



RESEARCH ARTICLE

Nonparametric Method for Estimation of Controlled Correlations in Studies of VEGF-Hypoxia Relationship

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Abstract

It is known that vascular endothelial growth factor (VEGF) expression is a response to hypoxia. On the other hand hypoxia may be detected by oximetry parameters including venous CO-oximetry indices or corresponding partial pressures of O₂ and CO₂. However significant correlation ties between VEGF levels and oximetry parameters were not found in groups of patients with ischemic stroke and transient ischemic attack. At that some effect related to the relationship between VEGF and sO₂ was observed at corresponding scatter plots. Correlation between VEGF and proteins S100 levels in serum existed only in group with severe hypoxia where sO₂ is less threshold close to 39-40%. So the relationship between VEGF level and saturation index sO₂ exists in conjunction with additional factor that is S100 level in serum. To assess statistical significance of observed regularity it is necessary to test three null hypotheses about independence of one of involved factors on two another. The relationship may be manifestation of hypoxia effect on VEGF. To assess significance of hypoxia effect as a whole all three null hypotheses were tested with the help of developed technique based on random permutations and involving nonparametric combinations of criteria related to single oximetry parameters. The statistical significance assessments also involved multiplicity adjustment aimed to take into account multiple search of additional factors among variety of biological indices from analyzed data set. As a result of developed technique application all three considered null hypotheses were rejected at adjusted level $p < 0.02$ when effect of hypoxia on correlation between VEGF and complement component C4 was evaluated.

Keywords

VEGF, Hypoxia, Controlled correlations, Permutation test, Nonparametric combinations, Multiple testing

Introduction

Angiogenesis may be a biological response to insufficient oxygen supply resulting in hypoxia. The key mediator of angiogenesis and probably neurogenesis [1] is vascular endothelial growth factor (VEGF) which is homodimeric glycoprotein with a molecular weight of approximately 45 kDa. VEGF expression is activated as a response to stabilization and nuclear translocation of hypoxia-inducible factor-1 (HIF-1) when intracellular oxygen level is reduced [2]. Existence of HIF-1, VEGF signalling pathways is confirmed by high levels of VEGF in patients with chronic obstructive pulmonary disease (CHOPD) [3] and asthma [4] or in subjects with "plateau red face" [5]. It is known that immune system is involved in angiogenesis via secreting VEGF and other pro-angiogenic factors by macrophages, neutrophils and other immune cells [6-8]. Oxygen saturation index sO₂ and other CO-oximetry parameters in venous blood reflect balance between oxygen delivery and oxygen consumption [9]. Low sO₂ correspond to tissue hypoxia [10]. However correlation coefficients between CO-oximetry

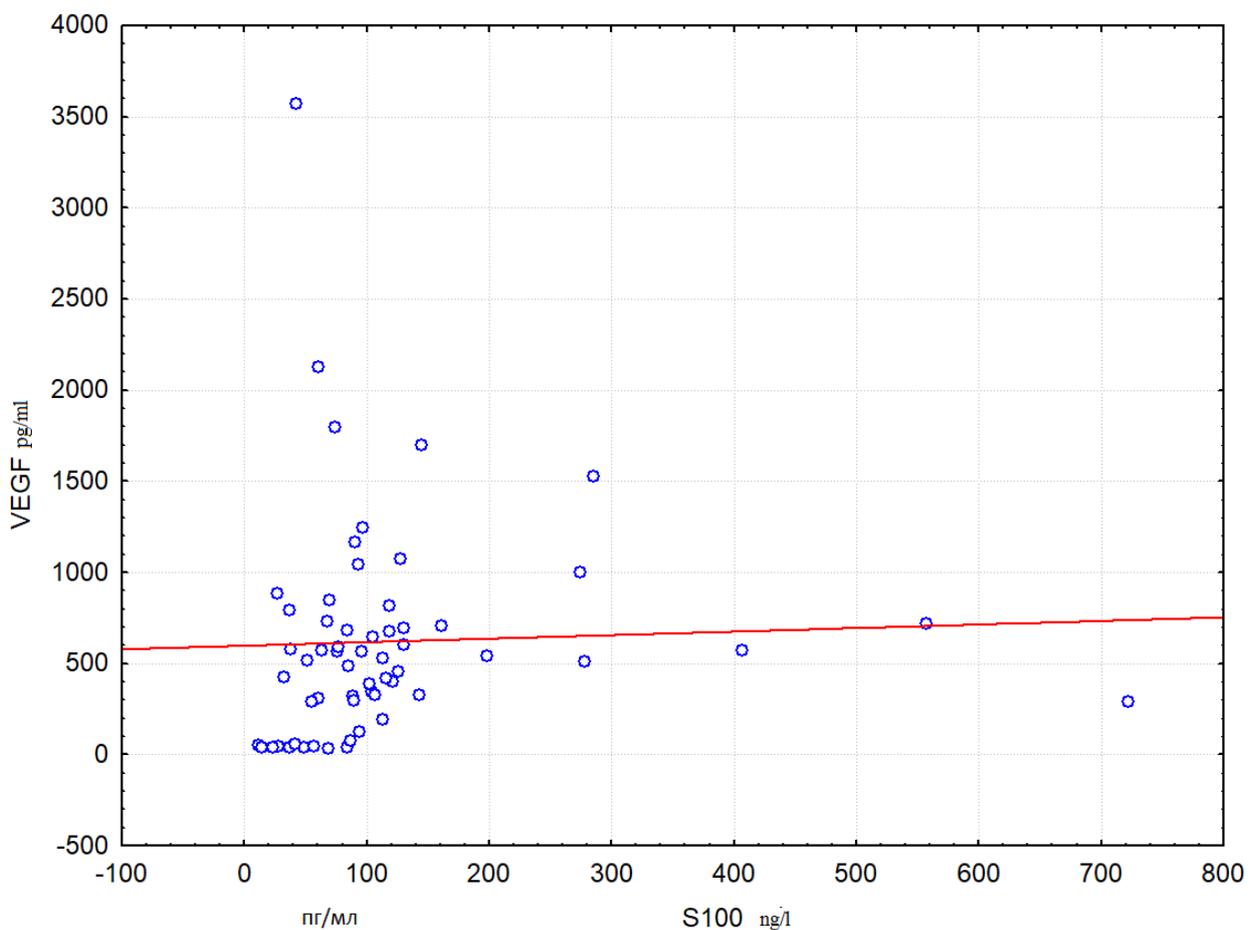
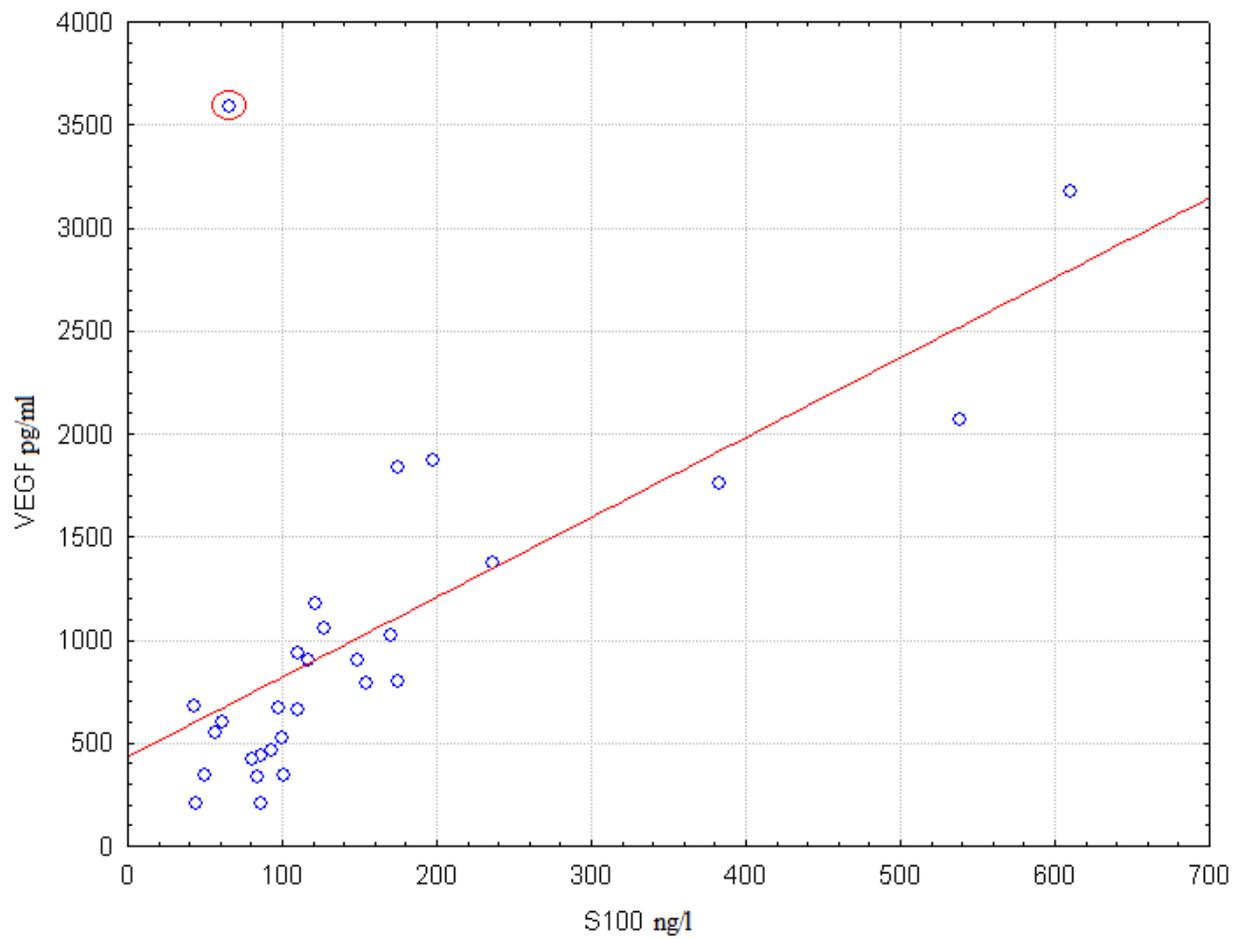


