Aus dem Zentralinstitut für Klinische Chemie und
Laboratoriumsdiagnostik
der Heinrich-Heine-Universität Düsseldorf
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# Autoantibodies against $M_5$-muscarinic and $beta_1$-adrenergic receptors in periodontitis patients

Dissertation

zur Erlangung des Grades eines Doktors der Zahnmedizin
der Medizinischen Fakultät der Heinrich-Heine-Universität
Düsseldorf

vorgelegt von
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2021

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez.:
Dekan: Univ.-Prof. Dr. med. Nikolaj Klöcker
Erstgutachter: Univ.-Prof. Dr. med. Fritz Boege
Zweitgutachter: Prof. Dr. Alfons Hugger

Meinen Eltern

Teile dieser Arbeit wurden veröffentlicht:

Scherbaum, I., Heidecke, H., Bunte, K., Peters, U., Beikler, T., Boege, F., (2020), Autoantibodies against $M_5$-muscarinic and $beta_1$-adrenergic receptors in periodontitis patients. Aging (Albany NY), (12) 16609-16620.

# Zusammenfassung

Autoantikörper gegen muskarinerge und beta$_1$-adrenerge Rezeptoren sind Risikofaktoren für eine chronische Herzinsuffizienz. Ein Zusammenhang zwischen Parodontitis, Autoantikörpern gegen beta$_1$-adrenerge Rezeptoren, sowie eingeschränkter Herzfunktion wurde bei Patienten, die der Chagas-Krankheit ausgesetzt sind, beobachtet. Die parasitäre Chagas-Krankheit kann eine Kardiomyopathie und eine Autoimmunisierung gegen beta$_1$-adrenerge Rezeptoren hervorrufen.

Um festzustellen, ob der Zusammenhang zwischen Parodontitis, erhöhter Rezeptor-Autoimmunisierung und Herzinsuffizienz unabhängig von der Chagas-Erkrankung ist, wurden deutsche Parodontitis-Patienten untersucht. Die Autoantikörper wurden durch IgG-Bindung an native, intakte muskarinerge und beta$_1$-adrenerge Rezeptoren oder an ein cyclisches Peptid bestimmt, das das krankheitsrelevante Konformationsautoepitop des aktiven Konformations-autoepitops des aktiven beta1-adrenergen Rezeptors nachahmt. Die Herzfunktion wurde auf der Grundlage der Serumspiegel von proBNP und Troponin I, der Entzündungsstatus auf der Grundlage von CRP bzw. IL-6 bewertet. Diese Parameter wurden bei gesunden und Parodontitis Patienten, sowie vor als auch nach der Parodontitis Therapie ermittelt.

Parodontitis Patienten wiesen signifikant höhere Autoantikörperniveaus gegen $M_5$-muscarinerge und beta$_1$-adrenerge Rezeptoren auf, zusätzlich stiegen die Autoantikörperspiegel nach der Therapie weiter an. Darüber hinaus zeigten Parodontitis Patienten einen signifikant erhöhten Entzündungsstatus und eine beeinträchtigte Herzfunktion. Die Rezeptor-Autoantikörper waren mit einem erhöhten Entzündungsstatus assoziiert, korrelierten jedoch nicht mit einer schlechteren Herzfunktion.

Unsere Ergebnisse deuten darauf hin, dass Parodontitis eine systemische Entzündung auslöst, die mit einer Autoimmunisierung des Rezeptors und unabhängig davon mit einer schlechteren Herzfunktion verbunden ist. Wir empfehlen eine koordinierte Behandlung von Parodontitis und Herzinsuffizienz. Zusätzlich empfehlen wir $M_5$R-Autoantikörper als möglichen Indikator für die Beurteilung des Parodontitis-Risikos.

# Abstract

Autoantibodies against muscarinic and beta$_1$-adrenergic receptors may be a risk factor for chronic heart failure. An association of periodontitis with autoantibodies against beta$_1$-adrenergic receptors and with impaired cardiac function has been observed in patients exposed to Chagas' disease. Chagas' disease is a parasitic disease which itself causes cardiomyopathy and autoimmunization against beta$_1$-adrenergic receptors.

To investigate whether the observation is independent of Chagas' disease, we examined the association between periodontitis, heart failure, and receptor autoimmunization in German periodontitis patients. In German patients, the cofounding impact of Chagas' disease can be safely excluded. The autoantibodies were determined by IgG binding to native, intact muscarinic and beta$_1$-adrenergic receptors or to a cyclic peptide that mimics the disease-relevant conformational autoepitope presented by the active beta$_1$-adrenergic receptor. The heart function was assessed on the basis of the serum levels of proBNP and troponin I, the inflammatory status of CRP and IL-6, respectively. These parameters were evaluated in healthy and periodontal patients as well as before and after periodontal therapy.

Patients with periodontitis had significantly ($p < 0.001$) higher autoantibody levels against $M_5$ muscarinic and beta$_1$ adrenergic receptors. Furthermore, these autoantibody levels continued to rise after periodontal therapy. In addition, periodontal disease patients showed a significantly increased inflammation status and impaired cardiac function. The receptor autoantibodies were associated with an increased inflammatory status but did not correlate with poorer cardiac function.

From this point onwards, our data indicate that periodontal disease triggers systemic inflammation that is associated with autoimmunization of the receptor and independently with poorer cardiac function. We recommend coordinated screening, monitoring and treatment of periodontitis and cardiac function, possibly with the help of serum proBNP determinations within standard dental care. In addition, we recommend $M_5$R autoantibodies as a serological marker for periodontitis, possibly as an early indicator for assessing the risk of periodontitis.

# List of abbreviations

| | |
|---|---|
| **$\beta_1$AR** | adrenergic beta$_1$-receptor subtype |
| **$\beta_1$AR-Aabs** | autoantibodies against adrenergic beta1-receptor subtype |
| **cAMP** | cyclic adenosine monophosphate |
| **CHF** | chronic heart failure |
| **CRP** | C-reactive protein |
| **CVD** | cardiovascular diseases |
| **DCM** | dilated cardiomyopathy |
| **GPCR** | G protein-coupled receptors |
| **HRV** | heartbeat variability |
| **IL-6** | interleukin 6 |
| **MR** | muscarinic acetylcholine receptors |
| **$M_5$R** | muscarinic acetylcholine receptor, subtype $M_5$ |
| **$M_5$R-Aabs** | autoantibodies against muscarinic acetylcholine receptor $M_5$ |
| **proBNP** | brain natriuretic peptides |
| **TpI** | Troponin I |

Contents

# 1 Introduction

## 1.1 Periodontitis

### 1.1.1 Etiology

Periodontitis is a chronic inflammatory disease of the supporting and surrounding tissues of the teeth. It belongs to the periodontal diseases which describe a wide range of oral infectious diseases of the periodontium. Up to 90 percent of the global world population suffers from periodontal diseases (Pihlstrom et al., 2005). By affecting up to 50 percent of the global world population, periodontitis is one of the most treated oral diseases and the sixth most prevalent chronic disease worldwide. In addition, it is the main reason of tooth loss in adults (Kassebaum et al., 2014, Bernabe et al., 2020, Benjamin, 2010).

Periodontal diseases communally start with gingivitis, a reversible inflammation of the gingiva caused by pathogenic microflora inside the dental plaque, a natural biofilm that forms on teeth and gingiva. As the inflammation of the gingiva progresses, there is the possibility of development into periodontitis. This occurs when the microbial shift in favor of the bacterial pathogens induces and continues inflammatory and immunological dysregulations (Kinane et al., 2017). On this occasion, degradation of the periodontium, formed by the alveolar bone, the gum, the periodontal ligaments and root cement of the teeth, follows (Hassell, 1993).

### 1.1.2 Prevalence

Globally, between 1990 and 2017, the age-standardized prevalence of severe periodontitis was static at 9.8 percent. Besides, the number of prevalent cases amount to 796 million (Bernabe et al., 2020).

The Fifth German Oral Health Study report published in 2014 announced that the number of people suffering from periodontal diseases is falling. Nonetheless, more

than every second young German adult suffers from periodontitis, with 43.4% suffering from moderate periodontitis and 10% from severe periodontitis. The study also depicted that periodontitis affects all age groups. With increasing age, both the prevalence and the severity of the disease increase. This observation leads to the prognosis that despite the falling prevalence, an increasing need for periodontal treatment is expected. On the one hand, this is due to the demographic changes within society. On the other hand, it is due to the shifting of chronic oral diseases to an older age, triggered by improved oral hygiene measures and thus a longer lasting dentition (Jordan et al., 2014).

### 1.1.3 Therapy

Periodontitis is a chronic disease whose course can in most cases be significantly improved with appropriate therapy. The correct and timely diagnosis of periodontitis represents an important factor for the success of the therapy. Usually, the degradation of periodontal tissues starts painless and is largely irreversible. For this reason, the dentist is generally responsible for the diagnosis of the disease, which is mainly based on a series of clinical measurements and radiographic findings (Kinane et al., 2017).

Once periodontitis is diagnosed, the therapy should be initiated. The main goal of periodontitis therapy represents the reducing of the periodontal bacterial pathogens. To control the microbial periodontal infection, the therapist removes bacterial biofilm, tartar and toxins from periodontally affected tooth surfaces. This combats acute periodontal inflammation and slows down periodontitis in its progression (Pihlstrom et al., 2005).

Generally, periodontal therapy starts with non-surgical periodontal therapy. During non-surgical therapy, the entire supra- and subgingival biofilm and calculus is removed mechanically by scaling and root planing. Therefore, various manual or powered instruments - hand scalers, curettes and ultrasonic instruments - can be used. Non-surgical periodontal treatment is effective for mild and moderate periodontitis cases but shows limitations in treating advanced periodontitis. In

patients with advanced periodontitis, further surgical therapy and/or supplemental application of local antiseptic drugs, local or systemic antibiotics are indicated to reduce the periodontal infection (Pihlstrom et al., 2005, Kinane et al., 2017).
Due to the chronic nature of the disease, compliance with a close recall is important for the success of the therapy. In addition, periodontitis patients must be informed about their possible contribution to an improvement of the disease course, including improved mouth hygiene and minimizing risk factors (Manresa et al., 2018).

