# Alteration of circulating natural antibodies against VEGFR1-derived peptide antigens in atherosclerosis

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**Research**

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# Abstract

Background Several lines of evidence suggest the protective role of natural antibody in common chronic disease like atherosclerosis and cancer. Vascular endothelial growth factor receptors 1 (VEGFR1) and 2 (VEGFR2) are important regulators of angiogenesis and their involvement in developing atherosclerosis cannot be ruled out.

Purpose The present study was designed to develop an in-house enzyme-linked immunosorbent assay to test if natural IgG for VEGFR1 and regulatory T cell markers CD25 and FOXP3 could be associated with atherosclerosis.

Methods A total of 218 patients with atherosclerosis and 200 healthy controls were recruited; all patients had atherosclerotic carotid plaque and carotid intima–media thickness was analyzed using a diagnostic ultrasound system.

Results Mann-Whitney U test demonstrated that plasma anti-VEGFR1 IgG levels were significantly lower in atherosclerosis patients than control subjects (Z=-2.46, P =0.014) although neither anti-CD25 IgG levels nor anti-FOXP3 IgG levels showed significant changes. Male patients mainly contributed to the decreased antiVEGFR1 IgG levels (Z=-2.45, P =0.014). Spearman correlation analysis failed to show any significant correlation between natural IgG levels and carotid intima–media thickness.

Conclusion Decreased levels of anti-VEGFR1 IgG may be involved in developing atherosclerosis-related conditions.


# Introduction

Atherosclerosis is a chronic inflammatory disorder developed within the wall of large and medium-sized arteries and responsible for several adverse clinical events including acute coronary syndrome and ischemic stroke [1, 2]. The disease is characterized by inflammatory infiltrates, subendothelial accumulation of oxidized lipids, angiogenesis and fibrosis. Both innate immunity and adaptive immunity contribute to a proatherogenic inflammatory response that occurs in every step of atherosclerosis development, from the beginning of the fatty streak to plaque rupture [3–6]. The pathogenic event of atherogenesis is a progressive process associated endothelial dysfunction, inflammatory cell adhesion and oxidative stress [7]. Vascular endothelial growth factor (VEGF) receptors 1 (VEGFR1) and 2 (VEGFR2) are important regulators of angiogenesis and their involvement in developing atherosclerosis cannot be ruled out [8]. In function, VEGFs stimulate the angiogenesis via activation of VEGFR2 while VEGFR1 has been thought to down-regulate the function of VEGFR2 [9, 10].

Regulatory T (Treg) cells were characterized as $CD4^{+}CD25^{+}$ T cells that could suppress excessive immune response and maintain immune tolerance in periphery [11]. Treg cells can also specifically express fork-head box P3 (FOXP3), a master regulator that is critical for their development and immunosuppressive function and is considered as one of the most reliable molecular markers for Treg

cells [12, 13]. Treg cells have been found to suppress atherosclerosis development or progression by down-regulating effector T cells (Teff)-mediated inflammatory response via multiple mechanisms, such as secretion of inhibitory cytokines interleukin (IL)10 (IL-10) and transforming growth factor beta (TGF-β) [1], cell-contact dependent suppression [14] and depletion of the IL-2 [15]. Several studies of experimental atherosclerosis showed that adoptive transfer of Treg cells prevented the development of atherosclerosis [16], while depletion of Treg cells by anti-CD25 antibody boosted the formation of atherosclerotic plaque [17].

Natural antibodies are immunoglobulin generated by innate B cells such as B-1 lymphocytes without any immunization and play an important role in maintaining immune homeostasis in the body [18–20]. The levels of natural antibodies are decreased with advancing age, so that an age-related loss in amount or efficacy of natural antibodies may increase risk of developing several diseases such as atherosclerosis, type-2 diabetes, Alzheimer's disease and malignancy [21]. To date, natural antibodies have found to be involved in several common chronic diseases such as amyloid proteins-related neurodegeneration [22] and cancer [23]. In a recent study, we found that decreased levels of natural antibodies against CD25-derived peptide antigens were associated with the development of lung cancer [24].

The present study was designed to determine the levels of natural antibodies against peptide antigens derived from CD25, FOXP3 and VEGFR1 in atherosclerosis and to confirm if these antibodies are associated with development of atherosclerosis.

