stress or "white coat" AH in some of these patients), the differences

between the control group and the group with HA (the AH group) would be less significant. The group with resistant hypertension (the RAH group) might also include some patients with non-resistant AH. Still, such a possible incorrect classification of some patients would only reduce the differences between the AH and the RAH groups, not increase them. Therefore, the small risk associated with incorrect patient classification to both AH groups (AH and RAH) in our study only reduced differences among the study groups and did not increase them. The equalization of the study groups in age achieved by eliminating patients under or equal to 50 years old instead of the process of matching the patients might also be considered as a limiting factor.

The lack of sodium sensitivity testing in our patients is another important study's limitation. However, our priority was examining the skin, which was only possible in patients who had undergone surgery with an abdominal incision. It would have been ethically debatable to perform a sodium sensitivity test on patients awaiting surgery. The precise protocol requires one week of low and then one week of high salt intake. A shorter procedure consisting of administering intravenous saline and then furosemide is not accurate.⁵² Moreover, a possible dietary interview with our patients about their salt intake would not have enabled us to identify a salt-sensitive group – it might have been more confusing than helpful. Most of our participants took several antihypertensive medications, including diuretics, usually for a long time. Determining the impact of such drugs on the concentration of Na⁺ in the skin and on the investigated mechanism was also not possible.¹¹ Moreover, every disease, taking any medications currently and in the past, the patient's hormonal state, and indication for surgery might impact the studied mechanism. The number of such influencing parameters is countless and completely unknown. Therefore, when we planned our study, we decided on a simple qualification of patients based on the division with and without hypertension after confirming the absence of exclusion criteria used in our research.

Conclusions

The results of our study, specifically the differentiation between the AH group and the respective C group, suggest that the NFAT5–VEGF-C–lymphangiogenesis pathway, previously described in experimental AH in rodent studies, might also be present in humans. However, it was only an observational study. Further experiments are needed to prove our findings. Regulation of this pathway also requires further research. Such investigations are worth the effort because establishing the exact role of the lymphatic system in the pathophysiology of AH would provide a basis for searching for new therapeutic agents for this disease.

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