Figure 1: Correlation between S100 and VEGF levels are compared in groups with sO2 < 38.4% (left scatter plot) and sO2 > 38.4% (right scatter plot).

parameters and VEGF levels were small and not statistically significant in groups of patients with severe neurological disorders. Corresponding data set is discussed below. Correlation coefficients between VEGF and partial pressures of O₂ and CO₂ were also not significant. Lack of reliable ties may be attributed among other things to complexity of existing dependence when relationship between two factors is controlled by the third one. Previous study of the data set with the help of OVP method [11,12] and visual analysis of related sparse diagrams uncover complex effect involving sO₂ and levels in serum of VEGF and proteins from S100 family.

Left scatter diagram from Figure 1 corresponds to group with sO₂ lower than 38.4%. This diagram conforms to existence of linear dependence of VEGF on S100 level. The only exception is one object that is marked with a red circle. At that there is no noticeable correlation between VEGF and S100 in the right diagram. This diagram corresponds to group with sO₂ greater than 38.4%. It may be suggested that the correlation observed in the left diagram may be caused by severe hypoxia existing when sO₂ is below 38.4%. The relationship from Figure 1 may be described by following equations:

$$\begin{aligned} y_j &= \beta_0^l + \beta_1^l x_{ij} + \epsilon_j^l \text{ if } z_{kj} < \delta \\ y_j &= \beta_0^r + \beta_1^r x_{ij} + \epsilon_j^r \text{ if } z_{kj} \geq \delta \end{aligned} \quad (1)$$

Model (1) evidently is equivalent to standard piecewise regression if X_i is equal to Z_k . It is supposed that the verification procedure must satisfy following demands:

- Verification procedure must include testing significance of both variables X and Z .
- Hypoxia is assessed by several oximetry parameters. It may be expected that statistical technique evaluating the effect of hypoxia on relationship between VEGF and additional factor X_i would be more powerful if it implements combination of statistical tests assessing effect each of oximetry parameters on relationship between Y and X_i .
- Effect from Figure 1 was found via testing variety of factors. So multiple testing must be taken into account when statistical significance is estimated.

Permutation test is an approach capable of meeting the listed demands due to following advantages. Permutation test may be implemented regardless whether or not underlying distributions of test statistics are known. There are no limitations on data sets sizes. Different permutation tests were proposed for assessing importance of each predictor in multiple regression models. Method based on testing significance of corresponding partial correlation coefficients may be mentioned in this regard [13]. Permutation test was used to make a choice between piece-wise regression and a simple linear regression [14]. However mentioned techniques are not suitable to evaluated significance of the

effect considered in the paper. Verification is possible with the help of discussed below procedure based on testing several null hypotheses.

Effective methods calculating nonparametric combination (NPC) of several dependent permutation tests [15] may be used for assessing significance of hypoxia effect by several oximetry parameters. Also permutation tests are widely used to control multiplicity in various applied tasks including high-dimensional tasks related to DNA microarray experiments [16-19]. At that there are different ways to control FWER. Single step or stepwise procedures may be used to receive adjusted p-values from previously received raw p-values by min P correcting procedure or to calculate adjusted p-values directly from distributions of test statistics by maxT technique [20-22]. Unlike the mentioned works our paper is focused at problems associated with multiplicity control and combinations of several criteria when more complicated multifactor regularities are studied.

Data Set and Preliminary Results

Effect of hypoxia on relationship between VEGF and 138 different biochemical, clinical or biophysical parameters was studied in a group of 88 patients of age from 33 to 88 with ischemic stroke and transient ischemic attack. Hypoxia level was assessed by partial pressures of O₂ and CO₂ and also by CO-oximetry parameters in venous blood that were measured with ABL80 FLEX CO-OX analyser. Serum levels of VEGF, S100 and complement component C4 were measured by the enzyme-linked immunosorbent assay (ELISA). CO-oximetry parameters together with partial pressures of O₂ and CO₂ in venous blood will be for the simplicity further referred to as oximetry parameters.

Results of standard correlation analysis are given in Table 1.

It can be seen from Table 1 that statistically significant linear ties between VEGF levels and hypoxia are absent.

It is quite possible that linear ties nevertheless may exist in combination with some additional factors. Such supposition is supported by the effect of oxygen satura-

Table 1: Correlation coefficients between partial pressures of O₂ and CO₂ and CO-oximetry parameters in venous blood and VEGF levels.

