Circulating Nampt and RBP4 levels in patients with carotid stenosis undergoing carotid endarterectomy (CEA)

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A B S T R A C T

Background: Obesity is a risk factor for atherosclerotic vascular disease. Altered adipokine secretion, including increased production of nicotinamide phosphoribosyltransferase (Nampt) and retinol binding protein 4 (RBP4) may link adipose tissue dysfunction to cardiovascular complications.

Methods: We determined Nampt and RBP4 serum concentrations in 193 consecutive patients with carotid stenosis prior to carotid endarterectomy (CEA) in relation to recently experienced ischemic events, markers of atherosclerosis and obesity, as well as anthropometric and clinical characteristics.

Results: Nampt but not RBP4 was significantly higher in symptomatic patients who experienced an ischemic event within 6 months before surgery compared to asymptomatic patients (p=0.001). In multivariate regression analysis Nampt was the only independent predictor of symptomatic carotid stenosis. Nampt correlated with peripheral leukocyte blood count (p<0.0001) and with the number of macrophages/foam cells within carotid plaques (p=0.042). However, Nampt and RBP4 serum concentrations did not correlate with the maximum percentage of carotid stenosis.

Conclusion: Our data suggest circulating Nampt as an independent predictor of recently experienced ischemic events in patients with carotid stenosis despite the lack of an association between Nampt and carotid atherosclerosis severity.

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1. Introduction

Obesity is a risk factor for complications of atherosclerotic vascular disease such as stroke and myocardial infarction [1–4]. Understanding the mechanism linking obesity to atherosclerosis is critical for its prevention and treatment. Reduced fat mass and improved fitness level after exercise interventions correlate with a decrease in many pro-inflammatory adipokines, adipose tissue derived factors that most likely play an important role in the atherosclerotic process [5,6]. Adipokines may directly affect vascular homeostasis by influencing the function of endothelial cells, arterial smooth muscle cells, macrophages, and foam cells in the vessel wall [7]. Pro-inflammatory adipokines are circulating mediators of inflammation participating in mechanisms of vascular insult and atherothrombotic complications. In pro-atherothrombotic and atherothrombotic states the secretion of pro-inflammatory adipokines is elevated [6]. Adipokines such as leptin, TNF-α, and IL-6 induce endothelial dysfunction and increase vascular inflammation thus driving the atherosclerotic process. They prompt the expression of adhesion molecules on the surface of endothelial cells, increase therefore the attachment and migration of monocytes into the vessel wall and enhance their conversion into macrophages and foam cells thus pushing inflammation [7]. In contrast to pro-inflammatory adipokines, adiponectin may act as an endogenous anti-inflammatory modulator of both innate and adaptive immunity in atherogenesis [9]. Adipokines were also found within atherosclerotic plaques, suggesting local in addition to endocrine effects of these mediators in atherosclerotic lesions.

Carotid atherosclerosis is a leading cause of cerebrovascular events. The control of vascular risk factors, i.e. obesity, hypertension,
dyslipidemia, diabetes and smoking, proved to reduce the number of fatal and non-fatal strokes [10,11]. The more recently described inflammation-associated adipokines nicotinamide phosphoribosyl-transferase (Nampt; also known as visfatin or pre-B-cell colony-enhancing factor, PBEF) and retinol binding protein 4 (RBP4) have been suggested as markers of carotid atherosclerosis measured as carotid intima–media thickness in patients with T2DM and in patients after cerebral infarcts, respectively [12,13].

However, the regulation of circulating Nampt and RBP4 in patients with either symptomatic or asymptomatic severe carotid stenosis prior to CEA has not been investigated. We therefore determined Nampt and RBP4 serum concentrations in relation to recently experienced ischemic events, the extent of atherosclerosis and obesity, circulating leptin and adiponectin, as well as anthropometric and clinical characteristics in individuals with severe carotid stenosis.