# Materials And Methods

*Subjects*

Plasma samples were collected from 218 patients with atherosclerosis, who were admitted to the Department of Neurology, Second Hospital of Jilin University, Changchun in the period between November 2015 and March 2017. Of these 218 patients aged 61.1±11.4 years, 127 were male and 91 were female. All patients had atherosclerotic carotid plaque and carotid intima–media thickness (CIMT) was analyzed using a diagnostic ultrasound system (iE Elite, Philips, USA). A total of 200 healthy subjects aged 60.9±11.7 years were simultaneously recruited from local communities, 109 of whom were male and 91 were female. Demographic information and clinical characteristics of these participants are given in Table 1. Those participants who had suffered from any type of malignancy and autoimmune disorders such as autoimmune thyroid disease, pernicious anemia, type-1 diabetes, celiac disease, multiple sclerosis, systemic lupus erythematosus and inflammatory bowel diseases were excluded from this study. All participants were of Chinese Han origin and all signed informed consent to donate their blood samples for this study as approved by the Ethics Committee of Second Hospital of Jilin University in accordance with the ethical guidelines of the Declaration of Helsinki.

*Detection of plasma IgG levels*

Seven linear peptide antigens, including three derived from CD25 (CD25a, CD25b and CD25c), two derived from FOXP3 (FOXP3a and FOXP3b) and two derived from VEGFR1 (VEGFR1a and VEGFR1b) were designed based on a computational epitope prediction software (http://www.iedb.org) and synthesized by solid-phase chemistry with a purity of ≥95%. The sequence information of these five peptide antigens is shown in Table 2. Enzyme-linked immunosorbent assay (ELISA) was developed in-house for detection of plasma IgG levels for the above three target molecules as described in previous reports [24, 25]. In order to minimize the effect of non-specific binding on the accuracy of experimental data, the specific binding ratio (SBR) was used to represent a relative level of plasma IgG for CD25, FOXP3 and VEGFR1. SBR was calculated as follows:

$$SBR = (OD_{sample} - OD_{NC})/(OD_{PC} - OD_{NC})$$

Pooled plasma from ≥100 unrelated healthy individuals was used as quality control (QC) sample and tested on every plate. The reproducibility of the in-house ELISA was evaluated by the inter-assay deviation with calculation of the coefficient of variation (CV).

*Statistical analysis*

All antibody test data were expressed as means ± standard deviation (SD). Statistical analysis was performed with the IBM SPSS 22.0 software. Based on Kolmogorov-Smirnov one-sample test for the normality of the distribution of plasma IgG levels, Mann-Whitney *U* test was used to examine the difference in plasma IgG levels between the patient group and the control group. Spearman correlation analysis was used to examine the relationship between the levels of plasma IgG for CD25, FOXP3 or VEGFR1 and carotid intima–media thickness. A *P*-value of <0.017 was considered statistically significant as three target molecules were independently tested in this study.

# Results

The in-house ELISA showed a good reproducibility with a CV value ranging from 7.37% to 21.15% (Table 3). As shown in Table 4, plasma IgG levels for VEGFR1b were significantly decreased in patients with atherosclerosis compared with control subjects (Z=-2.46, *P*=0.014) and male patients appeared to contribute to the decreased anti-VEGFR1b IgG levels (Z=-2.45, *P*=0.014). Plasma IgG levels for other six peptide antigens failed to show significant differences between the patient group and the control group.

There was no correlation between carotid intima-media thickness and plasma IgG levels for CD25, FOXP3 or VEGFR1 (Table 5).

# Discussion

The present study demonstrated that plasma IgG levels for VEGFR1-derived peptide antigen VEGFR1b were significantly lower in patients with atherosclerosis as compared with healthy controls, especially in male patients (Table 4), suggesting that dysfunction of VEGFR1 is likely to be involved in developing

atherosclerosis, although we failed to find a significant correlation between anti-VEGFR1b IgG levels and carotid intima-media thickness (Table 5). VEGFR family consists of three transmembrane receptors with tyrosine kinase activity, including VEGFR1, VEGFR2 and VEGFR3 [26]. VEGFR1 and VEGFR2 are highly expressed in vascular endothelial cells while VEGFR3 is mainly localized in lymphatic endothelial cells [27]. Because most VEGFR1 isoforms are soluble, they can block VEGF binding to VEGFR2 and influence the formation of blood vessels. It has been reported that bevacizumab that is anti-VEGF monoclonal antibody for treatment of solid cancer could produce cardiovascular toxicity [28]. Possibly, the imbalance between VEGFR1 and VEGFR2 contributes to the development of atherosclerosis.