Oximetry Parameter	Correlation Coefficient	P-values
Oxygen saturation index (sO ₂)	-0.158	> 0.1
Partial pressure of O ₂ (pO ₂)	-0.059	> 0.1
Partial pressure of CO ₂ (pCO ₂)	-0.035	> 0.1
Oxyhemoglobin fraction FCOHb	-0.058	> 0.1
Oxyhemoglobin fraction FO ₂ Hb	-0.146	> 0.1
Methemoglobin fraction (FMetHb)	0.13	> 0.1
Deoxyhemoglobin fraction (FHb)	0.196	> 0.05

tion index (sO2) on relationship between VEGF and proteins S100 levels in serum. This effect was discovered with the help of OVP technique [11] and visual analysis of corresponding scatter plots. Left scatter plot from Figure 1 presents strong linear relationship between VEGF and S100 in group with saturation level sO2 less than 38.4. It is seen from right scatter plot that noticeable linear dependence between VEGF and S100 is absent in the group with saturation level sO2 greater than 38.4.

It may be supposed from Figure 1 that hypoxia leads to correlation between VEGF (Y) and S100 (X_i). So S100 may be considered as additional factor. Our goal is to assess statistical significance of the assumed effect.

Verification of the Effect

Complex dependencies testing: The above supposition is too complex to test using a single null hypothesis. In fact the effect contradicts the following three hypotheses:

- a) $H_0^1(k, i)$ - Y is independent of vector (Z_k, X_i) ;
- b) $H_0^2(k, i)$ - X_i is independent of vector (Y, Z_k) ;
- c) $H_0^3(k, i)$ - Z_k is independent of vector (Y, X_i) .

All three hypotheses must be rejected to be sure that the supposition is perfectly correct. Otherwise the observed pattern may be explained simpler. For example it may be attributed to existence of linear relationship between Y and X_i when $H_0^3(k, i)$ is in fact true.

Testing single hypothesis: The discussed effect is associated with great difference between $\rho_l(Y, X_i)$ and $\rho_r(Y, X_i)$ great value of one of the two correlation coefficients. High correlation values are achieved randomly in small groups. So the two conditions above may be a convincing evidence of the effect only when sizes of both groups are great enough. Thus the mentioned conditions correspond to great values of the following functional:

$$Q_{2\rho}(\delta) = \frac{m_l m_r \left| |\rho_l(Y, X_i)| - |\rho_r(Y, X_i)| \right|}{1 - \max \{ \rho_l^2(Y, X_i), \rho_r^2(Y, X_i) \}}, \quad (2)$$

Where m_l is the number of patients in group \tilde{S}_l with $Z_k < \delta$ and m_r is the number of patients in group \tilde{S}_r with $Z_k > \delta$. The threshold δ is initially unknown. It is proposed to use the optimal threshold δ_0 that is calculated as $\delta_0 = \arg \max \hat{Q}_{2\rho}(\delta)$. Maximum of the functional $Q_{2\rho}(\delta)$ may be searched by trying all boundaries between distinct values of Z_k existing in full group \tilde{S} . Great values of $Q_{2\rho}(\delta_0)$ better testify against each of 3 null hypotheses $H_{01}^1(k, i)$, $H_0^2(k, i)$ and $H_0^3(k, i)$ if δ_0 is searched inside interval $(\delta_1; \delta_r)$ including only such thresholds for Z_k that provide simultaneous validity of inequalities $m_l > m_{thr}$ and $m_r > m_{thr}$. The narrowed search interval is used because of the high probability of great $Q_{2\rho}(\delta_0)$ values when null hypotheses are true but one of the groups is small. Probability of great correlation coefficients in

small groups evidently is higher. Full compensation of this probability increase is impossible by using multiplier $m_l m_r$ only.

Existence of outlying observations such as observation circled in red on the left part of Figure 1 reduces $Q_{2\rho}$ and so may hinder correct statistical evaluation of the studied effect. So it is better to use robust Pearson correlation coefficient.

Robust correlation coefficient: Suppose that dependence of variable U on variable V is studied on data set $\tilde{S} = \{(u_j, v_j) \mid j = 1, \dots, m\}$. At the first step simple linear regression model $U = \beta_0 + \beta_1 V + e$ is calculated by \tilde{S} . Let \tilde{S}_{out} be the set of all 3σ outliers or $\tilde{S}_{out} = \{(u_j, v_j) \mid |u_j - \beta_0 - \beta_1 v_j| > 3\sigma_e\}$, where σ_e is standard error of regression model. Robust correlation coefficient $\hat{\rho}$ is calculated as standard Pearson correlation coefficient ρ by set $\tilde{S} \setminus \tilde{S}_{out}$.

The robust version of the functional 2 can be written as

$$\hat{Q}_{2\rho}(\delta) = \frac{m_l m_r \left| |\hat{\rho}_l(Y, X_i)| - |\hat{\rho}_r(Y, X_i)| \right|}{1 - \max \{ \hat{\rho}_l^2(Y, X_i), \hat{\rho}_r^2(Y, X_i) \}}, \quad (3)$$

Where $\hat{\rho}_l$ and $\hat{\rho}_r$ are robust correlation coefficients in groups \tilde{S}_l and \tilde{S}_r . Optimal threshold δ_0 is calculated now as $\delta_0 = \arg \max_{\delta \in (\delta_1, \delta_r)} \hat{Q}_{2\rho}(\delta)$. Functional $\hat{Q}_{2\rho}(\delta_0)$ is used as statistics for testing null hypotheses $H_0^1(k, i)$, $H_0^2(k, i)$ and $H_0^3(k, i)$. Then p-values for these null hypotheses are calculated according to the following **Procedure I:**

- Calculate optimal threshold δ_0 on data set \tilde{S}_l as $\arg \max_{\delta \in (\delta_1, \delta_r)} \hat{Q}_{2\rho}(\delta)$. The observed statistics value T^0 is taken equal to $\hat{Q}_{2\rho}(\delta_0)$.

To test $H_0^1(k, i)$ repeat independently steps a_1 and a_2 for $r = 1, \dots, N$.

- a_1) Take a random permutation \tilde{S}_l^r of \tilde{S}_l . This is obtained by considering a random permutation $\mathbf{u}^r = (u_1^r, \dots, u_m^r)$ of objects labels $(1, \dots, m)$.

Let $\tilde{S}_l^r = \left\{ (y_{u_1^r}, x_{1i}, z_{1k}), \dots, (y_{u_m^r}, x_{mi}, z_{mk}) \right\}$.

- a_2) Calculate optimal threshold δ_0 on training set \tilde{S}_l^r as $\arg \max_{\delta \in (\delta_1, \delta_r)} \hat{Q}_{2\rho}(\delta)$. The statistics value T^r is taken equal to $\hat{Q}_{2\rho}(\delta_0)$.

- Then calculate estimate of p-value for the null $H_0^1(k, i)$ as $\hat{p}^1(k, i) = \frac{\sum_{1 \leq r \leq N} \mathbb{1}(T^r \geq T^0)}{N}$.

To $H_0^2(k, i)$ repeat independently steps a_3 and a_2 for $r = 1, \dots, N$.

- a_3) Take a random permutation \tilde{S}_l^r of \tilde{S}_l as $\tilde{S}_l^r = \left\{ (y_1, x_{u_1^r i}, z_{1k}), \dots, (y_m, x_{u_m^r i}, z_{mk}) \right\}$

Where $\mathbf{u}^r = (u_1^r, \dots, u_m^r)$ is a random permutation of $\{1, \dots, m\}$.

- Implement step (a_2).
- Then calculate estimate of p-value for the null

$$H_0^2(k, i) \text{ as } \hat{p}^2(k, i) = \frac{\sum_{1 < r < N} \mathbb{1}(T^r > T^0)}{N}.$$

To test $H_0^3(k, i)$ repeat independently steps a_4 and a_2 for $r = 1, \dots, N$.

