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Complement C3 as a potential drug target in periodontitis: Evidence from the *cis*-Mendelian randomization approach

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Abstract

Aim: Evidence from a Phase IIa trial showed that a complement C3-targeted drug reduced gingival inflammation in patients with gingivitis. Using drug-target Mendelian randomization (MR), we investigated whether genetically proxied C3 inhibition alters the risk of periodontitis.

Materials and Methods: We used multiple 'cis' instruments from the vicinity of the encoding loci of C3. Instrument selection was restricted to the drug target encoding loci (chromosome 19; 6,677,715–6,730,573 (GRCh37/hg19)). We selected three uncorrelated single-nucleotide polymorphisms (rs141552034, rs145406915, rs11569479) that were associated with serum C3 levels (p value $<1 \times 10^{-4}$) from a genome-wide association study (GWAS) of 5368 European descent individuals. We extracted association statistics from a GWAS of 17,353 clinical periodontitis cases and 28,210 European controls. Wald ratios were combined using inverse-variance weighted meta-analysis to estimate the odds ratio (OR) of the genetically proxied inhibition of C3 in relation to periodontitis.

Results: MR analysis revealed that the inhibition of C3 reduces the odds of periodontitis (OR 0.91 per 1 standard deviation reduction in C3; 95% confidence interval 0.87–0.96, p value = .0003).

Conclusions: Findings from our MR analysis suggest a potential protective effect of C3 blockade against periodontitis.

KEYWORDS
complement C3, drug discovery, immunomodulation, Mendelian randomization analysis, periodontitis

Clinical Relevance

Scientific rationale for study: Mounting evidence suggests that the complement system is dysregulated in periodontitis. The first human randomized clinical trial demonstrated promising effects mediated via the blockade of C3, a key factor of the complement system. Results from

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drug-target Mendelian randomization (MR) analysis can provide compelling evidence to predict the efficacy of pharmacological C3 blockage in future clinical trials in periodontitis patients.
Principal findings: Genetically proxied C3 inhibition reduced periodontitis risk.
Practical implications: Our MR analysis provides genetic evidence that C3-targeted drugs might be an efficient adjunct therapy in periodontitis.

1 | INTRODUCTION

Periodontitis is a prevalent chronic inflammatory disease affecting around 50% of adults, of which about 10% suffer from a severe periodontitis (Eke et al., 2015). Periodontitis is a complex multifactorial disease that arises from the intricate interplay of several genetic and environmental factors such as microbial communities and the host response. Dysregulated and excessive inflammatory response due to dysbiosis can lead to the destruction of the supporting tissues around the tooth (e.g., gingiva, periodontal ligament and alveolar bone) (Lamont et al., 2018). When left untreated, periodontitis can lead to tooth loss, and, consequently, impaired mastication and poor aesthetics, which affects the patients' quality of life (Chapple, 2014). In addition to the direct impact on oral health, this condition is associated with an increased risk of other systemic conditions, including cardiovascular diseases, diabetes mellitus, rheumatoid arthritis and Alzheimer's disease (Hajishengallis & Chavakis, 2021). Thus, treating periodontitis may also reduce the risk of periodontitis-associated comorbidities. Periodontal therapy reduces infection and inflammation by mechanically removing dental plaque and calculus, often with adjunctive antimicrobials, while optimizing the patients' biofilm control (Sanz et al., 2020). However, in highly susceptible cases, the treatment is deemed ineffective (Hajishengallis et al., 2021). It has been hypothesized that host modulation therapy can be incorporated into the management of periodontitis (Balta et al., 2021).

The complement system is a critical part of the innate immune system. Recent studies have shown that it not only acts as a first-line defence mechanism against pathogens and endogenous molecules but also modulates the host immune response by engaging in crosstalk interactions (Hajishengallis et al., 2017). In addition, complement dysregulation or excessive activation, whether caused by genetic factors or microbial virulence factors, emerges as a contributing factor in the aetiology and pathogenesis of a number of disorders, including periodontitis. Observational human studies, which reported elevated complement metabolites in gingival fluid during periodontal tissue inflammation, support this presumed role in the pathogenesis of periodontitis (Kajikawa et al., 2022). Moreover, the treatment that alleviated periodontal inflammation was associated with reduced complement C3 activation in gingival crevicular fluid (Niekrash & Patters, 1985).

