Tampere University

OLLI PATRAKKA

Studies on the Bacterial Microbiome in Thrombus Aspirates of Acute Ischemic Stroke Patients

Tampere University Dissertations 966

Tampere University Dissertations 966

OLLI PATRAKKA

Studies on the Bacterial Microbiome in Thrombus Aspirates of Acute Ischemic Stroke Patients

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the auditorium F114
of the Arvo Building, Arvo Ylpön katu 34,
on 8th March 2024, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finnish Cardiovascular Research Center Tampere
Finland

Responsible supervisor and Custos	Professor (emeritus) Pekka J. Karhunen Tampere University Finland	
Pre-examiners	Professor Resia Pretorius Stellenbosch University South Africa	MD, PhD, Professor h.c. Petri Kovanen Wihuri Research Institute Finland
Opponent	Adjunct Professor of Neurology Petra Ijäs University of Helsinki Finland	

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

Cover design: Roihu Inc.

ISBN 978-952-03-3314-0 (print)
ISBN 978-952-03-3315-7 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-3315-7

Carbon dioxide emissions from printing Tampere University dissertations have been compensated.

PunaMusta Oy – Yliopistopaino
Joensuu 2024

"It is also probable that infectious

diseases, especially the chronic ones, if associated

with an increase in cholesterol ester in the blood,

contribute to the fatty degeneration of the intima.

Exact data in this respect are still lacking."

Ruhl, A. Über die Gangarten der Arteriosklerose. *Veröffentlichungen aus der Kriegs- und Konstitutionspathologie*. 1929. Translated by James E. Young, 1944.

ACKNOWLEDGEMENTS

This doctoral dissertation was carried out at the Faculty of Medicine and Health Technology, Tampere University, Tampere. I thank Tampere University for providing me with the facilities where this research was conducted and the thesis written.

My deepest gratitude is dedicated to my supervisor, Professor Pekka J. Karhunen, with whom I have sat down countless times and revised my manuscripts. Pekka gently guided me and taught me modesty. When needed, he held me back and occasionally reminded me that there are more things in the world than just this one research field. I wish to thank all the co-authors and collaborators who have helped me with this project through the years. Thank you, Mika Martiskainen, for introducing me to this project in 2016 and taking me into the study group. Thanks are owed to Mervi Seppänen for teaching me the basics of laboratory work and to Sari Tuomisto for all the information on how to perform DNA extraction and qPCR analysis. I also thank Professors Terho Lehtimäki and Vesa Hytönen, as well as Markus Ojanen and Vili Lampinen, for the valuable talks and for inspiring me to take an even deeper dive into the subject. I also thank Heini Huhtala for helping me with statistical analyses when needed.

I wish to express my gratitude to my follow-up group for providing the needed guidance and for revising the manuscript – thank you, Professor Niku Oksala and Docent Irina Rinta-Kiikka! Irina is also to thank for lending me her personal work phone to recruit the stroke patients for this study and for encouraging me in the dissertation process. I would also like to thank neurologist Jyrki Ollikainen for teaching me acute neurology in such an inspiring way. I also acknowledge the fundings sources: the Ida Montin Foundation, the Finnish Medical Foundation, the Finnish Foundation for Cardiovascular Research, and the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, for making it possible for me to focus on the thesis. Thank you, Eeva Parviainen, for the professional revision of my English.

I want to thank my friends for taking me out and helping me to maintain a social life, as well as for asking out-of-the-box questions about my study. I would like to thank my loving parents, Kristiina and Jukka, as well as the rest of my family for being interested in my project, and for the provided help. Thanks to our German

shepherds Sanni and Pate for taking me out to get some fresh air whenever needed. Finally, I wish to extend special thanks to my partner and best friend Hanna-Sofia for always being interested in this project and for providing me with strength and a shoulder to lean on when needed, even when I did not realize I needed them.

ABSTRACT

Acute ischemic stroke and other cardiovascular diseases are the major causes of death in the world. For ischemic stroke, traditional risk factors are known, but it seems that they do not completely account for the pattern of epidemiology. Increasing evidence indicates that recent acute infections and chronic infectious diseases are important triggers or risk factors for stroke and other cardiovascular diseases. The role of oral bacteria in the pathogenesis of atherosclerosis and ischemic stroke has gained interest. However, the exact mechanisms of action remain uncertain.

This thesis investigates the role of oral bacteria, especially viridans streptococci, in the etiology of acute ischemic stroke. Viridans streptococci belong to the normal oral microbiome but may cause a severe disease if they enter the systemic circulation, such as in the case of infective endocarditis. In the first study, we studied the presence of bacterial DNA in thrombus aspirates of acute ischemic stroke patients treated with mechanical thrombectomy. Approximately 60% of the cases were considered to be due to atherosclerosis, while the rest were attributed to atrial fibrillation or other reasons. We found that the majority of the thrombi contained oral streptococcal DNA. In the second study, we assessed the oral health of these patients and combined the results with their bacterial DNA findings and grade of carotid stenosis. We found that an association between poor oral health and acute ischemic stroke was linked to carotid artery atherosclerosis. In the third study, we confirmed the presence of viridans streptococci in the same thrombus aspirates that were used in the first study, employing bacterial immunohistochemistry. In addition, we performed immunohistochemistry on endarterectomy samples taken from symptomatic patients and on postmortem carotid artery samples taken from asymptomatic individuals. The samples taken from symptomatic patients contained considerably more viridans streptococci than those taken from asymptomatic individuals. Most of the streptococci were detected inside neutrophil granulocytes, but remnants of a bacterial biofilm as well as free bacterial infiltrates were also found in some samples.

The mechanism of how oral pathologies are related to the pathogenesis of ischemic stroke has been unclear. Immune responses that have evolved to combat bacterial infections are shared with those involved in the immune response to the inflammatory components of atherogenesis. Our results suggest that, along with the classical known risk factors, oral streptococcal inflammation may play an important role in the pathogenesis of ischemic stroke and that there may be a bacterial biofilm inside the arterial intima, affecting the development and rupture of the carotid plaque, which results in an ischemic stroke. Also, following transient bacteremia, streptococci entering the systemic circulation might promote thrombosis by activating platelets, eventually leading to the formation of a thromboembolism and causing an ischemic stroke, particularly in patients with atrial fibrillation. The possibilities of primary prevention of acute ischemic stroke by means of antibacterial vaccines and timed antimicrobial treatment, as well as regular dental care, should be considered.

Keywords: viridans streptococci, atherosclerosis, acute ischemic stroke, inflammation, infection

TIIVISTELMÄ

Aivoinfarkti sekä muut sydän- ja verisuonitaudit ovat maailman johtavia kuolinsyitä. Aivoinfarktin perinteiset riskitekijät ovat tiedossa, mutta näyttää siltä, etteivät ne täysin selitä aivoinfarktin epidemiologista kuvaa. Lisääntyvä tutkimusnäyttö viittaa siihen, että äskettäiset infektiot ja krooniset tartuntataudit ovat tärkeitä laukaisijoita tai riskitekijöitä aivoinfarktille sekä muille sydän- ja verisuonitaudeille. Suun bakteerien rooli valtimonkovettumataudin ja aivoinfarktin synnyssä on kasvavan mielenkiinnon kohteena, joskin tarkka mekanismi on jäänyt epäselväksi.

Tässä väitöstutkimuksessa tarkasteltiin suun bakteerien, erityisesti viridans-ryhmän streptokokkien, roolia aivoinfarktin synnyssä. Viridans-ryhmän streptokokit kuuluvat normaaliin suun mikrobiomiin, mutta verenkiertoon päästessään ne voivat aiheuttaa vakavia sairauksia, kuten sydänläppien tulehduksen. Ensimmäisessä osajulkaisussa selvitimme bakteeri-DNA:n pitoisuuksia aivoveritulpissa, jotka oli kerätty mekaanisella trombektomialla hoidetuilta aivoinfarktipotilailta. Näistä aivoinfarkteista noin 60 prosenttia oli arvioitu johtuvan valtimonkovettumataudista, kun taas loput tapauksista liittyivät eteisvärinään tai muihin syihin. Havaitsimme, että suurin osa aivoveritulpista sisälsi suussa esiintyvien streptokokkien DNA:ta. Toisessa osajulkaisussa arvioimme samojen potilaiden suun terveyttä ja yhdistimme tulokset bakteeri-DNA-löydöksiin sekä kaulavaltimon ahtauman asteeseen. Huomasimme, että heikon suun terveyden ja akuutin aivoverenkiertohäiriön välinen yhteys liittyi kaulavaltimon valtimonkovettumatautiin. Kolmannessa osajulkaisussa varmistimme bakteeri-immunohistokemian avulla viridans-streptokokkien esiintymisen samoissa veritulpissa, joita tutkimme ensimmäisessä tutkimuksessa. Lisäksi teimme immunohistokemialliset tutkimukset kaulavaltimoiden endarterektomianäytteille, jotka oli kerätty oireisilta kaulavaltimoahtaumapotilailta, sekä *post mortem* -kaulavaltimonäytteille, jotka oli kerätty oireettomilta tutkittavilta. Näytteistä, jotka oli otettu oireilevilta henkilöiltä, löytyi huomattavasti enemmän viridans-streptokokkeja verrattuna oireettomien tutkittavien näytteisiin. Useimmat streptokokit havaittiin neutrofiilisten granulosyyttien sisällä, mutta joissain näytteissä oli myös jäännöksiä bakteeribiofilmeistä sekä vapaista bakteeri-infiltraateista.

Mekanismi, jonka kautta suun sairaudet lisäävät aivoverenkiertohäiriön riskiä, on ollut epäselvä. Bakteeri-infektioiden torjuntaan kehittyneet immuunivasteet ovat

samoja, jotka torjuvat tulehduskomponentteja valtimonkovettumataudin synnyssä. Tuloksemme viittaavat siihen, että muiden tunnettujen riskitekijöiden lisäksi viridans-ryhmän streptokokkibakteerien aiheuttama tulehdus saattaa olla tärkeässä roolissa akuutin aivoverenkiertohäiriön synnyssä ja että valtimon seinämässä voi olla bakteeribiofilmiä, joka voi vaikuttaa kaulavaltimon plakin kehittymiseen ja repeämiseen, mikä puolestaan johtaa aivoinfarktiin. Huonosti hoidetuista hampaista ajoittaisesti verenkiertoon päätyvä streptokokkikylvö saattaa lisätä tukostaipumusta aktivoimalla veren hyytymiskaskadia, johtaen veritulpan muodostumiseen ja aivoinfarktin syntyyn, erityisesti eteisvärinää sairastavilla potilailla. Suun streptokokkibakteerien aiheuttama tulehdus tulee ottaa huomioon aivoinfarktin ennaltaehkäisyssä säännöllisenä hammashuoltona. Sen lisäksi esimerkiksi oikea-aikaisesta antimikrobisesta hoidosta tai bakteerirokotteesta voi olla hyötyä, ja niiden vaikutusta on tärkeää tutkia tulevaisuudessa.

Asiasanat: viridans streptokokki, ateroskleroosi, aivoinfarkti, tulehdus, infektio

CONTENTS

ABBREVIATIONS

AF	atrial fibrillation
AI2	autoinducer 2
APC	antigen-presenting cells
apoB	apolipoprotein B
ATCC	American Type Culture Collection
BMG	Brain, Microbes and Genetics
CAC	carotid artery calcification
CD	cluster of differentiation
CI	confidence interval
COX-1	cyclooxygenase 1
COX-2	cyclooxygenase 2
CRP	C-reactive protein
CT	computed tomography
CVD	cardiovascular disease
DC	dendritic cell
DNA	deoxyribonucleic acid
GWAS	genome-wide association study
HDL	high-density lipoprotein
IBD	inflammatory bowel disease
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule 1
ICH	intracerebral hemorrhage
IFN-γ	interferon-γ
IgG	immunoglobulin G
IL	interleukin
LDL	low-density lipoprotein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
oxLDL	oxidized LDL

PAMP	pathogen-associated molecular pattern
Pcho	phosphocholine
PCR	polymerase chain reaction
Q	quartile
RBC	red blood cell
RCT	randomized controlled trial
SAH	subarachnoid hemorrhage
SCFA	short-chain fatty acid
SGLT 2	sodium-glucose cotransporter 2
SMC	smooth muscle cell
SR-A	scavenger receptor A
TH1	T helper type 1
TH2	T helper type 2
TLR	Toll-like receptor
TMAO	metabolite trimethylamine *N*-oxide
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
TSDS	Tampere Sudden Death Study
TVS	Tampere Vascular Study
VCAM-1	vascular cell adhesion molecule 1

LIST OF ORIGINAL PUBLICATIONS

I Patrakka O, Pienimäki JP, Tuomisto S, Ollikainen J, Lehtimäki T, Karhunen PJ, Martiskainen M. Oral Bacterial Signatures in Cerebral Thrombi of Patients with Acute Ischemic Stroke Treated with Thrombectomy. *Journal of the American Heart Association*. 2019;8(11):e012330.

II Patrakka O, Mehtonen H, Tuomisto S, Pienimäki JP, Ollikainen J, Huhtala H, Pessi T, Oksala N, Lehtimäki T, Järnstedt J, Martiskainen M, Karhunen PJ. Association between Oral Pathology, Carotid Stenosis, and Oral Bacterial DNA in Cerebral Thrombi of Patients with Stroke. *Stroke Research and Treatment*. 2021;2021.

III Patrakka O, Tuomisto S, Pienimäki JP, Ollikainen J, Oksala N, Lampinen V, Ojanen MJT, Huhtala H, Hytönen VP, Lehtimäki T, Martiskainen M, Karhunen PJ. Thrombus Aspirates from Acute Ischemic Stroke Patients are Infiltrated by Viridans Streptococci. *Journal of the American Heart Association*. 2023;12:e030639.

AUTHOR'S CONTRIBUTION

In the first publication, the author of this thesis contributed to the laboratory works and performed the majority of the bacterial DNA and statistical analyses. He wrote the initial manuscript. He was the corresponding author of the manuscript.

In the second publication, the author of this thesis contributed to the design of the study and recruited the study participants. He performed the statistical analyses, interpreted the results, and wrote the first draft of the manuscript. He was the corresponding author of the manuscript.

In the third publication, the author of this thesis contributed to the design of the study. He performed the statistical analyses and wrote the first draft of the manuscript. He was the corresponding author of the manuscript.

1 INTRODUCTION

Acute ischemic stroke is a medical emergency caused by decreased blood flow to the brain, which results in damage to brain cells. The signs and symptoms of stroke may include sudden-onset numbness or weakness in an arm or leg, facial droop, difficulty speaking or understanding speech, confusion, trouble with balance or coordination, and loss of vision. This is caused by an occlusion in the cerebral blood supply or a rupture of the cerebral arteries which causes intracranial bleeding. (Kuriakose & Xiao, 2020.) Cardiovascular diseases are the major cause of morbidity and mortality in Western countries, and stroke is the leading cause of adult long-term disability (Feigin et al., 2017; Katan & Luft, 2018). Carotid artery atherosclerosis stands as a significant factor contributing to large vessel stroke, and at least 20% of acute ischemic strokes may be due to embolism from ruptured atherosclerotic carotid arteries (Bos et al., 2014; Flaherty et al., 2012; Inzitari et al., 2000). Atherosclerosis was formerly considered to be a bland lipid storage disease of the arterial intima, but the evidence now supports the conclusion that inflammation plays a central role in all phases of the atherosclerotic process. (Libby, Ridker, & Maseri, 2002.)

Classic risk factors for acute ischemic stroke include age, male sex, hypertension, atrial fibrillation, diabetes, hypercholesterolemia, and smoking, but these do not completely account for the pattern of stroke epidemiology (Grau, Urbanek & Palm, 2010). Bacterial inflammation has been suggested to contribute to the development of atherosclerosis and atherothrombotic events, but the mechanism has remained unclear (Rosenfeld, 2013). Inflammation of an atherosclerotic plaque may affect its growth and contribute to plaque rupture, eventually leading to thrombosis (Lanter, Sauer & Davies, 2014). Moreover, previous or chronic infections are suggested to take part in the initiation and development of atherosclerosis (Elkind et al., 2010). The role of dental infections, in particular, as a risk factor for stroke has been investigated. Periodontal disease and tooth loss seem to be independently associated with an increased risk of ischemic stroke (Joshipura et al., 2003). A severe chronic dental infection has been found to predispose male patients to stroke, possibly by affecting blood coagulation and platelet function (Syrjänen & Peltola, 1989). Regular dental care may independently lower the risk of ischemic stroke (Sen et al., 2018).

The circulation is a closed system and the blood in healthy organisms was first believed to be a sterile environment. However, the principle of the presence of truly sterile blood in healthy humans has been challenged. In 2001 it was found that even 'healthy' blood specimens can contain bacterial 16S ribosomal DNA and nowadays it is known that a great many pathogens can survive in blood and inside erythrocytes. (Potgieter et al., 2015.) This is attributed to recent developments in molecular microbiological methods, which have also attracted new interest in the involvement of pathogens in cardiovascular diseases (Gerace et al., 2022; Guarner et al., 2006). One of the main interests is viridans group streptococci, which are found in a healthy oral cavity and which constitute the pioneers in the initiation of dental plaque formation (Kolenbrander et al., 2010). Viridans group streptococci are also known to cause infective endocarditis and possess thrombogenic properties (Kerrigan & Cox, 2009; Cahill & Prendergast, 2016).

Lehtiniemi and colleagues studied in 2005 oral bacterial DNA findings in autopsy samples of coronary arteries taken from sudden death victims. They used broad-range polymerase chain reaction (PCR) and were able to detect a wide palette of oral bacteria from coronary tissues. Bacterial DNA sequences of oral pathogens were detected in all cases regardless of the cause of death. (Lehtiniemi et al., 2005.) In addition, DNA of viridans group streptococcal bacteria have been detected in thrombus aspirates taken from acute myocardial infarction patients, as well as in patients with lower limb arterial thrombosis due to peripheral atherosclerosis (Pessi et al., 2013; Vakhitov et al., 2018). To our knowledge, there are no earlier studies depicting the role of oral bacteria in the thromboembolic events of cerebral arteries among patients who have suffered an acute ischemic stroke.

This dissertation investigates the role of bacteria, especially of oral origin, in the pathogenesis of acute ischemic stroke. We hypothesized that oral bacteria can be found in the cerebral arterial thrombi. Thus, in Study I, we explored whether oral bacterial DNA could be amplified using quantitative PCR from thrombus aspirates taken from acute ischemic stroke patients. In Study II, we investigated whether the bacterial DNA findings would correlate with the condition of the patients' teeth and carotid artery atherosclerosis, using computed tomography scans and ultrasonography. In Study III, we examined whether the presence of viridans streptococci group bacteria could be confirmed in the thrombus aspirates of stroke patients and in carotid artery specimens using bacterial immunohistochemistry. The studies were performed on patients who had suffered an acute ischemic stroke and patients with symptomatic carotid artery stenosis. Postmortem carotid artery samples comprising out-of-hospital deaths were also analyzed.

2 REVIEW OF THE LITERATURE

2.1 Epidemiology of stroke

Cardiovascular diseases are major causes of morbidity and mortality in Western countries. Cardiovascular diseases, including stroke, caused an estimated 17.9 million deaths in 2019, representing 32% of all deaths globally. Of these deaths, 85% were due to heart attack and stroke. (Roth et al., 2020; World Health Organization, 2021.) Stroke is the leading cause of adult long-term disability, with 26% new sufferers remaining disabled in basic activities of daily living (Katan & Luft, 2018). In the Unites States, approximately 795,000 people experience a stroke each year, roughly 692,000 (87.0%) of which are acute ischemic strokes (Benjamin et al., 2018). On average, every 40 seconds, someone in the United States has a stroke. The prevalence of stroke-related symptoms is found to be relatively high in a general population with no a prior diagnosis of stroke, suggesting that stroke may be underdiagnosed (Tsao et al., 2022). In Finland, the annual incidence of stroke is 61.7 per 100,000 inhabitants. It is estimated that the incidence will increase by 44% by the year 2035, mainly because of the continuously increasing lifespan. (Wafa et al., 2020.)

2.2 Subtypes of stroke

Strokes can be divided into hemorrhagic (intracerebral hemorrhage and subarachnoid hemorrhage) and ischemic strokes.

2.2.1 Hemorrhagic stroke

Hemorrhagic stroke occurs when a blood vessel ruptures and blood accumulates in the tissue around the rupture. Overall, roughly a fifth of all strokes are hemorrhagic, with intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) both accounting for 10% of strokes. For ICH, the median fatality rate at one month is 40%, which is significantly higher than for ischemic strokes. This is because of the

early problems with elevated intracranial pressure, herniation, and significant mass effect. (Montaño, Hanley, & Hemphill, 2021.) The strongest risk factors for ICH are uncontrolled hypertension and age-related amyloid angiopathy (Rymer, 2011). A subarachnoid hemorrhage is defined as the accumulation of blood in the space between the arachnoid membrane and the pia mater around the brain, which is referred to as the subarachnoid space. The majority of SAHs are nontraumatic, with 85% being secondary to the rupture of an intracranial aneurysm, while the remaining 15% are traumatic in nature. The typical locations for intracranial aneurysms are arterial bifurcations or abrupt vascular angles. Large unruptured aneurysms compress the adjacent cerebral tissue, causing neurological symptoms, and can thus be recognized and surgically treated before the rupture. (Ziu, Suheb, & Mesfin, 2023.)

2.2.2 Ischemic stroke

The most common type of stroke is an ischemic stroke. According to the generally used TOAST classification, there are five subtypes of ischemic stroke based on etiology: 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, 4) a stroke of other determined etiology, and 5) a stroke of undetermined etiology (Figure 1). (Adams et al., 1993; Fure, Wyller & Thommessen, 2005.)