- a_4) Take a random permutation \tilde{S}_i^r of \tilde{S}_i as $\tilde{S}_i^r = \left\{ \left(y_1, x_{11}, z_{u_1^r} \right), \dots, \left(y_m, x_{m1}, z_{u_m^r} \right) \right\}$

Where $\mathbf{u}^r = (u_1^r, \dots, u_m^r)$ is a random permutation of $\{1, \dots, m\}$.

- Implement step (a_2).
- Then calculate estimate of p-value for null hypothesis

$$H_0^3(k, i) \text{ as } \hat{p}^3(k, i) = \frac{\sum_{1 < r < N} \mathbb{1}(T^r > T^0)}{N}.$$

A regularity for combination of factors (Y, X_k, Z_j) similar to the one in Figure 1 is supposed to be significant at level α if all three inequalities $\hat{p}^1(k, i) < \alpha$, $\hat{p}^2(k, i) < \alpha$ and $\hat{p}^3(k, i) < \alpha$ hold. It is difficult to make a theoretical conclusion about unbiasedness and consistency of the described test. However its performance can be assessed in experiments with simulated data.

Experiments with simulated data

Design of experiments: Experiments were designed to imitate regularity from Figure 1 only for certain groups of variables while for the remaining ones such regularities were absent. Scenario includes generating variables $Y, Z_1, \dots, Z_7, X_1, \dots, X_n$. Variables Y and e_1, \dots, e_n were independently sampled from $N(0,1)$, variable X_1, U_g and variables U_2, \dots, U_7 are independently sampled from continuous uniform distribution $U(0,1)$. Variables X_1, \dots, X_n are calculated from Y, e_1, \dots, e_n, Z_1 and U_g . Variables Z_2, \dots, Z_7 are calculated from Z_1 and U_2, \dots, U_7 .

1. Variables X_1, \dots, X_{30} for an observation j were calculated as $X_{ij} = Y_j - \delta_1 * e_{ij}$ if $Z_{1j} < 0.4$ and as $X_{ij} = \delta_2 * e_{ij}$ otherwise. In groups with $Z_{1j} < 0.4$ correlation level between Y and X_i is determined by parameter δ_1 . Experiments with $\delta_1 = 0.75$ and $\delta_1 = 0.57$ were conducted. Choice $\delta_1 = 0.75$ provides generating data with $\rho(Y, X_i) = 0.8$ and choice $\delta_1 = 0.57$ corresponds to $\rho(Y, X_i) = 0.87$. Parameter δ_2 was taken equal to $\sqrt{1 + \delta_1^2}$. Observations with $Z_{1j} \geq 0.4$ were generated from distribution with $\rho(Y, X_i) = 0$.

Thus in the first experiment data was sampled from distribution with $\rho(Y, X_i) = 0.8$, $\sigma_Y = 1$ and $\sigma_{X_i} = \sqrt{1 + 0.75^2}$ when $Z_1 < 0.4$ and from distribution with $\rho(Y, X_i) = 0$, $\sigma_Y = 1$ and $\sigma_{X_i} = \sqrt{1 + 0.75^2}$ when $Z_1 \geq 0.4$.

2. In the second experiment data was sampled from distribution with $\rho(Y, X_i) = 0.87$, $\sigma_Y = 1$ and $\sigma_{X_i} = \sqrt{1 + 0.57^2}$ when $Z_1 < 0.4$ and from distribution

with $\rho(Y, X_i) = 0$, $\sigma_Y = 1$ and $\sigma_{X_i} = \sqrt{1 + 0.57^2}$ when $Z_1 \geq 0.4$. So data is generated to provide existence of effect that is similar to effect from Figure 1 for each combination from set $\{(Y, X_i, Z_k) | i = 1, \dots, 30, k = 1, \dots, 7\}$.

3. Variables X_{31}, \dots, X_{60} for the observation j were calculated as $X_{ij} = Y_j - \delta_1 * e_{ij}$ if $U_g < 0.4$ and as $X_{ij} = \delta_2 * e_{ij}$ if $U_g \geq 0.4$. So observations are generated from mixture of distributions with $\rho(Y, X_i) = 0.8$ and $\rho(Y, X_i) = 0$ in the first experiment and from mixture of distributions with $\rho(Y, X_i) = 0.87$ and $\rho(Y, X_i) = 0$ in the second one. Thus data is generated to provide existence of weak mutual correlation for pairs from $\{(Y, X_i) | i = 31, \dots, 60\}$ and independence of such pairs on Z_1, \dots, Z_k .
4. Variables X_{61}, \dots, X_{90} for the observation j were calculated as $X_{ij} = \delta_2 * e_{ij}$. So Y is independent on variables X_{61}, \dots, X_{90} and Z_1 .
5. Variables Z_2, \dots, Z_7 for the observation j were calculated as $Z_{ij} = Z_{1j}$ if $U_g \leq 0.9$ and as $Z_{ij} = 1 - Z_{1j}$ if $U_g > 0.9$. There are no regularities for combinations (Y, X_k, Z_i) when $31 \leq i \leq 90$ that are similar to regularity from Figure 1. Variables Z_1, \dots, Z_7 are included in scenario to imitate all 7 oximetry factors. At that for combinations (Y, X_k, Z_i) regularities are more distinct to compare with regularities for combinations (Y, X_k, Z_i) when $k > 1$. Sets $X_1, \dots, X_{30}; X_{31}, \dots, X_{60}; X_{61}, \dots, X_{90}$ will be referred to as $\tilde{C}_1, \tilde{C}_2, \tilde{C}_3$ correspondingly.

Results of experiment: Results of the first and second experiments are presented in Table 2. Columns of the table correspond to significance levels from $p < 0.0001$ to $p < 0.1$. Upper part of table corresponds to first experiment ($\delta_1 = 0.75$) and lower part of table corresponds to second experiment ($\delta_1 = 0.57$). Cell at intersection of row corresponding to significance level α and column corresponding to subset \tilde{C}_j contains number of triples (Y, X_k, Z_i) with $X_i \in \tilde{C}_j$ for which all three null hypotheses were rejected at least at level α . Number of triples from set $\{(Y, X_i, Z_k) | X_i \in \tilde{C}_j, 2 \leq k \leq 7\}$ for which all three null hypotheses were rejected at level α is given in the same cell in parentheses.

It may be seen from Table 2 that all three null hypotheses were rejected for the majority combinations from the set $\tilde{C}_1 = \{(Y, X_i, Z_k) | 1 \leq i \leq 30, k = 1, \dots, 7\}$. In the first experiment all null hypotheses were rejected at significance level $p < 0.01$ for all 30 combinations from \tilde{C}_1 when $k = 1$ and for 163 combinations from 180 when $k = 2, \dots, 7$. On the contrary all null hypotheses were rejected only for few combinations from the sets $\tilde{C}_2 = \{(Y, X_i, Z_k) | 31 \leq i \leq 60, k = 1, \dots, 7\}$ and $\tilde{C}_3 = \{(Y, X_i, Z_k) | 61 \leq i \leq 90, k = 1, \dots, 7\}$. All null hypotheses were rejected at significance level $p < 0.1$ only for one combination from \tilde{C}_2 and for one

Table 2: Results of experiments with simulated data.

δ_1	Set	< 0.0001	< 0.0002	< 0.001	< 0.0025	< 0.01	< 0.05	< 0.1
0.75	\tilde{C}_1	9 (20)	14 (31)	22 (95)	27 (125)	30 (163)	30 (174)	30 (178)
	\tilde{C}_2	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	0 (6)	1 (14)
	\tilde{C}_3	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	0 (3)	1 (6)
0.57	\tilde{C}_1	17 (53)	25 (78)	28 (133)	30 (155)	30 (171)	30 (174)	30 (174)
	\tilde{C}_2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (4)	1 (5)
	\tilde{C}_3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (5)	1 (6)

Table 3: Results of the null hypotheses testing when S100 or C4 are additional factors.