The complement cascade can be initiated by three distinct mechanisms: the classical, lectin and alternative pathways. Inflammation caused by any of these pathways involves the activation of C3, highlighting its integral role as a central mediator within the complement cascade (Mastellos et al., 2019). While C3 portrays a 'functional hub' where all complement activation pathways converge, rendering this protein a potential pharmacological target, it is important to acknowledge that other complement components, like anaphylatoxin C5a, have been implicated in the pathogenesis of periodontitis (see Figure 1). Animal models revealed that C3-deficient mice did not develop gingival inflammation and alveolar bone loss due to periodontitis (Maekawa et al., 2014). Cp40, an analogue to C3-inhibiting compstatin, blocks the binding of C3 to its convertase and thus interrupts the complement cascade. Cp40 treatment in non-human primates led to a decrease in periodontal inflammation and tissue destruction (Kajikawa et al., 2017; Maekawa et al., 2014, 2016). Consequently, Cp40 was clinically developed for human use as 'AMY-101' and revealed promising safety and efficacy end points in a Phase I safety trial (Mastellos et al., 2019) and a Phase IIa proof-of-concept study in gingivitis (Hasturk et al., 2021).

In our study, we employed a Mendelian randomization (MR) to genetically test whether inhibiting C3 interferes in the pathogenesis of periodontitis. This study design is based on the instrumental variable (IV) analysis approach where instruments are used to index an exposure and assess the hypothesized causal effect on a health outcome. We used genetic variants—single-nucleotide polymorphisms (SNPs)—that serve as IVs for the druggable protein target 'C3' and studied the corresponding potential effects of AMY-101 in periodontitis. Despite pre-clinical and clinical studies showing potential safety and efficacy of AMY-101, the failure rate of candidate drugs progressing through clinical trials is high, with increasing failure rates in Phase II and Phase III (Holmes et al., 2021). Our study thus aims to determine the potential causal role of C3 in periodontitis and to provide additional evidence for considering C3 inhibition in future clinical trials.

2 | MATERIALS AND METHODS

MR is an IV approach to determine whether there is a causal relationship between a modifiable risk factor and a disease. The MR study design aims to draw causal inference minimizing the impact of bias from confounding and reverse causation that conventional observational studies suffer from. Assuming that the random transmission of genetic traits from parents to their offspring is mirrored within a population, genetic variations can be used to distinguish sets of individuals who, on average, exhibit differences in a certain risk factor. This natural randomization makes this study design less prone to confounding and bias than conventional epidemiological approaches (Richmond & Davey Smith, 2022). Also, genetic associations are guarded against reverse causation as genetic variants remain fixed throughout one's lifetime