Several reports have demonstrated that oxidized low-density lipoprotein (oxLDL), a trigger of atherogenesis, may be involved in inhibiting Treg cells [29]. OxLDL can induce the apoptosis of Treg cells and hamper their immunosuppressive function through down-regulation of FOXP3 expression [30–32]. Recent work has suggested that activated Treg cells suppress the atherosclerosis progression and that FOXP3 genetically controls a transcriptional program that acts protectively in human atherosclerotic plaques [33]. Although our study has failed to show a significant change in circulating IgG for CD25 and FOXP3, there was a trend toward a decrease in anti-FOXP3b IgG levels in patients with atherosclerosis (Table 4). Further investigation is needed to test circulating IgG against a range of peptide antigens derived from FOXP3 protein.

It has long been noted that there is a gender difference in the pathophysiology of atherosclerosis [34, 35]. The gender differences in sex hormones and genetic background may be associated with susceptibility to atherosclerosis in men [36]. The present study found that there was a gender difference in circulating natural antibodies and a significant decrease in anti-VEGFR1b IgG levels was observed only in male patients (Table 4). This finding supports the hypothesis that males are more likely to develop atherosclerosis than females [36].

# Conclusion

Deficiency of plasma anti-VEGFR1 IgG is likely to contribute to the development of atherosclerosis; decreased anti-VEGFR1b IgG levels in the circulation may be a useful biomarker for identification of a subgroup of atherosclerosis-related conditions in which dysfunction of VEGFR1 may be involved.

# Abbreviations

BSA:Bovine serum albumin; CI:Confident interval; CIMT:Carotid intima-media thickness; CV:Coefficients of variation; ELISA:Enzyme-linked immunosorbent assay; FOXP3:Fork-head box P3; IL-10:Interleukin 10; NC:Negative control; OD:Optical density; oxLDL:Oxidized low-density lipoprotein; PBS:Phosphate - buffered saline; PC:Positive control; QC:Quality control; SBR:Specific binding ratio; SD:Standard deviation; TGF-β:Transforming growth factor beta; Treg:Regulatory T cells; VEGF:Vascular endothelial growth factor; VEGFR1:VEGF receptors 1; VEGFR2:VEGF receptor 2.

# Declarations

### Ethical approval and consent to participate

This work was approved by the Ethics Committees of the Second Hospital of Jilin University, Changchun, China, (IRB#: SHJU2017-099). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### Consent for publication

All patients or their surrogates provided informed consent for this study.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing Interests

The authors declare that they have no conflict of interests.


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### Author's contributions

PW and SL carried out laboratory work, data analysis and drafting the manuscript; ZW and HZ were responsible for identification of patients and healthy controls, and collection of samples and clinical information; XZ conceived of this study, supervised laboratory work and data analysis, and corrected the manuscript.


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# Tables

**Table 1**. Demographic and clinical characteristics of patients with atherosclerosis and control subjects

| Characteristics | Patients | Controls |
|---|---|---|
| Gender | | |
| Male | 127 (58.3%) | 109 (54.5%) |
| Female | 91 (41.7%) | 91 (45.5%) |
| Age (years) | 61.2±11.4 | 60.9±11.7 |
| Smoking history | | |
| Smoker | 106 (48.4%) | 98 (49%) |
| Non-smoker | 113 (51.6%) | 102 (51%) |
| Site of plaques | | |
| Carotid artery | 6 (2.7) | -- |
| Carotid bifurcation | 51 (23.3) | -- |
| Internal carotid artery | 39 (17.8) | -- |
| Internal carotid bifurcation | 2 (0.9) | -- |
| Common carotid artery | 100 (45.7) | -- |
| Subclavian artery | 20 (9.1) | -- |

**Table 2.** The sequence information for peptide antigens derived from three target molecules

| Antigen | Sequence(N→C) | NCBI Accession | Position (aa) |
|---|---|---|---|
| CD25a | kpghcrepppweneateriyhfvvgqmvy | NP_000408 | 99-126 |
| CD25b | iyhfvvgqmvyyqcvqgyralhrgpaesve | NP_000408 | 116-144 |
| CD25c | khtsqfpgeekpqaspegrpesetsch | NP_000408 | 167-187 |
| FOXP3a | dmfaffrnhpatwknairhnlslhkcd | NP_001107849 | 335-359 |
| FOXP3b | Kctfpnpsaprkdstlsavpqssyh | NP_001107849 | 134-156 |
| VEGFR1a | degvyhckatnqkgsvessayltvqgtsdk | NP_002010 | 725-754 |
| VEGFR1b | cqitwfknnhk iqqepgiilg pgsstd | NP_002010 | 691-715 |