Hyp.	Fact.	sO2	pO2	pCO2	FCO hb	FO2Hb	FMetHb	FHHb
H_0^1	S100	< 0.0001	0.0238	< 0.0001	0.17	0.0668	0.0027	< 0.0001
	C4	0.0001	< 0.0001	0.0122	0.0913	0.0001	0.0043	< 0.0001
H_0^2	S100	< 0.0001	0.0305	0.0001	0.12	0.0589	0.0016	0.0002
	C4	< 0.0001	0.0001	0.0081	0.0716	< 0.0001	0.0065	< 0.0001
H_0^3	S100	0.0362	0.728	0.018	0.888	0.857	0.1139	0.0564
	C4	0.0013	0.0007	0.1626	0.433	0.001	0.1049	0.0023

combination from \tilde{C}_3 when $k = 1$. At that number of combinations where all three null hypotheses were rejected equals 6 inside \tilde{C}_2 and 3 inside \tilde{C}_3 for $k = 2, \dots, 7$. In the second experiment number of combinations where three null hypotheses were rejected at level α were higher than number of such combinations practically for all significance levels. So the results of experiments strongly indicate unbiasedness of the developed criterion.

Experiments with clinical data

The developed technique was applied to find regularities similar to the one shown in Figure 1 on the described above clinical data set. Three null hypotheses were tested for combinations (Y, X_r, Z_k) where Y is concentration of VEGF in serum, Z_1, \dots, Z_7 were oxymetry parameters sO2, pO2, pCO2, FCOhb, FO2Hb, FMetHb, FHHb. All 138 variables different from VEGF concentration and oxymetry parameters were tried as additional factors X_1, \dots, X_n . The most significant effects were revealed if concentrations of S100 proteins or complement component C4 in serum are used as additional factor X . Table 3 present calculated p-values \hat{p}^1, \hat{p}^2 and \hat{p}^3 correspondingly for all such combinations.

It is seen from Table 3 that all null hypotheses are rejected at significance level $p < 0.001$ for combination (VEGF, pO2, C4), at significance level $p < 0.002$ for combinations (VEGF, sO2, C4) and (VEGF, FO2Hb, C4), at significance level $p < 0.05$ for combinations (VEGF, FHHb,

C4), (VEGF, sO2, S100), (VEGF, pCO2, S100). Regularity related to effect of hypoxia on correlation between VEGF and S100 is shown at Figure 2.

Pattern from Figure 2 is similar to the pattern from Figure 1. However boundary point for pattern from Figure 2 is received by procedure I. This boundary differs from boundary for the pattern from Figure 1 that was calculated by OVP method. Correlation coefficient between VEGF and S100 in group of 33 cases with sO2 < 39.75% equals 0.64. Correlation coefficient increases to 0.88 after removing an outlying object highlighted in the left scatter diagram by red circle. No relationship between VEGF and S100 exists in group of 55 cases with sO2 < 39.75% as it may be seen from right diagram. Corresponding correlation coefficient equals 0.03.

It may be seen from Figure 3 and Figure 4 that effect of hypoxia on relationship between VEGF and C4 is similar to the effect of hypoxia on relationship between VEGF and S100. Correlation coefficient between VEGF and C4 is equal 0.47 in group of 31 cases with sO2 < 39.25% which corresponds to left scatter diagram from Figure 3. Correlation coefficient increases to 0.76 after removing of outlying object which is highlighted at the left scatter diagram by red circle. No significant relationship between VEGF and C4 exists in group of 57 cases with sO2 > 39.25% as it may be seen from the right diagram. Corresponding correlation coefficient equals -0.11. Correlation coefficient increases to 0.05 after removing the highlighted at right diagram outlier.

Low saturation index sO2 corresponds to high FHHB

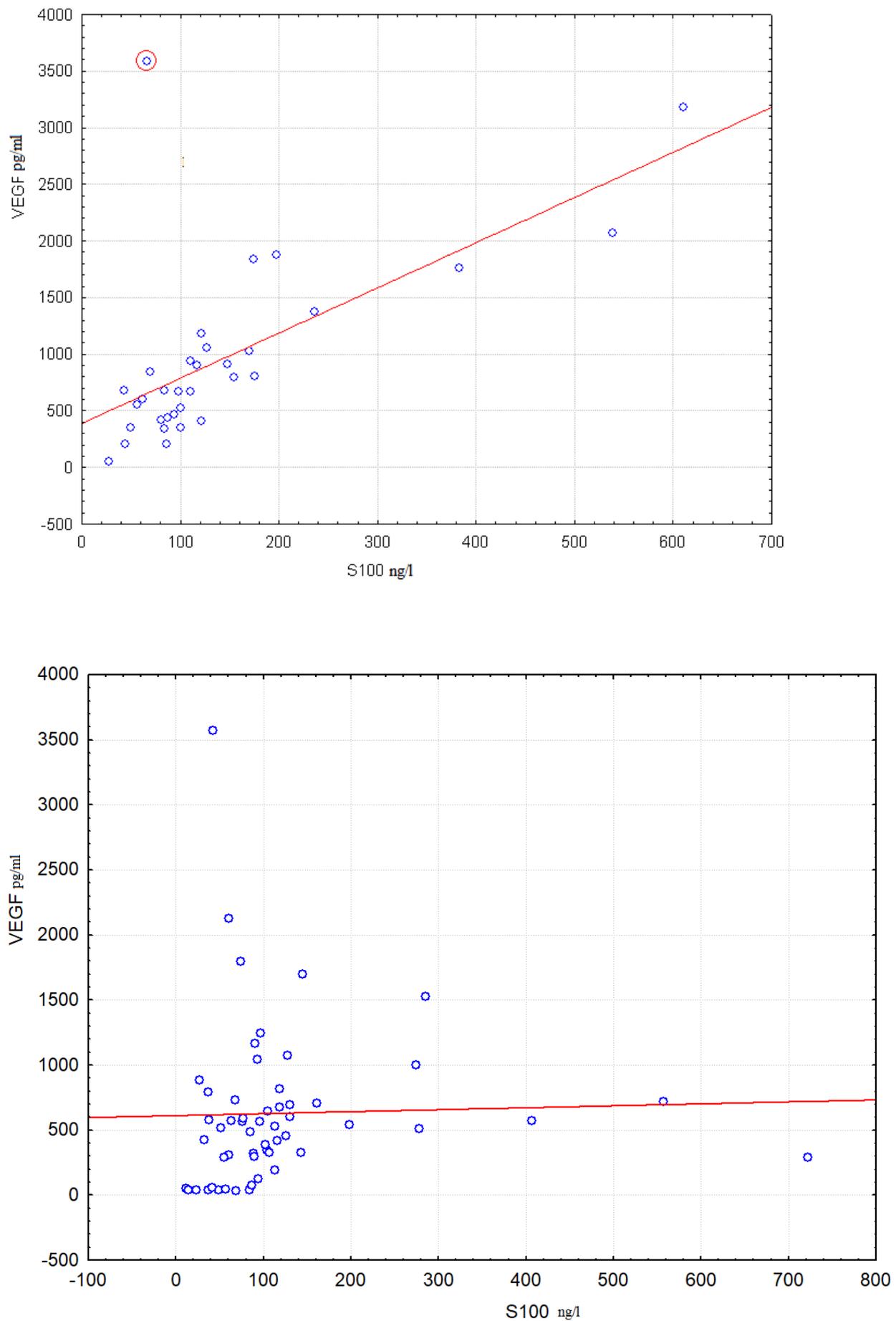


Figure 2: Correlations between S100 and VEGF levels are compared in groups with $sO_2 < 39.75\%$ (left scatter plot) and $sO_2 > 39.75\%$ (right scatter plot). Boundary point was calculated using Procedure I.

