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ORIGINAL ARTICLE

Correlation of crevicular fluid and serum levels of retinol-binding protein 4 and leptin in chronic periodontitis and obesity

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Abstract
Objective Retinol-binding protein 4 (RBP4) and leptin are both adipokines and involved in the pathophysiology of different vascular and inflammatory diseases and selectively elevated in patients with obesity. The aim of the present study was to determine and correlate the levels of RBP4 and leptin in gingival crevicular fluid (GCF) and serum in patients with chronic periodontitis (CP) and obesity.
Materials and methods A total of 70 patients with age group 25 to 45 years were divided into four groups based on gingival index (GI), probing depth (PD), clinical attachment level (CAL), body mass index (BMI) and radiographic evidence of bone loss. The groups were (1) group I (non-obese periodontally healthy), (2) group II (obese periodontally healthy), (3) group III (non-obese with chronic periodontitis) and (4) group IV (obese with chronic periodontitis). The GCF and serum levels of human RBP4 and leptin were quantified using ELISA.
Results An increase in RBP4 levels from group I to group IV was found in both GCF and serum. However, GCF leptin levels was found to be greatest in group II, then group I, group IV and group III showing the least while an increase in serum levels from group I to group IV was found. The GCF and serum values of the inflammatory mediator correlated with the evaluated periodontal parameters and with each other ($p < 0.05$).
Conclusion RBP4 and leptin can be considered as possible GCF and serum markers of inflammatory activity in CP and obesity, which further longitudinal studies are needed.

Keywords Chronic periodontitis · Inflammation · Obesity · Gingival crevicular fluid

Introduction

Obesity is defined as the abnormal accumulation and storage of fat in adipose tissue. It may have unfavourable effect on health leading to disability and death [1]. According to the WHO World Health Statistics Report 2014, overweight were more than 1.9 billion adults (18 years and older). More than 600 million of these were obese. Approximately about 13% (11% of men and 15% of women) were obese of the world's adult population. Anthropometric measures are used for the standard epidemiologic translation of these important clinical facts. Central obesity has been measured by waist circumference and waist/hip ratio (where visceral adipose tissue is stored), and body mass index (kg/m^2) has been used as a measure of general obesity.

Obesity is well known to be a significant risk factor for various diseases in adults, such as type 2 diabetes, hyperlipidemia, hypertension and cholelithiasis, which ultimately leads to cardiovascular disease [1]. Obesity might represent a systemic condition capable of influencing the initiation and progression of periodontal disease as noted first using a ligature-induced periodontitis model in the rat [2].

Periodontal disease, a chronic inflammatory condition, is mainly affected by dental biofilms, clinically characterized by periodontal pockets resulting from loss of attachment which progressively can lead to loosening and ultimately loss of teeth [3]. Periodontal destruction may be induced by local

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factors, such as dental biofilm, tooth anatomic factors, dental restorations, root fractures, cemental tears and systemic diseases, like diabetes, HIV infection or other factors that may depress the host immune response [4].

Retinol-binding protein 4 (RBP4) is a member of the lipocalin family, a transport protein for vitamin A, which is synthesized mainly by the hepatocyte and adipose tissue, secreted into the circulation bound to vitamin A and transthyretin [5, 6]. RBP4 is a novel adipocyte-secreted hormone that is upregulated in insulin-resistant condition associated with obesity, and also, RBP4 provokes insulin resistance [7].

It is well known that obesity is associated with low-grade inflammation, which is causally involved in the development of insulin resistance [8]. RBP4 appears to be correlated with some markers of low-grade inflammation. RBP4 can directly induce production of proinflammatory mediators involved in leukocyte recruitment and adherence, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) in both macrovascular and microvascular human endothelial cells [9]. Furthermore, serum RBP4 levels are positively correlated with circulating inflammatory factors, such as IL-6 and high-sensitivity C-reactive protein (hs-CRP) [10].

Serum RBP4 levels are positively correlated with body mass index (BMI) in obese patients with diabetes [11]. In a recent study, higher waist circumference (WC) and waist-to-hip ratio were associated with increased RBP4 levels and markers of systemic inflammation [12].