For large-artery atherosclerosis, evidence of intracranial or extracranial stenosis of greater than 50% is needed for an appropriate TOAST classification. These patients will usually have significant findings in a computed tomography (CT) or magnetic resonance imaging (MRI) scan, in addition to having clinical symptoms. Potential cardiogenic sources for embolisms should be excluded. It is estimated that large-artery atherosclerosis is the cause in 50% of all cerebral infarctions. For cardioembolism, at least one cardiac source for an embolism must be identified, and it is estimated as the cause in 20% of ischemic strokes. Patients with a small-vessel occlusion (25% of ischemic strokes), also known as a lacunar infarction, should have a normal CT/MRI examination with no source of cardiogenic embolism or have a large-artery atherosclerosis. These patients often have a history of diabetes mellitus or hypertension. A stroke of other determined etiology is a category which consists of rare causes, such as nonatherosclerotic vasculopathies, a hypercoagulable state, or hematologic disorders. These patients should have clinical and radiographical findings of an acute ischemic stroke. Cardioembolic and large-artery atherosclerosis should be excluded. A stroke is classified as a stroke of undetermined etiology, when

the cause of stroke cannot be determined with any degree of confidence. (Adams et al., 1993.)

In this thesis, we will focus on large-artery ischemic stroke and cardioembolism etiologies, as they are the major causes of acute ischemic stroke (Adams et al., 1993). Carotid stenosis and thrombosis are important causes of these stroke subtypes.

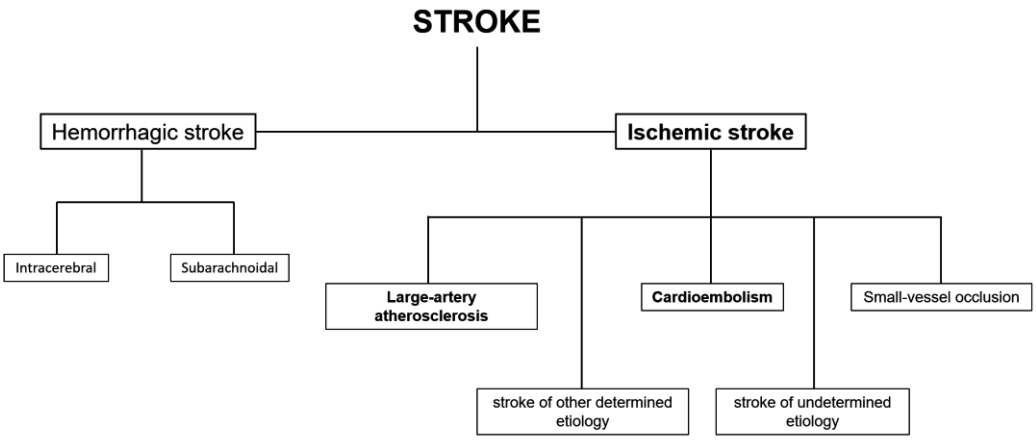


Figure 1. Classification of strokes according to the generally used TOAST criteria.

2.2.2.1 Carotid stenosis

Maintaining cerebral blood supply is crucial for all vertebrates and invertebrates (Dunton et al., 2021). Nearly 400 years ago, Thomas Willis described an arterial ring at the base of the brain. This anastomotic ring connects internal carotid arteries (ICAs) and vertebrobasilar circulation by communicating arteries and is now known as the circle of Willis. A similar structure can be found in reptiles, birds, and mammals, such as in monkeys, dogs, and rabbits. The evolutionary function of the circle of Willis is still debated. It protects cerebral arteries and the blood-brain barrier from hemodynamic stress by dissipating the pressure caused by an asymmetric arterial system. On the other hand, the circle of Willis maintains cerebral blood flow in the case of an acute ICA or basilar artery occlusion. (Kapoor, Kak, & Singh, 2003; Vrselja et al., 2014.)

Carotid artery atherosclerosis has been found to be an important cause of large vessel disease and ischemic stroke (Bos et al., 2014; Flaherty et al., 2012). The carotid artery bifurcation is a very common site for atherosclerotic plaque development.

This is because of the geometry of the bifurcation, which causes hemodynamic turbulence, thus accelerating atherosclerotic process. An acute ischemic stroke may be partially due to embolism from carotid arteries (Inzitari et al., 2000). The carotid artery seems to be the preferred site of onset of the atherosclerotic process in hypertensive patients. Patients with an acute ischemic stroke often have a history of untreated hypertension. (Fujihara et al., 2013.)

Vascular calcification is a well-known sign of atherosclerosis and can be found in 80%–90% of atheromas. Carotid artery calcification (CAC) is often found in asymptomatic patients and was reported to be present in 75% of males and 62% of females aged over 75 years in a review published in 2020. (Song et al., 2020.) The presence of inflammation in atherosclerosis leads to intimal microcalcifications and results in CAC. Small calcium deposits are the major source of plaque instability and are found in unstable and ruptured plaques. Intimal CAC can lead to carotid stenosis and subsequently cause ischemic symptoms if the artery is highly stenotic or occluded. On the other hand, a higher degree of carotid artery plaque calcification is associated with plaque stability. It is believed that microcalcifications cause plaque instability, whereas macrocalcification causes plaque stabilization. This suggests that there is a window of time during the calcification process when the risk of severe morbidity and mortality is temporarily increased. (Ahmed et al., 2021.) Intracranial CAC has been suggested to be a greater contributor to the stroke etiology than large-artery atherosclerosis in extracranial CAC in more proximally located vessel beds, independently of conventional cardiovascular risk factors, in an ethnically white population (Bos et al., 2014).

Endarterectomy, a surgical procedure to remove plaque from narrowed or blocked arteries, when performed for a carotid artery stenosis is not always beneficial. The European Stroke Organisation suggests endarterectomy for patients with 50%–99% symptomatic stenosis. For asymptomatic patients, carotid endarterectomy is recommended for patients with ≥ 60%–99% carotid stenosis, if the patients are considered to be at an increased risk of stroke on best medical treatment alone. (Bonati et al., 2021.)

2.2.2.2 Thrombosis

Thrombosis occurs when a blood clot forms inside a blood vessel, obstructing the blood flow in veins or arteries. In this thesis, we refer to arterial thrombosis, as it can cause an acute ischemic stroke.

Thrombosis is the pathologic counterpart of hemostasis. Rudolf Virchow stated in 1856 that thrombosis is caused by vessel damage, stasis, or hypercoagulability. This later came to be known as Virchow's triad and is still being used to describe the etiology and to assess the risk of thrombosis (Figure 2) (D. R. Kumar et al., 2010). Endothelial injury can lead to platelet activation over ulcerated plaques in atherosclerotic arteries. A damaged endothelium releases high levels of prothrombotic von Willebrand factor, which cross-links platelets via glycoprotein-1b and mediates their adhesion to the subendothelium, increasing the risk for thrombosis. (Blann, 2003.) Red blood cells (RBCs) have been historically regarded as passive bystanders in thrombosis. However, recent studies have suggested an active role of RBCs in arterial thrombosis. RBCs promote platelet margination, increase platelet–thrombus interactions, and increase platelet adhesion and activation. RBCs increase blood viscosity, but this effect is decreased by their high shear-induced shape change. (Byrnes & Wolberg, 2017.) Thrombosis is accelerated by many other factors, such as hypertension, bacterial endotoxins, and toxins absorbed from cigarette smoke (Martinelli, Bucciarelli & Mannucci, 2010).

Stasis is the disturbance or stagnation of normal blood flow. This may happen due to, for example, heart failure, which causes venous stasis. Malignance may increase the risk of thrombosis by activating the coagulation system, and a tumor can also externally compress the blood vessels. (Park et al., 2015.) In atherosclerosis, ulcerated plaques alter hemodynamic conditions and create turbulence, which leads to stasis. Stasis is a major contributor in the development of a cardioembolic stroke. (Cushman, 2007.) Hypercoagulability is a less frequent contributor to thrombosis. It is defined as any alteration of the coagulation pathways. The most common hypercoagulability state is a single-nucleotide mutation in factor V mutation (Leiden mutation), which is carried by 2%–15% of ethnically white people. Heterozygotes have a fivefold increased risk of thrombosis, while homozygotes have a 50-fold increase in risk. (Rosendorff & Dorfman, 2007.)

If the hemodynamic conditions are altered, activated platelets can take part in the formation of a cardioembolic stroke (Herzberg et al., 2005; Tomaiuolo, Brass & Stalker, 2017). Cardioembolic stroke accounts for approximately 20% of acute ischemic strokes, and the most important cause is a thrombosis caused by atrial fibrillation (Arboix & Alió, 2010). Other cardiac risk factors are mitral stenosis, annular calcification, and left ventricular hypertrophy (Sacco et al., 1997).

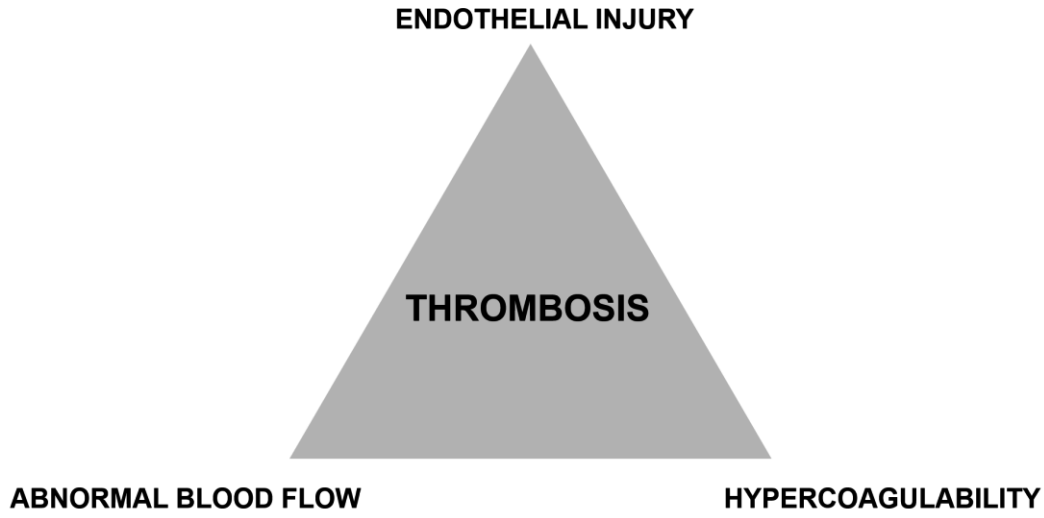


Figure 2. Virchow's triad describes the etiology and assesses the risk of thrombosis.

2.3 Risk factors for acute ischemic stroke

2.3.1 Traditional risk factors

Traditional etiological risk factors for acute ischemic stroke are age, male sex, hypertension, hypercholesterolemia, diabetes, atrial fibrillation, smoking, and obesity (Sacco et al., 1997; Allen & Bayraktutan, 2008).

According to a study published in 1999, hypertension is present in 84% of patients who suffer an acute stroke. There are many ways in which elevated blood pressure is involved in the development of a stroke. A high intraluminal pressure will lead to an extensive alteration in the endothelium and smooth muscle function of the intracerebral arteries. Endothelial damage can lead to local thrombus formation and ischemic lesions. Hypertension accelerates the arteriosclerotic process, thus increasing the likelihood of cerebral lesions related to stenosis and embolism originating from large extracranial vessels. (Johansson, 1999.) Blood pressure level is a simple physiologic parameter that is easily measured and modified (McManus & Liebeskind, 2016). Cohort studies have demonstrated that the strong association

between blood pressure and stroke appears to be continuous down to levels of at least 115/75 mmHg (Lawes et al., 2004).

Hypercholesterolemia is the fundamental risk factor for atherosclerosis and coronary heart disease, but for ischemic stroke the evidence is mostly indirect and not all meta-analyses have found clear association (Amarenco & Steg, 2007). The relationship between cholesterol levels and ischemic stroke can be bidirectional. While hypercholesterolemia is considered a risk factor for ischemic stroke, stroke events can also impact cholesterol levels due to changes in metabolism and lifestyle after a stroke. Untangling cause and effect can be challenging in observational studies. The longitudinal cohort studies were predestined to evaluate the role of cholesterol in coronary atherosclerosis, but not to investigate its role in stroke. (Piechowski-Jóźwiak & Bogousslavsky, 2004.) Low serum cholesterol levels increase the risk of fatal intracranial hemorrhage while higher levels increase the risk of death from ischemic stroke (Iso et al., 2010). Nonetheless, there is a consensus that lipid-lowering therapy significantly reduces the risk of ischemic stroke, and, therefore, lipid-lowering drugs, such as statins, are widely used as primary and secondary stroke prevention (Corvol et al., 2003; Kurth et al., 2007). For example, treatment with the cholesterol-lowering drug simvastatin reduced the ischemic stroke incidence by 30% compared to placebo treatment (Collins et al., 2002).

Diabetes is an independent risk factor for acute ischemic stroke (Allen & Bayraktutan, 2008). In the Honolulu Heart Program study, it was found that patients with diabetes mellitus had a twofold risk of thromboembolic stroke compared to patients without diabetes (Burchfiel et al., 1994). Diabetes causes various microvascular complications which can lead to ischemic stroke through vascular endothelial dysfunction, increased early-age arterial stiffness, systemic inflammation, and thickening of the capillary basal membrane (Chen, Ovbiagele & Feng, 2016). Intensive blood glucose control by either sulphonylureas or insulin decreases the risk of microvascular complications, but there is no evidence that it protects from macrovascular diseases, such as stroke, in patients with type 2 diabetes (Turner, 1998). A recent meta-analysis concluded that sodium-glucose cotransporter 2 (SGLT 2) inhibitors have a neutral effect on overall cerebrovascular events but may yield potential benefit in the prevention of hemorrhagic strokes in individuals with type 2 diabetes (Tsai et al., 2021). In 2023, it was stated that SGLT 2 inhibitors are associated with a lower stroke risk in patients with type 2 diabetes and atrial fibrillation (Chang et al., 2023). Yet, few studies to date address the role of SGLT 2 inhibitors in stroke prevention, and their role may thus be strengthened in the future.

Atrial fibrillation (AF) is the most important and treatable cardiac disease increasing the risk of stroke. In atrial fibrillation, blood can be stagnated in the left atrium, leading to thrombus formation and, consequently, to cerebral embolization. The lifetime risk of developing AF is approximately one in six, and among elderly individuals, the incidence of AF is 10%. (Choi et al., 2023.) Atrial fibrillation is independently associated with a fourfold increased risk of stroke (Wolf, Abbott & Kannel, 1991). It is estimated that AF is present in 35% of patients diagnosed with non-lacunar stroke and in 15%–24% of ischemic strokes. An AF-related ischemic stroke is almost twice as fatal as a non-AF stroke. (Choi et al., 2023.) Oral anticoagulant therapy can prevent up to 70% of strokes due to atrial fibrillation, but the medication has potential side effects (Kamel & Healey, 2017). Thus, the necessity of oral anticoagulant therapy is estimated using risk calculators, as the risk of AF-related stroke increases when other risk factors are present at the same time. If a patient with AF is at risk of hemorrhagic complications or drug interactions, left atrial appendage closure should be considered. The left atrial appendage is the major source of emboli in nonvalvular atrial fibrillation, so the closure of the appendage reduces stroke risk and anticoagulant therapy is no longer needed. (Contractor & Khasnis, 2011.) Improved diagnostic methods, such as a transesophageal echocardiography and prolonged hearth-rhythm monitoring that can recognize subclinical atrial fibrillation, have allowed the identification of different cardioembolic stroke etiologies that were previously determined as cryptogenic strokes (Di Tullio & Homma, 2002).

Cigarette smoking approximately doubles the risk of ischemic stroke according to large multivariable follow-up studies (Meschia et al., 2014). Also, some studies have provided evidence of a dose-response relationship, with the risk being ninefold in people smoking 40 cigarettes a day. Smoking contributes to an increased stroke risk through both short-term effects on the risk of thrombus formation and long-term effects related to an accelerated atherosclerotic process. Smoking causes endothelial dysfunction, increases fibrinogen concentration, reduces fibrinolytic activity, causes polycythemia, and increases platelet aggregability. Quitting smoking is linked to a significant decrease in the risk of a stroke and other cardiovascular events during the first 2–5 years, although former smokers never reach the same level as those who have never smoked. (Bhat et al., 2008; Meschia et al., 2014.)

Obesity is associated with all risk factors listed earlier, and the increased risk of stroke associated with obesity is partly mediated through other cardiovascular risk factors. Abdominal obesity is an independent risk factor for an ischemic stroke and more important than obesity involving the hips and thighs. Waist circumference and

the waist-to-hip ratio are better indicators of visceral fat accumulation and an adverse metabolic profile than is an elevated body mass index alone. Abdominal obesity is related to endothelial dysfunction and the initiation of atherosclerosis. It is also associated with blood flow disturbances and platelet activation through enhanced lipid peroxidation and inflammation. (Suk et al., 2003.)

2.3.2 Genetic risk factors

Genome-wide association study (GWAS) technology has had a major impact on our understanding of the genetics of stroke (Markus, 2011). It is suggested that there is a genetic component of stroke, with overall heritability estimates of 38% for all ischemic strokes. African Americans have a twofold greater risk of stroke and a higher frequency of post-stroke disability compared to European Americans (K. L. Keene et al., 2020). However, the inescapable conclusion is that not a single locus has a major effect on the risk of ischemic stroke overall (Matarín et al., 2007; Traylor et al., 2012). All of associations discovered seem to be specific to a stroke subtype. For example, genes *PITX2* and *ZFHX3* are risk factors for the cardioembolic stroke subtype (attributable risks 5.8 % and 7.0 %, respectively) because they increase the risk of atrial fibrillation. The associations of *HDAC9* and 9p21 are specific to large-vessel stroke (attributable risks 4.5 % and 7.2 %, respectively) and not present with other stroke subtypes (Traylor et al., 2012). Ischemic large-vessel stroke shares the same genetic risk factors with coronary artery disease. This is probably because the shared risk variants act through the atherosclerotic process (Deloukas et al., 2013).

2.3.3 Inflammatory risk factors

2.3.3.1 Systemic inflammation as a risk factor

Patients with autoimmune diseases, such as rheumatoid arthritis, have a greater risk of atherosclerosis. Also, their atherosclerotic plaques are more rupture-prone. One reason for this is that these two diseases have similarities in the cellular processes, and pathogeneses of the diseases share the same proinflammatory cells and cytokine activation. (Skeoch & Bruce, 2015.) There have been similar findings with other autoimmune diseases, such as systemic erythematous lupus, asthma, and inflammatory bowel disease (IBD). For example, the atherosclerotic plaque burden

was relevantly increased in asthma groups vs controls (severe asthma 43.1%, mild-to-moderate asthma 25.0%, control 14.3% of study participants). Patients with systemic lupus erythematosus have a 50-fold increased risk of cardiovascular diseases compared to the general population, which is due to dysfunctional immune and inflammatory mechanisms. Patients with autoimmune diseases have higher blood CRP and fibrinogen levels. A positive relationship has been shown between increased oxidative stress and carotid intima-media thickness in asthmatic patients. Patients with IBD have more endothelial dysfunction caused through a nitric oxide–mediated dilation of the vessels. Elevated cytokine levels, such as IL-1, IL-6, and tumor necrosis factor, are involved in both IBD and atherosclerosis. (Frostegård, 2005; Tuleta et al., 2017; Weissman et al., 2020.)

2.3.3.2 Infections as risk factors

Atherosclerosis has been considered to be associated with the Western lifestyle and shares the same risk factors as acute ischemic stroke (Lechner et al., 2020). A common assumption is that atherosclerosis is a predominately lifestyle-related disease. However, these traditional risk factors do not completely account for the pattern of stroke epidemiology. Increasing evidence indicates that recent acute infections and chronic infectious diseases are important triggers or risk factors for stroke (Grau, Urbanek & Palm, 2010). The possibility of infections contributing to atherosclerosis was suggested as early as in the early 1900s by several authors (Osler W, 1908; Klotz & Manning, 1912). Stroke is a common complication in severe bacterial infections, such as endocarditis and meningitis, but it also occurs in other, more common and milder infections (Syrjänen, 1993; Grau, Urbanek & Palm, 2010). It has been suggested that chronic or persistent latent infections, such as periodontitis, chronic bronchitis, or infections caused by *Chlamydia pneumoniae* and *Helicobacter pylori*, are related to the risk of stroke. Numerous studies with diverse research approaches have provided substantial evidence indicating that an acute infection caused by bacterial or viral agents is a significant and separate trigger for stroke. The temporal relationship between the infection and vascular events, observed in a gradual manner, along with experimental findings from animal models, strongly support a causal connection between infection and stroke. (Grau, Urbanek & Palm, 2010.) Increasing knowledge suggests that atherosclerotic process may be accelerated by bacterial infection (Morré et al., 2000; Valtonen, 1999).

The Horus study published in 2013 studied the presence of calcified plaques as markers of atherosclerosis by performing CT scans to more than 4,000-year-old

mummies. The study found probable or definite atherosclerosis of the aorta, iliac or femoral arteries, carotid arteries, and coronary arteries in 34% out of 137 mummies from four different geographical populations. The study stated that atherosclerosis was common in preindustrial populations, including preagricultural hunter-gatherers. The main cause of mortality throughout human evolution until the 20th century has been infections. Yet, among 20th century hunter-forager-horticulturalists, infections were a common aspect of daily life, and approximately 75% of mortality was attributed to infections. The high level of chronic infection and inflammation in premodern conditions might have promoted the inflammatory aspects of atherosclerosis. (Thompson et al., 2013.)

2.4 Atherosclerosis as an inflammatory disease

2.4.1 Pathogenesis of atherosclerosis

The present concept is that atherosclerosis is a complex chronic inflammatory disorder in the arterial intima, driven by oxidized or otherwise modified low-density lipoprotein (LDL) particles. Inflammation has a central role in all phases of the atherosclerotic process (Paoletti, Gotto & Hajjar, 2004; Andreou & Andreadou, 2009; Tousoulis et al., 2016). Atherosclerosis is a systemic vascular disorder involving multiple vascular beds.