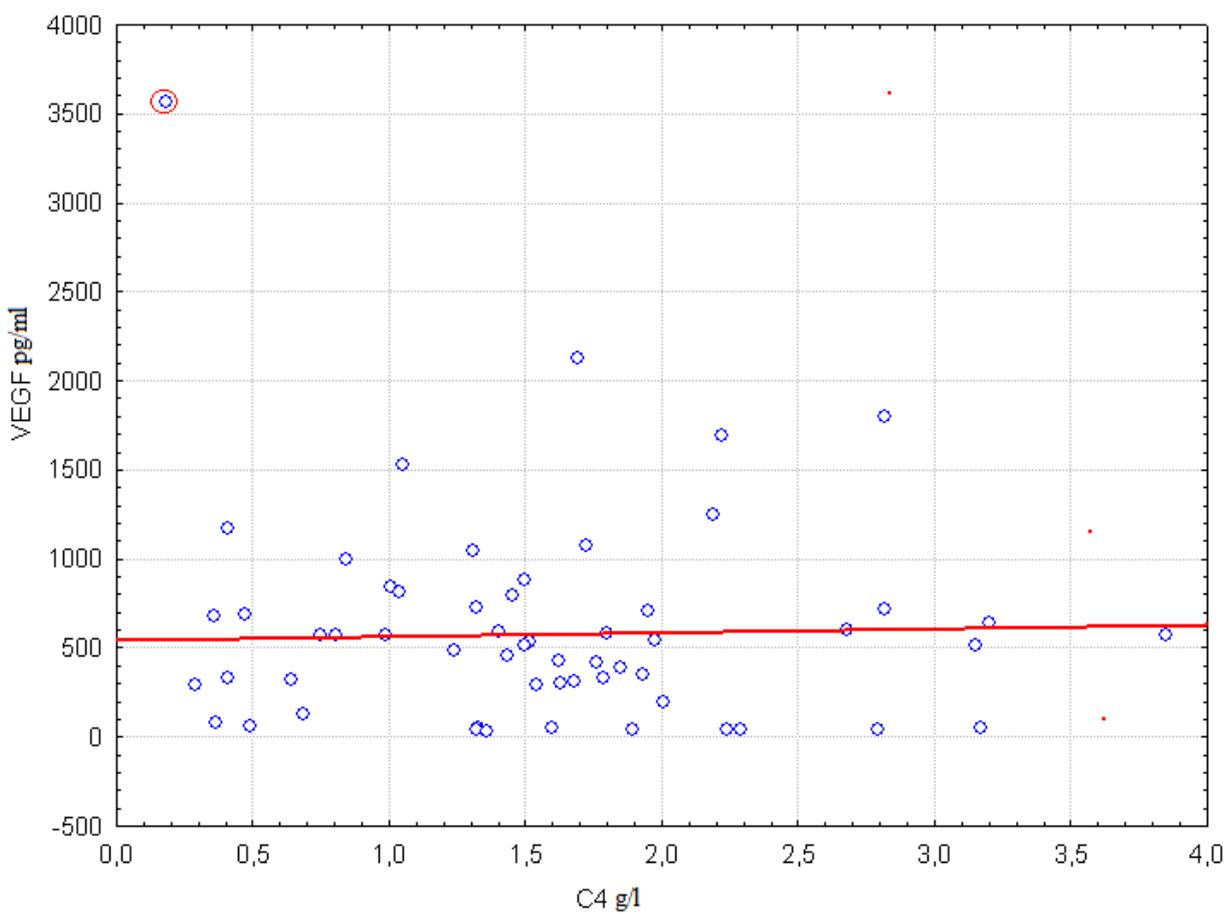
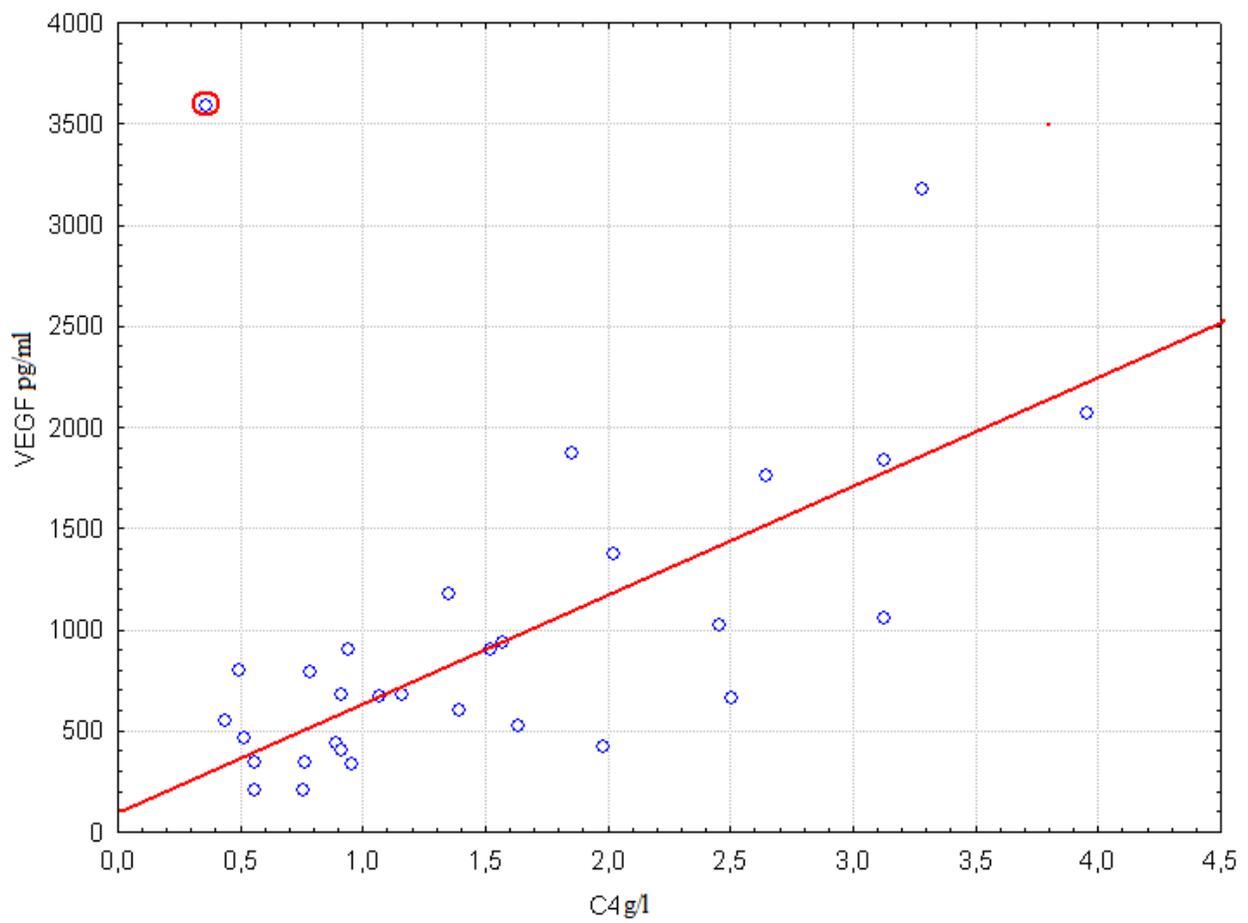


Figure 3: Correlation between VEGF and C4 levels are compared in groups with $sO_2 < 39.25\%$ (left scatter plot) and $sO_2 > 39.25\%$ (right scatter plot). Boundary point was calculated using Procedure I.