2. Subjects and methods

2.1. Subjects

The study was approved by the ethics committee of the University of Leipzig (148-2005) and the participating subjects signed an informed consent. A total of 193 consecutively recruited Caucasian men (n = 138) and women (n = 55) with extracranial carotid artery stenosis were included into the study. All patients, who were submitted for carotid endarterectomy (CEA) to the Carotid Stenosis Group of the Department of Surgery, University of Leipzig were recruited without any exclusion criteria. The severity of carotid atherosclerosis in each subject was assessed by the maximum percentage of stenosis. The exclusion criteria. The severity of carotid atherosclerosis in each

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical and anthropometric characteristics of carotid stenosis patients subdivided into symptomatic and asymptomatic subjects. Mean ± SD, for non-normally distributed parameters median and interquartile range are given; significant differences are in bold.</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Asymptomatic patients</td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.6 ± 9.2</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>75.6</td>
</tr>
<tr>
<td>Body mass index, BMI (kg/m²)</td>
<td>26.0 ± 3.7</td>
</tr>
<tr>
<td>Carotid artery stenosis (%)</td>
<td>87.1 ± 5.2</td>
</tr>
<tr>
<td>Patients with type 2 diabetes (%)</td>
<td>35.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.8 [5.5-6.4]</td>
</tr>
<tr>
<td>(Ex) smoker, n (%)</td>
<td>62.5</td>
</tr>
<tr>
<td>Patients with hypertension (%)</td>
<td>97.4</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>143.1 ± 22.1</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>78.3 ± 10.7</td>
</tr>
<tr>
<td>Serum RBP4 (ng/ml)</td>
<td>94.4 ± 68.8</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>8.4 [6.0-13.8]</td>
</tr>
<tr>
<td>Serum adiponectin (ng/ml)</td>
<td>17.9 [14.4-36.0]</td>
</tr>
<tr>
<td>Serum Nampt (ng/ml)</td>
<td>12.1 [9.0-15.6]</td>
</tr>
<tr>
<td>Concomitant medication</td>
<td>0.16 [0.10-2.24]</td>
</tr>
</tbody>
</table>

Leptin was measured by ELISA (Mediagnost, Reutlingen, Germany; sensitivity: 0.2 ng/ml; reference range of normal leptin values at a BMI of 25 kg/m²: males: 1.2–8.9 ng/ml; females: 8–24 ng/ml). Adiponectin was quantified using a Twin Plex ELISA (AdipoGen; sensitivity: 293 pg/ml; reference range of normal adiponectin values: 8.1–19.5 μg/ml). Serum C-reactive protein (CRP; immunoturbidometric latex test) and lipid analysis were performed with the Modular® Analytics EVO System (Roche Diagnostics GmbH, Mannheim, Germany). The leukocyte blood count was determined by the Sysmex XE-2100 analyzer (Sysmex GmbH, Bornbach, Germany).

2.2. Assays

RBP4 and Nampt serum levels were measured with an ELISA (AdipoGen; Inc.; Seoul, South Korea; sensitivity RBP4: 380 pg/ml, sensitivity Nampt: 30 pg/ml). The Nampt assay used in our study was evaluated carefully to be valid for Nampt measuring compared to other commercially available kits [14,15]. The Nampt sandwich ELISA used a monoclonal immobilized antibody and a polyclonal rabbit anti-human capture antibody directed to the full-length protein to determine Nampt within serum.

2.3. Histological assessment of carotid plaques

We studied consecutive plaques from 43 out of 193 (43/193) patients undergoing CEA. 22 out of 43 (22/43) patients were symptomatic. Plaques were scored as complicated (30/43) or non-complicated [16]. Serial cryostat sections of the plaques were stained for CD68 and Nampt as described [17]. Monoclonal antibodies directed to CD68 (clone KP1), and Nampt (clone OMN379) were purchased from Dako Deutschland GmbH (Hamburg, Germany) or Enzo Life Sciences GmbH (Lürrach, Germany), respectively. Inflammatory infiltrates containing CD68-positive macrophages/foam cells within the plaques were scored on a scale of 0 to 3 according to Redgrave et al. [18].
2.4. Statistical analyses

The study was powered to detect correlations >0.2 with 80% power and 5% significance threshold. Additionally, the study has 80% power to detect differences of 23 ng/ml RBP4 and of 1.4 ng/ml Nampt between symptomatic and asymptomatic patients (PASS 2008, version 08.0.5). Before statistical analysis, non-normally distributed parameters were logarithmically transformed to approximate a normal distribution (CrP, triglycerides, HDL cholesterol, HbA1c, RBP4, leptin, adiponectin). Mean and standard deviation (SD), for non-normally distributed parameters median and the interquartile range [25th–75th percentiles] were used. The following statistical tests were applied: unpaired Student’s t test, Chi quadrate test, Pearson’s simple correlation, partial correlation and linear regression.