### 1.1.4 Risk factors

Besides the interactions of pathogens in dental plaque, host risk and environmental factors are responsible for the development of periodontitis (Kinane et al., 2017).
These risk factors are divided in non-modifiable risk factors such as age and heredity, and modifiable risk factors in the likes of smoking, medication, stress, hormonal changes and systemic diseases (Nazir, 2017). The various causal factors interact and contribute in different forms to the development and severity of the disease (Loos and Van Dyke, 2020). Periodontitis patients usually exhibit one or more risk factors for the development of the disease. However, it should be emphasized that periodontitis patients with different severities of the disease may have identical risk factors (Genco and Borgnakke, 2013). Similarly, cases of severe periodontitis are known for which no acquainted risk factor has been identified. Still, the occurrence of one or more risk factors is a valuable sign of an increased periodontitis risk (Albandar, 2014).

## 1.2 Possible links between periodontitis and cardiovascular diseases

It is already established that the harmful host reaction of periodontitis is not limited to the oral cavity, but that periodontitis contributes to the pathogenesis of other systemic diseases and conditions. For instance, these include pneumonia, chronic

kidney disease, cognitive impairment, rheumatoid arthritis, obesity and cardiovascular diseases (CVD) (Linden and Herzberg, 2013).

CVD are the most frequent non-communicable diseases and globally the biggest cause of mortality and morbidity, responsible for around 17.8 million deaths in 2017 (Group, 2019). CVD is the combination of various diseases, all of which have a negative impact on the cardiovascular system. The diseases include for example ischemic heart disease, stroke, hypertensive heart disease and rheumatic heart disease, among which ischemic heart disease is the most frequent (Thomas et al., 2018).

Several epidemiological studies, including meta-analyses and systemic reviews, demonstrate a significant evidence for the association between periodontitis and CVD (Sanz et al., 2020a, Hansen et al., 2016, Dietrich et al., 2013, Lockhart et al., 2012, Blaizot et al., 2009). In addition, it is shown that periodontitis can increase the risk of CVD by up to 44 percent (Janket et al., 2003).

The mechanism of the relationship between both highly prevalent and chronic inflammatory diseases is a big part of current research. Various theories about the link between CVD and periodontitis exist (Kebschull et al., 2010, Sanz et al., 2020b). Main explanations are based on the common inflammatory character of both diseases (Schenkein and Loos, 2013, Carrizales-Sepulveda et al., 2018, Aarabi et al., 2018). Other attempts to explain the link are based on the high number of shared risk factors. For both pathologies, increased age, male gender, family history, smoking, diabetes mellitus, obesity, hypertension, and hypercholesteremia are common risk factors (Carrizales-Sepulveda et al., 2018, Friedewald et al., 2009, Lockhart et al., 2012).

In spite of the latter explanation, periodontitis is also propound as an independent risk factor for CVD (Dietrich et al., 2013, Dorn et al., 2010). Therefore, the destructive character of periodontal pathogens seems to play an important role. The periodontal pathogens lead to a subgingival destruction through which they may pass inside the blood circulation. This occurs during periodontitis treatment but also during daily-life activities, such as tooth brushing (Tomas et al., 2012). Through this bacteremia the periodontal pathogens may directly lead to CVD-inducing effects such as promoting

atheroma plaques (Armingohar et al., 2014, Padilla et al., 2006) or elevating thrombotic factors (Chandy et al., 2017). Instead of the direct mechanism it is also discussed that the pathogens lead to indirect effects by providing increased production of inflammatory mediators including interleukin-6 (IL-6) and C-reactive protein (CRP), which are known to contribute to an increased CVD risk (Glurich et al., 2002, Schenkein and Loos, 2013).
However, the theories still show limitations and the mechanism of the association between periodontitis and CVD is still not fully understood (Kebschull et al., 2010)

## 1.3 Receptor autoimmunization as a risk factor for cardiovascular diseases

Chronic heart failure (CHF) represents a leading public health issue (Bui et al., 2011). In 2018, CHF was still the main reason for a hospital stay and the third leading reason of death in Germany (Herzstiftung, 2019). The main symptoms of CHF are dyspnea, fatigue, and signs of volume overload, which may contain pulmonary rales and/or peripheral edema (Collaborators, 2018).
Despite the improved therapy concepts, the prevalence of CHF increases continuously (Virani et al., 2020). On the one hand, this depends on the increase in traditional risk factors within society. Traditional risk factors for example age, obesity, smoking, coronary heart disease and hypertension account for a considerable proportion of CHF. On the other hand, they are insufficient in being the only attempts to explain the high prevalence of CHF. Accordingly, more and more non-traditional risk factors are being identified (Virani et al., 2020).
It is known that autoantibodies against G-protein coupled receptors (GPCR) are risk factors for many diseases (Meyer and Heidecke, 2018). Autoantibodies against the adrenergic beta1-receptor subtype ($\beta_1$AR) and against muscarinic acetylcholine receptors (MR) seem to be related to CVD. Studies demonstrate that patients suffering from CHF often exhibit stimulatory autoantibodies against the beta$_1$-receptor subtype ($\beta_1$AR-Aabs) and autoantibodies against MR (Bornholz et al., 2014). Moreover, $\beta_1$AR-Aabs have been proven to cause CHF, in particular non-

ischemic CHF (Jahns et al., 2004). These and similar observations confirm that such autoantibodies are relevant risk factors of CHF (Boivin-Jahns and Jahns, 2018). Cross-reactions with viral or bacterial proteins are suspected causes of potentially cardiopathogenic autoimmunization (Boivin-Jahns and Jahns, 2018). In addition, it could be shown that agonistic autoantibodies activate different cellular pathways, without showing desensitization and internalization. The resulting chronical over-stimulation leads to negative effects to heart cells and worsens cardiac function, but the precise trigger remains unclear (Wallukat and Schimke, 2014, Boivin-Jahns et al., 2018).

Likewise, it has been pointed out that genetics, immune status, hormonal status, and environmental factors could equally well contribute to that autoimmune response (Nussinovitch and Shoenfeld, 2013).

### 1.3.1 $\beta_1$AR-Aabs:

For more than 50 years, autoantibodies against GPCR, which include $\beta_1$AR-Aabs, have been part of intensive research. Therefore, research has focused particularly on the association between $\beta_1$AR-Aabs and heart diseases. In 1956, Adams and Purves (Adams and Purves, 1956) at first detected GPCR autoantibodies in Graves' disease. In 1976, $\beta_1$AR-Aabs were first detected in patients suffering from Chagas' disease. Furthermore, it could be shown that the autoantibodies interact with myocardial cells. By interacting they lead to morphological and functional changes inside the myocardial cells (Sterin-Borda et al., 1976).

In 1987, Wallukat and Wollenberger (Wallukat and Wollenberger, 1987) first described $\beta_1$AR-Aabs in dilated cardiomyopathy (DCM). Confirmation of this observation (Magnusson et al., 1990, Limas et al., 1989) was followed by a still ongoing research into the association between DCM and $\beta_1$AR-Aabs. Animal immunization studies demonstrated that the $\beta_1$AR-Aabs can lead to cardiomyopathy phenotypes (Buvall et al., 2006), including left ventricular dilatation and dysfunction (Jahns et al., 2004) or arrhythmia (Fukuda et al., 2004). Clinical studies detected that more than 30% of the patients suffering from non-ischemic cardiomyopathy

show increased $\beta_1$AR-Aabs. Besides the DCM, $\beta_1$AR-Aabs are also present in around 10% of patients suffering from an ischemic cardiomyopathy (Jahns et al., 1999). Additionally, not only is the prevalence increased, but the prognosis is also worsened (Stork et al., 2006).

These observations lead to new therapy concepts, all of which aim to lower or completely remove the prevalence of the autoantibodies. Removal of the $\beta_1$AR-Aabs through extracorporeal immune adsorption (Dandel et al., 2012) or systemic neutralization reveal an improvement in the symptoms (Patel and Hernandez, 2013, Jahns et al., 2010). However, the improvement only occurs in $\beta_1$AR-Aabs positive patients (Dandel et al., 2012). Accordingly, a standardized, routinely applicable measurement procedure for $\beta_1$AR-Aabs, which is based on Immunoglobin G-binding to native human $\beta_1$ adrenergic receptors, was developed (Bornholz et al., 2016a).

### 1.3.2 $M_5$R-Aabs

Autoantibodies against the muscarinic acetylcholine receptor, subtype $M_5$ ($M_5$R-Aabs) are still relatively new to research. In comparison to other autoantibodies against MR, the $M_5$-subtype has been seldom determined in pathologies. The only pathological subgroup in which the presence of $M_5$R-Aabs seems to contribute to the disease course has been found in female patients with postural orthostatic tachycardia syndrome (Gunning et al., 2019).

The muscarinic acetylcholine receptor, subtype $M_5$ ($M_5$R) seems to be an important part of the vascular regulation. In the microcirculation of the brain, they were detected to cause a cholinergic dilation. In the vasculature of the heart, the $M_5$R was found to cause a vasorelaxation in epithelial cells (Harvey, 2012). A yet unpublished ongoing prospective cohort study points to the importance of $M_5$R-Aabs in the cardiovascular system. Here, the $M_5$R-Aabs reveal the strongest association of all other autoantibodies against MR-subtypes in worsening the heart function (Deubner et al., 2010). In addition, pre-experiments of this study detected a significant difference in $M_5$R-Aabs levels between periodontitis patients and healthy controls.

The clarity of these preliminary results led to the decision to pursue the $M_5$R-subtype in the study.

### 1.3.3 Embedding in research projects of the working group

Autoantibodies against $\beta_1$AR-Aabs, especially in connection with CVD, have long been a research focus of the institute of Prof. Boege. One research focuses on the pathological role of $\beta_1$AR-Aabs, especially in relation to CVD (Boivin-Jahns et al., 2018). A second focus is on researching the exact mechanisms of the biochemical pathology of $\beta_1$AR-Aabs (Bornholz et al., 2013b, Herda et al., 2012). Intensive work is also being carried out on the development and validation of a standardized and routinely applicable measurement method for potentially pathogenic $\beta_1$AR-Aabs. Therefore, existing assessment methods were examined (Bornholz et al., 2016c, Jahns and Boege, 2015). Consequently, a new assessment method was developed (Bornholz et al., 2016a). In this study, the new meanwhile CE certified assessment method was used to detect the $\beta_1$AR-Aabs of the cohort.