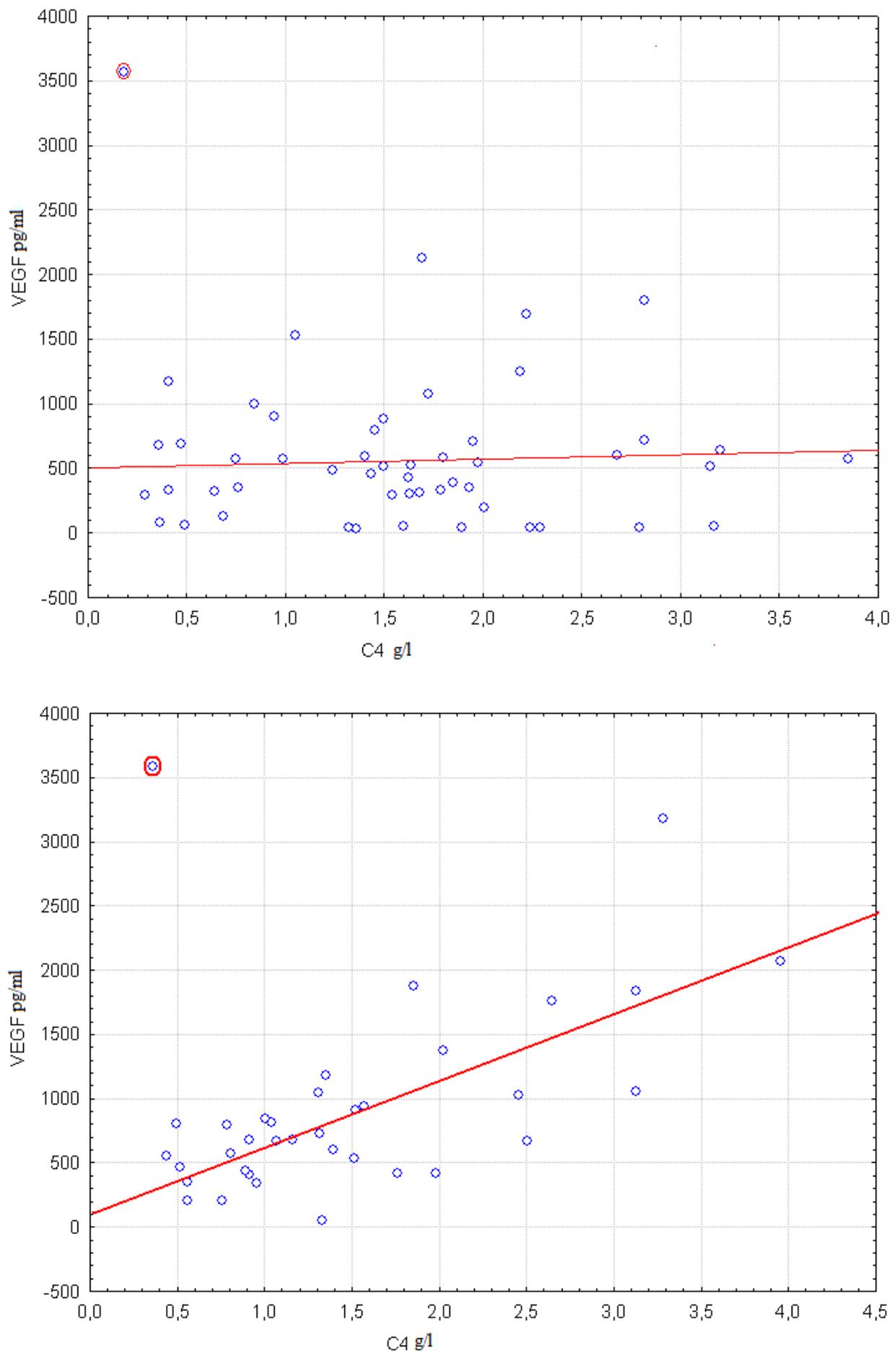


Figure 4: Correlation between VEGF and C4 levels are compared in groups with $FHHB < 56.3\%$ (left scatter plot) and $FHHB > 56.3\%$ (right scatter plot). Boundary point was calculated using Procedure I.

values. Strong correlation between VEGF and S100 exists when FHHB is greater than a certain threshold. At that correlation coefficient is close to zero when FHHB is lower than the threshold as can be seen in Figure 4. Correlation coefficient between VEGF and C4 in group of 36 cases with $FHHB > 56.3\%$ equals 0.45. Correlation coefficient increases to 0.73 after removing of outlier highlighted at right diagram. No significant relationship between VEGF and C4 exists in group of 52 cases with $FHHB < 56.3\%$ as it may be seen from left diagram. Corresponding correlation coefficient is equal -0.1. Correlation coefficient increases to 0.07 after removing of outlier highlighted at left diagram.

Our goal is testing if hypoxia has effect on VEGF production via controlling relationship between VEGF and some additional factor. Hypoxia effect is manifested via effects associated with different oximetry parameters. Existence of supposed hypoxia effect contradicts simultaneously to several of null hypotheses associated with different oximetry parameters. Hypoxia effect may be assessed by testing global null hypotheses $H_{0c}^1(i) = \bigcap_{k=1}^7 H_0^1(k,i)$, $H_{0c}^2 = \bigcap_{k=1}^7 H_0^2(k,i)$ and $H_{0c}^3 = \bigcap_{k=1}^7 H_0^3(k,i)$ [15]. Last global hypotheses may be tested with the help of nonparametric combinations (NPC) methodology [15]. To test global hypothesis $H_{0c}^g(i)$ ($g=1, \dots, 3$) on data set $\tilde{S}_t = \{(y_1, x_{1i}, z_{11}, \dots, z_{17}), \dots, (y_m, x_{mi}, z_{m1}, \dots, z_{m7})\}$ following NPC procedure was used.

Procedure II: Repeat steps $(b_1), \dots, (b_4)$ for $k = 1, \dots, 7$.

- b_1) Calculate T^0 according to step a_0 from Procedure I on data set $\{(y_1, x_{1i}, z_{1k}), \dots, (y_m, x_{mi}, z_{mk})\}$
- b_2) For $r = 1, \dots, N$ repeat step (b_2^g) if $H_{0c}^g(i)$ is tested, $g = 1, \dots, 3$.
 - b_2^1) Take random permutation
 - $\tilde{S}_t^r = \{(y_{u_i^r}, x_{1i}, z_{1k}), \dots, (y_{u_m^r}, x_{mi}, z_{mk})\}$ of \tilde{S}_t according to step (a_1) from Procedure I and implement step a_2 from Procedure I to calculate statistics T by \tilde{S}_t^r .
 - b_2^2) Take random permutation
 - $\tilde{S}_t^r = \{(y_1, x_{u_i^r}, z_{1k}), \dots, (y_m, x_{u_m^r}, z_{mk})\}$ of \tilde{S}_t according to step (a_3) from Procedure I and implement step a_2 from Procedure I to calculate

statistics T by \tilde{S}_t^r .