LDL oxidation is the most extensively studied mechanism related to the establishment of atherosclerotic cardiovascular disease. Atherosclerotic plaque formation begins when cholesterol-rich, apoB-containing lipoproteins are accumulated within the arterial intima in a dose-dependent manner. Physiological levels of serum LDL are considered to be 0.5–1.0 mmol/L, which are typical for newborns and a wide range of mammalian species. When LDL cholesterol increases above that level, the probability of the intimal retention of LDL and the progressive development of an atherosclerotic plaque is increased. (Ference et al., 2017.) This process often begins in childhood. Berenson and colleagues performed autopsies on 204 young persons who had died at the age of 2–39 years of various causes, principally trauma, and found that half of the cadavers had asymptomatic fatty streaks in their coronary arteries before the age of 16 (Berenson et al., 1998). People with genetic familial hypercholesterolemia have elevated levels of LDL cholesterol from birth and an increased risk of premature coronary heart disease. Interestingly,

cohort studies have reported that the risk of ischemic stroke is not higher for people with familial hypercholesterolemia compared to the general population. (Beheshti et al., 2018; Hovland et al., 2019)

The elevated LDL cholesterol level is a commonly used predictor of atherosclerosis and its clinical complications. If it is remarkably high, the development of early coronary artery disease does not require other risk factors. Sudden death may occur in a young person with only a single atherosclerotic lesion complicated by a coronary thrombus, without extensive vessel disease. Consequently, the extent of vascular lesions may not be directly related to the occurrence of clinical events, even in the presence of other risk factors. (Berenson et al., 1998.) An increased LDL cholesterol level is a risk factor for vascular mortality through the lifetime up to the age of 90 (MacMahon et al., 2007). High-density lipoprotein (HDL) has a reverse relationship with cardiovascular diseases, but there is no evidence that the agents targeted at HDL have a beneficial effect on mortality (D. Keene et al., 2014). The measurement of apolipoprotein B (apoB) is more useful clinically than measuring LDL cholesterol levels in the assessment of cardiovascular disease and stroke risk because apoB levels provide more information about atherogenic particles and are not influenced by the heterogeneity of particle cholesterol content. Bare LDL cholesterol measurement is insensitive to the accumulation of small LDL particles that are the most atherogenic. The heterogeneity of LDL particle cholesterol is increased in certain diseases, such as type 2 diabetes, because insulin resistance leads to the production of cholesterol-poor and dense LDL particles. Each LDL particle has one apoB molecule, and, regardless of density, the measurement of apoB levels detects the presence of these atherogenic particles, in contrast to LDL cholesterol, and may thus be better suited to guide lipid-lowering therapy. (Martin et al., 2009.) The trapping of apoB lipoprotein, which is the core protein of LDL particles, within the arterial wall drives the atherosclerotic process from the beginning to the end. The higher the concentration of apoB in serum is, the more apoB particles will be trapped within the arterial wall, and more lipoproteins are thereby phagocytized by macrophages and more foam cells are formed. Even though apoB levels can be measured accurately and inexpensively, apoB measurement has not yet been proven to be superior to LDL measurements, which is used in cardiovascular risk management according to the European Atherosclerosis Society. (Langlois et al., 2018.)

Atherosclerosis has a silent course for the first decades before clinical significance—e.g., acute coronary syndrome or stroke. Atherosclerotic plaque development can be identified by an early increase of serum inflammatory markers.

C-reactive protein (CRP) is a well-known protein whose circulating concentrations rise in response to inflammation, and it is related to the total cardiovascular risk. CRP promotes an increased expression of adhesion molecules, such as vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), on the endothelial surface. It induces the secretion of other pro-inflammatory factors and increases LDL oxidation. All of this leads to endothelial dysfunction and initiates the atherosclerotic process. (Balanescu et al., 2010.) Circulating monocytes migrate into the endothelium and subsequently differentiate into lipid-rich foamy macrophages (foam cells) by taking up oxidized LDL (oxLDL) by their scavenger receptors. Scavenger receptor family members scavenger receptor A (SR-A) and CD36 are the receptors for modified lipoproteins on macrophages, and their relevance to lipid uptake has been demonstrated *in vitro* and *in vivo*. Other scavenger receptors do not compensate for their absence. (Kunjathoor et al., 2002.) These foam cells may rupture and the cholesterol contained in them accumulates within the atherosclerotic lesion. The atherosclerotic process is progressed by intimal migration and the proliferation of vascular smooth muscle cells (SMCs), which express collagen and elastin. SMCs generate the fibrous cap at the atherosclerotic site, beneath the plaque endothelium. This process is promoted by the release of cytokines such as platelet-derived growth factor. Local inflammation is amplified by cytokines such as interferon-γ secreted by proinflammatory T-helper cells. A thick fibrous cap contributes to a plaque stability. SMCs in advanced lesions are generally considered to have plaque-stabilizing properties whereas macrophages are seen as detrimental to plaque stabilization (Bennett, Sinha & Owens, 2016). Moreover, more oxLDL, foam cells, and debris accumulate into the subendothelial space. Following this inadequate deposition of debris, more immune cells, such as monocytes, leukocytes, B-cells, T-cells, DCs, and mast cells, are recruited to the site (Chhibber-Goel et al., 2016). The lipid overload by macrophages and SMCs triggers apoptosis in the atherosclerotic plaque (Gui, Zheng & Cao, 2022). Apoptosis and necrosis of the foam cells and SMCs lead to the development of a lipid-rich necrotic core, weakening the fibrous cap. As the necrotic core grows and the fibrous cap diminishes, the advanced atherosclerotic lesion becomes vulnerable and is at risk of a plaque rupture. The rupture is caused when endopeptidases known as matrix metalloproteinases (MMPs), such as MMP-8 and MMP-9, are released by inflammatory cells and begin to degrade the extracellular matrix, including collagens and elastin. This collagenolysis leads to surface cap erosion and the activation of a coagulation cascade, which leads to thromboembolism and causes the acute clinical manifestations of atherosclerotic vascular disease. (Kelly, Lemmens, & Tsivgoulis,

2021; Linton et al., 2019.) Figure 3 demonstrates the basic phases and development of an atherosclerotic plaque.

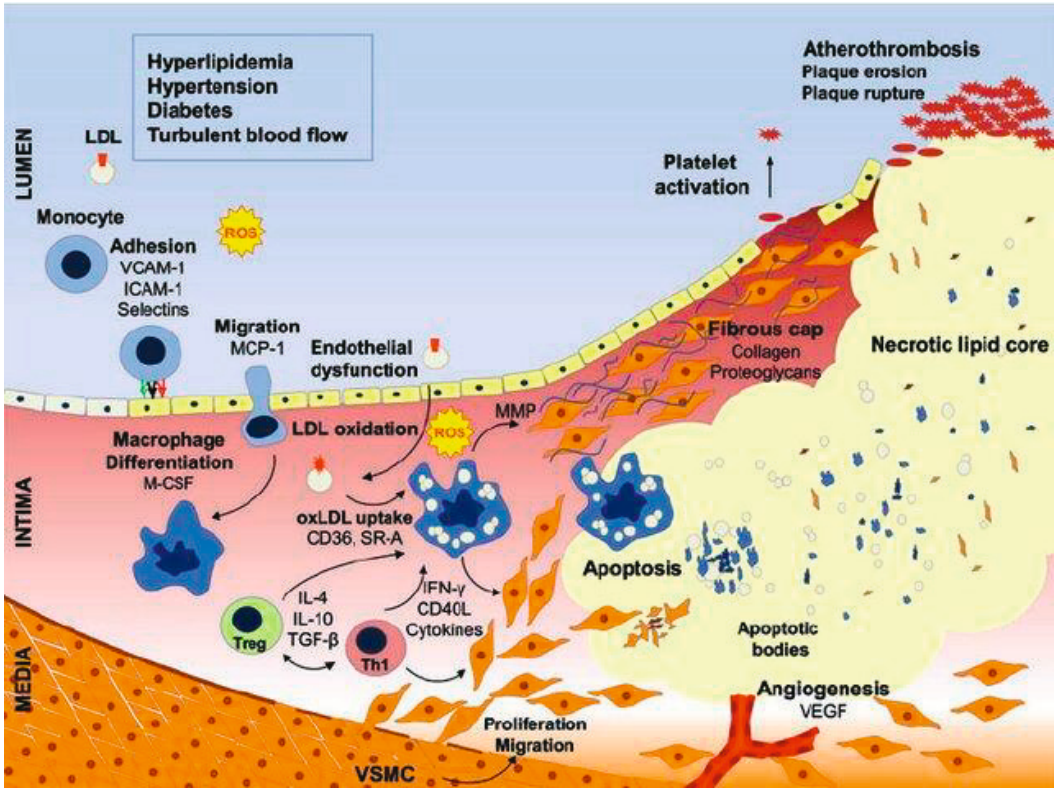


Figure 3. A schematic overview of the key mechanisms of atherosclerosis in an evolving atherosclerotic plaque. Prolonged exposure to risk factors induces endothelial dysfunction, causing lipid retention, adhesion molecule expression, and chemotactic substance secretion. This triggers immune cell recruitment and transmigration. Macrophages turn into foam cells by internalizing modified lipoproteins. Macrophages, T cells, and other immune cells release reactive species, growth factors, and cytokines, perpetuating inflammation. Vascular SMC migrate to the intima and form the fibrous cap by secreting extracellular matrix proteins. Apoptosis of foam cells and SMCs results in a necrotic core. Hypoxia-induced neovascularization and macrophage-secreted MMP can destabilize the fibrous plaque, leading to rupture. Republished with permission. Targeting the Ubiquitin-Proteasome System in Atherosclerosis: Status Quo, Challenges, and Perspectives. Antioxidants and Redox Signaling. 2014. (Wilck & Ludwig, 2014.) This copyrighted material was originally published by Mary Ann Liebert, Inc. publishers.

2.4.2 Role of immune cells in atherosclerosis

Macrophages have emerged as the protagonists of atherosclerosis. Monocytes infiltrate atherosclerotic plaque sites and are transformed into macrophages that form the major cell population present in the atherosclerotic plaques (Robbins et al., 2013). Nevertheless, macrophages are a heterogeneous group, and their subsets have either harmful or beneficial functions in atherogenesis. Macrophages can be divided to M1 and M2 phenotypes based on their activation. M2 macrophages are involved in tissue repair, have anti-inflammatory properties, and can stabilize vulnerable atherosclerotic plaques. M1 macrophages, on the other hand, are microbicidal and involved in the host defense response. (Wilson, 2010.) In the early stages of atherosclerosis, neutrophils migrate to the vascular wall. When neutrophils are no longer able to phagocytize, they become necrotic, thus adding to the inflammation. The ratio of neutrophils to lymphocytes is a marker of inflammation and is increased in atherosclerosis. (Mehu, Narasimhulu, & Singla, 2022.) In addition, T cells are also present in the atherosclerotic plaque. Both $CD4^+$ and $CD8^+$ T cells drive immune responses to peptides presented by major histocompatibility complex (MHC) class I on all nucleated cells and by MHC class II on antigen-presenting cells (APCs). *In vivo* and *in vitro* studies have shown that $CD4^+$ T cells recognize oxLDL and have pro-atherogenic roles. Regulatory T (Tregs) cells have anti-atherogenic roles, but they can be induced to switch their phenotype towards a pathogenic phenotype. It has been suggested that this is caused by increased intracellular cholesterol levels or by decreased IL-2 signaling, but the exact mechanism remains unknown. (Saigusa, Winkels, & Ley, 2020.) Due to the involvement of immunity cells in atherosclerosis, a vaccine against the cells present in atherosclerosis, such as Tregs, is under research. The problems are the instability of antigen-specific Tregs, because Tregs switching to other phenotypes could exacerbate atherosclerosis, and the vaccine could potentially increase the risk of autoimmune disorders due to the induction of a self-protein-specific immune response. (Kobiyama, Saigusa, & Ley, 2019.)

Mast cells are immune cells that have high reactivity to external allergens. Recently, it has been shown that mast cells are involved in different inflammatory diseases. (Kovanen, 2019.) Mast cells contribute to the atherosclerotic plaque progression and can be found in carotid artery plaque samples. It is implicated that mast cells take part in the plaque destabilization by thinning the fibrous cap. They increase intraplaque neovascularization by expressing basic fibroblast growth factor. Mast cells seem to be upregulated in unstable plaque regions. These are the reasons

why mast cells have been suggested as therapeutic targets against atherosclerosis. (Mekke et al., 2021.)

2.5 Microbiota associated with stroke

2.5.1 Immune response to bacteria

The human body is constantly exposed to microbes which usually only colonize the host harmlessly but which may cause harm if defense mechanisms break. The body's first line of defense against bacteria is the skin and the exposed epithelial surface, which have non-specific and innate protective systems. If organisms do enter the tissue, they will be combated by further elements of the innate immune system, which activates within hours of infection. Bacterial structures are identified, which leads to the activation of neutrophils, macrophages, and natural killer cells. This triggers cytokine and chemokine release and leads to a local inflammatory response. Neutrophils are characterized as being the first cell line that is recruited at the inflammation site. (Anaya, Shoenfeld & Rojas-Villarraga, 2013.) Neutrophils provide signals to other innate immune cells about the invading foreign threat. Neutrophils are loaded with various cytotoxic granules filled with powerful antimicrobial molecules, such as cationic peptides, proteases, lactoferrin, and myeloperoxidase. Neutrophils recruit and maturate macrophages, and they generate various chemotactic factors which attract more inflammatory cells to the inflammation site. The release of interferon-γ (IFN-γ) from activated neutrophils causes the activation of macrophages. Activated macrophages prolong the neutrophils' life span at the site of inflammation. (V. Kumar & Sharma, 2010.)

The more specific adaptive immunity, which consists of T- and B-lymphocytes, occurs with a delay. Dendritic cells (DCs) are critical for bridging the innate and adaptive immune responses. DCs capture invading pathogens and present their antigens to the corresponding lymphocytes. Following antigen uptake, DCs maturate and release cytokines and activate T cells. (Lecours et al., 2016.) Lymphocytes circulate in the bloodstream and lymphatic system and move into tissues as needed. $CD4^+$ T cells are important for the development of immunity to bacterial infections. The most important $CD4^+$ lineages are T helper type 1 (Th1), which promotes an immune response against intracellular pathogens, and Th2, which drives humoral responses. Th17 contributes to the elimination of extracellular pathogens, and

regulatory T cells suppress the immune response, thereby maintaining homeostasis and preventing the development of autoimmunity. (Lecours et al., 2016.) Gram-positive bacteria, such as viridans streptococci, are identified by their antigens, known as pathogen-associated molecular patterns (PAMPs). These include lipopeptides, peptidoglycan, flagellin, bacterial DNA, and lipoteichoic acid. Gram-negative bacteria are most often identified by their bacterial lipopolysaccharides. Toll-like receptors (TLRs) play a key role in the innate immune system. They recognize and bind a variety of bacterial PAMPs, which leads to the immune response. (Elson et al., 2007.) If the pathogens and the host survive the initial activation of innate and adaptive immune responses, the pathogenic bacteria may be capable of maintaining infections in a mammalian host even in the presence of inflammation. For example, bacteria such as *Helicobacter pylori* and *Mycobacterium tuberculosis* can establish life-long infections that can manifest as acute or chronic disease or be clinically asymptomatic. These bacteria can hide inside host cells, such as epithelial and endothelial cells or macrophages. Chronic *Streptococcus pneumoniae* and *Streptococcus pyogenes* pathogens can be found in the nasopharynx inside the nasopharyngeal-associated lymphatic tissue. (Monack, Mueller, & Falkow, 2004.)

2.5.2 Oral microbiota

In the literature, the mouth is often referred to as an island because of its unique environment. It is characterized by an almost constant presence of water (saliva), short-term temperature fluctuation, and exposed hard teeth surfaces. There is a wide and variable input of carbon and nitrogen through the food we eat. (Kolenbrander et al., 2010.) In a healthy situation, the oral cavity prevents bacterial invasion into tissues and the bloodstream by means of several barrier mechanisms: the surface epithelium, defensins, an electrical and immunological barrier, and a reticuloendothelial system (Li et al., 2000). Microbiologically, teeth are unique with two characteristics: they are the only non-shedding body surface, allowing bacteria to form structured biofilms and form on a specific epithelial-connective tissue seal (Darveau, 2009). The human oral cavity comprises of a minimum of 700 bacteria species, roughly half of which have not yet been cultured. The oral cavity has the second largest and diverse microbiota after the gut. Bacteria colonize all body surfaces in oral microbiota, creating a healthy and protective bacterial flora. The tongue houses the majority of the microbial burden in the oral cavity, with a high bacterial density. (Arweiler & Netuschil, 2016.) The composition of the oral

microbiome may change rapidly as the result of many factors, such as the temporal frequency of the host and diet, a response to the changes in pH, and interactions among the bacteria. There is a symbiotic relationship between the bacteria in our oral cavity, as the commensal populations do not cause harm and do not allow pathogenic species to adhere to the mucosa. The oral microbiome rests within biofilms throughout the oral cavity and forms an ecosystem that maintains health in a state of equilibrium. Bacteria become pathogenic only after having breached the barrier of the commensals, causing infection and disease. (Deo & Deshmukh, 2019.)

Streptococcus spp. are mostly found in the oral cavity of healthy individuals and in periodontal disease patients (Aas et al., 2005; Zheng et al., 2016). Viridans streptococci are a large group of alpha-hemolytic gram-positive bacteria species. They belong to the normal defensive oral microbiome but may, under certain circumstances, manifest as an acute disease. Viridans streptococci are the most common cause of infective endocarditis and sepsis (Cahill & Prendergast, 2016). The classification of viridans group streptococci has been revised regularly. According to the most recent classification updated in 2022, viridans group streptococci are divided into five subgroups based on the phenotypical characteristics of the bacteria, as seen in Figure 4 (Lakshmi & Leela, 2022). The groups *S. mutans*, *S. mitis*, and *S. sanguinis* are found in different parts of the oral cavity, as well as in the gastrointestinal tract. *S. mutans* is the major pathogen causing caries, and *S. mitis* causes bacteremia and infective endocarditis. *S. sanguinis* also causes infective endocarditis and takes part in dental biofilm formation. These gram-positive species are genetically close to each other and, in the clinical setting, not always easy to differentiate. (Basaranoglu et al., 2019; Kreth et al., 2005; Westling et al., 2008.) DNA of viridans group streptococcal bacteria have been detected in thrombus aspirates taken from myocardial infarction patients as well as from patients with lower leg thrombosis due to peripheral atherosclerosis (Pessi et al., 2013; Vakhitov et al., 2018). The presence of viridans streptococci in thrombus aspirates taken from acute ischemic stroke patients has not been studied previously.

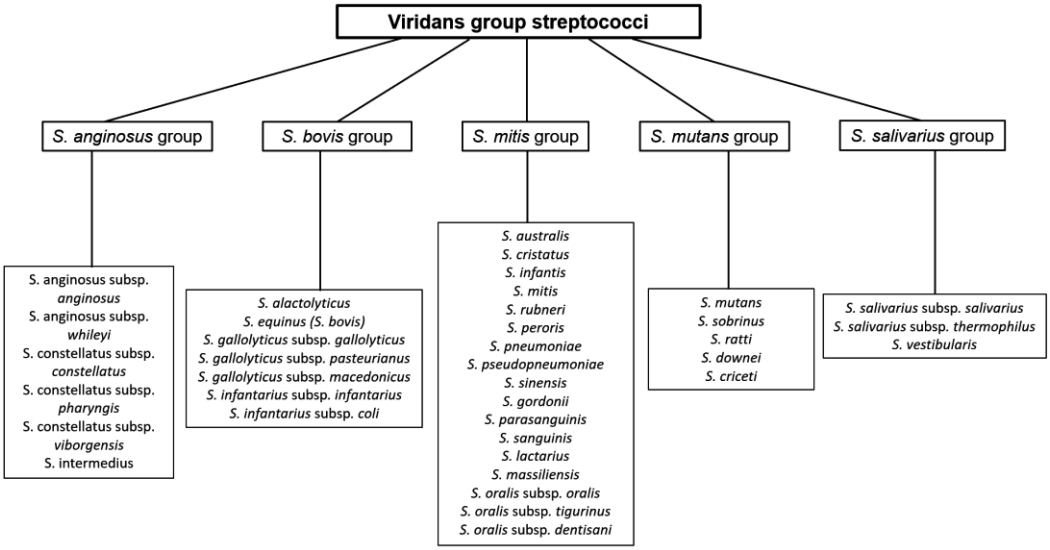


Figure 4. The most recent classification of viridans group streptococci. Data modified from Lakshmi & Leela, 2022.

Porphyromonas gingivalis is a gram-negative rod-shaped bacterium that is related to chronic periodontitis. Hayashi and colleagues infected ApoE-deficient mice with *P. gingivalis*. After 25 weeks' follow-up, a statistically significant increase in the size of atherosclerotic plaque was shown in the MRI analysis compared to the control group (Hayashi et al., 2011). In humans, the connection between *P. gingivalis* and cardiovascular disease is under dispute. A recent systemic review stated that *P. gingivalis* can induce a chronic periodontal infection and, consequently, be an important player in the formation of an atherosclerotic plaque (Fiorillo et al., 2019). Edentulousness is shown to be related to lowered *P. gingivalis* serum IgG levels (Aoyama et al., 2018).

Aggregatibacter actinomycetemcomitans is a small gram-negative rod that causes aggressive periodontitis and rapid destruction of the periodontal tissue. There are not many studies debating the role of *A. actinomycetemcomitans* in cardiovascular diseases. In a study conducted by Sakurai and colleagues (2007), they found that *A. actinomycetemcomitans* was found in 33% of the oral samples from acute coronary syndrome patients, while no *A. actinomycetemcomitans* was found in patients with coronary heart disease (Sakurai et al., 2007). *A. actinomycetemcomitans* is an oral bacterium that has also been linked with atherosclerosis and found in

endarterectomy samples obtained from patients with carotid stenosis (Haraszthy et al., 2000).

Further oral microbes that are linked with ischemic stroke are *Veillonella* spp., *Prevotella* spp., and *Fusobacterium nucleatum*. However, is it not certain whether these bacteria are major pathogens involved in the stroke pathogenesis or only bystanders. These gram-negative bacteria are also found in the gut. *F. nucleatum* plays a critical role in dental biofilm formation due to its abundant presence and its capability to coaggregate with other bacterial species in the oral cavity. *Veillonella* spp. produces succinate, a potential microbe-derived metabolite, which is associated with an increased risk of cardiovascular diseases. (Mashima & Nakazawa, 2014; Stingu et al., 2013.)