Leptin, a 16 kDa non-glycosylated peptide hormone is encoded by the obese (ob) gene and mainly produced by adipocytes. Structurally, it belongs to the type 1 cytokine superfamily [13]. Leptin acts as a proinflammatory cytokine by controlling several cytokine secretion patterns. Inflammatory stimuli such as interleukin (IL)-1, IL-6 or lipopolysaccharide (LPS) regulate leptin messenger RNA (mRNA) expression as well as circulating leptin levels [14]. It has been shown that leptin modulates cytokine production from monocytes/macrophages. An increase in LPS-induced production of tumour necrosis factor-α (TNF-α), IL-6 and IL-12 in murine peritoneal macrophages and human monocytes has been reported [15, 16].

In a previous study, assessment of human gingival crevicular fluid (GCF) and serum leptin levels in chronic periodontitis (CP) patients showed negative correlation between the GCF leptin concentration and the positive correlation between serum leptin concentration with periodontal disease progression [17].

Adipose tissue (AT) expression of RBP4 mRNA has been found to be positively correlated with leptin expression in women. In addition, it has reported leptin-dependent RBP4 secretion and association of RBP4 expression with leptin. It is confirmed in ex-vivo experiments, demonstrating that leptin dose dependently increased the RBP4 secretion in visceral adipose tissue (VAT) explants [18]. Also, serum RBP4 levels have been found to be correlated positively with leptin levels in polycystic ovary syndrome (PCOS) in obese women [19].

To date, no study has reported levels of RBP4 in GCF and serum and its correlation with leptin levels in periodontal health and CP among non-obese and obese individuals. In this context, this clinico-biochemical study was undertaken to determine the GCF and serum levels of RBP4 and its correlation with leptin levels in healthy and CP patients with and without obesity.

Materials and methods

The study was conducted from October 2014 to January 2015 and performed in full accordance with ethical principles. Institutional Ethical Committee and Review Board (IRB) of the Government College of Dentistry and Research Institute, Bangalore, India, approved the study protocol. After ethical clearance, 70 patients (35 males and 35 females) with age group 25 to 45 years and minimum 20 natural teeth were recruited from the Periodontology Department OPD section, Bangalore, India. Patients were informed for participation, and written informed consent was obtained.

Exclusion criteria: patients with diabetes, aggressive periodontitis, hypertension, gross oral pathology, rheumatoid arthritis, cardiovascular disease, tumours or any other systemic illness which can influence periodontal disease course. Also, the patients with history of smoking, pregnant or lactating females or those patients who received any medication which affects periodontal status such as, cyclosporins, phenytoin or calcium channel blockers or antibiotics, anti-inflammatory drugs (NSAIDS) or had undergone periodontal therapy in the previous 6 months were excluded from the study.

For each patient, a full-mouth periodontal probing and charting was done. Using the WHO guidelines [20], BMI charting was done and intraoral periapical radiographs were taken for each patient using the paralleling technique. Patients with BMI in the range of 18.5–22.9 kg/m^2 were considered in the non-obese groups and in the obese groups BMI ≥ 25 kg/m^2 with waist circumference (WC) ≥90 cm in men and ≥80 cm in women were included. Bone loss was recorded on a radiograph to segregate CP patients from other groups.

The patients were classified into four groups based on gingival index (GI); probing depth (PD); clinical attachment level (CAL), with radiographic evidence of bone loss; BMI and WC as follows: (1) group I (non-obese periodontally healthy group) includes 15 patients with clinically healthy periodontium with no evidence of disease (PD ≤3 mm, GI = 0, CAL = 0 and BMI = 18.5–22.9 kg/m^2), with absence of any crestal bone loss as seen on radiograph; (2) group II (obese

periodontally healthy group) included 15 patients with periodontium which was clinically healthy with no evidence of disease (PD ≤3 mm, GI = 0, CAL = 0 and BMI ≥25 kg/m^2) with absence of crestal bone loss; (3) group III (non-obese with chronic periodontitis group) included 20 patients, who manifested signs of gingival inflammation clinically (PD ≥5 mm, GI >1, CAL ≥3 mm and BMI = 18.5–22.9 kg/m^2) bone loss seen radiographically and (4) group IV (obese with chronic periodontitis group) included 20 patients, who manifested signs of gingival inflammation clinically (PD ≥5 mm, GI >1, CAL ≥3 mm and BMI ≥25 kg/m^2) with bone loss seen radiographically.