– b_2^3) Take random permutation

$\tilde{S}_t^r = \{(y_1, x_{1i}, z_{u_i^r, k}), \dots, (y_m, x_{mi}, z_{u_m^r, k})\}$ of \tilde{S}_t according to step (a_4) from Procedure I and implement step a_2 from Procedure I to calculate statistics T by \tilde{S}_t^r .

- b_3) Calculate $\hat{p}(k,i) = \frac{\sum_{1 \leq j \leq N} \mathbb{1}(T^j(i) \geq T^0(i))}{N}$.
- b_4) Calculate $\lambda(k,r,i) = \frac{\sum_{1 \leq j \leq N} \mathbb{1}[T^j(i) \geq T^r(i)]}{N}$ $r = 1, \dots, N$
- Calculate statistics $T_c^0(i) = \psi[\hat{p}(1,i), \dots, \hat{p}(7,i)]$
- Calculate statistics $T_c^r(i) = \psi[\lambda(1,r,i), \dots, \lambda(7,r,i)]$
- Then calculate $\hat{p}_c(i) = \frac{\sum_{1 \leq j \leq N} \mathbb{1}[T_c^r(i) \geq T_c^0(i)]}{N}$ that is p-value testing hypothesis H_{0c}^g when step b_2^g .

Test statistics in Procedure II is calculated as a combining function of p-values related to partial tests. Several combining functions are discussed in [15]. The best performance is achieved according to our experiments when slightly modified Fisher combining function ψ is used. Let $\hat{p}_1, \dots, \hat{p}_k$ are some p-values calculated by permutation test with N random permutations. Then $\psi(\hat{p}_1, \dots, \hat{p}_k) = -2 \sum_{i=1}^k \log \hat{p}_i'$, where $\hat{p}_i' = \hat{p}_i$ if $\hat{p}_i > 0$ and $\hat{p}_i' = 1/2N$ otherwise.

Results of the Procedure II applied to the studied data set are presented in Table 4. It can be seen that the global null hypotheses H_{0c}^1 and H_{0c}^2 are rejected at level $p < 0.0005$ when additional factors are C4 and S100 concentrations. Global null hypothesis H_{0c}^3 is rejected at $p = 0.0001$ when additional factor is C4 concentration. But H_{0c}^3 is not rejected when additional factor is S100 concentration.

Multiplicity Control

It was necessary to test global null hypotheses from

Table 4: Results of the global null hypothesis testing with NPC and multiplicity control with Procedure III.

Hypothesis	Additional Factor	Fisher Function	Combined p-value	Corrected p-value
H_{0c}^1	S100	82.06	0.0001	0.0127
	C4	100.95	0.0001	0.0127
H_{0c}^2	S100	87.96	0.0004	0.0481
	C4	96.04	< 0.0001	< 0.0001
H_{0c}^3	S100	25.4	0.091	Not significant
	C4	63.9	0.0001	0.0127

set $\{H_{0c}^1, H_{0c}^2, H_{0c}^3 | i = 1, \dots, n\}$ to reveal the supposed effect of hypoxia on relationship between concentration of VEGF and concentrations of C4 or S100. So a multiple testing procedure must be used to assess true statistical significance of revealed effects. It is sufficient to use a single-step procedure because only two global null hypotheses associated with C4 and S100 as initial factors were rejected. Methods min P and max T are effective tools of multiplicity control [22]. More universal min P procedure is preferable for discussed task because max T technique is based on unjustified supposition about approximate equality of test statistics distribution.

Adjusted p -values were calculated by the represented below **Procedure III**.

Repeat steps (c_1) , (c_2) and (c_3) for $i = 1, \dots, n$.

- c_1) Use Procedure II to calculate statistics $T_c^0(i), T_c^1, \dots, T_c^N$ by \tilde{S}_i and random permutations $\{\tilde{S}_i^r | r = 1, \dots, N\}$ correspondingly. At that step b_2^g is implemented when $H_{0c}^g(i)$ is tested, $g = 1, \dots, 3$.
- c_2) Calculate $\lambda_c(0, i) = \frac{\sum_{1 \leq j \leq N} \mathbb{1}(T_c^j(i) \geq T_c^0(i))}{N}$
- c_3) Calculate $\lambda_c(r, i) = \frac{\sum_{1 \leq j \leq N} \mathbb{1}(T_c^j(i) \geq T_c^r(i))}{N}$
- c_4) Calculate $\hat{p}_{mt} = \frac{\sum_{1 \leq r \leq N} \mathbb{1}[\min_{i \in \{1, \dots, n\}} \lambda_c^r(i) \leq \hat{p}_c^0(i)]}{N}$
- c_5) \hat{p}_{mt} is equal to \hat{p}_{mt}^g that is adjusted p -value testing global null hypothesis H_{0c}^g when (b_2^g) is used at step (c_1) .

The described Procedure III provides weak FWE control. Procedure III was applied to calculate adjusted p -values for global null hypotheses H_{0c}^1, H_{0c}^2 and H_{0c}^3 . Results are presented in Table 4.

It is seen from Table 4 that the global null hypotheses H_{0c}^1 and H_{0c}^2 are rejected when concentrations of C4 and S100 are additional factors. At that H_{0c}^3 is not rejected if additional factor is concentration of S100. So it is possible that pattern from the Figure 2 may be related only to existence of linear relationship between VEGF and S100 levels in serum which is not controlled by hypoxia. On the other hand set of relationships that are represented at Figure 3 and Figure 4 or are mentioned in Table 3 where concentrations of C4 is additional factor cannot be explained with the help of some simpler effect. These relationships cannot be reduced to linear correlation between VEGF and C4. Also they cannot be explained by effect of hypoxia on VEGF only or on C4 only. So supposition that relationship between VEGF and C4 levels in serum is controlled by hypoxia is

in accordance with data.

Conclusion

Results may be shortly summarized as follows. A method was developed which is aimed to discover relationships of the following type in data: Significant linear correlation between two factors Y and X_i exists only if third factor Z_k belongs to interval from one side of some threshold δ . At that from another side of δ Pearson correlation coefficient is close to zero.

It was suggested to consider such three-factor relationship as statistically significant when rejecting three null hypotheses: H_{0c}^1, H_{0c}^2 and H_{0c}^3 . Nonparametric permutation tests with statistics that is optimal value of special quality functional were used to test these hypotheses.

Performance of the method was evaluated in tasks with simulated data. Good concordance between found regularities and patterns provided by the experiment scenario is seen from Table 2.

The method was applied to test supposition that hypoxia control relationship between serum VEGF concentration and some factor from the analyzed clinical database. It was supposed that hypoxia is manifested by oximetry parameters. Three null hypotheses were rejected for set of triples (Y, X_i, Z_k) where Y is VEGF concentration, X_i is some additional factor and Z_k is some of oximetry parameters.

Significance of hypoxia effect on correlation between VEGF level and additional factor X_i may be assessed as combined significance of effects related to different oximetry parameters. Combined significance was evaluated with the help of NPC method testing intersection of null hypotheses related to set of triples $\{(Y, X_i, Z_k) | k = 1, \dots, 7\}$. A single-step permutations based FWE control was implemented to take into account that additional factor is searched among 138 variables.

It was shown that three combined null hypotheses were rejected at significance level $p < 0.02$ when concentration of complement C4 is the additional factor. Developed technique may be used in variety of biomedical tasks where it is necessary to assess effect of some factor or some group of factors on existing linear ties.

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