2.5.2.1 Dental biofilm

Dental plaque, a subgingival biofilm, is a complex polymicrobial community. It can form if the teeth are not mechanically taken care of. This community has coevolved with their human host to establish a highly sophisticated relationship, in which both pathogenic and mutualistic bacteria coexist in a homeostasis. Retention on the tooth surface is essential for bacteria to survive, as they are otherwise swallowed with the saliva. Through retention, these bacteria can form organized, intimate, multispecies communities referred to as dental plaque. The bacterial species can be placed in two general categories, namely early and late colonizers. The bacteria representing early colonizers coaggregate with only a specific set of other early colonizers and generally not with any of the late colonizers. Plaque formation is initiated by adherence of the pioneer *Streptococcus* spp. (e.g., *S. gordonii*, *S. oralis*, *S. mitis*) in the salivary pellicle, which is a layer of proteins and glycoproteins of salivary origin that coats the surfaces of oral tissues and is formed just seconds after a tooth is cleaned. Streptococci are efficient colonizers. (Kolenbrander et al., 2002.) According to a study conducted by Nyvad and Kilian, streptococci constitute 60% to 90% of the bacteria that colonize the teeth within the first four hours after professional cleaning using ultrasonic disintegration (Nyvad & Kilian, 1987). Streptococci recognize receptors such as statherin, proline-rich proteins, salivary alfa-amylase, and salivary agglutinin in the permanent salivary pellicle. The process is continued when other early colonizer bacteria, such as *Veillonella*, *Prevotella*, and *A. actinomycetemcomitans*, are subsequently attached to polysaccharide or protein receptors on the pioneer bacterial cell surface and aggregate to them. (Huang, Li, & Gregory, 2011.) *Streptococcus* species are able to format lactate, which is a favored substrate of the *Veillonella* species. Moreover,

Veillonella species are not capable to colonizing the tooth surface without *Streptococcus* species; these two species form a mutualistic relationship in a bacterial biofilm. (Mashima & Nakazawa, 2014.) *Fusobacterium nucleatum* belongs to the border between early and late colonizers. It coaggregates with all of the early and late colonizers and acts as a bridge between early and late colonizers. Thus, it is the most numerous gram-negative species in healthy sites, and its numbers increase markedly in periodontally diseased sites. *F. nucleatum* is always present when *P. gingivalis* is also present. (Kolenbrander et al., 2002.) Other late colonizers are, for example, *A. actinomycetemcomitans* and *Eubacterium* spp. Late colonizers do not usually coaggregate together, with the exception of *Treponema denticola* and *P. gingivalis* that coaggregate together. A schematic presentation of a dental biofilm can bee see in Figure 5. The signal molecule autoinducer 2 (AI2) has an important role in the formation of multispecies biofilms. Many oral bacteria produce less AI2 compared with oral pathogens when grown as monoculture. The presence of AI2 in two-species communities containing *Streptococcus oralis* and *Actinomyces oris* enables mutual growth of both organisms. (Kolenbrander et al., 2010.)

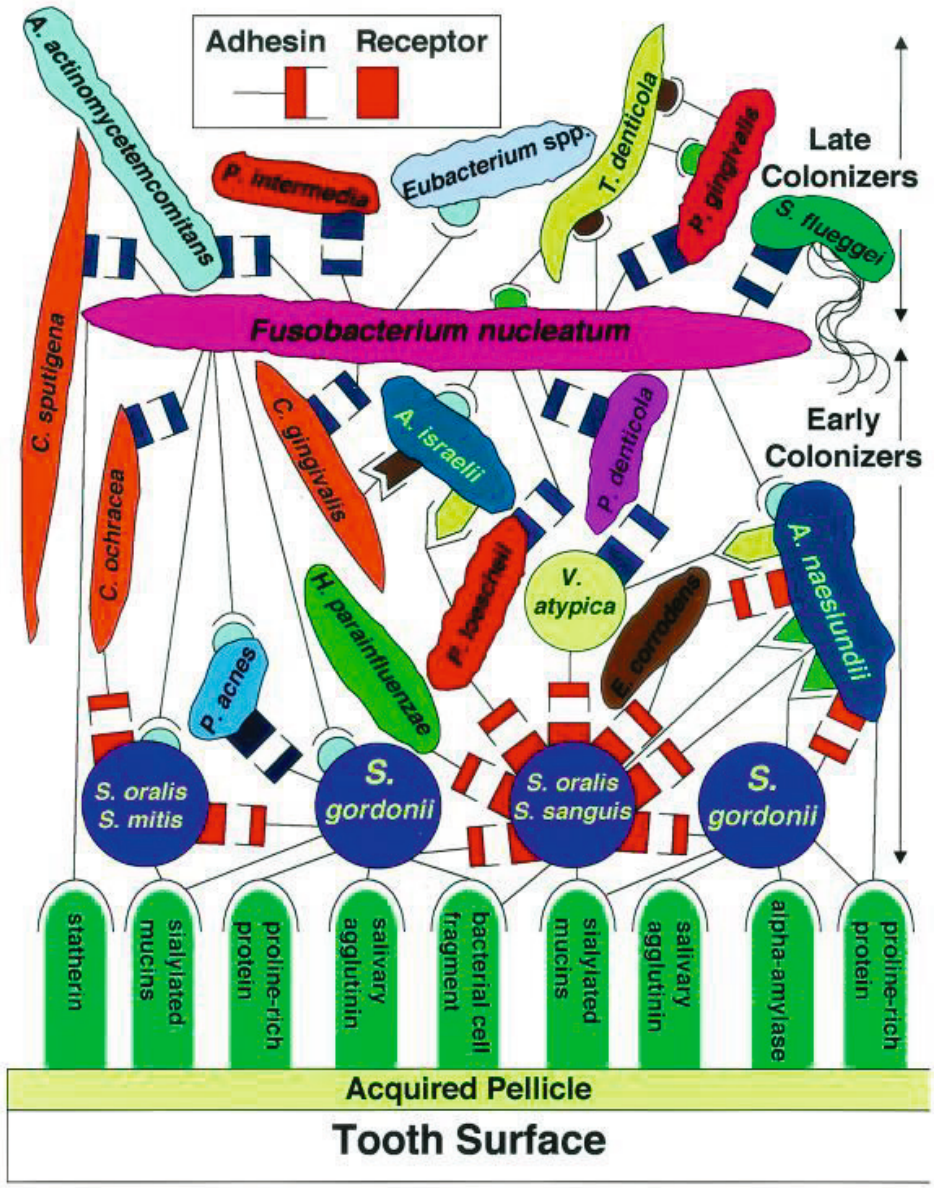


Figure 5. Spatiotemporal model of oral bacterial colonization on the tooth surface. *Streptococcus* species are considered as the pioneer colonizers in the early stages of dental plaque formation. Published with permission. (Kolenbrander et al., 2002.)

2.5.2.2 Periodontal disease and ischemic stroke

Periodontal disease is a chronic inflammatory condition affecting approximately 20%–50% of people worldwide. Advanced periodontal disease, periodontitis, results in severe loss of supporting structures and substantial tooth loss, affecting 10%–15% of the population globally. Estimates of the prevalence of periodontitis vary across populations because the definition of a case of periodontitis depends on which thresholds are used and no sets of specific thresholds have been consistently applied in epidemiological studies. Periodontal disease is manifests clinically through the detection of gingival inflammation, clinical attachment loss, resorption of alveolar bone, periodontal pockets, and tooth mobility (Figure 6). The localized inflammation of the gingiva that is initiated by bacteria in the dental plaque, known as gingivitis, is the early state of periodontitis. Chronic periodontitis occurs when gingivitis is left untreated and progresses to the loss of the gingiva, bone, and ligaments. It is classified as generalized chronic periodontitis when it affects more than 10 of the 32 teeth. Deep periodontal pockets are the hallmark of the disease. Epithelial cells function as a physical barrier against pathogens. Dendritic Langerhans cells within the epithelium present microbial antigenic material to lymphocytes, and an immune response is generated. However, neutrophils, granulocytes, and lymphocytes are overwhelmed by the magnitude and chronic persistence of the microbial biofilm. This inflammatory response to the accumulation of microbial plaque and its byproducts leads to alveolar bone resorption by osteoclasts. (Kinane, Stathopoulou, & Papapanou, 2017.) It can potentially cause irreversible damage to the teeth-supporting tissues. In advanced periodontitis, after the destruction of the structural components of teeth-supporting apparatus, tooth loss can occur. The risk factors for periodontitis include poor oral health care habits, smoking or chewing tobacco, hormonal changes, obesity, and poor nutrition. Genetic predispositions have been considered to be an important cause of periodontitis, with heritability estimates of as high as 50%. However, GWAS studies suggest that genetic predisposition to chronic periodontitis is conferred collectively by hundreds or thousands of genes and there is no specific single-nucleotide polymorphisms. (Di Stefano et al., 2022; Kinane et al., 2017.)

If bacteria invade the dental pulp, an endodontic infection, such as a periapical abscess, can occur. Bacteria enter through either a dental cavity or a crack in the tooth and spread all the way down to the root and may eventually end up in the systemic circulation. Periodontitis in the furcation area, where the roots of a multirooted tooth diverge, is known as furcation lesions. These infections are usually

asymptomatic and cause a chronic inflammatory response, giving oral bacteria a convenient route into the bloodstream. Consequently, these infections lead to bone resorption around the root apex, which is the typical radiographic feature of apical periodontitis. (Siqueira & Rôças, 2013.) The microbiological characteristics and inflammatory responses are mostly similar to those of chronic periodontitis and periapical infection (Kerekes & Olsen, 1990; Rupf et al., 2000). Endodontic infections are associated with at least 150 and periodontal with 350 bacterial species, many of which are anaerobic (Debelian, Olsen & Tronstad, 1994). The accumulation of anaerobic bacteria forms a subgingival biofilm, dental plaque, close to the highly vascularized tissues below the gum line on the surface of the teeth. The total amount of bacteria in dental plaque can be as high as 10^{11} micro-organisms per milligram, and poor oral hygiene can increase the amount of bacteria colonizing the tooth surfaces by as much as tenfold compared to a situation with normal oral health (Loesche, 1997). Therefore, mechanical cleaning and flossing helps to provide oxygen to prevent the formation of anaerobic dental plaque.

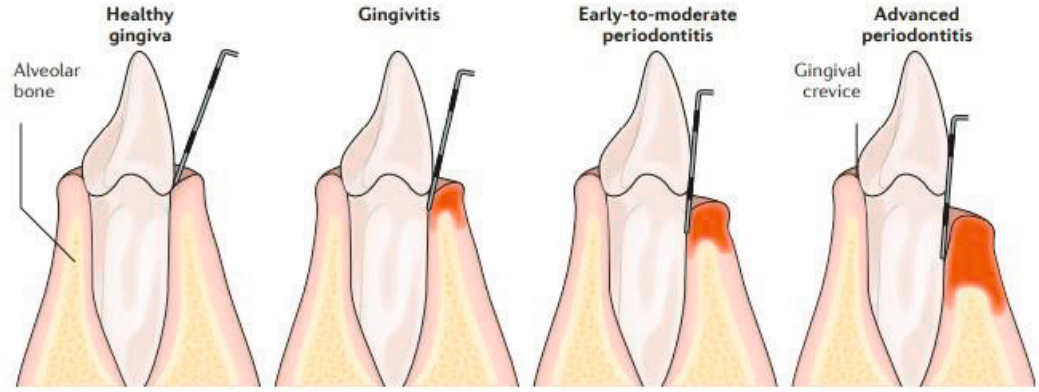


Figure 6. The development of periodontal disease. Adapted from Kinane et al., 2017.

Oral conditions such as periodontitis and gingivitis not only affect the mouth and its supporting structures but can also have implications for various systemic health conditions. The link between cardiovascular disease and periodontitis was suggested as early as in 1989, when Mattila and colleagues found that patients with an acute myocardial infarction had poorer dental health compared to healthy individuals, even after adjustment for age, social class, smoking, serum lipid concentrations, and the presence of diabetes (Mattila et al., 1989). Ischemic stroke and myocardial infarction often share a similar pathophysiologic process—in both arterial thrombosis may

occur in the background of atherosclerosis. Later on, periodontal disease was found to be a risk factor for stroke, with the direct mechanism still remaining unclear (Grau, Becher, et al., 2004; Joshipura et al., 2003). Severe periodontitis increased the risk of ischemic stroke fourfold among middle-aged men, but not among women, after adjusting for age, sex, number of teeth, vascular risk factors and diseases, childhood and adult socioeconomic conditions, and lifestyle factors (Grau, Becher, et al., 2004). In an Indian population, periodontitis was an even stronger risk factor for ischemic stroke than hypertension and smoking (Pradeep et al., 2010).

Four basic mechanisms have been proposed for how oral infections are linked to the pathogenesis of atherosclerosis: 1) low-level bacteremia, 2) systemic inflammation, 3) host immune response, and 4) atherogenic effects caused by oral pathogens (Aarabi, Heydecke & Seedorf, 2018).

1) In low-level bacteremia, oral bacteria enter the systemic circulation and end up on the arterial wall, contributing directly to the pathophysiology of atherosclerosis through immune cell activation. Inflammation of an atherosclerotic plaque may affect its growth and contribute to plaque rupture, leading to thrombosis (Koren et al., 2011; Lanter, Sauer & Davies, 2014).
2) Systemic inflammation refers to the inflammation induced by inflammatory mediators (such as IL-6) that are released from the sites of oral inflammation into the blood stream. Oral infections such as gingivitis and periodontitis consistently elevate systemic levels of CRP. CRP has correlation with the incidence of cardiovascular events and stroke. (Ridker et al., 2002; Rost et al., 2001.) CRP increases the adhesiveness of platelets to the endothelium (Grad, Pachino & Danenberg, 2011). Inflammation at nonvascular sites, such as in teeth, may also affect the progression of atherosclerotic lesions via circulating chemical mediators (Charo & Taubman, 2004).
3) A host immune response refers to when autoimmunity to host proteins results from the immune response to specific components of oral pathogens. Heat shock proteins are involved in stress protection. Some oral bacteria, such as *Porphyromonas gingivalis*, contain homologs to human heat shock proteins and can induce humoral and cellular immune responses in humans. These heat shock proteins may cross-react with T cells and contribute to the atherogenesis. Nevertheless, atherosclerosis at a young age has been observed in patients with other autoantibody-associated diseases, such as rheumatoid arthritis and systemic lupus erythematosus. (Frieri & Stampfl, 2016; Skaggs, Hahn, & McMahon, 2012.)

4) The final proposed mechanism refers to pro-atherogenic effects resulting from specific bacterial toxins that are produced by pathogenic oral bacteria. Gram-negative bacteria such as *Aggregatibacter actinomycetemcomitans* produce an endotoxin called lipopolysaccharide (LPS), which is one of the most potent innate immune-activating stimuli known. LPS has severe pro-inflammatory effects. Some proteins secreted by oral bacteria can induce lipid peroxidation and modify both LDL and HDL particles. (Aarabi et al., 2018.) Interestingly, Kallio and colleagues concluded in their study that inflammation associated with periodontitis may enhance very low density lipoprotein particles pro-atherogenic properties by increasing their ability to induce macrophage activation and foam cell formation (Kallio et al., 2013).

Yet, there is no consensus about the mechanism of action as to how oral bacteria participate in the development of an atherosclerotic plaque. Most likely, all of the mechanisms discussed here are involved in the process (Pillai et al., 2018).

2.5.3 Gut microbiota

Tuomisto and colleagues reported in 2019 that intestinal *Enterobactericeae* bacteria were found in 12.1%, *Clostridium leptum* in 2.4%, and *Lactobacillus* spp. in 2.4% of post-mortem coronary plaque samples (n = 67) (Tuomisto et al., 2019). Current data support the complex relationship between diet, the gut microbiota as well as their metabolites, and stroke. Gut microbiota dysbiosis, which is caused by Western diets, the lack of dietary fiber and an increased red meat intake, may have a role in the development of stroke. Gut bacteria generate proatherogenic metabolites. For example, the metabolite trimethylamine *N*-oxide (TMAO) is generated from phosphatidylcholine, which can be found in red meat, eggs, and shellfish. Increased levels of TMAO are linked to cardiovascular diseases. TMAO increases macrophage cholesterol accumulation and the formation of foam cells, and it activates platelets. Fiber fermentation by the gut microbiota results in the release of metabolites known as short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. According to animal studies, SCFAs prevent the progression of cardiovascular diseases. Patients who have suffered an ischemic stroke with severe stroke outcomes have been shown to have a lower abundance of SCFA-producing bacteria. (Peh et al., 2022.) Furthermore, there is evidence that the metabolic activity of the gut microbiota is related to blood pressure levels and may thus contribute indirectly to the overall cardiovascular disease and stroke risk (Holmes et al., 2008). With most of

the evidence being indirect, however, there is little to suggest a direct causative relationship between gut bacteria and cardiovascular disease. The causality is difficult to establish, as an ischemic stroke alters the composition of the gut microbiota composition. (Jonsson & Bäckhed, 2017; Shen et al., 2021.)

2.5.4 Other candidate bacteria

Chlamydia pneumoniae is gram-negative bacterium that can cause persistent infections of the respiratory and gastrointestinal tract. In the late 1980s and early 1990s, an association was found between raised IgG and/or IgA titers against *C. pneumoniae* species and asymptomatic carotid stenosis, acute myocardial infarction, and coronary heart disease (Melnick et al., 1993; Saikku et al., 1988; Thom et al., 1992). It was suggested that a chronic chlamydial infection could be a factor in the pathogenesis of atherosclerosis (for more detail please refer to Chapter 2.6.2). Also, it was found that *C. pneumoniae* bacteria can be found in atherosclerotic plaques, but not in healthy arteries (Farsak et al., 2000). *Helicobacter pylorii* is a common cause of chronic gastritis and a risk factor for gastric cancer. Furthermore, *H. pylori* DNA has been found in atherosclerotic plaques (Rosenfeld & Campbell, 2011). Although many studies have failed to establish an association, some seroepidemiological studies have confirmed a relationship between *H. pylori* serology and atherosclerosis. Similar findings have also been demonstrated with antibodies related to *Mycoplasma pneumoniae*. In general, it is presumable that any chronic bacterial infection can cause a systemic inflammation that may contribute to the atherosclerotic process. Moreover, it has been suggested that the infectious burden, rather than any single pathogen, contributes to the development of atherosclerosis through different mechanisms. (Sessa et al., 2014.)

2.6 Treatment of acute ischemic stroke

2.6.1 Acute treatment

The treatment of an acute ischemic stroke has undergone major developments during the last three decades. Prior to 1995, it was customary for stroke patients to remain in emergency departments without the necessity of immediate brain imaging. This approach was justified by the belief that even a basic distinguishing between an

intracerebral hemorrhage and ischemic stroke would not alter the course of treatment. (Campbell et al., 2015.) Recombinant tissue plasminogen activator was approved in 1996 as a treatment for acute ischemic stroke. Later, the intravascular catheter-based revascularization of cerebral vessels begun to evolve. In 2004, the first clot retriever device was approved by the U.S. Food and Drug Administration. Subsequently, the instruments evolved and many trials demonstrated the benefit of the use of direct aspiration of cerebral arterial clots as the primary treatment for acute ischemic stroke among thrombolytic agents. (Berkhemer et al., 2015; Turk et al., 2018; Wahlgren et al., 2016.) Thrombolysis is performed by using an intravenous recombinant tissue plasminogen activator that, if administered within 4.5 hours of symptom onset, significantly increases the likelihood of recovery to independence. A large vessel occlusion is relatively resistant to dissolution by plasminogen activators, which is why endovascular mechanical thrombectomy should be considered.

Mechanical thrombectomy is usually performed within a six-hour window. In recent years, it has been demonstrated that patients with a late-presenting large vessel occlusion (from 6 to 24 hours) may still receive clinical benefit from mechanical thrombectomy selected by perfusion scans (El Tawil & Muir, 2017). In Australia, it was estimated that mechanical thrombectomy would be applicable to 7%–13% of all ischemic stroke patients. In Finland, 407 mechanical thrombectomies were performed in 2016, which means the real demand for thrombectomies would be almost twice as high as the number of procedures that are currently performed (Lindsberg et al., 2017). In addition, it is important to assay the etiology to establish proper secondary prevention, such as starting statins, antihypertensive drugs, or oral anticoagulants.

The treatment of each potential stroke patient should be regarded with priority and urgency. Guidelines recommend a door-to-needle time of 60 minutes or less. Acute stroke teams greatly improve the flow of the events within the emergency room and are widely used across the world. (Kamal et al., 2017.) In Tampere, Finland, the average door-to-needle time is 20 minutes, and mechanical thrombectomy is performed within 90 minutes of the patient's arrival at the hospital in the majority of cases (Tampere University Hospital, 2020).

2.6.2 Anti-inflammatory treatments against ischemic stroke

Even though it is now widely accepted that inflammation has a role in atherosclerosis, only a few trials have shown that reducing inflammation reduces the rates of cardiovascular events. Some medications currently used for ischemic stroke prevention have known anti-inflammatory effects. Low-dose aspirin (acetylsalicylic acid, ASA), which is commonly used for stroke prevention, inhibits inducible cyclooxygenase 1 (COX-1) and reduces thromboxane A2 synthesis, which is the major antithrombotic effect of aspirin. At high doses, such as those used in primary treatment of an acute myocardial infarction, aspirin also inhibits COX-2, resulting in decreased prostaglandin synthesis in response to inflammatory cytokines. (Kelly et al., 2021.)