## 1.4 Relevance of receptor autoimmunization in periodontitis patients

Periodontal disease is a probable candidate to induce autoimmunization against $\beta_1$AR and MR and thereby induce or promote poorer heart function known to be associated with that disease (Hansen et al., 2016, Dietrich et al., 2013, Lockhart et al., 2012, Blaizot et al., 2009).

In connection with periodontitis, previous study results from Argentina reported that periodontitis patients exhibit significantly increased $\beta_1$AR-Aabs levels than periodontal healthy individuals. Equally important, the periodontitis patients also display a reduced heartbeat variability (HRV). Based on their research results, the Argentinean researchers concluded that periodontitis could lead to autoimmunization against $\beta_1$AR and as a result the poorer cardiac function of the patients develops (Segovia et al., 2011, Segovia et al., 2012, Reina et al., 2012).

However, the little available data that associate periodontitis and potentially cardiopathogenic autoimmunization against $\beta_1$AR and MR are viewed critically. In Argentina, autoimmunization against $\beta_1$AR consists of endemic Chagas' disease with a very high background preference (Munoz-Saravia et al., 2012).

Chagas' disease is provoked by the protozoan parasite *Trypanosoma cruzi*. The disease is endemic in 21 Latin American countries, and Argentina represents the second most commonly affected country after Bolivia (Pérez-Molina and Molina, 2018). The usual transmission route to humans occurs through the feces of infected bloodsucking insects. Occasionally, a transmission by indirect mechanism as in the case of blood transfusion takes place (Rassi et al., 2010). The clinical course of Chagas' disease usually displays an acute phase and a chronic phase. During acute phase, patients show non- or light symptoms for example fever or an inflammation at the inoculation site. Around 30–40% of chronically infected patients may develop an organ involvement. For instance, cardiomyopathy, mega oesophagus or megacolon (Pérez-Molina and Molina, 2018). Cardiomyopathy, also called Chagas' heart disease, represents the most common comorbidity and occurs in around 14–45% of chronically infected Chagas' patients (Pérez-Molina and Molina, 2018). Interestingly, more than 95% of the symptomatic patients show increased $\beta_1$AR-Aabs levels. Besides, 30% of the non-symptomatic patients exhibit increased $\beta_1$AR-Aab levels as well (Munoz-Saravia et al., 2012)

## 1.5 Scope of the dissertation

The aim of the study is to collectively investigate the effects of periodontitis and its treatment on the prevalence of the circulation of $\beta_1$AR-Aabs and $M_5$R-Aabs. In addition, the study is carried out to investigate the possible involvement of these autoantibodies in cardiac injury and generalized inflammation.

The only studies on possible cardiopathogenic autoimmunization against $\beta_1$AR in periodontitis patients come from Argentina. The Argentinian studies propound an association between periodontitis, autoimmunization against $\beta_1$AR and worsened cardiac function. However, they are viewed critically because Chagas' disease is an

endemic disease in Argentina. The parasitic disease is known for causing an autoimmunization against $\beta_1$AR and in this consequence a worsened cardiac function. In addition, the Argentinean researchers measured the heart function by HRV and concluded that the heart function was poorer from a reduced HRV.

Consequently, we wanted to examine these possible and clinically relevant connections in a German cohort, where the obfuscated effects of endemic Chagas' disease can safely be excluded. In addition, we wanted to investigate the heart injury of the cohort by determining accredited and established laboratory parameters.

Therefore, we examined sera of periodontitis patients, drawn in the University hospital of Münster and control sera of non-periodontitis patients, drawn in the University hospital in Düsseldorf. A permit for the study from the ethics committee of the University of Münster (Reg. ID: 1VBei, decision of 12.06.2001) and approval from the ethics committee of the University of Düsseldorf (Internal study number 5541R, Reg. ID:2016045065, decision of 20.06.2016) is available.

# 2 Published original article

Autoantibodies against $M_5$-muscarinic and beta$_1$-adrenergic receptors in periodontitis patients, Scherbaum, I., Heidecke, H., Bunte, K., Peters, U., Beikler, T., Boege, F., Aging (Albany NY), 12: 16609-16620, (2020)

www.aging-us.com AGING 2020, Vol. 12, No. 16

Research Paper



## Autoantibodies against $M_5$-muscarinic and beta$_1$-adrenergic receptors in periodontitis patients

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**Keywords:** parodontitis, autoantibodies, chronic heart failure, beta1-adrenergic receptor, M5-muscarinic receptor
**Received:** May 27, 2020 **Accepted:** July 21, 2020

**Published:** August 28, 2020

### ABSTRACT

**Autoantibodies against muscarinic and beta$_1$-adrenergic receptors are considered a potential cause and/or risk factor for chronic heart failure. Association of periodontitis with such autoantibodies and with impaired heart function has been observed in patients exposed to endemic Chagas' disease, which triggers by itself cardiomyopathy and receptor immunization.**

**Here we studied the association between periodontitis, markers of cardiac injury and receptor autoimmunization in periodontitis patients (n = 147) not exposed to Chagas' disease. The autoantibodies were determined by IgG binding to native intact muscarinic and beta$_1$-adrenergic receptors or to a cyclic peptide mimicking the disease-relevant conformational autoepitope presented by the active beta$_1$-adrenergic receptor. Possible cardiac injury and inflammatory status were judged by serum**

**levels of proBNP/Troponin I and CRP/IL6, respectively. These parameters were analysed in healthy and periodontally diseased individuals as well as before and after periodontal therapy.**

**Patients with periodontitis had significantly ($p < 0.001$) higher levels of autoantibodies against $M_5$-muscarinic and beta$_1$-adrenergic receptors, which further increased following periodontal therapy. Receptor autoantibodies were associated with increased inflammatory status but not with increased markers of cardiac injury. Thus, our data indicate that periodontitis triggers systemic inflammation, which is associated with receptor autoimmunization, and, independently thereof, with cardiac injury.**

## INTRODUCTION

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues that leads to tooth loss if left untreated [1]. It is the sixth most common chronic disease, affecting up to 50% of the global population [2]. Oral dysbiosis, characterized by a microbial shift in favour of bacterial pathogens, initiates and perpetuates inflammatory and immunological dysregulations that cause the breakdown of periodontal tissues. However, these detrimental host responses are not only confined to the oral cavity but contribute to the pathogenesis of several other systemic diseases and conditions, e.g. diabetes, chronic kidney disease, obesity and cardiovascular diseases [3, 4].

Cardiovascular diseases (CVD) are the most common non-communicable diseases worldwide [5]. Periodontitis and cardiovascular diseases share common risk factors such as smoking and diabetes [6]. Moreover, periodontitis is suggested as an independent risk factor for CVD. In this regard, either direct effects, i.e. the invasion of periodontal bacteria into oral and non-oral tissues (such as atheroma plaques), and indirect effects, i.e. the increased production of inflammatory mediators like interleukin-6 and C-reactive protein have been found to contribute to the increased CVD risk in periodontitis patients [6–11].

Chronic heart failure (CHF) affects over 26 million people globally with an increasing prevalence [12]. Stimulatory autoantibodies against the adrenergic beta1-receptor subtype ($\beta_1$AR-Aabs) and autoantibodies against muscarinic receptors (MR-Aabs) are frequently found in patients with CHF [13]. Periodontitis patients were reported to frequently exhibit circulating autoantibodies against the adrenergic beta1-receptor subtype ($\beta_1$AR) associated with poorer cardiac function [14–16]. The presence of these antibodies is generally considered a relevant risk factor for CHF [17]. It is associated with poorer heart function [18] and prognosis [19] in non-ischemic chronic heart failure. Immunization against $\beta_1$AR has been demonstrated to cause CHF compatible with human syndromes of non-ischemic chronic heart failure [20]. The presence of these autoantibodies and their cross-reactions with viral or bacterial proteins are suspected causes of a potentially cardiopathogenic autoimmunization. Furthermore, genetic predisposition, the immune and hormonal status, and a variety of environmental factors are thought to contribute to that autoimmune response [21]. However, the trigger mechanism remains unclear [17].

Periodontal disease is a plausible candidate for triggering autoimmunization against $\beta_1$AR and muscarinic receptors (MR). Along that road, periodontitis could compromise heart function and induce CHF [7–10]. However, data that correlate periodontitis with a potentially cardiopathogenic autoimmunization against $\beta_1$AR and MR are ambiguous. Previous reports of an association of periodontitis with high levels of $\beta_1$AR and MR have been conducted in the South of Argentina, where a high prevalence of such autoimmunization is mainly attributed to the very high prevalence of endemic Chagas' disease [22]. Therefore, it is necessary to investigate the possible link between periodontitis, cardiac injury and autoimmunization against $\beta_1$AR and muscarinic receptors under conditions where the confounding impact of endemic Chagas' disease can be excluded, i.e. a population living in mid-Europe. In the present study we follow up on this notion, assessing markers of cardiac injury and markers of inflammation in periodontitis patients and healthy controls and determining the impact

of periodontal therapy on $\beta_1$AR-Aabs and $M_5$R-Aabs levels in a European population.

## RESULTS

Before periodontal therapy, periodontitis patients exhibited statistically significant higher levels of $\beta_1$ARAabs and $M_5$R-Aabs than the healthy controls. Median $\beta_1$AR-Aab levels as determined by IgG-binding to intact native receptor were about twice as high in patients not yet having undergone treatment (17.12 ± 10.02 Units/mL) as compared to healthy controls (8.22 ± 5.13 Units/mL). This difference was highly significant ($p < 0.001$), and could be confirmed by IgGbinding to a cyclic peptide representing the conformational auto-epitope within the second extracellular loop of the receptor associated with the active receptor conformation (Table 1). Recent evidence indicates that autoimmunity to this epitope is causally involved in the pathogenesis of dilated cardiomyopathy [23]. The latter, more specific assay revealed three-fold higher $\beta_1$AR-Aab levels in patients not yet having undergone treatment (6.34 ± 2.55 ng/mL) as compared to healthy controls (2.31 ± 1.28 ng/mL) ($p < 0.001$).