Site selection and GCF fluid collection

Clinical examiners were well trained and calibrated before examination and sample collection procedure. The first examiner (ARP) carried out all the clinical examinations, intraoral radiographs, group allotment and sampling site selection on the first day; and the second examiner (DK), who was blinded to the groups allotted, did the sample collection on the following next day. This was done to prevent the GCF contamination with blood at the time of probing. In order to obtain intraexaminer reproducibility, all the clinical assessments were performed with a periodontal probe i.e., University of North Carolina (UNC)-15 (Hu-Friedy, Chicago, IL, USA). Based on the highest scored sites in the oral cavity, i.e. sites showing most severe inflammatory signs and greater amount of attachment loss (in chronic periodontitis cases with or without obesity), two test sites for GCF sample collection were selected. One of the selected sites was used for RBP4 analysis while other for leptin. In the healthy group, to standardize site selection and obtain adequate fluid volume, sampling was predetermined to be from one site, i.e. the mesiobuccal region of the maxillary right first molar, in the absence of which the left first molar was sampled (site with GI = 0).

Initially, the patients were made to sit comfortably on the dental chair, in an upright position, after which, air drying of the selected test site was performed. This was followed by its isolation using cotton rolls. Supragingival plaque was then removed gently using Universal Gracey curette #4R/4L[1] to avoid contamination of the paper strips[2], and GCF was collected using the intracrevicular 'superficial' method developed by Loe and Holm-Pederson [21]. Periotron 8000[3] was used to determine the absorbed GCF volume of each strip by electronic impedance. Four hundred microlitre of phosphate-buffered saline was used to place the periopaper strips. These were then stored at −70 °C till the assay procedure. Periopaper strips visually contaminated with blood or saliva were discarded. After GCF collection, periodontal treatment (scaling and root planing) was carried out for periodontitis patients at the same appointment.

Serum collection

Following disinfection of skin over the antecubital fossa, 2 ml of blood was collected in 2-ml syringes by venipuncture using 20-gauge needle and transferred immediately to the laboratory. Blood sample was allowed to clot at room temperature, and after 1 hour, it was centrifuged at 1000 rpm for 15 min to separate serum component. Serum was extracted from blood and stored at −70^o C till the assay procedure.

RBP4 and leptin analyses

A technician who was masked from the groups allocated and the sample collection determined RBP4 and leptin levels by using the collected GCF and serum samples and enzyme-linked immunosorbent assay (ELISA) kit. The samples were assayed for RBP4 using human RBP4 ELISA kit[4] and for leptin using human leptin ELISA kit[5] at the Department of Microbiology, Kempegowda Institute of Medical Sciences, Bangalore. The RBP4 and leptin levels in GCF and serum were determined by ELISA according to the manufacturer's instructions.

Statistical analysis

Based on power analysis, sample size was determined at a confidence interval of 95% ($p < 0.05$). To achieve 90% power and detect mean differences between groups, 12 patients per group were required. A statistical software program[6] was used to analyse the segregated data.

Normality assumption test was carried out, and it was found that the assumption is valid. Comparison of GCF and serum levels of RBP4 and leptin between the groups was tested using analysis of variance (ANOVA). Scheffe's test was used for multiple comparisons in order to find out the pair or pairs which differ significantly at 5% significance level. Pearson's correlation coefficient was used to assess the relationship between GCF and serum concentrations of RBP4 or leptin, and their relationship with clinical parameters were analysed using a software program. If p value <0.05, it was considered to be statistically significant.