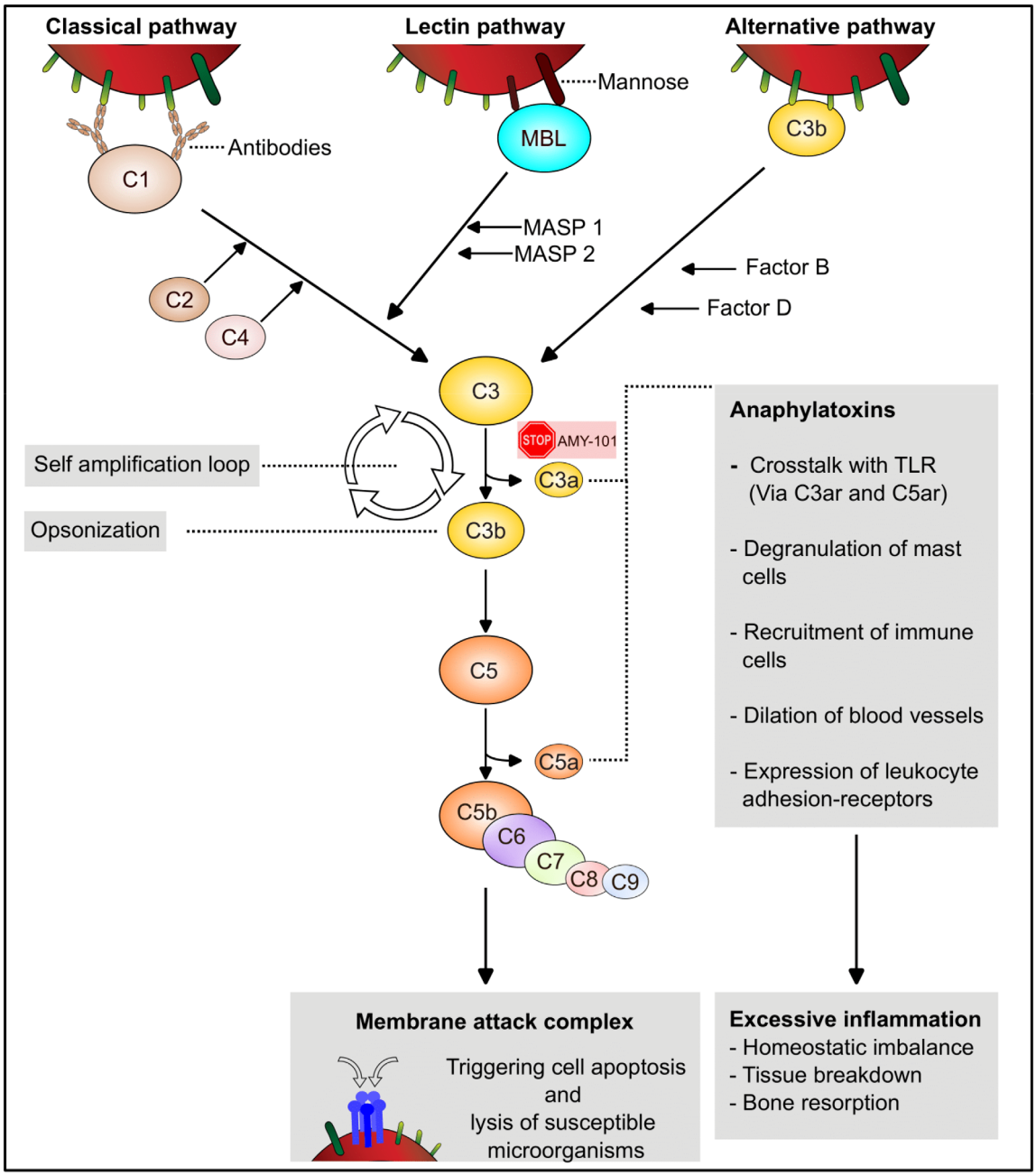

FIGURE 1 Simplified schematic of the complement cascade (inhibition). Three different pathways can activate the complement system. Classical activation occurs when antibodies bind to antigens and is mediated by complement factor C1. The lectin pathway requires the binding of mannose-binding lectin (MBL) to mannose residues of microbial polysaccharides. It is triggered in the absence of antibodies but follows the classical pathway. The alternative pathway begins with the spontaneous breakdown of C3 into C3a and C3b. C3b is rapidly inactivated unless a microbial surface is nearby. In this case, C3b and additional factors form the C3 convertase. Each of these pathways leads to the cleavage of the central complement C3, followed by the cleavage of C5 by the so-called C5 convertases with the subsequent formation of the C5b-9 membrane attack complex (MAC). The MAC is the final constituent of the terminal complement cascade that forms pores in the membrane of susceptible pathogens or targeted cells, leading to osmolysis. The released C3a and C5a are highly potent anaphylatoxins and exert immunomodulatory and pro-inflammatory effects. Precisely these effects are associated with the excessive inflammation of the periodontium and the resulting pathophysiological changes (Hajishengallis et al., 2021).

and are not altered by the disease process (Lawlor et al., 2008). The natural randomization also makes MR analysis analogous to randomized clinical trials (see Figure 2). In genetic association studies, the measured effect corresponds to the presence of the 'effect allele' in the genetic variant (equivalent to a drug in a randomized clinical trial), compared with a 'baseline allele' variant (similar to a placebo in a randomized clinical trial) (Roberts, 2018). The standard MR approach selects SNPs from the whole genome regardless of the location of