Moreover, serum levels of $\beta_1$AR-Aabs (as determined by IgG-binding to the cyclopeptide) exhibited only a marginal overlap between controls and patients (Figure 1, middle). Given the apparent superior discriminative power of the cyclopeptide-assay, $\beta_1$AR-Aab levels derived from this assay were selected for subsequent analyses (Table 2 and Figure 2). An even more pronounced difference between healthy controls and periodontitis patients was observed for the levels of circulating $M_5$R-Aab. Periodontitis patients presented five-fold higher circulating $M_5$R-Aab levels (24.14 ± 17.10 Units/mL) before periodontal therapy, compared to corresponding levels in healthy controls (4.90 ± 3.04 Units/mL) ($p < 0.001$). The values of circulating $M_5$RAabs exhibited only a marginal overlap between controls and patients (Figure 1, d left). It should also be noted that in the patients, serum levels of $M_5$R-Aabs and $\beta_1$AR-Aabs were significantly correlated with each other (Supplementary Figure 2).

In the next step, we examined the impact of periodontal therapy on $\beta_1$AR and $M_5$R autoimmunization. For this purpose, we compared within the periodontitis group baseline levels of $\beta_1$AR- and $M_5$R-Aabs (pre-therapy and on the day of therapy) with corresponding values measured at each of the post-therapy follow-up visits. Autoantibody levels did not significantly change from pre-therapy to follow-up determinations at 8 and 17 weeks after therapy. However, at 30 weeks and more after therapy, $\beta_1$AR- and $M_5$R-Aabs were significantly ($p < 0.001$) increased by about 40% above pre-therapy levels, and these increases remained stable until 112 weeks after therapy (Figure 2). It should be noted that data from all patients, who left the study at any time

**Table 1. Baseline characteristics of periodontitis patients and healthy individuals.**

| Group Variables | Periodontitis (n=146) | Healthy (n=60) | P value[1] |
|---|---|---|---|
| proBNP (ng/l) | 49.46 (± 63.07) | 35.33 (± 46.56) | = 0.002 |
| TpI (ng/l) | 3.30 (± 2.16) | 3.00 (± 0.00) | < 0.001 |
| CRP (mg/dl) | 0.11 (± 0.22) | 0.07(± 0.13) | = 0.160 |
| IL-6 (ng/l) | 1.60 (± 1.11) | 1.50 (± 0.00) | < 0.001 |
| □1AR-Aab[2] (U/ml) | 17.12 (± 10.02) | 8.22 (± 5.13) | < 0.001 |
| □1AR-Aab[3] (ng/ml) | 6.34 (± 2.55) | 2.31 (± 1.28) | < 0.001 |
| $M_5$R-Aab[2] (U/ml) | 24.14 (± 17.10) | 4.90 (± 3.04) | < 0.001 |
| Gender (% female) | 67.81 | 66.7 | > 0.99 |
| Age (years) | 47 (± 16.11) | 28 (± 11.66) | <0.001[4] |

Median values of non-normally distributed data (± interquartile range)

[1]Significance of difference between groups

[2]IgG-binding to intact receptor

[3]IgG-binding to cyclopeptide representing the conformational auto-epitope within the second extracellular Loop of the receptor

[4]Poor age-match of control group inevitable due to age-associated prevalence of periodontitis

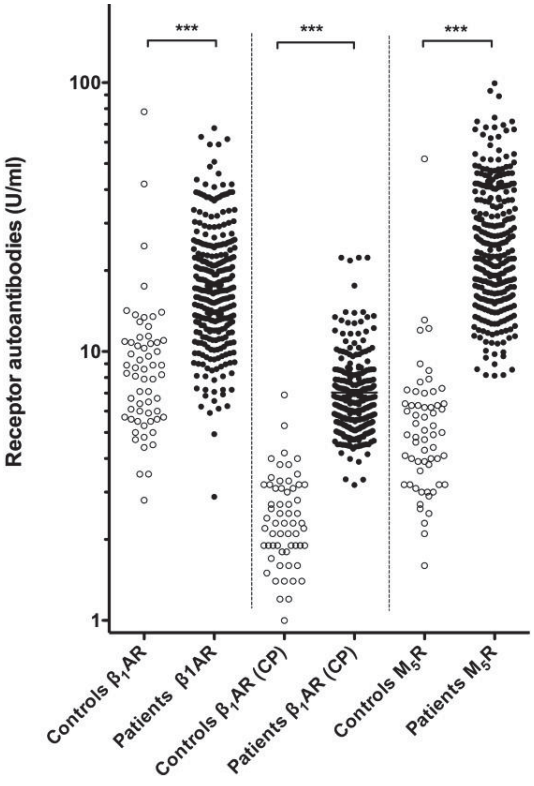


**Figure 1. Levels of circulating □1AR-Aabs and $M_5$R-Aabs of patients (before therapy) and controls.** Left and Right:

Serum levels of □1AR-Aabs and $M_5$R-Aabs were measured by IgGbinding to the respective native receptors (CellTrend GmbH). Middle: □1AR-Aabs were determined by IgG-binding to a cyclic peptide providing a valid representation of the presumed pathogenic conformational auto-epitope within the second extracellular loop of the receptor associated with the active receptor conformation (indicated CP). ***: differences at p < 0.001 significance.

during post-therapy follow-up for undisclosed reasons (N = 80) were excluded from the above longitudinal analyses.

In the next step, we addressed the link between periodontitis, cardiac injury and generalized inflammation suggested by various studies, e.g. [24]. For that purpose, we measured the serum-levels of cardiac (proBNP, TpI) and inflammatory markers (CRP, IL-6) and compared these values between healthy individuals and periodontitis patients in pre-therapy samples. Median values of cardiac markers were significantly higher in the periodontitis group compared to the control (see Table 1). Likewise, the periodontitis group showed significantly higher serum levels of IL-6, while the CRP levels did not differ significantly between the groups (Table 1). In summary, these results confirm previous studies [6–11, 25, 26]. In addition, we noticed that in post-therapy follow up of periodontitis patients (5 weeks and more) TpI increased (by 8.5%, p < 0.001). proBNP exhibited a similar, albeit insignificant, trend to increase during post-therapy follow. In contrast, the inflammation

markers exhibited a weak and insignificant trend to decrease upon therapy (not shown).

Finally, we addressed the question whether cardiac injury, generalized inflammation and autoimmunization against $\beta_1$AR and/or $M_5$R were interrelated or at least co-incident in the patients. To test this hypothesis, we analyzed correlations between the levels of the cardiac and inflammatory markers and the serum levels of circulating $\beta_1$AR-Aabs or $M_5$R-Aabs. These data are summarised in Table 2. $\beta_1$AR-Aabs and $M_5$R-Aabs were significantly correlated with the level of inflammation markers. Interestingly, this correlation was much more pronounced during therapy follow-up.

**Table 2. Correlation between receptor autoantibodies, cardiac markers and inflammation markers in periodontitis patients before and after therapy.**

| | $\beta_1$AR-Aab[1] | | $M_5$R-Aab[2] | |
|---|---|---|---|---|
| | Pre-therapy | Post-therapy[3] | Pre-therapy | Post-therapy[3] |
| proBNP | -.042 | .041 | -.027 | .044 |
| TpI | -.024 | .026 | -,036 | .035 |
| CRP | .192* | .223* | .159 | .195* |
| IL-6 | .140 | .280* | .074 | .214* |

Values given as correlation coefficients *significance of correlation ($p < 0.05$)

[1]IgG-binding to cyclopeptide representing the conformational auto-epitope within the second extracellular loop of the receptor

[2]IgG-binding to intact receptor [3]Five weeks or more, all follow ups summarized

In contrast, $\beta_1$AR- and $M_5$R-Aabs were not correlated with the cardiac markers (neither before nor after therapy). In summary these findings suggest that

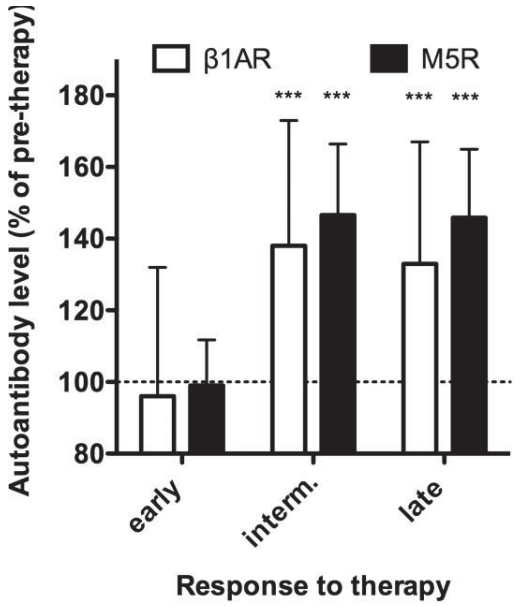

**Figure 2. Response of $\beta_1$AR- and $M_5$R-Aabs to therapy.** Serum levels of $\beta_1$AR-Aabs (white) were measured by IgGbinding to a cyclic peptide representing the presumed pathogenic conformational auto-epitope within the second extracellular loop of the receptor. $M_5$R-Aabs (black) were measured by IgG-binding to the native receptors (CellTrend GmbH). Values obtained at 5 and 17 weeks after therapy (early), 30 and 44 weeks after therapy (intermediate) and 58 to 112 weeks after therapy (late) are normalized to pretherapeutic values (dotted line). Data of n=66 patients undergoing complete follow up are given as median ± interquartile range. *** indicate differences to pre-therapy values at $p < 0.001$ significance.

receptor-autoimmunization is a by-product of the inflammatory response to periodontitis (and the therapy thereof), but not directly associated with the extent of cardiac injury in these patients.