[1] Universal gracey curette #4R/4L, Hu-friedy, Chicago, IL, USA
[2] Periopaper, Ora Flow Inc., Amityville, NY, USA
[3] Periotron 8000, ProFlow Inc., Amityville, NY, USA
[4] Human RBP 4 Elisa kit, RayBiotech Inc., USA
[5] Human Leptin Elisa kit, RayBiotech Inc., USA
[6] SPSS statistical software, SPSS version 22.0, Chicago, IL, USA

Results

Descriptive statistics and the mean RBP4 and leptin concentrations obtained from all groups are shown in Table 1. All the samples in each group tested positive for RBP4 and leptin. RBP4 levels in GCF and serum: The mean RBP4 concentration was greatest for group IV then group III, group II, with the group I showing the least concentration for both GCF and serum. Leptin levels in GCF and serum: The mean leptin concentration in GCF was greatest for group II followed by group I, then group IV, with the group III showing the least concentration while in serum it was greatest for group IV followed by group III, group II, then group I. ANOVA test was done to find out the equality of means between all the four groups, whereby a significant difference in the GCF and serum levels were found between the four groups for both the molecules (p value <0.05). When pairwise comparison was made for RBP4 and leptin between the groups, means were statistically significant in both GCF and serum in all groups (Table 2). Pearson's correlation coefficient between RBP4 and leptin concentration (GCF and serum) and the various clinical parameters are shown in Table 3. Significant correlation was found between GCF and serum RBP4 and leptin concentration with body mass index and periodontal clinical parameters (GI, PD and CAL), except with GI and CAL in groups I and II. The Pearson's correlation coefficient test found the correlation between GCF and serum levels of RBP4 and leptin to be statistically significant (Table 4).

Discussion

The present clinico-biochemical study was carried out to estimate the levels of RBP4 and leptin in GCF and serum in chronic periodontitis patients with and without obesity and to assess the correlation between RBP4 and leptin in these patients. The study also aimed to investigate the probability of using RBP4 as a biomarker of inflammation, linking chronic periodontitis and obesity and its comparison with leptin.

There may be a multidirectional association between obesity and chronic periodontitis in which proinflammatory cytokines may play a critical role [22]. Many inflammatory adipokines and cytokines like TNF-α, resistin, IL-6 and RBP4 have been shown to have increased in serum of patients with obesity [11, 23]. Some studies in the past have shown a notable association between obesity and periodontitis in relation to body fat and BMI [24].

By recruiting the equal number of males and females and by choosing patients between 25 and 45 years, we tried reduce the effect of the patient's age and gender on RBP4 and leptin levels. The levels of RBP4 and leptin were notably higher in obese patients (group II and group IV) compared to non-obese patients (group I and group III, respectively), in both GCF and serum. This represents the influence of obesity on RBP4 and leptin secretion in GCF and serum.

Graham et al. found in their study that serum RBP4 levels are positively correlated with BMI in obese non-diabetic and diabetic patients [11]. In a recent study,

Table 1 Descriptive statistics of study population (mean ± SD)

Study group	Group I (N = 15)	Group II (N = 15)	Group III (N = 20)	Group IV (N = 20)	p value
Age (years)	33.1 ± 6.28	35.33 ± 6.70	35.10 ± 6.24	34.75 ± 6.01	0.760
Sex (M/F)	8/7	8/7	10/10	9/11	
GI	0.00	0.00	1.91 ± 0.44	1.98 ± 0.49	<.001*
PD (mm)	2.06 ± 0.70	2.13 ± 0.74	6.05 ± 0.94	6.25 ± 0.96	<.001*
CAL (mm)	0.00	0.00	4.2 ± 0.95	4.3 ± 1.03	<.001*
BMI (kg/m^2)	20.44 ± 1.29	28.18 ± 1.70	20.45 ± 1.32	29.09 ± 2.10	<.001*
RBP4 GCF conc.(ng/ml)	3.53 ± 1.06	9.00 ± 0.75	16.75 ± 1.65	23.50 ± 1.63	<.001*
RBP4 serum conc.(ng/ml)	6.53 ± 1.12	12.20 ± 1.37	19.75 ± 1.51	27.30 ± 1.80	<.001*
Leptin GCF conc.(pg/ml)	231.33 ± 9.02	330.93 ± 8.31	33.00 ± 9.61	132.00 ± 10.01	<.001*
Leptin serum conc.(pg/ml)	81.07 ± 11.02	178.00 ± 12.64	272.70 ± 11.54	375.55 ± 11.86	<.001*

Group I—non-obese periodontally healthy; group II—obese periodontally healthy; group III—non-obese with chronic periodontitis; group IV—obese with chronic periodontitis