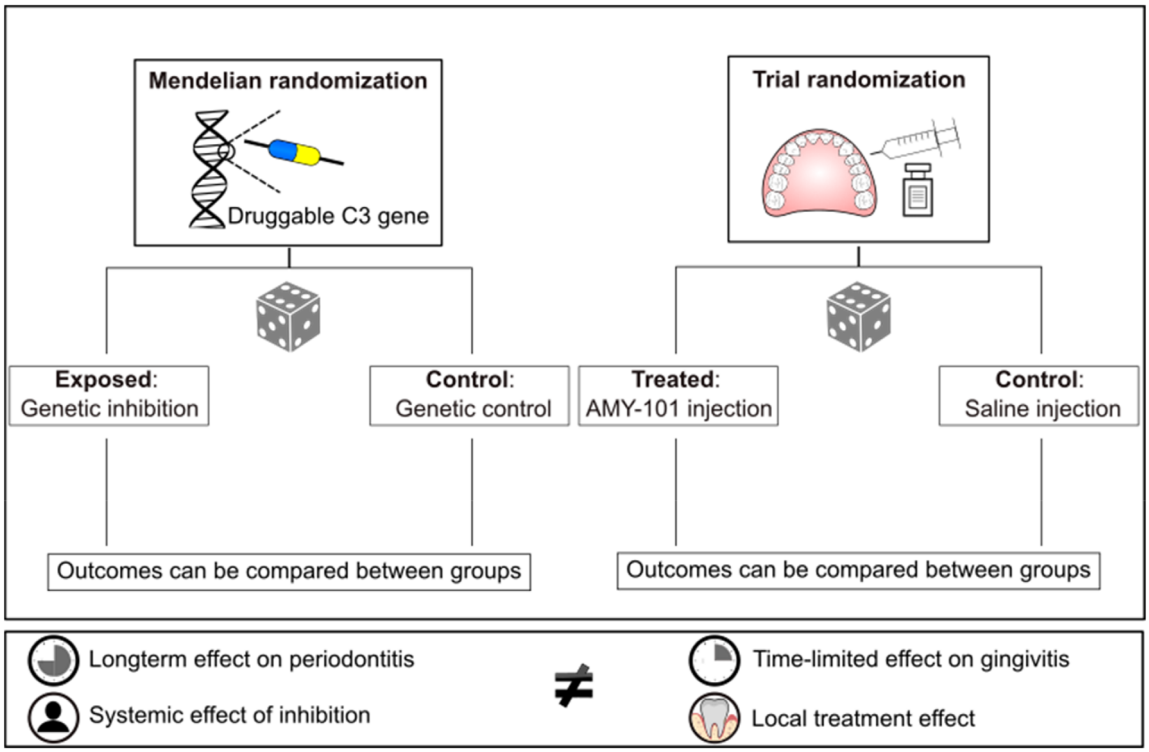

FIGURE 2 A comparison of study principles. This overview compares and contrasts parallels between the clinical trial of AMY-101 and our Mendelian randomization (MR) approach. In the clinical trial, randomization was achieved by random allocation of drug-application sites, whereas in MR it was achieved by random allocation of alleles. The clinical trial examined the local and time-limited effect of AMY-101, while the MR approach examined the effect of lifelong exposure to a hypothetical drug (i.e., genetic variant) that targets the encoded protein (Lawlor et al., 2008).

these variants with respect to the encoding loci. Drug-target MR (or *cis*-MR) utilizes SNPs from within and around a gene known to encode a druggable protein, like C3. While standard MR elucidates the causal relationship between a modifiable risk factor and an outcome of interest, *cis*-MR determines whether altering a certain drug target changes the risk of a disease (Schmidt et al., 2020). To validly assess the causal relationship, instruments must satisfy the relevance, independence and exclusion restriction/no horizontal pleiotropy assumptions. The first assumption requires that the instruments are strongly associated with the risk factor. The second assumption states that there are no common causes of the instruments and outcome. The third assumption entails that the instruments alter the outcome only through the exposure (Burgess et al., 2019) (see Figure 3).

2.1 | C3 indexing GWAS

Variants were derived from a genome-wide association study (GWAS) of serum concentration of C3 from a large-scale proteogenomic study ($N = 5368$) from the age, gene/environment susceptibility cohort. Association estimates in this GWAS were derived from participants of European descent. C3 in serum was detected and quantified by the Slow-Off rate Modified Aptamer proteomic profiling technology (Gudjonsson et al., 2022).

2.2 | Periodontitis GWAS

Summary statistics data for periodontitis were obtained from the Gene-Lifestyle Interactions in Dental Endpoints consortium. A total of 17,353 participants of European ancestry were classified as clinical periodontitis cases and 28,210 as controls. Periodontitis was defined by either the Centers for Disease Control and Prevention/American Academy of Periodontology classification or the Community Periodontal Index case definition (Shungin et al., 2019).