## DISCUSSION

This study was carried out, in order to investigate the impact of periodontitis and its treatment on the prevalence of circulating of $\beta_1$AR-Aab and to address the possible involvement of these autoantibodies in coincident cardiac injury. Autoantibodies against MR were included in the study, given the involvement of such autoantibodies in many autoimmune-diseases including CHF (see below). We focused here on autoantibodies against the $M_5$-subtype, because among all muscarinic receptor subtypes tested, $M_5$Rautoantibodies exhibited the strongest association with the decline of cardiac function in a cohort of postmyocarditis patients (unpublished results from a prospective cohort study on etiology and titer-course of cardiac autoantibodies and their effect *on* survival (ETiCS-study) [27], mentioned with kind permission of the study coordinator). Moreover, we observed in preexperiments that $M_5$R-Aabs levels exhibited a clear difference between periodontitis patients and healthy controls.

### Salient findings

We confirm previous reports [14–16] of an association between periodontitis and circulating $\beta_1$AR-Aabs. We could exclude the suspected confounding impact of endemic Chagas' disease on the previously mentioned study. Beyond that, we observed that periodontitis is also, and even more clearly, associated with increased levels of $M_5$R-Aabs. When drawing that conclusion, it must, however, be taken into account that the control group was much younger, which is inevitable due to the high prevalence of periodontitis in the elderly. Since autoimmune status changes with age, it is difficult to conclude that the higher levels of autoantibodies in the patients are solely due the disease. Moreover, $\beta_1$ARAabs and $M_5$R-Aabs were highly coincident (i.e. were increased in the same patients, see Supplementary Figure 2) in the periodontitis patients of our cohort and serum levels of both autoantibody species further increased upon periodontal therapy. Our data support previous reports of an association of periodontitis with systemic inflammation and impaired cardiac function. However, there is no indication that an enhanced receptor-autoimmunity is linked to increases in serum markers of cardiac injury, which is the case in other etiologies of CVD. Thus, increased prevalence of $\beta_1$ARAabs and in particular of $M_5$R-Aabs could be a distinct feature of periodontitis and periodontitis-associated autoimmunization against these two receptors, which is possibly boosted upon periodontal therapy. However, these phenomena may not directly be associated with increases in serum markers of cardiac injury inferring a direct link to impaired cardiac function.

### Possible links between periodontitis and receptor autoimmunization

Chagas' disease is the model for cardio-pathogenic receptor autoimmunization. In this disease immunization against the ribosomal P2beta protein of *T. cruzi* induces humoral autoimmunity against the $\beta_1$AR, and the $\beta_1$AR-Aabs thus induced are the cause of CVD occurring 10-20 after infection with *T cruzi* [28]. Interestingly, Chagas' disease is only seen in the western hemisphere. It still remains unclear what triggers receptor-directed humoral autoimmune responses in CVD unrelated to Chagas' disease [17]. It has been suspected for a very long time that the trigger could be bacterial or viral antigens [29]. On the other hand, it is known that the invasive interventions in periodontal therapy entail release of bacterial antigens into the blood stream on a large scale [6]. Consequently, it is tempting to speculate that such exposure of the

immune system to periodontal bacteria and increased circulating proinflammatory markers could be the cause of the high prevalence of autoimmunization against $\beta_1$AR and $M_5$R observed in periodontitis. The changes in circulating levels of $\beta_1$AR- and $M_5$R-Aabs after therapy seen here are in line with this notion, as serum levels of receptor autoantibodies remained stable immediately after therapy, but increased within several weeks post-therapy, which is consistent with the typical time window of immuniszation e.g. vaccination. Serum levels of receptor autoantibodies were highest in the intermediate observation window, in which follow-up frequency (and associated re-exposure of the immune system to periodontal bacteria) was the highest (every three months). The levels decreased again in the late observation window when the follow-up frequency was reduced to twice a year. In summary, these observations indicate that repeated release of bacterial antigens and the periodontitis-associated release of inflammatory markers into the blood stream possibly induce and maintain autoimmunization against $\beta_1$AR and $M_5$R in periodontitis patients. This notion is also supported by the apparent correlation between posttherapy levels of receptor autoantibodies and inflammation markers.

**Etiological implications of $M_5$R-Aabs**

Autoantibodies against muscarinic acetylcholine receptors have been implied in many diseases. Autoantibodies against the $M_2$ subtype play a role in a variety of cardiovascular diseases [30–39]. Autoantibodies against the $M_3$-subtype are involved in Sjögren's syndrome [40]. Autoantibodies against the $M_3$- and $M_4$-subtypes cause the chronic fatigue syndrome [41, 42]. Autoantibodies against the $M_5$subtype have rarely been observed in the context of diseases. The only published disease-associated incidence of $M_5$R-Aabs has been reported from a small subgroup of female patients suffering from postural orthostatic tachycardia syndrome. These patients also exhibited increased levels of autoantibodies against all other MR-subtypes and various adrenergic receptors [43]. Interestingly, in an ongoing prospective cohort study on titer-course *of cardiac autoantibodies and their effect on* survival (ETiCS-study) [27], which included analysis of autoantibodies against all MR subtypes, the $M_5$R-autoantibodies exhibited the strongest association with decline of cardiac function (unpublished results mentioned with kind permission of the study coordinator). The $M_5$R plays a crucial role in cholinergic dilation of the microcirculation most notably of the cerebrum [44, 45]. In mice, loss of this receptor leads to cognitive deficits [46]. If periodontitisassociated autoantibodies had the potency to inhibit or stimulate the $M_5$R (yet to be confirmed), one of their most probable biological effect would be an interference with the regulation of cerebral microcirculation. Interestingly, autoantibodies against various other regulatory receptors of cerebral microcirculation have been implicated in the pathogenesis of Alzheimer's disease and vascular dementia in humans [47]. Moreover, large population-based association studies suggest a link between periodontitis and dementia [48]. Thus, $M_5$R-Aabs could provide a mechanistic link between periodontitis and the associated risk of vascular dementia, therefore it may be of high value to follow up on this marker in future population-based studies.

**Etiological implications of $\beta_1$AR-Aabs**

The potential of $\beta_1$AR-Aabs to cause or promote cardiovascular pathogenesis is firmly established by the Chagas' disease paradigm, as well as animal models of passive and active immunization and clinical therapy trials of removal and/or neutralization of these autoantibodies [17]. A variety of mechanisms have been demonstrated by which $\beta_1$AR-Aabs can possibly harm the cardiovascular system [49]. Current belief holds that the common denominator of cardiotoxicity of $\beta_1$AR-Aabs is the stabilization of an active conformation of the $\beta_1$AR-molecule, entailing a chronic stimulation and/or hyper-sensitization of $\beta_1$-adrenergic signal transduction in the heart [50] and related target tissues [49].

In the present study, we investigate another example of an epidemiological association between cardiac injury (evidenced by an increase in serum proBNP) and increased serum levels of $\beta_1$AR-Aabs. The increased levels of $\beta_1$AR-Aabs levels detected in case of periodontitis plausibly belong to the cardio-

noxious variety, because $\beta_1$AR-Aabs bind to a cyclic peptide, which was recently demonstrated to mimic the conformational epitope related to the active conformation of the receptor molecule, which constitutes the cardio-pathogenic autoepitope [23]. Nevertheless, our data do not support the conclusion that these autoantibodies are indeed the cause of the observed increases in cardiac markers (indicating cardiac injury and inferring impaired cardiac function in the periodontitis patients), because increased circulating levels of $\beta_1$AR-Aabs (reacting with the presumed cardio-pathogenic autoepitope) in the periodontitis group were not correlated to increased levels of cardiac markers. Interestingly, this lack of correlation was also observed following therapy although median levels of both cardiac markers and $\beta_1$AR-Aabs increased upon therapy. Thus, it seems plausible to assume that receptor-autoimmunization and cardiac injury are independent consequences of enhanced inflammation. One must also take into consideration that age distribution in the periodontitis group was much higher than in the control group. Therefore, the poorer cardiovascular function apparent in the patients may at least in part be due to age-related risk factors [8, 51].

### Practical conclusions

Currently, monitoring of periodontitis relies mostly on the mechanical probing of dental pockets, followed by mechanical debridement of the intraoral hard and soft tissues. The association between periodontitis and CVD, that is evidenced by multiple studies [6–11] and supported here by the observation of associated increases in serum markers of cardiac injury, is to our knowledge only sparsely monitored or even considered in routine dental healthcare. Conversely, monitoring of cardiac function in the elderly does not stringently include surveillance of the periodontal status. Several studies suggest that screening and therapy of periodontitis and cardiac function should be coordinated in the elderly [6–11, 25, 26]. The data presented here confirm this notion. We suggest that coordinated surveillance of CVD and periodontitis could *inter alia* be achieved by introducing determinations of serum proBNP into standard dental healthcare. Conversely, $M_5$R-Aabs could provide a distinct serological marker of periodontitis, which in clinical settings not encompassing dental care possibly would allow for a preliminary stratification of periodontitis risk. Most notably, it should be considered to evaluate $M_5$R-Aabs as a possible marker for the risk of vascular dementia in the context of periodontitis.

## MATERIALS AND METHODS

### Study participants

Study subjects were recruited from a prospective cohort study that included 147 initially untreated periodontitis patients at University of Münster, Germany. Periodontitis was diagnosed using case definitions in population-based studies [52] in accordance with the previously used 1999 Workshop classification [53]. 60 systemically and periodontally healthy individuals were recruited as controls at University of Düsseldorf. Absence of periodontitis was determined by probing pocket depths ≤ 2mm.

The study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Boards of University of Münster (IRB approval Nr. 1VBei) and University of Düsseldorf (IRB approval Nr. 3786). All participants have given their written consent to participate, were residents in Germany and none of them had a history of Chagas' disease and/or lived for the past 20 years in areas, where Chagas' disease is endemic. Exclusion criteria included the use of systemic antibiotics within six months prior to study enrolment, requirement of antibiotic prophylaxis, history of endocarditis, bleeding disorders, history of organ transplantation, dialysis, pregnancy and lactation.