As mentioned earlier, Saikku and colleagues (1988) published a novel study in Lancet that demonstrated a strong serological association between *C. pneumoniae* and coronary heart disease, and this sparked the interest in whether cardiovascular diseases could be prevented with antimicrobial treatment (Saikku et al., 1988). However, attempts to replicate the original observation have failed. A meta-analysis comprising 14 prospective studies with more than 3,000 patients did not find a statistically significant association between *C. pneumoniae* and coronary heart disease (Danesh et al., 2000). In small pilot trials, antibiotics against *C. pneumoniae* reduced cardiovascular events, but in later large studies antimicrobial treatments were not distinguishable from placebo and the academic interest in antibiotic treatment against cardiovascular diseases diminished for decades (Campbell & Kuo, 2004; Gelfand & Cannon, 2004; Danesh, 2005).

Statins, which are used to treat hypercholesterolemia, also have anti-inflammatory properties. They seem to decrease the number of inflammatory cells in atherosclerotic plaques. Overall, this can be seen as the plasma levels of high-sensitivity-CRP (hs-CRP) decrease. Statins lower hs-CRP levels even in the absence of hyperlipidemia, and this is related to a better prognosis. (Blake & Ridker, 2000; Diamantis et al., 2017.) The use of low-dose methotrexate and colchicine, both nonspecific anti-inflammatory agents, for stroke prevention has also been studied, but no trial to date has proved the benefit and safety of these medications (Kelly, Lemmens & Tsivgoulis, 2021).

Ridker and colleagues studied the inflammatory hypothesis in 2017 by administering canakinumab, a therapeutic monoclonal antibody targeting interleukin-1β, to patients with a previous myocardial infarction and a hs-CRP level of more than 2 mg/L. The study was placebo-controlled, randomized, and double-

blinded. Canakinumab was chosen because the drug reduced plasma levels of IL-6 and hs-CRP without lowering the level of LDL cholesterol. As the results, canakinumab at a dose of 150 mg every 3 months led to a significantly lower rate of recurrent cardiovascular events to patients with a history of myocardial infarction than placebo, independently of lipid-level lowering. When the end point was incidence of stroke, no statistical significance was found. However, the high cost of canakinumab and concerns about an increased risk of fatal sepsis have currently prevented its widespread adoption in clinical practice. (Ridker et al., 2017.)

3 AIMS OF THE STUDY

The aim of this study was to assess the relationship between oral bacterial infections and the development of atherosclerosis and ischemic stroke. Due to earlier findings of viridans streptococcal DNA in coronary atheromas and thrombi, the present study focuses on the role of oral streptococci in the pathogenesis of acute ischemic stroke. The specific aims were:

To evaluate whether oral bacterial DNA could be amplified using bacterial qPCR from thrombus aspirates taken from acute ischemic stroke patients (Study I).

To establish whether the bacterial findings would correlate with the condition of the patients' teeth and carotid artery atherosclerosis, using computed tomography scans and ultrasonography (Study II).

To confirm the oral bacterial findings in thrombus aspirates and carotid artery samples using bacterial immunohistochemistry (Study III).

4 MATERIALS AND METHODS

4.1 Materials

In this thesis, we included participants from the Brain, Microbes and Genetics (BMG) study, Tampere Vascular Study (TVS), and Tampere Sudden Death Study (TSDS). In Study I, we included samples from the BMG study. In Study II, we studied BMG samples, as well as TVS samples. In Study III, we investigated samples from all three study series (Figure 7).

4.1.1 Brain, Microbes and Genetics (BMG) study

Thrombus aspirates were collected from 75 acute ischemic stroke patients (mean age 66.9 years, 69.3% men) treated with mechanical thrombectomy between December 2013 and January 2017 in the Acute Stroke Unit of Tampere University Hospital, Tampere, Finland. The thrombus was aspirated from the middle cerebral artery in 98.4% of the cases. The only excluding criterion for recruiting patients was unsuccessful retrieval of the thrombus using mechanical thrombectomy. A neurologist examined all patients when they arrived at the hospital and evaluated the possibility of revascularization using thrombectomy together with a neurointerventional radiologist. The etiology of large-vessel occlusions of the brain in patients treated with endovascular thrombectomy during the study period in Tampere University Hospital was cardioembolic in 38% and atherosclerotic in 62% of the cases (personal communication, Dr. Jyrki Ollikainen). The median delay between the onset of an ischemic stroke and hospital arrival was 2 hours and 20 minutes (range 0–16 h). Medical history was collected from Tampere University Hospital's digital patient archives. None of the patients had been treated with antibiotics or had experienced severe infections or septicemia during the stroke.

4.1.2 Tampere Vascular Study (TVS)

Endarterectomy samples were collected from 20 symptomatic patients (mean age 66.2 years, 65% men) diagnosed with an ipsilateral carotid artery stenosis. All open vascular surgical procedures were performed at the Division of Vascular Surgery and the Heart Center at Tampere University Hospital between 2006 and 2009. The patients had suffered amaurosis fugax, a transient ischemic attack, or a stroke. Eight (40%) patients had suffered an acute ischemic stroke. The severity of the carotid stenosis was histologically classified, the average being 82% (range 69%–99%). All samples were classified as either a fibrotic or calcified atheroma (AHA type V) or a complicated atheroma with rupture and thrombosis/hemorrhage (AHA type VI), according to the American Heart Association classification (Stary et al., 1995; Virmani et al., 2000). Medical history was collected from Tampere University Hospital's digital patient archives.

In the study, a small, 2 x 2–3 mm endarterectomy sample that could be extracted without endangering the patient was immediately placed in sterile 10% buffered formalin. From the initial 96 samples obtained for histology, 20 (20.8%) were selected because they were large enough for immunohistochemical studies.

4.1.3 Tampere Sudden Death Study (TSDS)

Postmortem carotid artery samples were collected from 48 autopsy cases (mean age 66.4 years, 62.5% men) during medico-legal autopsies at the Department of Forensic Medicine at the University of Tampere between 2010 and 2015, representing a cross-section of the population. The selection criteria for the cases were: out-of-hospital death, time elapsed postmortem of less than 6 days, intact middle torso and bowel, no signs of bacterial infections or drug addiction, and no visible wounds or necrosis. None of the cases had died of a stroke.

The time interval between death and storage of the body in the mortuary was less than 24 hours in all cases. In the mortuary, the bodies were kept at 4°C. Low temperatures prevent bacterial growth, and bacterial populations are unlikely to alter during such storage conditions (Tuomisto, Karhunen & Pessi, 2013). Based on hospital records, incident police reports with data on drugs found in the home and possible treatments, as well as on physician's admission notes, none of the victims of a sudden out-of-hospital death had used antibiotics within two weeks prior to their death. The relatives of the deceased were sent a structured interview concerning risk factors and other background information.

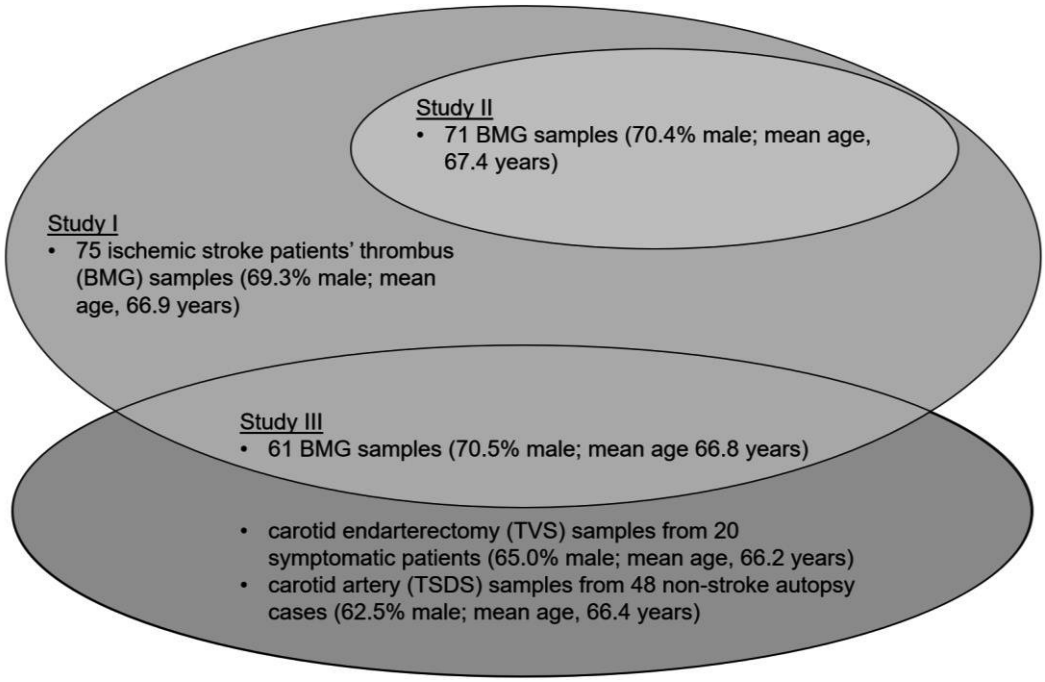


Figure 7. Subjects included in the studies.

4.2 Methods

4.2.1 Mechanical thrombectomy and thrombus sample collection (Studies I, II, and III)

An introducer sheet was placed into a femoral artery, and a control blood sample for bacterial genetic analysis was collected through the sheet. A guiding catheter of up to 9 Fr (Merci®, Stryker neurovascular, USA) with a tip balloon was then navigated into the carotid artery proximally to the occluded site. The micro-catheter (0.021") with the guide wire was used to navigate through the occluded site and to deploy the stent retriever (Trevo®, Stryker neurovascular, USA) over the thrombus. An additional distal access catheter was used to achieve the thrombus if needed. Forceful aspiration through proximal catheters was acquired with a 60 cc syringe while retrieving the deployed stent. In a minority of the cases, only direct thrombus aspiration was used. Different device settings were selected by the operator on a case by case basis. The thrombectomy was repeated until the angiologic result was

satisfactory. The gathered thrombus was divided into a 1.5 cc Eppendorf microcentrifuge tube for qPCR analysis and a histological sample in a formalin container.

4.2.2 Quantitative bacterial PCR (Studies I, II, and III)

Quantitative PCR was performed on 75 thrombus aspirate and arterial blood samples taken from the same patients. Bacterial DNA was extracted from the samples using a commercially available QIAamp DNA Mini Kit (Qiagen, Germany) according to the instructions provided.

The presence of bacterial DNA in thrombus and blood samples from the same patients was determined by using published oligonucleotide primers and probes for *Streptococcus* spp., mainly the *S. mitis* group, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and universal bacterial primers and probe with RNaseP (Applied Biosystems, Foster City, CA), as a reference (Nonnenmacher et al., 2004; Tuomisto et al., 2013; Yang et al., 2002).

Quantitative PCR assays were performed using specific Taqman allele hybridization with the AbiPrism 7900 HT Sequence Detection System (Taqman, Applied Biosystems, Carlsbad, CA, USA) under standard conditions, with the following cycle profile: 50°C for 2 minutes, 95°C for 10 minutes, and 60 cycles of 95°C for 15 seconds and 58°C for 1 minute. MasterMix was prepared using Maxima Probe/ROX qPCR MasterMix (Thermo Fisher Scientific®, Waltham, Massachusetts), adding at final concentrations of 900 nM of each primer and 250 nM of each fluorescence-labeled probe. All amplifications and detections were carried out as duplicates or quadruples (in uncertain cases) depending on test runs in a MicroAmp optical 384-well reaction plate with optical caps (Sarsted, Nümbrecht, Germany) in a reaction volume of 5 µL with 1 µL of non-diluted DNA.

A human housekeeping gene, RNaseP, was used as a reference measurement to determine the relative amount of bacterial DNA in the sample. The determination was done by the comparative threshold cycle (Ct) method. The critical threshold cycle (Ct) is the cycle at which a statistically significant increase in ΔRn is first detected and at which the fluorescence becomes detectable above the background. Ct is inversely proportional to the logarithm of the initial number of template molecules—that is, the initial amount of sample DNA. Calculation with the Ct method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$) was carried out with a simplification. (Livak & Schmittgen, 2001; Suzuki, Yoshida, & Nakano, 2005; Tuomisto et al., 2014;

Yoshida et al., 2003.) Firstly, the differences in the Ct values between candidate bacteria and reference gene measurement (candidate bacteria – RNaseP [ΔCt]) for each sample were calculated; then the comparative Ct (thrombus – patient's own blood, [ΔΔCt]) was calculated. The samples were marked as bacterial positive, if $2^{-\Delta\Delta Ct} \geq 2$ (Bubner, Gase & Baldwin, 2004; Tichopad et al., 2010), or if there was amplified bacterial DNA in the thrombus but not in the control sample. DNA was extracted from the entire thrombus in most of the cases. If the aspirated thrombus was large, a small part of it was sent to histological analyses, while DNA was extracted from rest of the thrombus.

4.2.3 Imaging and assessment of dental pathology using brain computed tomography (Study II)

Non-contrast computed tomography scans (Lightspeed VCT, GE Medical Systems, United States) were taken of all patients when upon arrival at the hospital to exclude an intracranial hemorrhage, and the scans were used for the assessment of oral health. The parameters of the CT scans were as follows: slice thickness of 0.63 mm, field of view 320.0 mm, voltage of 120 kV, and current of 649 mA. With the Philips Brilliant™ Workspace (Philips Healthcare, The Netherlands), the CT images were reconstructed into 1mm multi-slice axial, sagittal, and coronal planes. Furthermore, synthetic panorama and 1mm multi-slice reconstructions of the right and left oblique sagittal and plane in accordance with dental arches were reconstructed.

CT reconstructions were inter- and intra-observed by two evaluators, an experienced (Jorma Järnstedt, DDS) and a trainee oral and maxillofacial radiologist (Helena Mehtonen, DDS), using Carestream Vue PACS software (Carestream Health, United States) and diagnostic monitors (Barco, Belgium). The assessments were performed in dim lighting. Scores that differed between the observers were assessed jointly. The total number of teeth, the number of missing teeth, and the following parameters for each tooth were registered: periapical condition, horizontal and vertical alveolar bone loss, caries, furcation lesions, and the condition of pericoronal spaces.

The condition of a tooth was first registered as sound, filled, caries with pulp exposed, or tooth being decayed as residual root, but due to resolution issues and artifacts deteriorating the diagnostic accuracy, we eventually combined the groups as sound/filled and caries with pulp exposed/residual root. Periapical infection was registered if an osteolytic finding was observed surrounding the root apex (Figure 8).

A distance of > 2 mm from the cementoenamel junction was regarded as vertical bone loss. Scores for horizontal bone loss were difficult to assess from CT scans, and because the scores differed between the evaluators, they were left out from the analyses. A furcation lesion was registered if an osteolytic finding at the furcation was observed. The pericoronal space was defined to be infected if the pericoronal space was ≥ 3 mm and/or the surrounding bone showed signs of infection.

The combined pathology sum was calculated to assess the overall oral health status in the same way as has been done previously (Janket et al., 2004; Karhunen et al., 2006; Mattila et al., 1989). The amounts of vertical bone loss as well as periapical, furcation, caries, and pericoronitis lesions were summed per each tooth. The patients were then divided into three equal groups based on the sum per tooth: normal to slight pathology (n = 21, sum 0–0.06), moderate pathology (n = 21, sum 0.07–0.23), and severe pathology (n = 22, sum 0.24–2.00). Edentulous patients (n = 7) were excluded from the analysis.

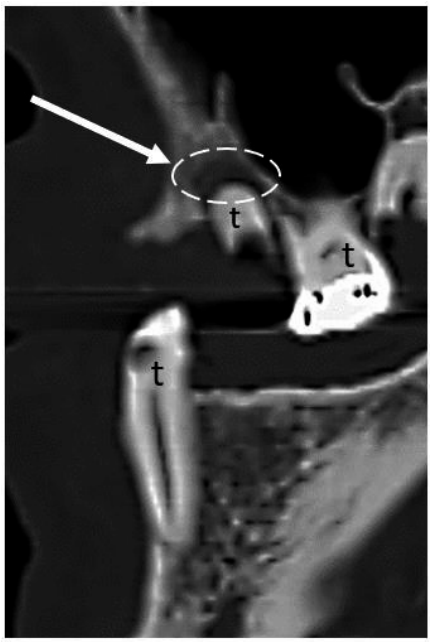


Figure 8. A tooth's periapical lesion as seen on a CT scan (t = tooth). Osteolytic finding can be observed surrounding the root apex.

4.2.4 Bacterial immunohistochemistry (Study III)

Antibodies against three major viridans streptococcal species—*S. sanguinis* (ATCC 10556), *S. mitis* (ATCC 49456), and *S. gordonii* (ATCC 10558)—were raised in rabbits (Thermo Fisher Scientific®, Waltham, Massachusetts). The performance of the resulting antibodies was confirmed with Western blot by staining the respective streptococcal lysates (data not shown). All histological samples were immunostained with an antibody cocktail containing antibodies raised against the three species of viridans streptococci. The immunostaining intensity was assessed with a semiquantitative score (+/++/+++). ImageJ software (github.com/fiji/fiji) was used to create color convolution in order to increase the contrast in the immunostained samples for better visualization of the results. Control stainings were performed with an immunoglobulin G (IgG) isotype control antibody (ab37415, Abcam®), as well as with an IgG anti-*Escherichia coli* antibody (ab137967, Abcam®) raised in rabbits. The specificities of the antibodies were tested in a histological tissue section by inoculating (+37°C, 5 hours) coronary artery samples with a suspension of streptococcal bacteria species (obtained from ATCC) and performing an immunohistochemical study using an antibody against the same bacteria (Figure 9). Gut and liver samples from the TSDS autopsy cases were stained with a streptococcal cocktail to show the ability of the antibodies to detect streptococci in feces as well as in liver Kupfer cells (Figure 10). To validate the specificity of the immunohistochemical stainings, we used several controls. The rabbit IgG isotype control is a primary antibody that lacks the specificity to the target. Isotype controls are used as negative controls to differentiate a non-specific background signal from a specific antibody signal. An *E. coli*–specific antibody was used to show the specificity of the viridans streptococci antibody cocktail. All IgG isotype control–stained samples as well as the *E. coli* immunostainings were negative.

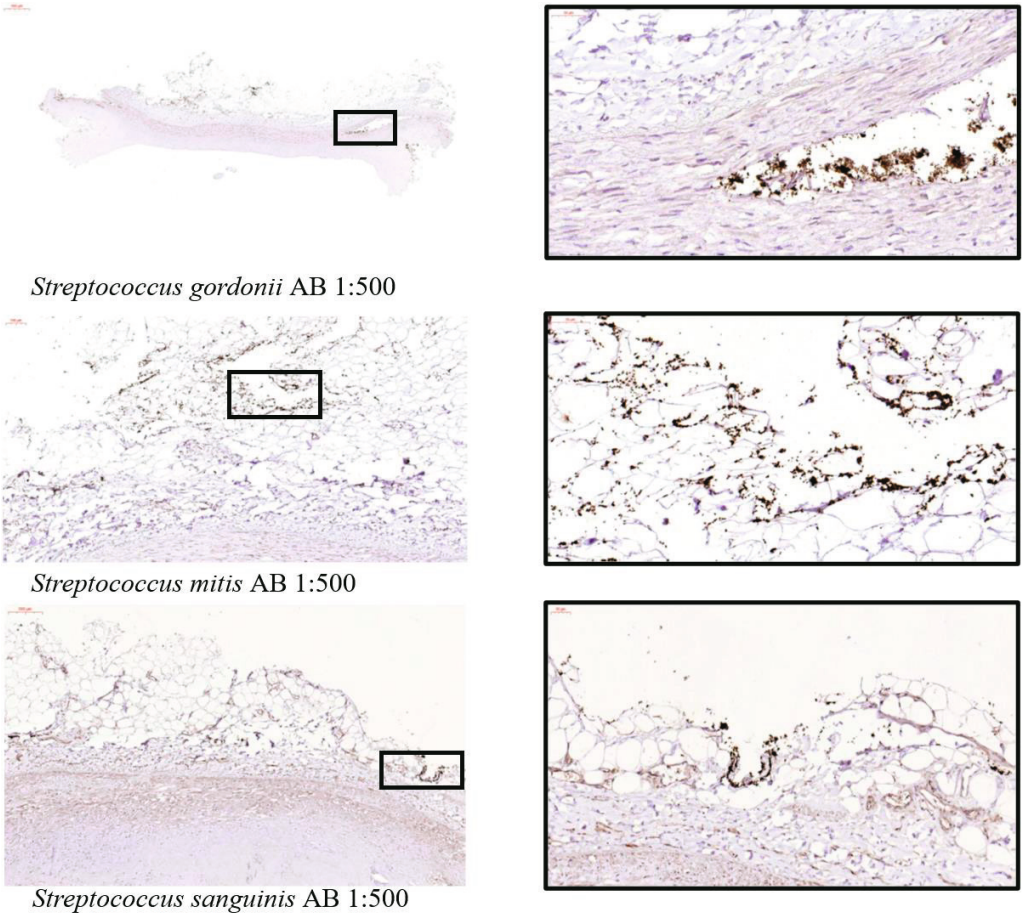


Figure 9. All antibodies stained the corresponding ATCC bacteria intensively with no (*S. gordonii* and *S. mitis*) or weak (*S. sanguinis*) background.

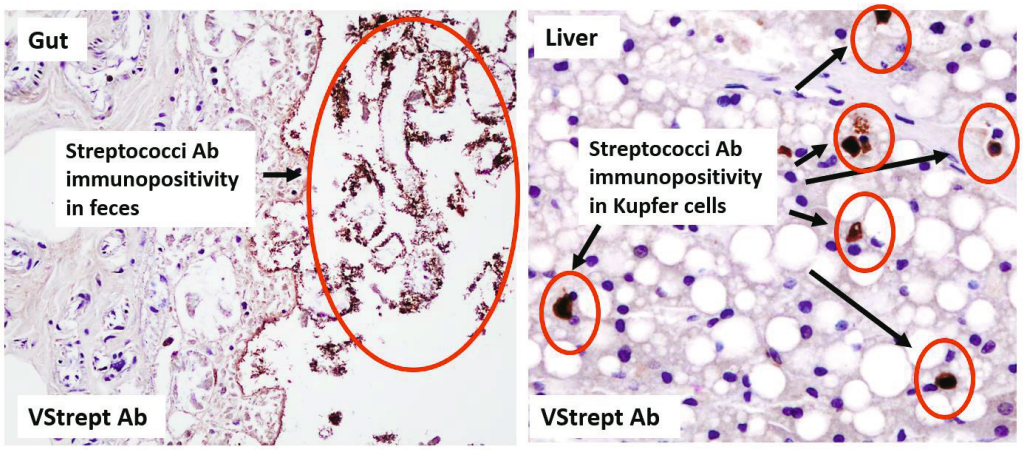


Figure 10. Viridans streptococcal immunostaining revealed masses of streptococci in gut samples, as well as strong and specific positivity in liver Kupfer cells.