*Significant at p value <0.05

Table 2 Pairwise comparison using the Scheffe's test for GCF and serum RBP4 and leptin concentration

		GCF		Serum	
Marker	Group	Mean difference	*p* value	Mean difference	*p* value
RBP4	I and II	5.571	<0.001*	5.700	<0.001*
	I and III	13.321	<0.001*	13.250	<0.001*
	I and IV	20.071	<0.001*	20.800	<0.001*
	II and III	7.750	<0.001*	7.550	<0.001*
	II and IV	14.500	<0.001*	15.100	<0.001*
	III and IV	6.750	<0.001*	7.550	<0.001*
Leptin	I and II	98.648	<0.001*	97.714	<0.001*
	I and III	199.286	<0.001*	192.414	<0.001*
	I and IV	100.286	<0.001*	295.264	<0.001*
	II and III	297.933	<0.001*	94.700	<0.001*
	II and IV	198.933	<0.001*	197.550	<0.001*
	III and IV	99.000	<0.001*	102.850	<0.001*

Group I—non-obese periodontally healthy; group II—obese periodontally healthy; group III—non-obese with chronic periodontitis; group IV—obese with chronic periodontitis

*Significant at *p* value <0.05

higher waist circumference and waist-to-hip ratio were associated with higher RBP4 levels and markers of systemic inflammation [12]. Another study demonstrated that serum RBP4 levels were elevated in insulin-resistant mice and humans with obesity and type 2 diabetes [7]. These findings suggest RBP4 to be closely associated with obesity.

RBP4 is an adipokine, synthesised mainly by the hepatocyte and adipose tissue [5, 6]. The production of proinflammatory molecules involved in leukocyte recruitment and adherence by RBP4 is retinol independent and occurs, in part, via activation of NADPH oxidase and NF-κB [9]. The correlation between elevated serum RBP4 level and subclinical inflammation revealed that RBP4 induces inflammation in macrophages [10, 25]. Macrophages contribute to chronic adipose inflammation and insulin resistance present in adipose tissue during obesity. The proinflammatory effects of RBP4 on macrophages are mediated through stimulation of toll-like receptor 4 (TLR4) and c-Jun N-terminal protein kinase (JNK) signalling [26].

The verdict that RBP4 provokes proinflammatory pathways in macrophages, combined with previous studies, found that serum RBP4 levels correspond significantly with the appearance of inflammation [10]. Since, both obesity and periodontal disease are a state of chronic inflammation, the increased levels of GCF and serum RBP4 in obesity and CP can be justified.

In the present study, the obese group (group II: 9.00 ± 0.75 ng/ml) had significantly higher GCF RBP4 concentration with respect to non-obese group (group I: 3.53 ± 1.06 ng/ml). Similarly, group IV (23.50 ± 1.63 ng/ml) had significantly higher GCF RBP4 concentration than group III (16.75 ± 1.65 ng/ml). The possible reason for elevated levels of RBP4 could be due to overproduction from increased systemic inflammatory response present in obesity to disease process in the periodontal pocket.

Table 3 Pearson's rank correlation coefficient test comparing GCF and serum RBP4 and leptin concentration to GI, PD, CAL and BMI

Marker	Sample	Parameters	Group I	Group II	Group III	Group IV
RBP4	GCF	GI	–	–	<0.001*	<0.001*
		PD	<0.001*	<0.001*	<0.001*	<0.001*
		CAL	–	–	<0.001*	<0.001*
		BMI	<0.001*	<0.001*	<0.001*	<0.001*
	Serum	GI	–	–	<0.001*	<0.001*
		PD	<0.001*	<0.001*	<0.001*	<0.001*
		CAL	–	–	<0.001*	<0.001*
		BMI	<0.001*	<0.001*	<0.001*	<0.001*
Leptin	GCF	GI	–	–	<0.001*	<0.001*
		PD	<0.001*	<0.001*	<0.001*	<0.001*
		CAL	–	–	<0.001*	<0.001*
		BMI	<0.001*	<0.001*	<0.001*	<0.001*
	Serum	GI	–	–	<0.001*	<0.001*
		PD	<0.001*	<0.001*	<0.001*	<0.001*
		CAL	–	–	<0.001*	<0.001*
		BMI	<0.001*	<0.001*	<0.001*	<0.001*