### Study design and sampling

The healthy group was subjected to a one-time supragingival debridement and blood sample collection at the time of recruitment. Periodontitis group also received supragingival debridement at the time of recruitment-Supragingival debridement was followed with non-surgical periodontal therapy after 6-8 weeks. Non-surgical periodontal therapy consisted of

supra- and subgingival debridement of oral hard and soft tissues, and adjunctive antimicrobial therapy with 0.2% chlorhexidin-containing mouthrinse used three times daily for 10 days. Surgical periodontal therapy and tooth extractions were performed 10-14 weeks after non-surgical periodontal therapy. The periodontitis group was followed up for a total period of two years after non-surgical therapy. The follow-up was done every 3 months during the first year and every 6 months during the second year. Follow-up examinations comprised of a comprehensive oral examination, supportive periodontal therapy (supra- und subgingival debridement) and blood sample collection. Baseline values of periodontitis group were derived from two samplings carried out at 2 weeks before non-surgical periodontal therapy (hereafter referred to as pretherapy) and on the day of non-surgical periodontal therapy (referred to as therapy). Data of early therapy responses were collected from two samplings carried out 5 and 17 weeks after therapy. Intermediate responses were derived from two subsequent samplings 30 and 44 weeks after therapy. Late responses were summarised from subsequent samplings at 58 to 112 weeks after therapy. A total of 50 ml venous blood was collected from each participant by antecubital vein puncture at each visit.

### Laboratory tests

Pre-analytical handling of blood samples and determination of established parameters of generalized inflammation (CRP and IL-6), myocardial ischemia (TpI), and cardiac wall tension (proBNP) in sera followed routine diagnostic procedures accredited according to DIN EN ISO 15689. $\beta_1$AR-Aabs, and autoantibodies against the muscarinic acetylcholine receptor $M_5$ ($M_5$R-Aabs), were measured with commercially available ELISAs (CellTrend GmbH, Luckenwalde, Germany) according to the instructions of the manufacturer. Both these assays provide native receptors presented in their physiological membrane environment as immunogenic targets for IgG binding. In addition, $\beta_1$AR-Aabs were determined by IgG-binding to a cyclic peptide providing a valid representation of the conformational epitope within the second extracellular loop of the receptor associated with the active receptor conformation. It has been demonstrated that preabsorption with this peptide neutralises the cardiopathogenic potency of stimulatory receptor antibodies in mice [23]. The cyclic peptide was coated onto microtiter plates by established procedures and these plates were processed in a similar manner as the above commercial assays. The two assays for $\beta_1$AR-Aabs exhibited a reasonable correlation (Suppl. Figure 1) with just a few extreme outliers, which, most probably, are due to the presence of $\beta_1$AR-Aabs not directed against the second extracellular loop of the receptor.

### Statistical methods

All data analyses were performed using IBM SPSS Statistics 26.0 software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). Normal distribution was tested by the method of Shapiro-Wilk. Median values and interquartile range (median ± IQR) are stated, when parameters exhibited non-normal distribution. Otherwise mean values and standard deviation (mean ± SD) are stated. The Mann-Whitney-U test was used to analyze differences between baseline values of periodontitis patients (before therapy) and controls. Friedman's test was used to detect an influence of periodontal therapy on $\beta_1$AR and $M_5$R autoimmunization and post-hoc tests with Bonferroni correction were carried out to determine which therapy time points differed significantly. Wilcoxon's signed rank-test was used for longitudinal analyses of the patient group before and after therapy. Spearman's correlation was performed to compare methods and assess parameter correlations at pre- and post-therapy within the periodontitis group. Bivariate comparisons were performed to avoid increased type II error probability arising from multiple comparisons with adjusted p values. All tests were performed with the standard 0.05 level of statistical significance.

## Abbreviations

$\beta_1$AR: adrenergic beta1-receptor subtype; $\beta_1$AR-Aab: autoantibody against the adrenergic beta1-receptor subtype; CHF: chronic heart failure; CRP: C-reactive protein ; CVD:

cardiovascular disease; ELISA: enzyme linked immune assay; IL-6: interleukin 6 ; $M_5R$: muscarinic acetylcholine receptor, subtype $M_5$; $M_5RAab$: autoantibody against muscarinic acetylcholine receptor $M_5$ ; proBNP: brain natriuretic peptides; TpI: Troponin I.

## ACKNOWLEDGMENTS

Technical assistance is gratefully acknowledged to Birgit Hanzen.

## CONFLICTS OF INTEREST

Harald Heidecke is chief executive officer of CellTrend GmbH, Luckenwalde, Germany. All other authors declare no potential conflicts of interest financial or otherwise.

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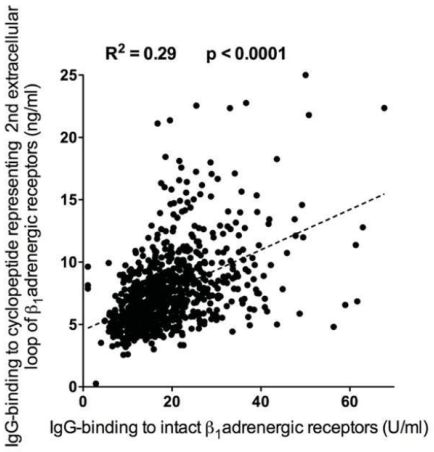
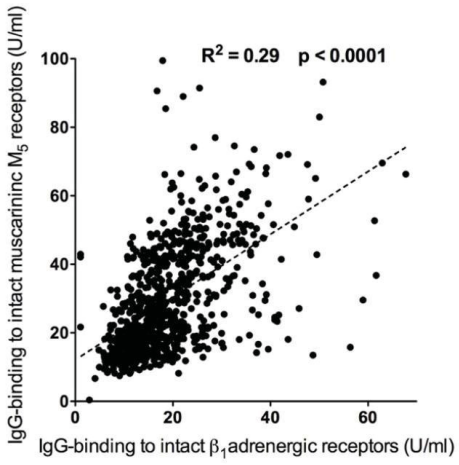
# SUPPLEMENTARY MATERIALS

## Supplementary Figures




**Supplementary Figure 1. Comparison of $\beta_1$AR-Aabs measured by IgG-binding to the native intact receptor (CellTrend GmbH) or to a cyclic peptide providing a valid representation of the presumed pathogenic conformational auto-epitope within the second extracellular loop of the receptor associated with the active receptor conformation [23].**




**Supplementary Figure 2. Comparison of serum levels of $\beta_1$AR-Aabs and $M_5$R-Aabs in periodontitis patients.** Both autoantibodies were measured by IgG-binding to the native intact receptor (CellTrend GmbH).

# 3 Discussion

## 3.1 Central results

The study demonstrates that also in a German population, periodontitis patients display increased levels of $\beta_1$AR-Aabs. Therefore, we validate the preceding results from the Argentinian study group (Segovia et al., 2012, Segovia et al., 2011, Reina et al., 2012) and point out that the increase in circulation of $\beta_1$AR-Aabs is independent of endemic Chagas' disease. Moreover, we observed an even more plain increase in levels of $M_5$R-Aabs in periodontitis patients. Beyond that, the circulation of $\beta_1$AR-Aabs and $M_5$R-Aabs increased through periodontitis therapy and it is positively correlated to cardiac injury and generalized inflammation. *"When drawing that conclusion, it must, however, be taken into account that the control group was much younger, which is inevitable due to the high prevalence of periodontitis in the elderly. Since autoimmune status changes with age, it is difficult to conclude that the higher levels of autoantibodies in the patients are solely due the disease"* (Scherbaum et al., 2020). It has been reported that higher age leads to increased autoimmunization against GPCR, by example $\beta_1$AR (Liu et al., 1999, Cabral-Marques et al., 2018). In addition, there is evidence that individuals without CVD also show increased GPCR autoantibody levels. However, the GPCR autoantibody levels of healthy individuals seem to be distinctly lower than levels of individuals suffering from CVD (Liu et al., 1999, Boivin-Jahns and Jahns, 2018, Jahns et al., 1999).

*"Moreover, $\beta_1$AR-Aabs and $M_5$R-Aabs were highly coincident (i.e. were increased in the same patients, see Suppl. Fig. 2) in the periodontitis patients of our cohort and serum levels of both autoantibody species further increased upon periodontal therapy. Our data support previous reports of an association of periodontitis with systemic inflammation and impaired cardiac function"* (Scherbaum et al., 2020). In this regard, several meta-analyses prove that periodontitis patients exhibit a significant higher risk of developing CVD than periodontal healthy patients (Janket et al., 2003, Blaizot et al., 2009, Bahekar et al., 2007). Moreover, a recent systemic review and meta-analysis showed that periodontitis treatment statistically significantly reduces parameters representing a cardiovascular risk

(Roca-Millan et al., 2018). Furthermore, the actual consensus report (Sanz et al., 2020b), from the European Federation of Periodontology and the American Academy of Periodontology, proclaims that there is evidence that periodontitis patients exhibit higher CRP and IL-6 levels. Moreover, periodontal treatment seems to decrease the inflammation parameters (Sanz et al., 2020b, Freitas et al., 2012, Demmer et al., 2013).

*"However, there is no indication that an enhanced receptor-autoimmunity is linked to increases in serum markers of cardiac injury, which is the case in other etiologies of CVD. Thus, increased prevalence of $\beta_1$AR-Aabs and in particular of $M_5$R-Aabs could be a distinct feature of periodontitis and periodontitis-associated autoimmunization against these two receptors, which is possibly boosted upon periodontal therapy. However, these phenomena may not directly be associated with increases in serum markers of cardiac injury inferring a direct link to impaired cardiac function"* (Scherbaum et al., 2020).

## 3.2 Possible link between periodontitis and receptor autoimmunization

First, we were able to show that, also in a German cohort, periodontitis patients exhibit a higher autoimmunization against $\beta_1$AR and $M_5$R. Likewise, an earlier investigation from Argentina demonstrated for the first time that periodontitis patients display increased $\beta_1$AR-Aab levels than patients without periodontitis (Segovia et al., 2012, Segovia et al., 2011). Nevertheless, the results of the study are questioned because they come from Argentina, where Chagas' disease is endemic. *"Chagas' disease is the model for cardio-pathogenic receptor autoimmunization. In this disease immunization against the ribosomal P2beta protein of T. cruzi induces humoral autoimmunity against the $\beta_1$AR, and the $\beta_1$AR-Aabs thus induced are the cause of CVD occurring 10-20 after infection with T cruzi (Lopez Bergami et al., 2005). Interestingly, Chagas' disease is only seen in the western hemisphere. It still remains unclear what triggers receptor-directed humoral autoimmune responses in CVD unrelated to Chagas' disease (Boivin-Jahns and Jahns, 2018). It has been suspected for a very long time that the*

*trigger could be bacterial or viral antigens (Hoebeke, 1996)"* (Scherbaum et al., 2020).