4.3 Statistics

Statistical analyses were carried out using IBM SPSS Statistics 28 (Armonk, NY: IBM Corp.). In Study I, associations between the bacterial findings and nominal parameters were analyzed using Pearson's Chi-squared test. Age was treated as normally distributed, and, therefore, mean and sample standard deviation values were calculated, and Student's t-test used. The Mann–Whitney U-test was applied to analyze the associations between bacterial findings and the median arrival time at the hospital, as these values were not normally distributed. In Study II, associations between bacterial DNA findings and nominal dental parameters were analyzed using Pearson's Chi-squared test. Age-adjusted logistic regression analysis was employed to estimate the odds ratio (OR) and 95% confidence interval (CI) for associations between bacterial DNA findings, grade of carotid artery stenosis, and combined dental pathology. The number of teeth was not normally distributed, and, therefore, median values and quartiles (Q1 and Q3) were calculated. In Study III, associations between bacterial immunohistochemistry and sample frequencies were analyzed using the $\chi 2$ test. $P < 0.05$ was considered statistically significant.

4.4 Ethical issues

All participating patients gave their written consent to be included in the study. The TVS study was approved by the Ethics Committee of Pirkanmaa Hospital District (R99204). All clinical investigations were conducted according to the principles of the Declaration of Helsinki. The BMG study was approved by the Ethics Committee of Pirkanmaa Hospital District (R13093), and the study was explained to the patients. In the TSDS study, informed consent was given by relatives. The TSDS study was approved by the Ethics Committee of Pirkanmaa Hospital District (R09097).

5 RESULTS

5.1 Presence of bacterial DNA in thrombus aspirates (Study I)

Characteristics of the study population are presented in Chapter 5.6, in Table 1. For the 75 patients who underwent thrombectomy to treat an acute stroke, 84.0% (n = 63) of their aspirated thrombi were positive for bacterial DNA in qPCR, while 16.0% (n = 12) of the thrombi were bacteria-negative. In addition, 78.7% (n = 59) of the aspirated thrombi were positive for *Streptococcus* spp., mainly the *S. mitis* group. Bacterial DNA of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* was not found in the thrombi. Of the arterial blood samples that were collected during the thrombectomy procedure, 9.33% (n = 7) were positive for both bacteria and *Streptococcus* spp., mainly *S. mitis* group, and 1.33% (n = 1) were positive for both *P. gingivalis* and *A. actinomycetemcomitans*.

The median factor of the presence of *Streptococcus* spp., mainly *S. mitis* group, DNA in the thrombus aspirates was 5.10-fold and that of total bacteria DNA 7.93-fold in comparison to the control blood samples taken from the same patients. The N-fold values for qPCR findings are presented in Figure 11. Patients who were positive for any bacterial DNA were more often males ($p = 0.067$) and more often had diabetes ($p = 0.074$) and a previous cerebrovascular disease ($p = 0.046$) than the patients who tested negative for bacterial DNA. However, there were no differences in the patients' demographic parameters between those positive and negative for *S. mitis* group bacterial DNA. For these data, please see original publication I.

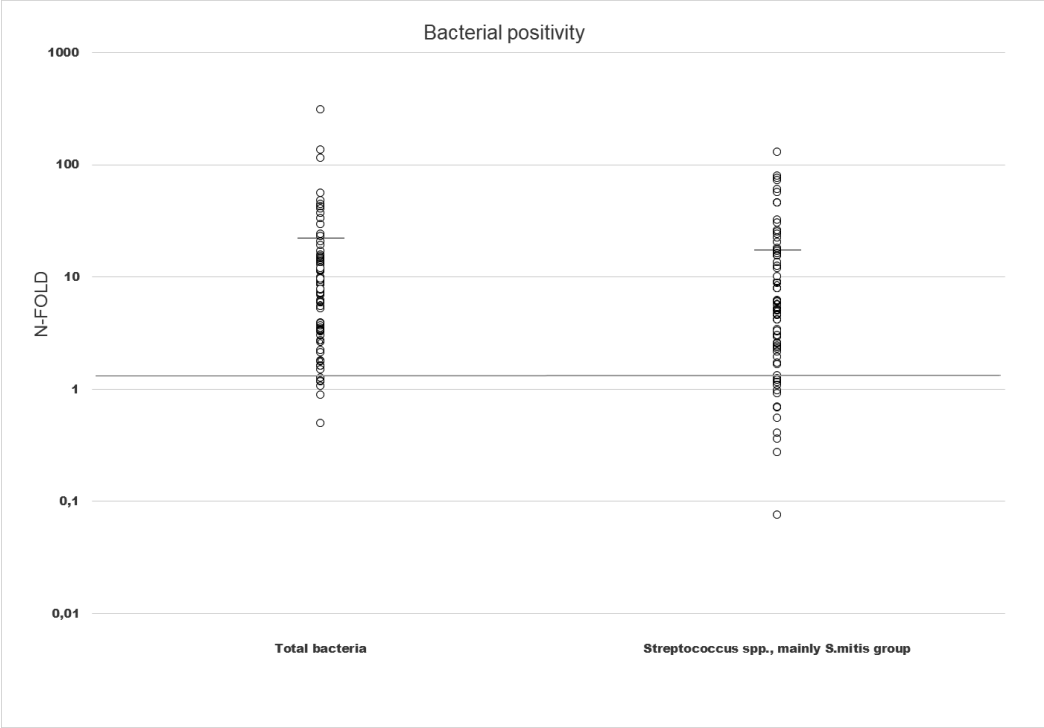


Figure 11. N-fold values of over 2.0 are significantly positive for bacterial DNA. The line at the 2.0 y-axis level indicates that most thrombi collected during thrombectomies of acute stroke patients are positive for bacterial DNA. Median N-fold values are illustrated with black lines.

5.2 Association between dental pathology and bacterial DNA findings in thrombus aspirates (Study II)

The study population comprised 50 (70.4%) men and 21 (29.6%) women. The mean age of the patients was 67.4 years. The participants were the same as in Study I, but four were excluded because their dental pathology could not be assessed due to poor CT reconstructions. Also, seven edentulous patients were excluded from the dental pathology analyses. The characteristics of the study population are detailed in original publication II. Using universal bacterial primers, bacterial DNA was found in 59 (83.1%) of the thrombus aspirates, at a median of 8.6-fold rate compared to the control peripheral blood sample from the same patient. Viridans streptococcal DNA was found in 56 (78.9%) of the thrombus aspirates (median 5.1-fold). Carotid stenosis was found in 54 (81.2%) patients and carotid dissection in 4 (5.6%) patients. Eleven (19.0%) patients had $\geq 50\%$ stenosis and 43 (74.1%) patients $< 50\%$ stenosis.

In general, the patients' oral health was poor. They had an average of 17.3 ± 9.8 teeth (range 0–32). Of the total of 71 patients, periapical lesions were found in 32 (45.1%), vertical bone loss in 20 (28.2%), furcation lesions in 25 (35.2%), caries in 21 (29.6%), and enlarged pericoronary spaces in 3 (4.2%) patients. There was no association between the number of teeth and bacterial DNA counts in thrombus aspirates. Connections between dental pathology and bacterial or oral DNA findings in thrombus aspirates were not statistically significant when the dental variables were treated individually. This data can be seen in original publication II. However, when pathologies were summed up, a linear correlation between oral streptococcal bacterial DNA findings and the condition of the teeth was discovered ($p = 0.032$) in the age-adjusted analysis, whereas this association was not found for total bacterial DNA amounts ($p = 0.197$). In the age-adjusted analysis, patients with better oral health had more oral streptococcal bacterial DNA in their thrombus aspirates than the group with the worst pathology ($p = 0.028$). On average, the group with normal to slight pathology had a 25.8-fold difference, the moderate pathology group a 13.0-fold difference, and the severe pathology group a 5.84-fold difference in the amount of oral streptococcal DNA between their thrombus aspirates and arterial blood samples (Figure 12).

5.3 Association between carotid artery stenosis and bacterial DNA findings in thrombus aspirates (Study II)

There was no association between carotid stenosis and total amounts of bacterial DNA in thrombus aspirates. However, patients with ≥ 50% stenosis had slightly more (18.1- vs 13.9-fold) oral streptococcal DNA in their thrombus aspirates than did patients with < 50% stenosis, but this difference was not statistically significant ($p = 0.578$) in the age-adjusted logistic regression analysis, due to high variation in the DNA folds and the small number of the cases (Figure 13).

5.4 Association between carotid stenosis and dental pathology (Study II)

There was no difference ($p = 0.604$) in the number of teeth between those with ≥ 50% carotid stenosis (18.7 ± 8.9) and those with < 50% stenosis (17.0 ± 10.1). We found that there were more cases (45.5% vs 25.0%) with severe dental pathology among patients with ≥ 50% carotid stenosis than among those with < 50% stenosis. In logistic regression analysis with age and the amount of oral streptococcal DNA as covariates, there was a trend (odds ratio [OR] 7.122, $p = 0.083$) towards an association of ≥ 50% carotid stenosis with more severe dental pathology, but age (OR 1.044, $p = 0.295$) or the amount of streptococcal DNA (OR 1.023, $p = 0.188$) were not significant covariates (Figure 14).

5.5 Oral bacterial DNA in carotid stenosis samples (Study II)

Oral streptococcal DNA (viridans group streptococci, mainly *Streptococcus mitis*) was detected in 2 (33%) of the 6 surgically collected sterile atherosclerotic carotid endarterectomy samples showing advanced atherosclerosis. In one of these cases (17%), DNA from *Porphyromonas gingivalis* was also amplified. Cases that were negative for oral bacterial DNA also showed severe atherosclerosis.

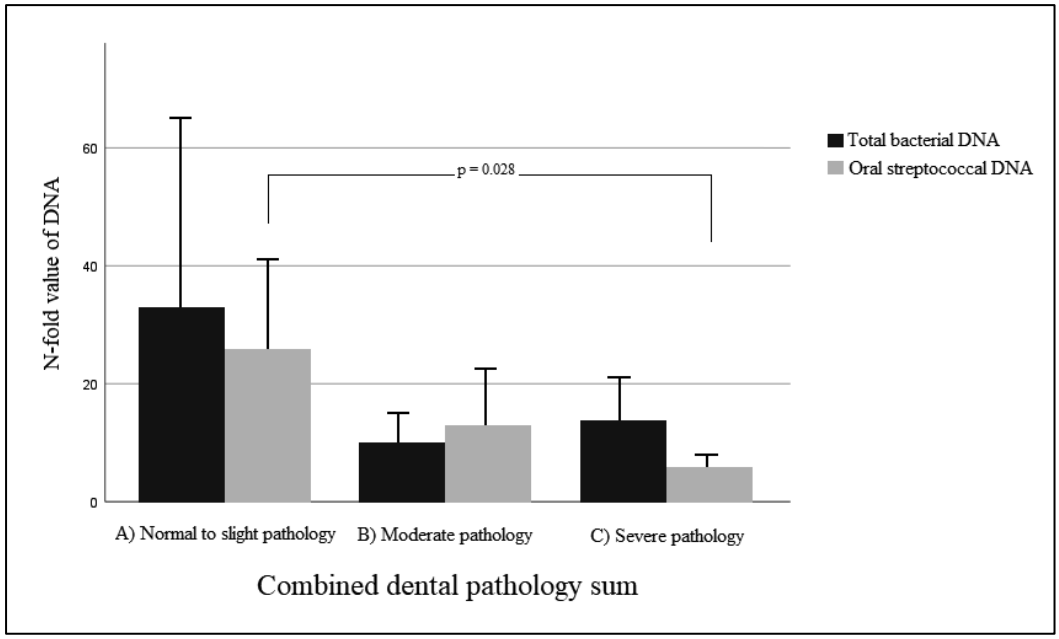


Figure 12. N-fold value of total bacterial (p = 0.197) and oral streptococcal (p = 0.032) DNA between the thrombus and arterial blood samples from the same patient and the relationship with the sum of dental pathologies. A) Normal to slight pathology (n = 21), B) moderate pathology (n = 21), C) severe pathology (n = 22).

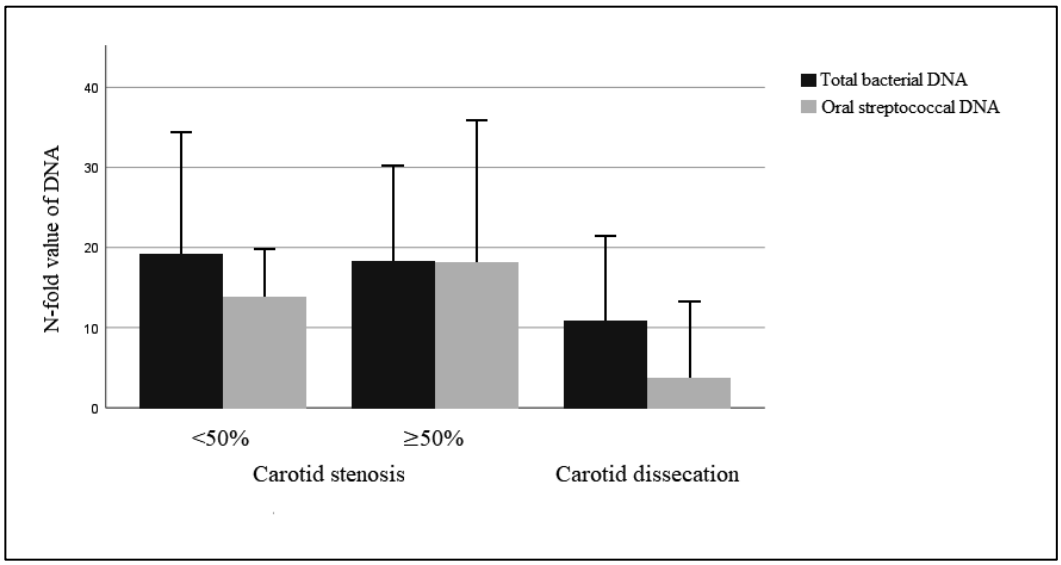


Figure 13. N-fold value of total bacterial DNA (p = 0.983) and oral streptococcal DNA (p = 0.701) between the thrombus and arterial blood samples from the same patient and the relationship with < 50% (n = 43) and ≥ 50% (n = 11) stenosis. DNA amounts were lower in patients with carotid artery dissection (n = 4).

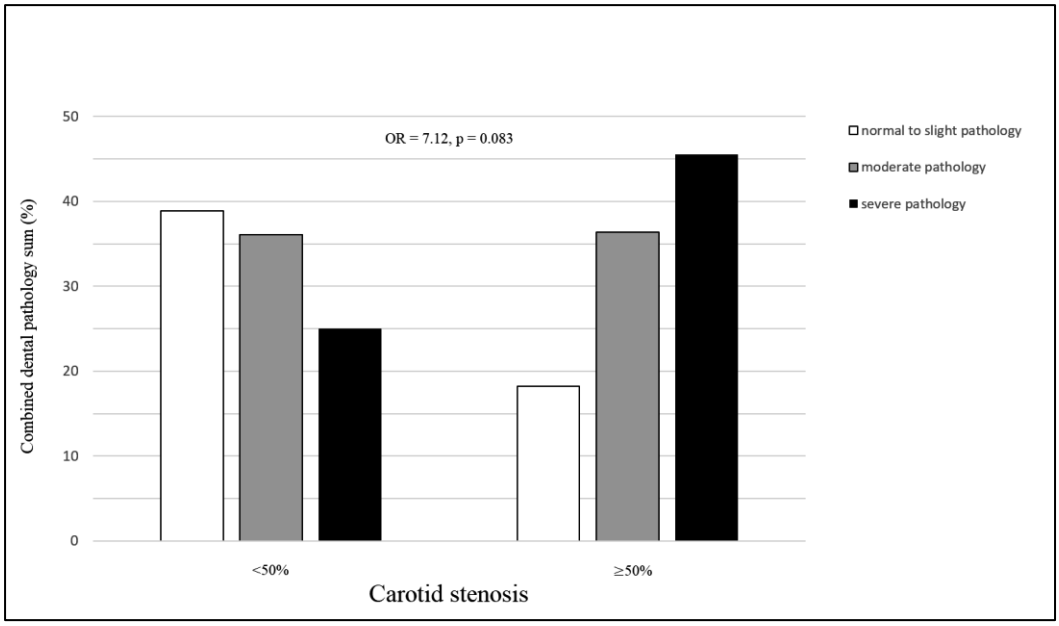


Figure 14. Association between the severity of carotid stenosis and combined dental pathology. In logistic regression analysis with age and the amount of oral streptococcal DNA as covariates, there was a trend (OR 7.122, p=0.083) towards an association of ≥ 50% carotid stenosis with more severe dental pathology.

5.6 Immunohistochemistry results of the three different study series (Study III)

The study protocol is presented in Figure 15. The characteristics of the three different sample series are presented in Table 1. The mean age and sex of the participants did not differ between the three study groups. Cerebrovascular disease was significantly less frequent and coronary heart disease significantly more common in the TSDS series. Patients with symptomatic carotid stenosis in the TVS series were more often smokers compared to the other groups. Hypertension was reported more frequently as a risk factor in symptomatic BMG and TVS series patients compared to the TSDS autopsy series.

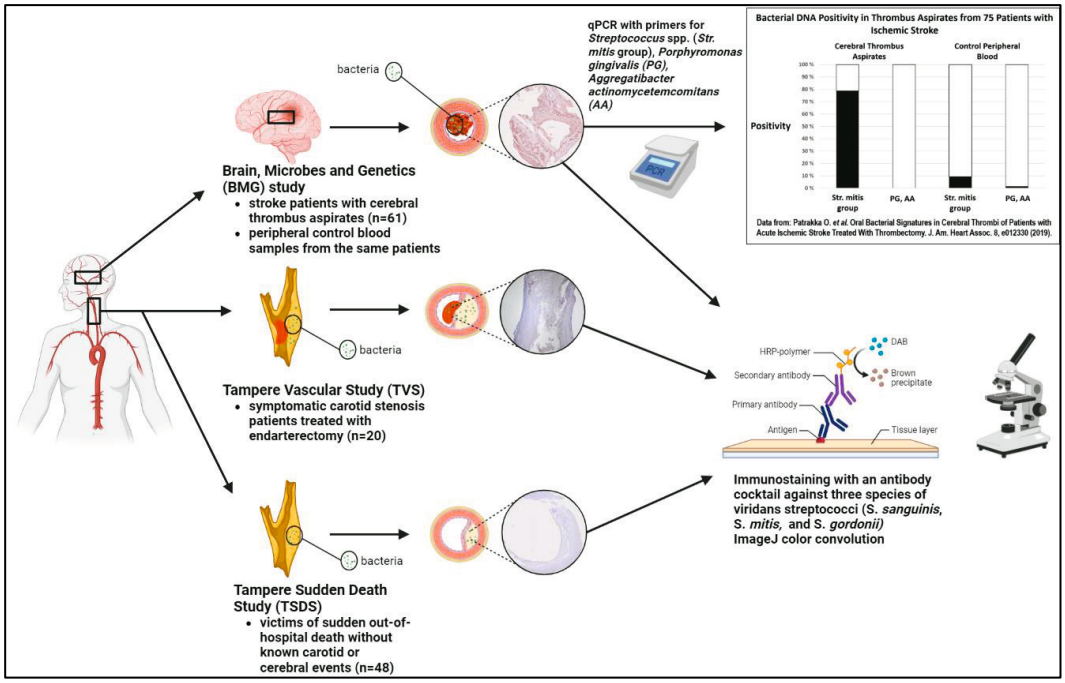


Figure 15. Overview of the study protocol of Study III.

Table 1. Clinical characteristics of the study series.

Characteristics	Brain, Microbes and Genetics (BMG) N=61	Tampere Vascular Study (TVS) N=20	Tampere Sudden Death Study (TSDS) N=48
Age (mean±SD)	66.8±10.9	66.2±8.42	66.4±15.6
Male sex, n (%)	43 (70.5)	13 (65.0)	30 (62.5)
Diabetes, n (%)	11 (18.0)	4 (20.0)	7 (14.6)
Dyslipidemia, n (%)	24 (39.3)	17 (85.0)	N/A
Arterial hypertension, n (%)	31 (50.8)	15 (75.0)	15 (31.3)
Coronary heart disease, n (%)	13 (21.3)	6 (30.0)	26 (54.2)†
Cerebrovascular disease, n (%)	61 (100)	19 (95.0)	2 (4.2)†
Atrial fibrillation, n (%)	39 (63.9)	N/A	N/A
Heart failure, n (%)	9 (14.8)	1 (5.00)	N/A
Smoking status, n (%)	13 (36.1)*	15 (75.0)	18 (37.5)

*Data available from 36 subjects; †Cause of death, N/A = data not available.

As regards the aspirated cerebral artery thrombi of stroke patients, DNA from *Streptococcus* spp., mainly the *S. mitis* group, was found in 78.7% of the cases participating in the BMG study (Figure 15), whereas DNA from *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* was not detected. Of the control arterial blood samples collected during the thrombectomy procedure, 9.33% were positive for *Streptococcus* spp. and 1.33% for both *P. gingivalis* and *A. actinomycetemcomitans*. These findings have been described in detail in Study I.

Out of the 61 thrombus aspirate samples from the stroke patients in the BMG study, 39 (84.8%) were histologically positive (+/++/+++) for viridans streptococci group bacteria (Figure 16). Most streptococci were detected inside neutrophil granulocytes, but there were also remnants of bacterial biofilm as well as free bacterial infiltrates in some samples (Figure 17, A–B). High numbers of neutrophilic granulocytes were present in the samples.