2.3 | Instrument selection

**We identified *cis*-acting variants as proxies for C3 inhibition within a region 300 kB 5′ to 300 kB 3′ of the drug target encoding loci (chromosome 19; 6,677,715–6,730,573 (GRCh37/hg19)) that were associated with serum C3 levels (p value $<1 \times 10^{-4}$). We clumped the genetic variants by applying a conservative threshold (<0.001) to assure that instruments are not in linkage disequilibrium. We estimated the F statistics to measure instrument strength. A magnitude of 10 is considered a reasonable threshold to rule out weak instrument bias (Burgess et al., 2019). It should be noted that *cis*-acting variants have a substantial effect on protein expression compared with other potential downstream biomarkers, and this ensures the validity of the relevance assumption.

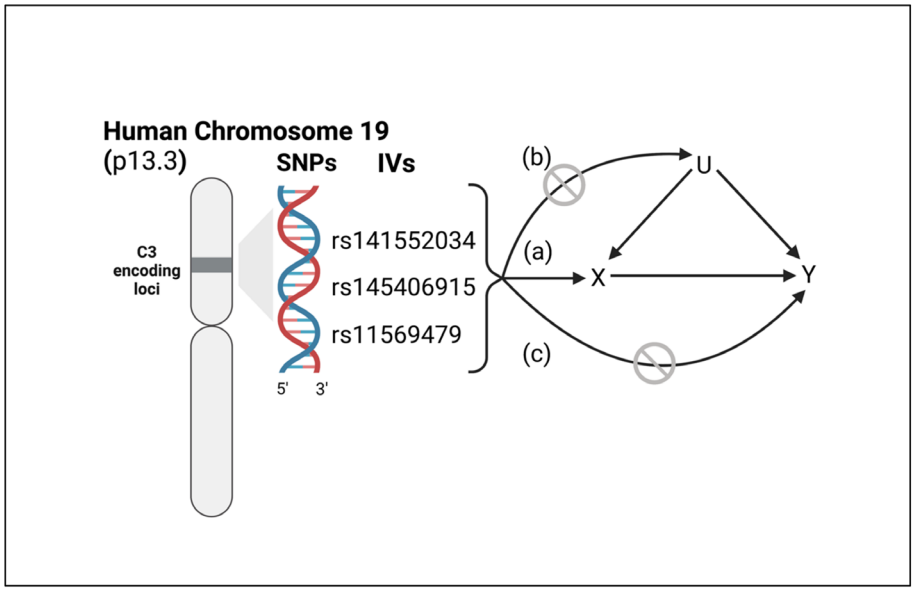

FIGURE 3 Directed acyclic graph (DAG) illustrating the causal assumptions. 'Created with BioRender.com'. This DAG represents the causal relationship and assumptions underlying our analysis. We used single-nucleotide polymorphisms (SNPs) that serve as instrumental variables (IVs) for the druggable protein target 'C3'. Three assumptions must be satisfied to ensure a Mendelian randomization study is valid, which include: (a) SNPs should be associated with the exposure (X) (i.e., the relevance assumption), (b) SNPs must not be associated with confounders (U) of the X–Y association ('exchangeability' or 'no correlated pleiotropy' assumption) and (c) SNPs must not be associated with the outcome (Y) other than via its association with X ('exclusion restriction' or 'no horizontal pleiotropy') (Burgess et al., 2018; Labrecque & Swanson, 2018).

Furthermore, we searched the instruments in Phenoscanner to confirm their independence from any known confounders (Kamat et al., 2019). Because it is a protein–disease relationship rather than a downstream biomarker–disease relationship, our MR study is less prone to violate the assumption of 'no horizontal pleiotropy' (Schmidt et al., 2020).

2.4 | Statistical analysis

The instruments selected based on their association with serum C3 levels were then searched in the outcome GWAS, data for the exposure and outcome were harmonized according to the effect allele, and none of the instruments were palindromic. It is not required for these SNPs to be associated with the outcome, and if not present a proxy SNP can be used instead. The individual causal effect for each instrument was calculated as the ratio of the instrument–outcome association to the instrument–exposure association. Ratio estimates were pooled using inverse-variance weighted (IVW) meta-analysis. Additionally, we performed a leave-one-out analysis to assess whether the causal effect substantially changes upon removal of a single instrument (Burgess et al., 2019). All analyses were performed in R version 4.1.2 using the TwoSampleMR and MendelianRandomization packages.