Second, we demonstrated that periodontitis patients exhibit higher levels of $\beta_1$AR-Aabs and $M_5$R-Aabs after periodontitis therapy, and that they were highest in middle response. To the best of our knowledge, no other study has yet investigated the development of $\beta_1$AR and $M_5$R autoimmunization through periodontal therapy. *"It is known that the invasive interventions in periodontal therapy entail release of bacterial antigens into the blood stream on a large scale (Sanz et al., 2020b). Consequently, it is tempting to speculate that such exposure of the immune system to periodontal bacteria and increased circulating proinflammatory markers could be the cause of the high prevalence of autoimmunization against $\beta_1$AR and $M_5$R observed in periodontitis. The changes in circulating levels of $\beta_1$AR- and $M_5$R-Aabs after therapy seen here are in line with this notion, as serum levels of receptor autoantibodies remained stable immediately after therapy, but increased within several weeks post-therapy, which is consistent with the typical time window of immunization e.g. vaccination. Serum levels of receptor autoantibodies were highest in the intermediate observation window, in which follow-up frequency (and associated re-exposure of the immune system to periodontal bacteria) was the highest (every three months). The levels decreased again in the late observation window when the follow-up frequency was reduced to twice a year In summary, these observations indicate that repeated release of bacterial antigens and the periodontitis-associated release of inflammatory markers into the blood stream possibly induce and maintain autoimmunization against $\beta_1$AR and $M_5$R in periodontitis patients. This notion is also supported by the apparent correlation between post-therapy levels of receptor autoantibodies and inflammation markers"* (Scherbaum et al., 2020).

Nevertheless, it is not fully clear if there are other factors which could contribute to the higher levels of $\beta_1$AR-Aabs and $M_5$R-Aabs in periodontitis patients. Nussinovitch and Shoenfeld (Nussinovitch and Shoenfeld, 2013) declare that genetics, immune status, hormonal status, and environmental factors all have contributing effects to the autoimmune response. Furthermore, they convey that the exact reasons for the induction of the immune answer are not clarified. Further

research is necessary to detect the exact mechanism of the increased serum levels of $\beta_1$AR-Aabs and $M_5$R-Aabs in periodontitis patients and through periodontal treatment.

## 3.3 Etiological implications of $M_5$R-Aabs

Since they have been associated with many diseases, autoantibodies against MR are part of intensive research. Several clinical and animal studies reveal that autoantibodies against the $M_2$ subtype play a central role in various CVD, such as DCM and CHF (Gurses et al., 2015, Yoshizawa et al., 2012, Pei et al., 2012, Stavrakis et al., 2011, Baba et al., 2004, Matsui et al., 2001, Liu et al., 1999, Fu, 1996, Fu et al., 1993, Li et al., 2016). To illustrate this, an animal study demonstrated that induced autoimmunization against the $M_2$ subtype causes typical symptoms of DCM, like increased heart weight in combination with lower intraventricular diameter, less systolic function and a longer action potential duration (Yoshizawa et al., 2012). Other studies indicate that autoantibodies against the $M_3$-subtype play a role in Sjögren's syndrome, an autoimmune disease, affecting exocrine glands, in particular the salivary and lacrimal gland (Yu et al., 2018). Furthermore, autoantibodies against the $M_3$- and $M_4$-subtypes were detected to be involved in the chronic fatigue syndrome (Loebel et al., 2016, Scheibenbogen et al., 2018). *"Autoantibodies against the $M_5$-subtype have rarely been observed in the context of diseases. The only published disease-associated incidence of $M_5$R-Aabs has been reported from a small subgroup of female patients suffering from postural orthostatic tachycardia syndrome. These patients also exhibited increased levels of autoantibodies against all other MR-subtypes and various adrenergic receptors (Gunning et al., 2019). Interestingly, in an ongoing prospective cohort study on titer-course of cardiac autoantibodies and their effect on survival (ETiCS-study) (Deubner et al., 2010), which included analysis of autoantibodies against all MR subtypes, the $M_5$R-autoantibodies exhibited the strongest association with decline of cardiac function (unpublished results mentioned with kind permission of the study coordinator). The $M_5$R plays a crucial role in cholinergic dilation of the microcirculation most notably of the cerebrum (Elhusseiny et al., 1999, Yamada et al., 2001). In mice, loss of this*

*receptor leads to cognitive deficits (Araya et al., 2006). If periodontitis-associated autoantibodies had the potency to inhibit or stimulate the $M_5R$ (yet to be confirmed), one of their most probable biological effect would be an interference with the regulation of cerebral microcirculation. Interestingly, autoantibodies against various other regulatory receptors of cerebral microcirculation have been implicated in the pathogenesis of Alzheimer's disease and vascular dementia in humans (Karczewski et al., 2012). Moreover, large population-based association studies suggest a link between periodontitis and dementia (Lee et al., 2017). Thus, $M_5R$-Aabs could provide a mechanistic link between periodontitis and the associated risk of vascular dementia, therefore it may be of high value to follow up on this marker in future population-based studies"* (Scherbaum et al., 2020).

## 3.4 Etiological implications of $\beta_1$AR-Aabs

*"The potential of $\beta_1$AR-Aabs to cause or promote cardiovascular pathogenesis is firmly established by the Chagas' disease paradigm, as well as animal models of passive and active immunization and clinical therapy trials of removal and/or neutralization of these autoantibodies (Boivin-Jahns and Jahns, 2018). A variety of mechanisms have been demonstrated by which $\beta_1$AR-Aabs can possibly harm the cardiovascular system (Boivin-Jahns et al., 2018) "* (Scherbaum et al., 2020). On the molecular level, it could be shown that $\beta_1$AR-Aabs may bind and therefore lead to activation of the $\beta_1$AR (Bornholz et al., 2013a). In addition, the $\beta_1$AR-Aabs seem to increase the sensitization for endogenous catecholamines (Bornholz et al., 2013a). Not only on molecular level, but also the cell signaling is affected by $\beta_1$AR-Aabs. For instance, $\beta_1$AR-Aabs seem to stimulate cyclic adenosine monophosphate (cAMP), respectively cAMP depending signaling cascades (Jahns et al., 2000). Likewise, $\beta_1$AR-Aabs appear to exhibit direct toxic effects on cardiac cells, as in the cases of positive chronotropic and/or dromotropic effects on isolated cardiomyocytes or atrial preparations (Magnusson et al., 1994, Sterin-Borda et al., 1976). *"Current belief holds that the common denominator of cardiotoxicity of $\beta_1$AR-Aabs is the stabilization of an active conformation of the $\beta_1$AR-molecule, entailing a chronic stimulation and/or hyper-sensitization of $\beta_1$-adrenergic signal transduction in the heart (Bornholz et al., 2016b) and related*

*target tissues (Boivin-Jahns et al., 2018)*" (Scherbaum et al., 2020). However, it must be taken into consideration that regardless of all acquainted mechanisms, the exact cardio pathological effects of $\beta_1$AR-Aabs still remain unclear (Boivin-Jahns et al., 2018).

*"In the present study, we investigate another example of an epidemiological association between cardiac injury (evidenced by an increase in serum proBNP) and increased serum levels of $\beta_1$AR-Aabs. The increased levels of $\beta_1$AR-Aabs levels detected in case of periodontitis plausibly belong to the cardio-noxious variety, because $\beta_1$AR-Aabs bind to a cyclic peptide, which was recently demonstrated to mimic the conformational epitope. This epitope is related to the active conformation of the receptor molecule, which constitutes the cardio-pathogenic autoepitope (Wolfel et al., 2020)"* (Scherbaum et al., 2020). Likewise, in a recent study, Lund et al. (Lund et al., 2018) reported a correlation between several receptor autoantibodies and parameters of myocardial damage as well as parameters of inflammation. Other studies failed to find a positive correlation between the $\beta_1$AR-Aabs and heart parameters (Ernst et al., 2019, Ernst et al., 2018).

*"Nevertheless, our data do not support the conclusion that these autoantibodies are indeed the cause of the observed increases in cardiac markers (indicating cardiac injury and inferring impaired cardiac function in the periodontitis patients), because increased circulating levels of $\beta_1$AR-Aabs (reacting with the presumed cardio-pathogenic autoepitope) in the periodontitis group were not correlated to increased levels of cardiac markers. Interestingly, this lack of correlation was also observed following therapy although median levels of both cardiac markers and $\beta_1$AR-Aabs increased upon therapy"* (Scherbaum et al., 2020). In terms of elevated cardiac troponin I (TpI) levels, it has been reported that extent and severity of periodontitis positively correlates with levels of cardiac TpI (Marfil-Alvarez et al., 2014). Moreover, patients suffering from acute myocardial infarct show a significant correlation to periodontal destruction; even if the common risk factors diabetes mellitus, or diabetes mellitus and smoking together, were excluded (Kodovazenitis et al., 2014, Kodovazenitis et al., 2011). Focusing on the cardiac injury measured by brain natriuretic peptides (proBNP) levels of periodontitis patients, a recent study also observed that periodontitis patients

exhibit increased levels of proBNP in comparison to non-periodontitis individuals (Leira and Blanco, 2018). The study coordinator proclaimed that next to the cardiac injury, the increase could be based on higher inflammation in patients suffering from periodontitis. In relation to this, it is revealed that inflammation could play a role as a non-cardiac factor for elevated proBNP levels (Jensen et al., 2010). *"Thus, it seems plausible to assume that receptor-autoimmunization and cardiac injury are independent consequences of enhanced inflammation. One must also take into consideration that age distribution in the periodontitis group was much higher than in the control group. Therefore, the poorer cardiovascular function apparent in the patients may at least in part be due to age-related risk factors (Dietrich et al., 2013, Dorn et al., 2010)"* (Scherbaum et al., 2020).