Most (80.0%) carotid endarterectomy samples obtained from the symptomatic patients included in the TVS study were positive for oral streptococci (Figure 16). In contrast, the carotid artery samples from the TSDS study comprising autopsy cases of out-of-hospital deaths, mainly due to coronary heart disease, showed considerably less (31.3%) immunopositivity, with 16.7% of the samples being clearly immunopositive (++/+++). In the TVS endarterectomies and TSDS carotid artery sample series, oral streptococci were found in clusters of streptococcal antibody–positive neutrophilic granulocytes, as well as remnants of bacterial biofilm (Figure 17, E–J). The immunopositivity scores were highest in the BMG thrombus aspirates and TVS carotid endarterectomies ($p < 0.001$; Figure 16).

Staining of the samples with an IgG isotype antibody or *E. coli* antibody resulted in a negligible signal, suggesting the reliability of the viridans streptococcal antibody cocktail staining (Figure 17, C–D).

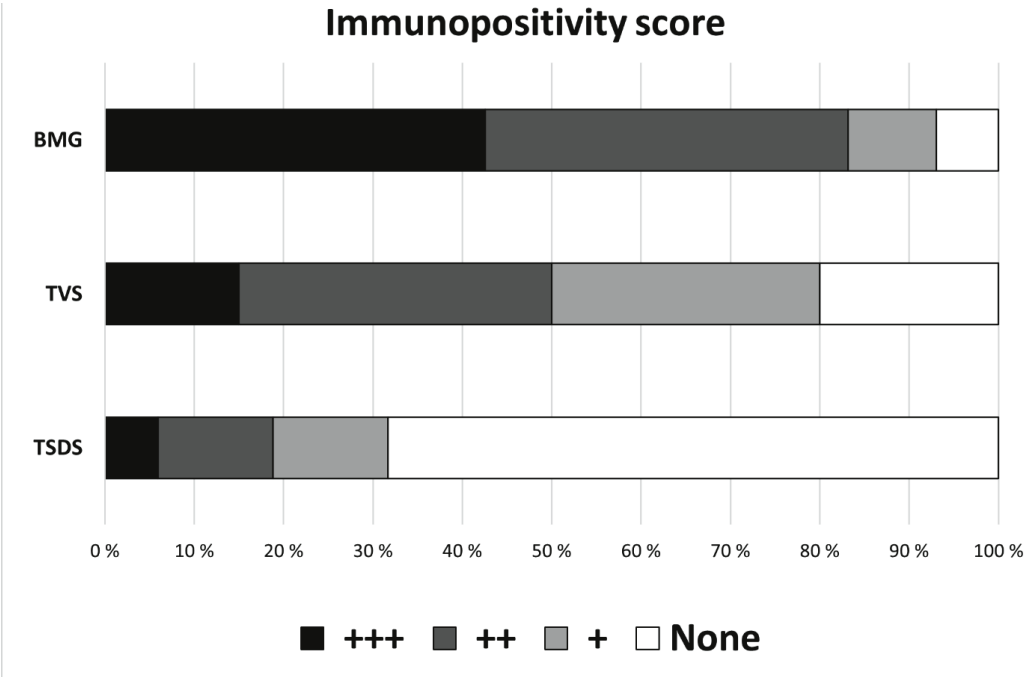
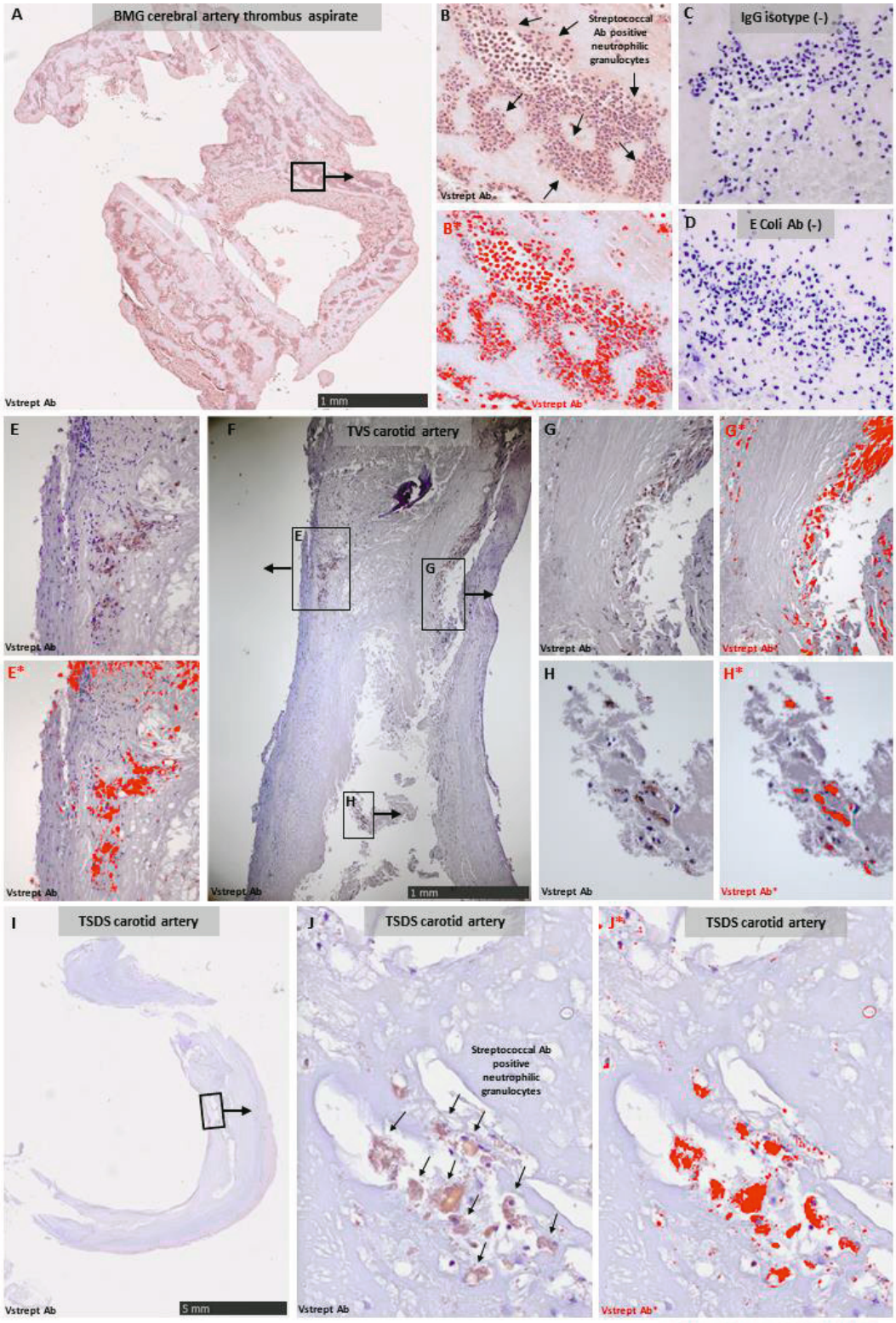


Figure 16. Sample frequencies and a three-step classification of intensity of immunopositivity ($p < 0.001$).



* ImageJ color convolution

Figure 17. Viridans streptococcal immunostaining showing diaminobenzidine (DAB; brown) and color convoluted (red) microscopic photographs of bacterial infiltrates in thrombus aspirates from the BMG series (A–B), ruptured carotid atheroma samples from the TVS study (E–H), and carotid artery samples from the TSDS series (I–J). Thrombus aspirates were also stained with IgG isotype (C) and *E. Coli* (D) antibodies to show the specificity of the bacterial immunostaining.

6 DISCUSSION

6.1 General considerations

We found oral bacterial DNA, mainly viridans streptococcal DNA, in over 4/5 of the thrombus samples collected from acute ischemic stroke patients. DNA of *P. gingivalis* and *A. actinomycetemcomitans* was not found in the thrombus aspirates. In 2014, Fernandes and colleagues studied the presence of oral bacterial DNA in atherosclerotic plaques of 13 patients with carotid stenosis or an aortic aneurysm. They found *S. mutans*, belonging to viridans group streptococci, in 100% of the samples, but all samples were negative for *P. gingivalis*. Most (77%) patients were edentulous. (Fernandes et al., 2014.) In another study, DNA of *P. gingivalis* was found in 54% and *A. actinomycetemcomitans* in 46% of atheromatous plaques of coronary arteries taken from patients with periodontitis, while DNA findings were significantly lower in periodontally healthy individuals (Gaetti-Jardim et al., 2009). It has been found that elderly patients with chronic periodontitis more often have periodontal bacteria in their atheromas than do young patients (Kozarov et al., 2006).

We confirmed the presence of viridans streptococcal bacteria using bacterial immunohistochemistry. Almost all of the thrombus aspirates contained oral streptococci, located mainly inside neutrophilic granulocytes. Remnants of bacterial biofilm were also present. Our study population suffered from poor oral health and had, on average, 17/32 (53%) teeth left. Patients with better oral health had more oral streptococcal DNA in their thrombi than the group with the worst pathology. We also found a trend towards an association of ≥ 50% carotid artery stenosis with more severe dental pathology. In symptomatic patients subjected to carotid endarterectomy, most carotid samples were strongly immunopositive for viridans group streptococci. We found strong streptococcal immunopositivity in 17% of the carotid artery samples obtained from non-stroke autopsy cases, representing a cross-section of the population. To our knowledge, only one study has previously reported the presence of viridans group streptococci in carotid artery endarterectomies using immunolocalization (Chiu, 1999), but the presence of viridans group streptococci in cerebral thrombi has not been shown previously. Our results suggest that there may

be a connection between symptomatic inflammation in the carotid arteries and a cerebral thrombus, and that oral pathologies may be related to it.

6.2 How do bacteria end up in an atherosclerotic plaque?

The most frequently found bacteria in cerebral thrombus aspirates in our study, namely viridans group streptococci, are common oral bacteria associated with the development of dental plaque (Kolenbrander et al., 2010). Oral bacteria can enter the bloodstream after dental procedures—for example, root canal treatment, tooth extraction, or daily tooth brushing—causing transient bacteremia. In tooth extraction, most of the bacteria translocated into the circulation have been found to be viridans streptococci (Lockhart et al., 2008; Narayanan & Vaishnavi, 2010). Amoxicillin medication in connection with tooth extraction decreased the frequency of positive streptococci findings in the patients' peripheral blood samples (Lockhart et al., 2008). Furthermore, it has been found that small amounts of bacteria can be found in the systemic circulation without any clinical symptoms (Whittle et al., 2019). Chronic periodontitis begins as a gingivitis in the soft tissues resulting in inflammation. This is caused by a resident biofilm that forms on the tooth surfaces at the gingival margin. If left untreated, this leads to the damage of connective tissue, periodontal ligament, and bone. Gingival ulceration increases the risk for bacteremia. (Chhibber-Goel et al., 2016.) After accessing systemic circulation, bacteria might end up in an atherosclerotic lesion via a circulating monocyte or directly through neovasculature developing inside the carotid atherosclerotic plaque (Camaré et al., 2017).

6.3 Biofilm-related theory: is it applicable to atherosclerotic plaques?

Oral bacteria are known to form biofilms on hard tooth surfaces but also on soft epithelial tissue (Kolenbrander et al., 2010; Huang, Li & Gregory, 2011), as discussed in Chapter 2.5.2. Viridans streptococci are the initial colonizers in the development of a dental plaque (Kolenbrander et al., 2010). Streptococci and other oral bacteria produce autoinducer 2 (AI2), which is a key molecule in the establishment of a multispecies biofilm (Cuadra-Saenz et al., 2012). It is suggested that bacteria also form biofilms in the atherosclerotic plaque (Snow et al., 2016). In 2012, Wolcott and

colleagues studied the presence of bacterial DNA in atherosclerotic plaques using qPCR. They identified over 100 unique taxonomic signatures, with DNA from *Streptococcus* spp. being among the most predominant. They observed a heterogeneous distribution of bacteria in the plaque samples, similar to the spatial heterogeneity seen in other biofilm infections, such as wounds. This suggests the presence of a biofilm phenotype infection in the plaques. (D. Wolcott, J. Wolcott, & Palacio, 2012.) This could contribute to the persistent inflammation due to atherosclerosis. It is suggested that biofilm structure remains stable if the external circumstances do not change. A study by Lanter and colleagues demonstrated *in vitro* that a biofilm may disperse when challenged with norepinephrine in the presence of transferrin. The biofilm dispersion and the release of free bacteria and degradative enzymes secreted by the bacteria could influence the integrity of the surrounding arterial tissues, leading to an increased risk of plaque rupture. (Lanter et al., 2014.)

Atherosclerotic plaques contain calcium hydroxyapatite and carbonate apatite that contribute to the calcification of the plaque, which happens frequently in advanced atherosclerosis. The molecular mechanisms behind the calcification process are not yet fully understood. The calcified atherosclerotic arterial plaque is histomorphologically indistinguishable from bone tissue, and the calcification process shares similarities with osteogenesis in bone with added chronic inflammation. Oksala and colleagues demonstrated in 2010 that there is on-going osteoclastogenesis in human atherosclerotic plaques, which leads to the calcification (Oksala et al., 2010). This process is similar to the formation of dental calculus on the surface of the teeth. It may be that the bacterial biofilm inside an atherosclerotic plaque causes inflammation that may take part in the calcification process. In addition, some animal model studies have shown that repetitive inoculation with oral bacteria that participate in biofilm formation (*S. sanguinis*) leads to large calcifications of the aortic valve. In the same study, groups inoculated with normal flora bacteria *Corynebacterium matruchotii* had no evidence of calcification. (Akcalı & Lang, 2018; Cohen et al., 2004.)

Biofilm-phenotype bacteria protected by an extracellular matrix are much more resistant to antibiotics than the same bacteria in circulating planktonic form. This might be one reason why antimicrobial treatment for *Chlamydia pneumoniae* in patients with coronary heart disease is ineffective (Andraws, Berger & Brown, 2005). The originally chosen antimicrobial treatment against *C. pneumoniae* was clarithromycin. Clarithromycin has an effect against *C. pneumoniae*, but it is not the adequate treatment against periodontal bacteria. It is hypothesized that *C. pneumoniae* might have been the wrong suspect behind the infectious origin of atherosclerosis and that

periodontitis is the culprit behind the failure of previous antimicrobial treatments to prevent cardiovascular events and stroke. Further trials to evaluate the benefit of periodontal treatment in prevention of stroke are needed. (Paju et al., 2007.)

6.4 Poor oral health as a risk factor for stroke

In Finland, dental infections are common, with the prevalence of caries being almost 100%, that of severe periodontitis 15%–20%, and that of periapical lesions up to 27% (Suominen-Taipale et al., 2004). Periodontitis and stroke share several risk factors, and the diseases often coexist, which is why the subject is challenging to study. People with good oral health are often healthier overall and have fewer unhealthy habits that affect the progression of atherosclerosis. Still, the causality is gaining more and more evidence, as noted previously. According to recent studies, periodontitis and tooth loss have an independent direct association with stroke (Pillai et al., 2018; Virtanen et al., 2017). Regular dental care lowers the risk of ischemic stroke (hazard ratio 0.77, 95% confidence interval 0.63–0.94) (Sen et al., 2018). Fluoride in drinking water prevents dental disease, which in turn has been suggested to lower the risk of coronary heart disease. This was studied in Finland by Kaipio and colleagues from 1961 to 1995, and it was stated that the geographical pattern of coronary heart disease was consistent with the concentration of fluoride in drinking water. Mortality from coronary heart disease was the highest in the north-east Finland, where the fluoride content of drinking water was low among other many potential risk factors for coronary heart disease. Later, the widespread use of fluoridated toothpastes, soft drinks, and certain food items have reduced the significance of drinking water as a source of fluoride. (Kaipio, Näyhä, & Valtonen, 2004.)

After the antimicrobial trials against *C. pneumoniae* failed in the prevention of cardiovascular diseases, the interest in treating chronic bacterial infections to reduce cardiovascular diseases was diminished. Now, as the association between dental pathologies, oral bacteria, and acute ischemic stroke has been shown in multiple studies, the subject is again gaining interest. Still, to the best known, there is only one clinical trial registered concerning the subject, namely the PREMIERS study. Registered in 2015, it is a multicenter randomized controlled trial that aims to test whether intensive periodontal treatment reduces the risk of recurrent vascular events among ischemic stroke and TIA survivors in comparison with standard periodontal treatment. However, the phase II trial found no significant difference between the

two treatments, but larger study groups will be enrolled for phase III, which will also have a longer follow-up. (Redd et al., 2019; Sen et al., 2023.) In 2019, Lin and colleagues published a 14-year retrospective population-based cohort study observing the relationship between periodontitis with and without specific treatment and the incidence of ischemic stroke. Their study demonstrated that dental scaling and the intensive treatment of periodontal disease could possibly help to reduce the risk of developing stroke. Also, patients who underwent tooth extraction therapy had a higher risk of stroke. (Lin et al., 2019.) On the other hand, intensive periodontal therapy induces a moderately more acute systemic inflammatory response and is associated with endothelial cell activation. This is likely attributed to the fact that intensive periodontal therapy is often performed to patients with severe periodontitis. The release of cytokine tumor necrosis factor alpha (TNF-α) into serum has been associated with the severity of periodontal disease. (Francesco D'Aiuto, Parkar, & Tonetti, 2007.) This was later studied in a clinical self-controlled case series. The results were that the rate of ischemic stroke and myocardial infarction was increased in the first four weeks after invasive dental treatment. The association remained positive after the exclusion of patients with classical risk factors for vascular diseases. The incidence of vascular events gradually returned to the baseline rate within six months. Nevertheless, the long-term benefits of invasive dental treatment as regards vascular health will probably outweigh the short-term adverse effects. (Minassian et al., 2010.)

Tooth loss is most often caused by oral diseases. Untreated dental and periodontal infections will eventually result in intentional or spontaneous tooth loss. Periodontal disease is considered to be the main cause of tooth loss in adults worldwide (Kinane, Stathopoulou & Papapanou, 2017). In our Study II, 60,6% of the patients suffered from a moderate or severe dental pathology and had an average of 17.3 (±9.8 SD) remaining teeth. The number of teeth is similar to the numbers in a Finnish national survey, where the average number of teeth was 17.0 in individuals aged 65–74 years, while the average number of remaining teeth in a Swedish population of the same age was 26.0 (Koskinen, Lundqvist & Ristiluoma, 2012; Ljung et al., 2019). Although the number of dentists does not differ significantly between Sweden and Finland, the practice of preferring tooth extraction instead of the treatment of caries applied in Finland may explain the differences in the numbers of teeth preserved (Suominen-Taipale et al., 2004). Interestingly, stroke prevalence in Finland (395.9 strokes per 100,000 inhabitants) is higher compared to Sweden (368.6 strokes per 100,000 inhabitants), even though the countries share a similar

economic and social structure, as well as a similar health care system (Kristiansen, 2000; Wafa et al., 2020).

Our findings on the association between poor oral health and low bacterial DNA findings in the thrombus aspirates of the same patients might be explained by studies reporting that the longer the periodontal disease exposure, the fewer focal infection focuses there are present. With only a few teeth left in the oral cavity as a result of long-lasting periodontal disease, the infectious pathway is closing and the continuous flow of oral bacteria through the root canal decreases (Desvarieux et al., 2003). On the other hand, if tooth loss is caused during the early years of life or is due to other factors, such as caries or trauma, the patients might be affected by periodontal disease for the rest of their life (Shahi et al., 2022). Several previous studies have shown the positive association between ischemic stroke and tooth loss, especially in younger age groups (Syrjänen et al., 1989; Wu et al., 2000; Joshipura et al., 2003; Grau, Becher, et al., 2004; Pihlstrom & Michalowicz, 2018). In our Study II, where the mean age of the patients was 67 years, we found no association between tooth loss and bacterial DNA counts in thrombus aspirates. Among elderly people, several studies have demonstrated no connection between tooth loss and atherosclerotic vascular diseases (Beck et al., 1996; Grau, Becher, et al., 2004; Syrjälä et al., 2009). In contrast, Heitmann and colleagues concluded that edentulous patients had a threefold increased risk of developing stroke compared to those with most of their teeth remaining. Interestingly, the risk of coronary heart disease was not significantly increased. (Heitmann & Gamborg, 2008.)

6.4.1 Oral infections and the risk of ischemic stroke—mechanism of action

The decline in stroke incidence during the 20th century can only incompletely be explained by conventional risk factors and their temporal trends (Lindsberg & Grau, 2003; Grau, Urbanek & Palm, 2010). As noted, it has been long suspected that bacterial infection may play a role in the pathogenesis of stroke, with infections of oral origin being studied extensively (Grau, Becher, et al., 2004; Joshipura et al., 2003; Morré et al., 2000; Sen et al., 2018; Syrjänen, 1993; Valtonen, 1999). Increased levels of antibodies against several bacteria, such as streptococci, staphylococci, and enterobacteria, were found in 44% of young stroke patients, but in only 9% of controls (Syrjänen et al., 1986). The peripheral blood neutrophil count is an independent predictor of stroke severity upon admission, a greater degree of disability at discharge, and 30-day mortality (Furlan et al., 2014; Grau, Boddy, et al.,

2004). A severe chronic dental infection was found to predispose to stroke in males, possibly by affecting blood coagulation and platelet function (Syrjänen et al., 1989).

Periodontitis has been shown to elevate the overall infectious burden in a generally healthy population, apical periodontitis with a dental root abscess is suggested to be associated with increased levels of systemic inflammation, and chronic low-grade oral infection and inflammation have been related to unfavorable systemic cardiovascular effects (D'Aiuto et al., 2005; Gomes et al., 2013). Poor oral health may contribute to the progression of the atherosclerotic lesions via circulating chemical mediators (DeStefano et al., 1993; Danesh et al., 2000; Cotti et al., 2010).