3 | RESULTS

Three SNPs (rs11569479, rs141552034, rs145406915) were employed as IVs to convey the effect of genetically proxied C3 blockade on periodontitis risk (Table S1). *F* statistics ranged between 13 and 45, indicating no weak instrument bias. None of the SNPs were associated with any common cause of C3 and periodontitis. Our MR analysis found that the inhibition of C3 reduces the odds of periodontitis (odds ratio 0.91 per 1 standard deviation reduction in C3; 95% confidence interval [CI] 0.87–0.96) (Table 1). In a leave-one-out analysis, we showed that the direction of effect was not primarily influenced by a single instrument (Figure S1).

TABLE 1 Mendelian randomization estimate for effects of complement C3 inhibition on periodontitis using genetic variants as instruments.

Outcome	Method	No. SNPs	OR	CI	*p* value
Periodontitis	IVW	3	0.91	0.865; 0.958	.0003

Abbreviations: CI, confidence interval; IVW, inverse-variance weighted analysis based on genetic associations of three uncorrelated SNPs (rs11569479, rs141552034 and rs145406915) with complement C3 and periodontitis risk; OR, odds ratio per one standard deviation decrease in C3.

4 | DISCUSSION

The present study used a *cis*-MR approach to investigate the potential therapeutic effect of C3 blockade on periodontitis. Our results showed that downregulation of C3 lowered the odds of periodontitis. This finding is in line with proof-of-concept studies of C3 inhibition

in pre-clinical models of periodontitis (Kajikawa et al., 2017; Maekawa et al., 2014, 2016) and with a randomized controlled clinical trial (Phase IIa) of C3 inhibition in patients with gingivitis (Hasturk et al., 2021).

The first in-human clinical trial recruited 50 healthy males to investigate the safety and tolerability of a single ascending dose and multiple doses of AMY-101 (Mastellos et al., 2019). Up to 21 days after treatment, none of the participants experienced treatment-related adverse events. Also, the pharmacokinetic and pharmacodynamic profiles of AMY-101 proved its suitability for further testing in clinical trials (Mastellos et al., 2019). In 2019, the US Food and Drug Administration approved AMY-101 as an investigational new drug, for a Phase IIa clinical trial to evaluate its safety and efficacy in patients with gingival inflammation (Hasturk et al., 2021). Forty participants (50% female) were included in a placebo-controlled, double-blinded, split-mouth study design. In the dose selection phase, 12 participants were randomized to three dosing groups (0.025, 0.1 and 0.5 mg per interdental papilla) to identify a safe and effective dose. Consequently, 0.1 mg per interdental papilla was chosen for the main study and given to the efficacy population of 32 patients. All participants ($N = 40$), regardless of the AMY-101 dose, represented the safety population and demonstrated the desired safety profile. The inflammation-related clinical indices, 'modified gingival index' (MGI) and 'bleeding on probing' (BOP), served as primary and secondary outcomes, respectively. Four weeks after treatment initiation, the MGI (measured at six sites on the tooth) showed a greater improvement in the treatment group than in the placebo group (least squares mean difference of −0.181, 95% CI −0.248 to −0.114). Similarly, BOP was greatly reduced in the treated sites (Hasturk et al., 2021). Tissue destruction-related clinical measures, such as pocket depth (PD) or clinical attachment loss (CAL), form the basis of periodontitis diagnosis (Tonetti et al., 2018) and did not change greatly from baseline measurements. This observation can be due to the lack of the minimum number of participants needed to see the clinically relevant changes. In our study, the outcome of interest was periodontitis; thus, it demonstrates the impact of C3 inhibition on a broader range of clinical indices including PD and CAL. In comparison with our results, pre-clinical studies revealed less alveolar bone loss in sites injected with AMY-101 than in placebo-treated sites (Maekawa et al., 2014). These pre-clinical features of C3 blockade, in addition to our findings, warrant further investigation of AMY-101 in clinical trials.