Receiver Operating Characteristic (ROC) analyses were used to assess the diagnostic power of RBP4 and Nampt for discrimination between symptomatic and asymptomatic patients. The observed area under the ROC curve (AUC) is tested against the null-hypothesis (AUC = 0.5). Optimal cut-offs are derived from the ROC curves by maximizing the sum of sensitivity and specificity (Youden-Index).

Additionally, the effect of Nampt and RBP4 on the symptomatic status of patients was assessed using multivariate logistic regression techniques. All statistical computations were performed using SPSS version 18.0 (Chicago, IL, USA). P values less than 5% were considered as significant.

3. Results

3.1. Nampt and RBP4 serum concentrations in patients with carotid stenosis prior to CEA

Clinical and anthropometric characteristics of individuals included in our study are summarized in Table 1. We confirmed within our patients the well-described correlations of circulating leptin to age (p < 0.001), gender (p < 0.0001), BMI (p < 0.0001), and T2DM (p = 0.005) and of circulating adiponectin to age, gender, triglycerides, and HDL cholesterol (all p < 0.0001) suggesting that we examined a representative patient group. In univariate regression analyses Nampt serum concentration strongly correlates with leukocyte blood count, moderately with circulating adiponectin and slightly with HDL-cholesterol (Table 2); each parameter correlated independent of age, sex, and BMI with Nampt. We found significant well-known correlations between circulating RBP4 and CrP, total cholesterol, triglycerides, adiponectin and leptin (Table 2). Triglycerides and leptin correlated independent of age, sex, and BMI with RBP4.

Concomitant medication with statins had no significant effect on Nampt (statins 8.6 ± 3.3 ng/ml, no statins: 8.4 ± 3.9 ng/ml, p = 0.757) and RBP4 (statins 95.4 [66.0–123.0] μg/ml; no statins: 92.2 [67.6–129.4] μg/ml; p = 0.805) when analyzing all patients or comparing symptomatic and asymptomatic patients. The same was true for medication with antithrombotic agents (data not shown).

3.2. Serum Nampt was higher in symptomatic compared to asymptomatic patients

A relation between the maximum percentage of carotid stenosis and serum levels of all four examined adipokines, i.e. RBP4, Nampt, leptin, and adiponectin, was missing.

To further elucidate the relationship between circulating RBP4 and Nampt and the clinical severity of carotid stenosis, we divided our patients into two subgroups of either symptomatic or asymptomatic carotid stenosis patients. Serum Nampt concentrations were significantly higher in symptomatic compared to asymptomatic individuals.

Construction of ROC curves confirmed that Nampt serum concentration but not RBP4 significantly discriminates between groups of symptomatic and asymptomatic patients (Nampt: AUC = 0.64, p = 0.004, RBP4: AUC = 0.47, p = 0.270). The detection level for Nampt is about 7 ng/ml resulting in a sensitivity of 80% and a specificity of 44%. Interestingly, Nampt serum concentrations decreased with increasing time since the last experienced symptom of carotid artery stenosis. Patients who had symptoms 91–180 days prior to hospitalization had a Nampt serum concentration indistinguishable from asymptomatic patients.

We performed multivariate linear regression analyses to test the hypothesis that high pre-operative Nampt levels predict recent symptoms of carotid stenosis independent of age, sex, BMI, percentage carotid artery stenosis, T2DM, hypertension, (ex)smoking, CrP, leukocyte blood count, serum lipids, and the other adipokines. Multivariate regression analysis identified circulating Nampt as the only significant and independent predictor of previous neurological symptoms of carotid artery disease (p = 0.010).

3.3. Histological assessment of carotid plaques

We studied consecutive plaques from 43/193 carotid patients by histology [16,18]. Symptomatic and asymptomatic patients showed a comparable percentage of complicated plaques (68.2% vs. 71.4%).