Group I—non-obese periodontally healthy; group II—obese periodontally healthy; group III—non-obese with chronic periodontitis; group IV—obese with chronic periodontitis

*Significant at *p* value <0.05

Table 4 Correlations of serum and GCF concentrations of RBP4 and leptin in each group using Pearson's correlation coefficient test

Study groups	GCF (RBP4 vs leptin)		Serum (RBP4 vs leptin)	
	Correlation coefficient	*p* value	Correlation coefficient	*p* value
Group I	−0.796	<0.001*	0.446	<0.001*
Group II	−0.159	<0.001*	0.543	<0.001*
Group III	−0.826	<0.001*	0.738	<0.001*
Group IV	−0.770	<0.001*	0.811	<0.001*

Group I—non-obese periodontally healthy; group II—obese periodontally healthy; group III—non-obese with chronic periodontitis; group IV—obese with chronic periodontitis

*Significant at *p* value <0.05

Leptin, a 16 kDa protein is secreted mainly by white adipose tissue, and its levels are positively correlated with the amount of body fat [13]. Even leptin from adipose tissue is triggered by proinflammatory cytokines such as TNF-1α and IL-1β, suggesting that these cytokines stimulate short-term release of stored leptin [15].

In the present study, the group II had significantly higher GCF leptin level with respect to group I. Similarly, group IV had significantly higher GCF leptin concentration than group III. The findings are in accordance with a previous study done by Karthikeyan and Pradeep who found inverse correlation between the GCF leptin concentration and periodontal disease progression [17]. Carlson et al. suggested that GCF leptin levels in healthy sites of patients with periodontitis may play a preventive role in periodontal pocket [27]. Leptin receptor expression is greatly affected by cytopathic changes that occur on endothelial cells during inflammation. More leptin-leptin receptor complex formation in the gingival tissue caused by greater leptin receptor expression is responsible for decreased detectable GCF leptin levels. Thus, the decreased GCF leptin levels with progression of periodontitis in the present study can be justified.

Conversely, there was a rise in serum leptin concentration from group I to group IV. This could be attributed to two mechanisms. First, during gingival inflammation, vascular endothelial growth factor causes expansion of vascular network, which may accelerate the net rate of leptin removal from the gingival tissue and could elevate levels of leptin in serum. Thus, gingiva, along with adipose tissue, could be a potential originator of circulating leptin in periodontal disease patients [28]. Secondly, it could be a body defence mechanism to counteract periodontal inflammation as leptin is a part of the immune response and host defence mechanism [29]. Therefore, in the present study, the increase in serum leptin levels with periodontal disease progression and obesity is fully justified.

Both RBP4 and leptin are proinflammatory mediators and are thought to play an important role in local and systemic inflammatory responses. Since GCF and serum, RBP4 and leptin levels are elevated in patients with obesity and CP, suggesting that RBP4 and leptin levels are modulated by factors associated to subclinical inflammation. Also, increased serum leptin concentration may cause the increased concentration of RBP4, suggesting leptin-dependent RBP4 secretion and association of RBP4 expression with leptin.

The present cross-sectional study showed that the levels of RBP4 are positively correlated with BMI and periodontal parameters. Also, a significant correlation between RBP4 and leptin levels has been found; therefore, it can be proposed that RBP4 and leptin are a novel molecular link between obesity, chronic inflammation and periodontal disease. Though the present study did provide a correlation between RBP4 and leptin levels in GCF and serum, however, further studies with a larger sample size from different demographics will further strengthen the findings. In the present study, RBP4 and leptin levels in patients having periodontitis have not been evaluated post treatment. This evaluation may further give us information on the relation of RBP4 and leptin levels to periodontal treatment.

Conclusion

Thus, within the limitations of the present study, RBP4 and leptin may be an important inflammatory biomarker which may help link obesity and CP. Further longitudinal prospective, multicentre studies involving larger population are required to affirm the findings of present study and to better understand the role of RBP4 in the pathogenesis of periodontal disease and obesity and its association with leptin.

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Compliance with ethical standards The study is in compliance with ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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