The complement system is an important and powerful actor in the host defence system. However, recent discoveries showed that its excessive stimulation causes tissue damage. Once activated, the complement system follows a cascade of opsonization of the target (e.g., a bacterial cell), self-amplification, generation of effector molecules and immune crosstalk (see Figure 1) (Ricklin et al., 2016). C3, a central node in all relevant pathways, presents itself as a promising therapeutic target (see Figure 1). In 2007, the first C3 inhibitor 'pegcetacoplan' was approved for use in patients with nocturnal haemoglobinuria. This discovery revealed the relevance of this treatment strategy and set the scene for the development of therapeutic C3 antagonists in other diseases like retinal diseases, neurodegenerative diseases, severe coronavirus disease and periodontitis (Lamers et al., 2022). AMY-101 still has to overcome Phase IIb (to set the optimal dose to show biological activity with minimal side effects) and Phase III (to assess the therapeutic effectiveness) before being approved for patient use. Acknowledging that the main issue in drug development is failure due to lack of efficacy in Phases II and III (Holmes et al., 2021), findings from MR studies provide compelling evidence of the causal relationship between protein drug targets and diseases, increasing the probability of candidate drugs to succeed in Phase III clinical trials (Nelson et al., 2015). On several occasions, MR studies predicted the therapeutic effects of candidate drugs prior to their testing in clinical trials (Holmes et al., 2021). In the Phase IIa clinical trial by Hasturk et al., randomization was achieved by the random allocation of drug-application sites, whereas in our MR, the random allocation of the genetic variants acts as a randomization device. A key difference, however, is that whereas the Phase IIa clinical trial examined the local short-term effect of AMY-101, this drug-target MR study examined the long-term inhibition of C3 (Lawlor et al., 2008).

A key strength of our study is that we applied a protein drug-target MR analysis. In an attempt to ensure the validity of our instruments and minimize the risk of violating the 'no horizontal pleiotropy' assumption. We selected IVs based on their proximity to the C3 gene and in relation to C3 levels rather than an association with downstream biomarkers. Thus, we ensured the validity of our IVs and we minimized the risk of violating the 'no horizontal pleiotropy' assumption. Nevertheless, the study has limitations. First, the exposure and outcome association estimates were derived from individuals with European ancestry. Linkage disequilibrium patterns can differ between populations and may not extend to other ethnic groups, therefore limiting the generalizability of our findings to other ethnicities (Burgess et al., 2019). Although plausibly selected, we could not validate our IVs based on mRNA expression due to the unavailability of such data. Additionally, since we selected the SNPs from the vicinity of a single gene region, we were unable to apply pleiotropy-robust MR methods. Future GWAS with even larger sample size, standardized periodontitis definitions and information on the severity of the disease hold the potential to further enrich our understanding and validate the findings presented in our article. Finally, it is important to mention that effect estimates obtained from MR studies are often interpreted as the lifetime effect. However, in a clinical setting, time is a relevant aspect that must be taken into consideration. This represents a challenging aspect of interpreting the implications of lifetime estimates derived from MR in the context of pharmaceutical interventions on the severity of the disease hold the potential to further enrich our understanding and validate the findings presented in our article (Sanderson et al., 2022).

5 | CONCLUSIONS

Drug-target MR facilitates an early assessment of a drug candidate and is gaining interest as a fundamental tool in drug development. Our study suggests a beneficial effect of pharmacologically targeting

C3 in periodontitis and recommends further testing of AMY-101 in a Phase III clinical trial.

AUTHOR CONTRIBUTIONS

Zoheir Alayash, Birte Holtfreter, Hansjörg Baurecht, Michael Nolde and Stefan Lars Reckelkamm were involved in conception and design. Zoheir Alayash, Sebastian-Edgar Baumeister, Hansjörg Baurecht, Michael Nolde and Stefan Lars Reckelkamm were involved in the development of methodology. Zoheir Alayash, Sebastian-Edgar Baumeister, Michael Nolde and Stefan Lars Reckelkamm were involved in acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.). Zoheir Alayash, Sebastian-Edgar Baumeister, Hansjörg Baurecht, Michael Nolde and Stefan Lars Reckelkamm were involved in analysis and interpretation of data (e.g., statistical analysis, biostatistics and computational analysis). Zoheir Alayash, Sebastian-Edgar Baumeister, Birte Holtfreter, Thomas Kocher, Hansjörg Baurecht, Benjamin Ehmke, Michael Nolde and Stefan Lars Reckelkamm were involved in writing, review and/or revision of the manuscript. Zoheir Alayash, Michael Nolde and Stefan Lars Reckelkamm were involved in administrative, technical or material support (i.e., reporting or organizing data and constructing databases).

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The authors did not receive funding for this study.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Summary genetic data for complement C3 protein are deposited at the GWAS Catalog with accession ID: GCST90088016. The periodontitis summary data are available at https://data.bris.ac.uk/data/dataset/2j2rqgzedxlq02oqbb4vmycnc2.

ETHICS STATEMENT

All analyses were based on publicly available summary statistics without accessing individual-level data; hence, ethical approval was not required. The included GWAS received informed consent from the study participants and were approved by pertinent local ethical review boards.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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