Patients with complicated plaque tended to show higher Nampt levels (9.6 ± 3.0 ng/ml) compared to patients with uncomplicated plaques (6.7 ± 3.4 ng/ml). Patients with a high number of CD68-positive macrophages/foam cells (scores 2 and 3) showed higher Nampt levels (8.9 ± 3.0 ng/ml) compared to patients with no or low numbers of these cells (scores 0 and 1; 6.4 ± 1.9 ng/ml, p = 0.042). Serial cryostat sections of 22/43 carotid plaques were stained for CD68 and Nampt (Fig. 1). Nampt was localized in carotid atherosclerotic plaques only in CD68-positive lipid-laden macrophages and foam cells. The expression level of Nampt within these cells varied between the patients but did not correlate to any characteristic of complicated or non-complicated plaques (data not shown).

There was no correlation between circulating RBP4 and the numbers of macrophages/foam cells within the plaques in our patients.

3.4. Serum RBP4 decreased after CEA

Comparison of RBP4 one day before (80.0 [62.5–105.6] μg/ml) and four days after surgery (61.3 [42.6–74.6] ng/ml; p < 0.0001) revealed a dramatic decrease of RBP4. Both, i.e. pre- and post-CEA RBP4 values correlated well (r = 0.44; p = 0.001). There were no differences between sex, symptomatic and asymptomatic patients, or patients with and without T2DM with respect to early postoperative changes in RBP4 levels.

Table 2

Correlation matrix of study variables with Nampt and RBP4 serum concentrations. The same parameters as in Table 1 have been examined, but only significant correlations are shown. Pearson’s correlation coefficient (r) and level of significance (P); significant differences are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Nampt</th>
<th>RBP4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Symptomatic (%)</td>
<td>0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>Serum CrP (mg/l)</td>
<td>0.106</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leukocyte count (Gpt/l)</td>
<td>0.313</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>−0.089</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.146</td>
<td>n.s.</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol (mmol/l)</td>
<td>−0.132</td>
<td>n.s.</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol (mmol/l)</td>
<td>−0.195</td>
<td>0.023</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>−0.033</td>
<td>n.s.</td>
</tr>
<tr>
<td>Serum adiponectin (µg/ml)</td>
<td>−0.213</td>
<td>0.006</td>
</tr>
</tbody>
</table>
We tested whether circulating RBP4 levels one day before surgery were influenced by factors associated with surgery like psychological stress. Sera of 16 out of these 65 (16/65) patients were available 3.0 ± 0.78 months before surgery. However, the three month and one day pre-surgery levels did not differ significantly (three months 79.6 [69.8–107.7] μg/ml, one day 78.0 [64.7–113.3] μg/ml) and correlated well (r=0.726).

In contrast to RBP4, post-surgery Nampt (7.2±2.9 ng/ml) was not decreased after CEA compared to pre-surgery Nampt (8.4±3.2 ng/ml). The same was true for CrP before (4.2 [1.5–9.9] mg/l) and after (3.5 [1.3–10.2] mg/l) CEA and for the leukocyte blood count before (7.8±2.2 Gpt/l) and after (7.6±2.0 Gpt/l) CEA. Thus, the decrease of RBP4 after CEA is not associated with a simultaneous decrease of inflammatory markers.

4. Discussion

In the present study we examined the association of circulating Nampt and RBP4 with the extent of atherosclerosis, and anthropometric as well as clinical characteristics. We found higher Nampt levels in symptomatic patients with recent neurological symptoms compared to asymptomatic patients. Nampt was the only parameter associated with a preceding symptomatic event. Recently, we described circulating leptin as such a predictor in 107 carotid stenosis patients [19]. This result was confirmed in a group of 74 patients by Bountouris et al. [20], but could not be replicated in the present study although both of our patient groups did not differ significantly in relevant parameters. Contradictory results were also described for other circulating parameters discriminating between symptomatic and asymptomatic patients including CrP [19,21–24] or TNF-α [19,23,25]. Other factors such as matrix metalloproteinases, neurofilament protein heavy chain, or IL-6 have not yet been validated by many groups. Thus, robust biomarkers reflecting recent neurological events in carotid stenosis patients are currently not available. The relevance of Nampt as a biomarker must be evaluated in further studies.