**Table 3**. Inter-assay deviation of the in-house ELISA for plasma IgG antibodies

| Antibody | No of plates | Coefficient of variation (%) |
|---|---|---|
| CD25a | 20 | 19.1 |
| CD25b | 20 | 12.2 |
| CD25c | 20 | 21.2 |
| FOXP3a | 20 | 12.0 |
| FOXP3b | 20 | 10.8 |
| VEGFR1a | 20 | 7.4 |
| VEGFR1b | 20 | 11.9 |

**Table 4**. The levels of plasma IgG against CD25, FOXP3 and VEGFR1 in patients with atherosclerosis and control subjects

| IgG | Group | Patient (n) | Control (n) | Z[a] | *P* [b] |
|---|---|---|---|---|---|
| CD25a | Male | 0.73±0.20(127) | 0.72±0.19(109) | 0.40 | 0.686 |
| | Female | 0.75±0.21 (91) | 0.70±0.21 (91) | 1.41 | 0.158 |
| | Both | 0.73±0.20(218) | 0.71±0.20(200) | 1.20 | 0.231 |
| CD25b | Male | 0.81±0.21(127) | 0.80±0.23(109) | 0.43 | 0.669 |
| | Female | 0.84±0.19(91) | 0.82±0.19(91) | 0.99 | 0.323 |
| | Both | 0.82±0.20(218) | 0.81±0.21(200) | 0.92 | 0.359 |
| CD25c | Male | 1.32±0.43(127) | 1.31±0.51(109) | 0.49 | 0.622 |
| | Female | 1.37±0.46 (91) | 1.29±0.48 (91) | 1.26 | 0.208 |
| | Both | 1.34±0.44(218) | 1.30±0.49(200) | 1.22 | 0.222 |
| FOXP3a | Male | 0.93±0.28(127) | 0.92±0.24(109) | -0.15 | 0.878 |
| | Female | 0.97±0.27 (91) | 0.92± 0.20(91) | 1.09 | 0.274 |
| | Both | 0.94±0.27(218) | 0.93±0.23(200) | -0.53 | 0.594 |
| FOXP3b | Male | 0.85±0.25(127) | 0.92±0.25(109) | -2.17 | 0.03 |
| | Female | 0.91±0.25 (91) | 0.93±0.21 (91) | -0.74 | 0457 |
| | Both | 0.87±0.25(218) | 0.92±0.23(200) | -2.224 | 0.025 |
| VEGFR1a | Male | 1.56±0.45(127) | 1.65±0.42(109) | -1.83 | 0.067 |
| | Female | 1.67±0.57 (91) | 1.67±0.47 (91) | -0.53 | 0.595 |
| | Both | 1.60±0.50(218) | 1.66±0.44(200) | -1.82 | 0.069 |
| VEGFR1b | Male | 1.44±0.39(127) | 156±0.38(109) | -2.45 | 0.014 |
| | Female | 1.59±0.52 (91) | 1.60±0.41 (91) | -0.81 | 0.416 |
| | Both | 1.50±0.45(218) | 1.58±0.39(200) | -2.46 | 0.014 |

Plasma IgG levels are expressed as mean ± SD in SBR. [a] Mann-Whiteney *U* test; [b] $P < 0.017$ was considered statistically significant.

**Table 5**. Spearman correlation analysis of the relationship between carotid intima-media thickness and plasma IgG levels for CD25, FOXP3 and VEGFR1

| Antibody | df | Coefficients of correlation (r) | *P* |
|---|---|---|---|
| CD25a | 216 | -0.011 | 0.870 |
| CD25b | 216 | -0.057 | 0.405 |
| CD25c | 216 | -0.026 | 0.698 |
| FOXP3a | 216 | 0.015 | 0.829 |
| FOXP3b | 216 | -0.020 | 0.765 |
| VEGFR1a | 216 | 0.018 | 0.788 |
| VEGFR1b | 216 | -0.020 | 0.765 |