## 3.5 Further statistical results

Within the statistical analysis, we obtained additional results not listed in the paper. In addition to the impact of periodontal therapy on autoimmunization against $\beta_1$AR and $M_5$R, we also examined the course of the heart injury and inflammatory markers of the periodontitis patients during the different stages of periodontal treatment. For that purpose, we compared within the periodontitis group baseline levels of the heart injury and inflammation markers (pre-therapy and on the day of therapy) with corresponding values measured at each of the post-therapy follow-up visits. In between the four timepoints of the periodontitis therapy, none of the cardiac injury or inflammatory parameters changed significantly (data not shown).

Furthermore, we also addressed the question of whether there is a correlation between cardiac injury and inflammatory response in periodontitis patients. To test this hypothesis, we correlated the markers of heart injury (proBNP, TpI) within the inflammatory markers (CRP, IL-6). ProBNP was highly significantly correlated with the inflammatory parameters CRP and IL-6, while TpI just showed a high significance with IL-6 and was not correlated to CRP (data not shown).

## 3.6 Disposition of the reviewers concerns and suggestions

First, one reviewer noted the missing age-match between the periodontitis patients and controls. Since autoimmune status changes with age, it is difficult to conclude that the higher levels of autoantibodies in periodontitis patients than in controls are only due to the disease. We fully agree to this concern, but we refer to the condition of an unavoidable age difference between periodontitis and healthy controls, due to the strong relation between periodontitis and increased age. Second, the reviewer asked why only $M_5$R-Aabs were studied, since autoantibodies against multiple MR might be associated with heart function. In our study, we focused on the $M_5$R-Aabs because a yet unpublished cohort study in post-myocarditis patients (Deubner et al., 2010) revealed that autoantibodies against the $M_5$-subtype show the strongest association with reduction of cardiac function. Moreover, pre-experiments determined a significant difference in $M_5$R-Aabs between periodontitis patients and healthy controls.

Another reviewer objected in an earlier version of the paper the use of the term “cardiac function” in the absence of data on blood pressure, ejection fraction, ventricular volume or ECG results is inappropriate. As suggested of the reviewer, we have replaced such wording throughout the manuscript by the terms “cardiac injury” and “markers of cardiac injury”. In addition, the reviewer asked why only IL-6 but no other inflammatory markers by example interleukin 1, interleukin 10 or tumor necrosis factor α were measured. He expressed that it would be interesting to be able to compare cytokines seen in Chagas’ disease with more inflammatory markers in periodontitis. We fully agree that inclusion of more cytokines would have been interesting and highly desirable. However, our limited funds have forced us to spare on the absolute essential minimum of biomarkers required to judge generalized inflammation. According to current clinical guidelines of almost all chronic inflammatory diseases, that would be IL-6 and CRP.

Additionally, the reviewer asked for a more precise interpretation of the increasing levels of $M_5$R-Aabs following therapy. They called into question, whether antibiotic mouthwash for 10 days was the only therapy. Besides, they questioned why the concentration of the autoantibodies rises in mid-course of the study and

drops at longer intervals from the beginning of the study. First, adjunctive antimicrobial therapy using a 0,2% chlorhexidine-containing mouthrinse, three times daily, for 10 days is the standard adjunctive therapy according to the current clinical guidelines. Systemic antibiotic prophylaxis is currently not heralded due to risk of thereby promoted resistance. Second, we argue that the time lapse between first treatment and the increase in autoantibodies is in line with the typical time course of immunization, as for vaccination. Besides, in our opinion the increase of the titer mid-course of the study reflects the higher frequency of follow up treatments (and associated re-exposure of the immune system to periodontal bacteria) during that period. Along the same lines we believe that the subsequent decrease of titers is due to the lesser revision frequency and the much longer intervals between exposure to bacterial antigens and necrotic tissue. We are grateful for the constructive objections of the reviewers and for the associated opportunity of improving the manuscript.

## 3.7 Limitations and strengths of the study

The study has several limitations. First, as also noted by the reviewers, the higher age distribution in the periodontitis group in comparison to the healthy control group displays one limitation.

Second comes the circumstance in which we just investigated the receptor autoimmunization against $M_5R$ and $\beta_1AR$, the inflammatory status by CRP and IL-6 and the cardiac injury by proBNP and TpI. A more extensive examination of the cohort using further parameters would have resulted in an even more detailed insight of the link between periodontitis, cardiac injury and receptor autoimmunization

Third, the high amount of periodontitis patients who left the study prematurely is another limitation. During the post-therapy follow-up, 80 periodontitis patients left for undisclosed reasons. Moreover, the smaller cohort size of the control group and the different ages of the bloodsera are shortcomings. On top of that, we only collected data from the control group at one timepoint.

Finally, our study is limited by the lack of precise knowledge about the increase and function of the autoimmunization against $M_5R$ and $\beta_1AR$. Further studies are necessary to contribute to a deeper understanding.

Regardless of the several limitations, the accomplished study also has strengths. To the best of our knowledge, no other study in Europe yet examined the association of periodontitis with receptor autoimmunization, cardiac injury and systemic inflammation. Therefore, the influence of Chagas' disease on the receptor autoimmunization can be safely ruled out in our study.

Although many patients left the study during the follow-up, we still exhibit a comparatively large cohort size of 66 patients in the end. Furthermore, the long follow-up of up to 112 weeks, divided into eight follow-up phases represents a strength of the study. Consequently, we were able to observe and analyze the exact course of the changes within receptor autoimmunization, inflammation status and cardiac injury of periodontitis patients through the different stages of therapy.

## 3.8 Conclusion

In this study, we investigated the association between periodontitis, cardiac insufficiency and autoantibodies against $M_5R$ and $\beta_1AR$. Our results demonstrate that, in periodontitis patients, the autoimmunization against $M_5R$ and $\beta_1AR$ is associated with increased inflammatory status as well as periodontal therapy, but not with serum biomarkers for cardiac insufficiency. The study helps our understanding of the link between periodontitis, indications of cardiac injury and autoimmunization against $M_5R$ and $\beta_1AR$.

*"Currently, monitoring of periodontitis relies mostly on the mechanical probing of dental pockets, followed by mechanical debridement of the intraoral hard and soft tissues. The association between periodontitis and CVD, that is evidenced by multiple studies (Sanz et al., 2020b, Hansen et al., 2016, Dietrich et al., 2013, Lockhart et al., 2012, Blaizot et al., 2009, Bahekar et al., 2007) and supported here by the observation of associated increases in serum markers of cardiac injury, is to our knowledge only sparsely monitored or even considered in routine dental healthcare. Conversely, monitoring of cardiac injury in the elderly does not stringently include surveillance of the periodontal status. Several studies suggest that screening and therapy of periodontitis and heart injury should be coordinated in the elderly (Sanz et al., 2020b, Hansen et al., 2016, Dietrich et al., 2013, Lockhart et al., 2012, Blaizot et al., 2009, Bahekar et al., 2007, Schenkein and Loos, 2013, Aarabi et al., 2018). The data presented here confirm this notion "* (Scherbaum et al., 2020).

We therefore recommend raising attentive awareness of the close connection between periodontitis and CVD in cardiologists and dentists. We advise cardiologists and dentists to consult together for the screening and treatment of CVD- and periodontitis patients. In this sense, CVD patients may be advised to check closely with the dentist, especially regarding periodontal changes. CVD patients could be advised to perform a periodontal examination, especially when no explicable cause for their symptoms can be found. In addition, it is also important to inform patients about the possible connection between periodontitis and CVD. Only by creating an awareness of the possible influence of both diseases, patients get the opportunity to actively influence the course of their own diseases.

In connection with infectious endocarditis, antibiotic prophylaxis is already carried out in dental practice. Before invasive dental treatments, patients which are the most likely to have severe or fatal infectious endocarditis such as those with severe congenital heart defects, previous endocarditis or valve replacement, get an antibiotic prophylaxis. The antibiotic prophylaxis aims at a reduction of an infectious endocarditis through bacteremia. Therefore, it is recommended for dental treatments which manipulate the gingiva, the periapical tooth region and/or which enable a perforation of the oral mucosa, as these are all known to pose the risk of inducing a bacteremia. Taking an appropriate antibiotic orally 30-60 minutes before the treatment should reduce the risk of an infectious endocarditis caused by bacteremia (Naber et al., 2007). Our study implies that antibiotic prophylaxis for CVD patients could also be important during periodontitis therapy. The possible benefit of antibiotic prophylaxis during periodontal treatment is supported by the fact that periodontitis and its treatment trigger systemic inflammation, which is combined with receptor autoimmunization against $M_5R$ and $\beta_1AR$, and regardless of that with cardiac injury. Therefore, periodontal therapy puts CVD patients at risk of worsening their symptoms.

Additionally, *"we suggest that coordinated surveillance of CVD and periodontitis could inter alia be achieved by introducing determinations of serum proBNP into standard dental healthcare. Conversely, $M_5R$-Aabs could provide a distinct serological marker of periodontitis, which in clinical settings not encompassing dental care possibly would allow for a preliminary stratification of periodontitis risk. Most notably, it should be considered to evaluate $M_5R$-Aabs as a possible marker for the risk of vascular dementia in the context of periodontitis"* (Scherbaum et al., 2020)

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# 5 Acknowledgments

First, I would like to express my gratitude to my doctoral supervisor Univ.-Prof. Dr. F. Boege for giving me the opportunity to work on this exciting dissertation topic and for providing the framework conditions that made this work possible. With competent, motivating and friendly support, he enabled me to learn scientific thinking and working.

I wish to express my appreciation to Univ.-Prof. Dr. Dr. T. Beikler for kindly providing the sample material, as well as the great support with the publication.

I want to thank Dr. H. Heidecke and his team from CellTrend GmbH for measuring the receptor autoantibodies and always being open to answer my questions.

I would like to thank Univ.-Prof. Dr. A. Hugger for being my co-referee.

I want to thank K. Bunte and Dr. U. Peters for the great cooperation and helpfulness while writing the publication.

I want to thank Birgit Hanzen for helping me to familiarize with the laboratory work and for all her friendly advices.

I also wish to thank Dr. E. Cieslik-Köchling for the very helpful statistical advices.

To Francesco, thank you for the correction of my English and for all your understanding.