Beside the fact that Nampt may be a predictor for recent neurological symptoms in individuals with carotid stenosis, circulating Nampt correlated strongly to peripheral leukocyte blood count in our patients. Nampt mRNA expression pattern (http://biogps.gnf.org)
and very recent publications [14,26] revealed the highest Nampt levels in peripheral blood supporting our observation that circulating Nampt correlates strongly with peripheral leukocyte number. Nampt is also localized in lipid-laden macrophages and foam cells within atherosclerotic lesions where it has been suggested to play a role in plaque destabilization [27]. Bone-marrow derived macrophages and macrophages differentiated from peripheral blood monocytes secrete Nampt [28]. In foam cells obtained from monocyctic THP-1 cells treated with oxidized low-density lipoprotein (ox-LDL) high levels of Nampt mRNA were determined [27]. These results indicate a contribution of accumulated lipid-laden macrophages and foam cells in carotid plaques to increased levels of circulating Nampt. As shown here in histology, within carotid atherosclerotic plaques CD68-positive lipid-laden macrophages and foam cells were the only potential cellular source for Nampt. Although the expression level of Nampt within these cells varied between our patients, scoring the number of CD68-positive lipid-laden macrophages and foam cells represents the number of Nampt expressing cells in plaques well. Accordingly, we found higher serum Nampt levels in patients that showed high numbers of lipid-laden macrophages/foam cells within the carotid plaques compared to patients with low numbers of such cells. Whether Nampt secretion by these cells affects circulating Nampt has yet to be investigated.

We did not find any significant correlation between Nampt serum concentration and BMI further suggesting that adipose tissue cells do not represent the main source of circulating Nampt. Within visceral fat, macrophages were identified as a source of Nampt [29]. The absence of an association between Nampt serum levels and BMI may also be explained by its multifactorial regulation and function as a nicotinamide phosphoribosyltransferase [14,30,31]. In addition, the use of non-valid assays for the measurement of Nampt in other studies may have led to contradictory results regarding the association between circulating Nampt and parameters of fat mass and distribution [14].

Several studies revealed consistently higher circulating Nampt in patients with chronic CAD, acute coronary syndrome (ACS) or carotid stenosis, i.e. clinically relevant atherosclerosis, compared to healthy controls [12,32,33] but whether there is a correlation between Nampt and the extent of atherosclerosis in patients with relevant plaques is unclear. Here, we did not see an association between Nampt and the percentage of carotid stenosis in patients undergoing CEA, thus with advanced plaques (see also Limitations of the study). In patients with CAD Nampt levels did not vary across the Gensini score, the angiographical index for luminal narrowing, or the number of narrowed coronary arteries [33]. These facts and our data indicate a missing correlation between Nampt and the extent of atherosclerosis.

Retinol-binding protein 4 (RBP4) has gained a lot of attention after its multifactorial regulation and function as a nicotinamide phosphoribosyltransferase [14,30,31]. In addition, the use of non-valid assays for the measurement of Nampt in other studies may have led to contradictory results regarding the association between circulating Nampt and parameters of fat mass and distribution [14].

5. Limitation of the study

There was no correlation between serum Nampt as well as RBP4 and the severity of carotid stenosis. This may be caused by the fact that almost all of our patients undergoing CEA had advanced stenosis and the carotid stenosis phenotype showed low variance. Thus, our patients represent the most affected individuals, not the full range of clinically encountered carotid stenosis. Moreover, the severity of carotid atherosclerosis was evaluated by the maximum percentage stenosis only. Plaque burden defined as the sum of the maximal thickness of all plaques in the bilateral carotid arteries determined by high-resolution ultrasound [50] would give a second important index for evaluation of severity of atherosclerosis. Both facts limit our statement on the missing relationship between serum Nampt as well as RBP4 and the severity of carotid stenosis.
Acknowledgments

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[8] Berg AH, Scherer PE. Adipose tissue, in... [rest of the reference is not visible].