Bacterial surface proteins of viridans streptococci can directly bind to various platelet receptors and activate them (Bensing, Rubens & Sullam, 2001; Kerrigan & Cox, 2009). Activated platelets induce the recruitment of proatherosclerotic cells such as monocytes and lymphocytes, which speeds up the atherosclerotic processes (Herzberg et al., 2005; Arman et al., 2014; Tomaiuolo, Brass & Stalker, 2017). There can also be indirect activation via plasma immunoglobulin G (Naik, 2014). Oral streptococci may initiate or contribute to platelet aggregation in the coronaries (Herzberg et al., 2005). In 2002, Okahashi and colleagues found that *S. sanguinis* can induce foam cell formation of macrophages, therefore promoting the earliest events of atherogenesis (Okahashi et al., 2011). Moreover, viridans group streptococci are known to stimulate endothelial cells to produce various proinflammatory cytokines, such as IL-6 and IFN-γ. Elevated cytokine levels and systemic inflammation are related to the initiation, development and rupture of an atherosclerotic plaque. (Chia et al., 2002; Hahn, Best, & Tew, 2000; Ramji & Davies, 2015.) Odontogenic *P. gingivalis* can secrete gingipains proteinase, which persuades an increase in platelets intracellular [Ca^{2+}] and leads to the activation and aggregation of platelets (Klarström Engström et al., 2015; Lourbakos et al., 2001). Oral pathogens may promote the development and rupture of the plaque through cytokine activation and inflammation responses (Figure 19) (Chia et al., 2002; Hahn et al., 2000; Ramji & Davies, 2015). Furthermore, following bacteremia, a bacteria-induced thrombus might be trapped in the left atrial appendage of the heart, causing a cardioembolic stroke in patients with altered hemodynamics, such as in atrial fibrillation (Herzberg et al., 2005; Langer & Gawaz, 2008). In our BMG series, 64% of the patients had atrial fibrillation and this mechanism can account for part of the bacterial findings in thrombi. Despite this, however, 62% of the cases were considered to be due atherosclerosis, suggesting uncertainty in determining the etiology.

6.4.2 Systematic reviews on the connection between periodontal disease and stroke

Several reviews have been published on the connection between periodontal disease and stroke. Figure 18 demonstrates the currently held concept of the relationship. However, most of the reviews are based on epidemiological and cohort studies.

In 2012, Lafon and colleagues published a systematic review comprising nine cohort studies with results demonstrating that the risk of stroke was significantly increased by the presence of periodontitis (relative risk 1.63, 95% CI 1.25–2.00). Tooth loss was also reported to be a risk factor for stroke (relative risk 1.39, 95% CI 1.13–1.65). Gingivitis was not reported as a significant risk factor. Pillai and colleagues reported similar findings in their systematic review in 2018 with 30 studies included. (Lafon et al., 2014; Pillai et al., 2018.)

An association between stroke and oral diseases has been established, but the causality is more difficult to determine. Stroke patients generally have poorer oral hygiene practices and oral health. The American Heart Association stated in their systematic review published in 2012 that observational studies do not support a causative relationship between periodontal diseases and atherosclerotic disease (Lockhart et al., 2012). The lack of causative relationship has been proposed to be due to different reasons, such as tooth extraction for other reasons besides periodontal disease, a change in diet after tooth loss, selection bias, as well as biological and behavioral factors (Johansson et al., 1994; Joshipura, Douglass & Willett, 1998).

A meta-analysis published in 2020, comprising nine studies, stated that there is an association between tooth loss and stroke, but the included studies had a risk of bias and the presented evidence was of low statistical power. The authors concluded that the causality is difficult to investigate but also suggested screening for stroke in edentulous patients. (Fagundes et al., 2020.)

Sanz and colleagues published a consensus report in 2020 organized by the European Federation of Periodontology and the World Heart Federation. In summary, it was stated that the progression of cardiovascular diseases and stroke may be influenced by successful periodontal treatment independently of traditional cardiovascular risk factor management. Furthermore, it was concluded that it is unlikely that cardiovascular disease is a risk factor for periodontitis. (Sanz et al., 2020.)

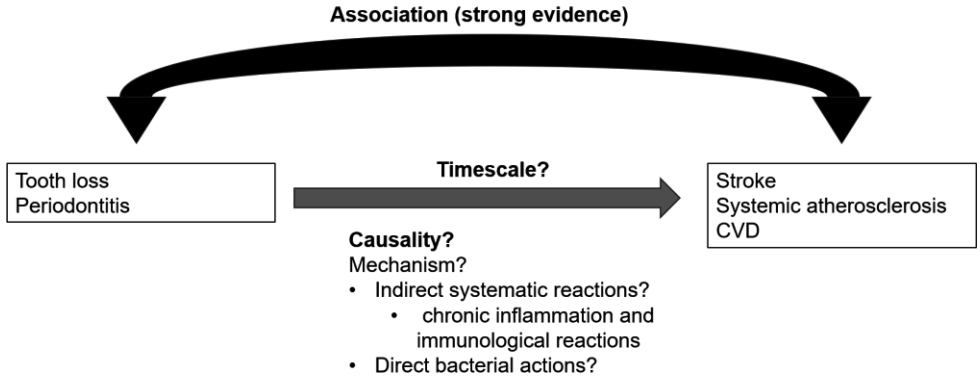


Figure 18. Relationship of chronic oral diseases with cardiovascular diseases (CVD) and stroke.

6.4.3 Immunological similarities between oxLDL and Streptococci

Both oxidized LDL and bacteria activate TLR-mediated pathways in macrophages and neutrophilic leukocytes, leading to the elevation of cytokine levels and systemic inflammation, which are related to the initiation, development, and rupture of an atherosclerotic plaque (Chia et al., 2002; Curtiss & Tobias, 2009; Hahn et al., 2000; Hajishengallis et al., 2002; Kurita-Ochiai et al., 2015; Ramji & Davies, 2015). TLRs are mainly expressed on first-line immune cells, such as macrophages, neutrophils, and dendritic cells. It has been found that especially TLR2 and TLR4 are expressed in high numbers on macrophages in human atherosclerotic lesions. Macrophages lacking TLR4 are protected against cholesterol intake and foam cell formation. In LDL receptor knockout mice that were administered TLR2 agonists, atherosclerosis was dramatically increased. In addition to oxLDL intake, both gram-negative and gram-positive bacteria act as ligands to TLR2 and TLR4. (Miller, 2005; Singh et al., 2020.) Also, scavenger receptor CD36 functions as a phagocytic receptor for a variety of bacteria in addition to oxLDL (Kunjathoor et al., 2002).

Oxidized or otherwise modified LDL is highly immunogenic, as discussed by Binder and colleagues (Binder et al., 2003). OxLDL contains a variety of oxidation-specific neoepitopes that are recognized by the innate and adaptive immune system. Receptors of macrophages, such as scavenger receptors A (SR-A) and CD36, bind to oxidation-specific ligands, including phosphorylcholine-containing oxidized

phospholipids, and promote an unregulated uptake of oxLDL. It is believed that LDL and viable cells contain an epitope known as phosphorylcholine that is revealed by oxidation or when the cells undergo apoptosis. Phosphorylcholine, also known as phosphocholine (PCho), is the hydrophilic polar head group of some phospholipids. It exists in all eukaryotes, ranging from yeast to humans, including *Streptococcus* spp. This epitope is interesting, as *in vitro* studies have suggested molecular mimicry between phosphorylcholine epitopes of oxidized phospholipids of oxLDL and the phosphorylcholine of common microbial pathogens. Indeed, pneumococcal immunization of mice decreased the extent of atherosclerosis. (Binder et al., 2003.) Moreover, it has been shown that plasma IgA antibodies against both PCho and streptococci cell wall polysaccharide are significant predictors of the long-term risk of cardiovascular diseases (Kankaanpää et al., 2018). The immune responses that have evolved to combat bacterial infections are similar as those involved in the immune response to the inflammatory components of atherogenesis (Figure 19) (Binder et al., 2002).

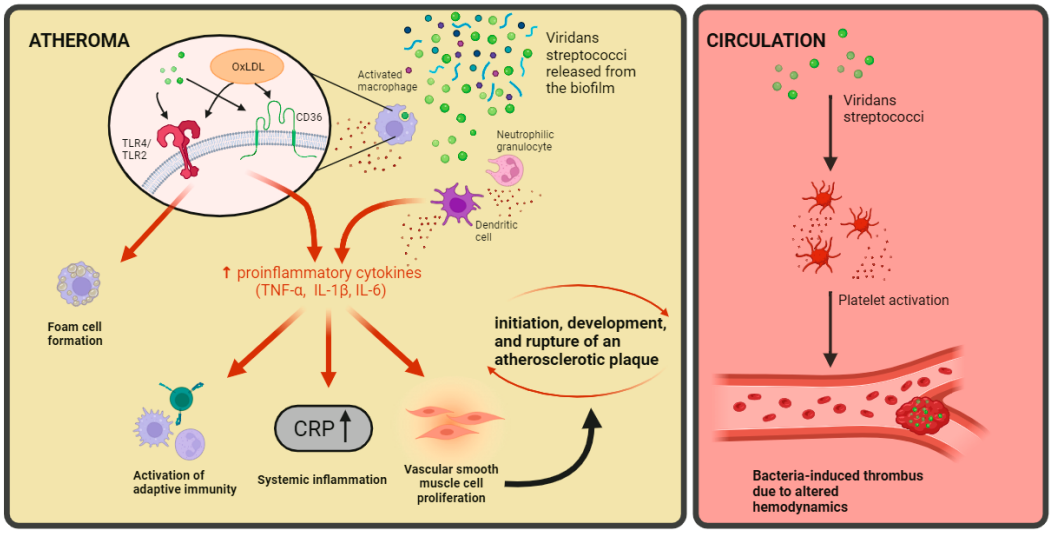


Figure 19. Schematic view of the possible relationship between the pathogenesis of atherosclerosis and oral streptococci. TLR = Toll-like receptor, oxLDL = oxidized LDL, CD36 = a scavenger receptor. Created with BioRender.com.

6.5 Carotid stenosis and oral pathology

We found streptococcal DNA in 33% of the carotid endarterectomy samples from surgical patients suffering from peripheral artery disease. Using immunohistochemistry, we found that 80% of the carotid endarterectomy samples of symptomatic patients were immunopositive, whereas the samples taken from asymptomatic patients were more often immunonegative (31%). Previously, the presence of odontogenic bacteria, such as viridans group streptococci (*S. sanguinis*) and *P. gingivalis*, has been demonstrated in atherosclerotic plaques of human carotid arteries histologically and by means of polymerase chain reaction (Chiu, 1999; Haraszthy et al., 2000). Previous infections or chronic exposure to common infective agents are suggested to contribute to carotid atherosclerosis (Elkind et al., 2010). Oral infections seem to contribute to increased carotid artery intima-media thickness, leading to subclinical atherosclerosis (Pussinen et al., 2019). A significant association has been reported between the degree of tooth loss and the prevalence of carotid artery plaques (Desvarieux et al., 2003). It has been proposed that bacteria present in carotid artery plaques contribute to an enhanced risk of plaque rupture, leading to thrombosis (Lanter, Sauer & Davies, 2014). Viridans group streptococci have been found to be the most common gram-positive bacteria persisting after intracanal disinfection procedures and after root canal treatment (Narayanan & Vaishnavi, 2010). Koren and colleagues showed that an abundance of *Veillonella* sp. and *Streptococcus* sp. in the oral cavity was linked to their abundance in carotid atherosclerotic plaques (Koren et al., 2010).

We found that ≥ 50% carotid stenosis is related to more severe dental pathology. Our findings are in line with previous studies (Desvarieux et al., 2003; Schillinger et al., 2006; Kamak, Yildirim & Rencber, 2015). Our patients with ≥ 50% stenosis had slightly more oral streptococcal DNA in their aspirated thrombi compared to those with < 50% stenosis. We may hypothesize that the oral bacteria found in thrombi originate from ruptured carotid artery plaques (see Figure 17, E–H), as oral bacterial inflammation may be related to the development of the atherosclerotic plaque via the inflammatory mechanism in the arterial wall and through cytokine activation (Hahn, Best & Tew, 2000; Chia et al., 2002; Bartova et al., 2014; Ramji & Davies, 2015). We also demonstrated that a high number of neutrophils can be detected in the carotid artery wall when oral bacteria are present. A high neutrophil count is associated with histopathologic features of rupture-prone carotid atherosclerotic lesions and can be found at the rupture-site of a plaque (Ionita et al., 2010; Soehnlein, 2012). A high peripheral blood leukocyte count associates with stroke severity and

outcome (Furlan et al., 2014; Grau, Boddy, et al., 2004). It can be hypothesized that both bacteria and neutrophils contribute to the inflammation and the induction of cytokine release, in addition to the inflammation related to oxLDL and atheromatous debris inside the plaque. Therefore, neutrophils might be recruited by the bacteria inside the plaque and end up being released from the ruptured atherosclerotic carotid plaque into the circulation. This might be one mechanism of how viridans streptococci are involved in acute ischemic stroke.

6.6 Strengths and limitations

Our study has some strengths. We had the opportunity to study unique and clinical patient material. We had antemortem and postmortem samples from different series. In the BMG study, we did not sort the patient material, which means that the risk for selection bias is low. As mechanical thrombectomy is performed as urgent care, we had patients from across Finland and not only from one region. Both quantitative PCR and immunohistochemistry are very sensitive, and when used with positive and negative controls, the risk of false positivity or negativity is low. All samples were treated with the same reagents and DNA extraction kit in the same laboratory, according to the same protocol.

One of the limitations of Study I is its small sample size, which prevents further subgroup analyses. The exact impact of living bacteria on thrombosis cannot be declared using PCR. In addition, the PCR method we used detects the presence of bacterial DNA in the examined samples but is unable to separate living bacteria from phagocytized bacterial DNA. Culturing and staining are the most frequently performed techniques when detecting bacterial species. Nonetheless, the PCR method seems to be more accurate and cost-effective in comparison with traditional culturing. (Aly et al., 2012; Atieh, 2008.) Although our analysis revealed the absence of DNA from *P. gingivalis* and *A. actinomycetemcomitans* in the thrombi, it does not exclude the possibility of their role in the pathogenesis of acute ischemic stroke. The presence of bacterial DNA was defined by an artificial cutoff value: the sample was considered positive if it contained 2 times more bacterial DNA compared to the control sample from the same patient. The samples contained a median of ≈5 times more *S. mitis* DNA than the control samples. However, the validity is also affected by the inhomogeneity of the thrombus material (De Meyer et al., 2017; Muñoz et al., 2018). In Study II, one of the limitations was that the grade of carotid artery stenosis was measured using either computerized tomographic angiography or Doppler

ultrasound, the concordance of which is 79% (Titi et al., 2007). Tooth conditions were estimated from CT reconstructions. Dental and periodontal disease can be recognized in dental CT, but it is not as accurate as the gold standard cone-beam computed tomography (Steinklein & Nguyen, 2013; Lechuga & Weidlich, 2016). The limitations of Study III include the fact that we used three different sets of patient samples and that the endarterectomy samples and thrombus aspirate samples were taken from different patients. We only used an immunohistochemistry antibody cocktail against viridans streptococci species, and it is possible that other bacteria were also involved in the pathogenesis of thrombosis.

6.7 Future directions

As we have demonstrated, the causal relationship between oral pathologies and acute ischemic stroke seems complicated to demonstrate and the time scale between these two factors is hard to determine. This transversal study is a hypothesis-generating study and cannot demonstrate any etiological relation. In the future, it would be interesting to collect endarterectomy and cerebral thrombus samples from the same patients and see whether there are differences in the bacterial findings. In addition, next-generation sequencing performed on the samples would reveal more information about the localization of other bacterial DNA in the plaque.

It is challenging to establish a proper and ethical clinical intervention investigating the relationship between periodontitis and acute ischemic stroke. All patients with active periodontitis need treatment, and it is therefore unethical to randomize study participants into groups receiving or not receiving active treatment. The formation of a bacterial biofilm inside the artery wall probably happens over decades. The progression of biofilm formation seems to be initiated by oral streptococci. Vaccination against oral streptococci could theoretically reduce the risk of the formation of a bacterial biofilm and thrombosis. Secondly, the administration of penicillin during the acute treatment of a large vessel occlusion might be worth considering. During atherosclerotic plaque rupture, the pathway to the plaque is open, allowing antimicrobial agents to affect the bacteria within the biofilm and, consequently, eliminate the biofilm. Thirdly, it would be interesting to study whether mechanical dental care, such as flossing in addition to regular tooth brushing, to break the anaerobic polymicrobial community of periodontal bacteria would be beneficial in the prevention of stroke and other cardiovascular diseases. Finally, invasive dental procedures, such as root canal treatment, dental extraction, and

calculus removal, should be considered to be performed under the cover of antibiotics, such as penicillin or amoxicillin, to counteract the consequences of procedure-related bacteremia in selected patient groups.

7 CONCLUSIONS

The mechanism behind the association between ischemic stroke and infections has remained uncertain. We demonstrated that large amounts of oral streptococci can be found in the aspirated thrombi of acute ischemic stroke patients, as well as in carotid artery plaque samples taken from symptomatic patients, using both bacterial qPCR and immunohistochemistry. Oral streptococci are living organisms affecting the human metabolism and immune system. Immune responses that have evolved to combat bacterial infections are shared with those involved in the immune response to the inflammatory components of atherogenesis.

Our results suggest that, along with the classical known risk factors, oral streptococcal inflammation may play an important role in the pathogenesis of ischemic stroke. Streptococci may infiltrate carotid artery plaques through neovasculature developing inside the plaque, initiating the formation of bacterial biofilm. Subsequently, this biofilm may affect the development of the plaque. The dispersion of the biofilm and the release of free bacteria, along with degradative enzymes secreted by the bacteria, can influence the integrity of the surrounding arterial tissues. This, in turn, increases the risk of plaque rupture, ultimately resulting in an ischemic stroke. During bacteremia, streptococci may promote thrombosis by activating platelets, especially in patients with atrial fibrillation. The possibilities of primary prevention of acute ischemic stroke using antibacterial vaccines and timed antimicrobial treatment, as well as regular dental care, should be considered.

DISCLOSURES

The author states no disclosures or conflicts of interest.

SOURCES OF FUNDING

This thesis was supported with grants from the Ida Montin Foundation, the Finnish Medical Foundation, the Finnish Foundation for Cardiovascular Research, and the Pirkanmaa Regional Fund of the Finnish Cultural Foundation.

REFERENCES

Aarabi, G., Heydecke, G., & Seedorf, U. (2018). Roles of Oral Infections in the Pathomechanism of Atherosclerosis. *International Journal of Molecular Sciences*, *19*(7). https://doi.org/10.3390/IJMS19071978

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–5732. https://doi.org/10.1128/JCM.43.11.5721-5732.2005

Adams, H. . P., Bendixen, B. . H., Kappelle, L. . J., Biller, J., Love, B. . B., Gordon, D. . L., & Marsh, E. . E. (1993). Classification of Subtype of Acute Ischemic Stroke. *Stroke*, *23*(1), 35–41. https://doi.org/10.1161/01.STR.24.1.35

Ahmed, M., McPherson, R., Abruzzo, A., Thomas, S. E., & Gorantla, V. R. (2021). Carotid Artery Calcification: What We Know So Far. *Cureus*, *13*(10). https://doi.org/10.7759/CUREUS.18938

Akcalı, A., & Lang, N. P. (2018). Dental calculus: the calcified biofilm and its role in disease development. *Periodontology 2000*, *76*(1), 109–115. https://doi.org/10.1111/PRD.12151

Allen, C. L., & Bayraktutan, U. (2008). Risk factors for ischaemic stroke. In *International Journal of Stroke* (Vol. 3, Issue 2, pp. 105–116). Int J Stroke. https://doi.org/10.1111/j.1747-4949.2008.00187.x

Aly, B. H., Hamad, M. S., Mohey, M., & Amen, S. (2012). Polymerase Chain Reaction (PCR) Versus Bacterial Culture in Detection of Organisms in Otitis Media with Effusion (OME) in Children. *Indian Journal of Otolaryngology and Head and Neck Surgery*, *64*(1), 51–55. https://doi.org/10.1007/s12070-011-0161-6

Amarenco, P., & Steg, P. G. (2007). The paradox of cholesterol and stroke. *Lancet (London, England)*, *370*(9602), 1803–1804. https://doi.org/10.1016/S0140-6736(07)61751-6

Anaya JM, Shoenfeld Y, & Rojas-Villarraga A. (2013). Autoimmunity: From Bench to Bedside. *El Rosario University Press*. https://doi.org/10.1371/JOURNAL.PONE.0055545

Andraws, R., Berger, J. S., & Brown, D. L. (2005). Effects of antibiotic therapy on outcomes of patients with coronary artery disease: A meta-analysis of randomized controlled trials. In *Journal of the American Medical Association* (Vol. 293, Issue 21, pp. 2641–2647). JAMA. https://doi.org/10.1001/jama.293.21.2641

Andreou, D. E., & Andreadou, I. (2009). Atherosclerosis: An inflammatory disease. In *Pharmakeftiki* (Vol. 22, Issue 3, pp. 83–96). Curr Pharm Des. https://doi.org/10.2174/138161212802481237

Aoyama, N., Suzuki, J. I., Kobayashi, N., Hanatani, T., Ashigaki, N., Yoshida, A., Shiheido, Y., Sato, H., Minabe, M., Izumi, Y., & Isobe, M. (2018). Associations among tooth loss, systemic inflammation and antibody titers to periodontal pathogens in Japanese patients with cardiovascular disease. *Journal of Periodontal Research*, *53*(1), 117–122. https://doi.org/10.1111/jre.12494

Arboix, A., & Alió, J. (2010). Cardioembolic Stroke: Clinical Features, Specific Cardiac Disorders and Prognosis. *Current Cardiology Reviews*, *6*(3), 150. https://doi.org/10.2174/157340310791658730

Arman, M., Krauel, K., Tilley, D. O., Weber, C., Cox, D., Greinacher, A., Kerrigan, S. W., & Watson, S. P. (2014). Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood*, *123*(20), 3166–3174. https://doi.org/10.1182/blood-2013-11-540526

Arweiler, N. B., & Netuschil, L. (2016). The Oral Microbiota. *Advances in Experimental Medicine and Biology*, *902*. https://doi.org/10.1007/978-3-319-31248-4_4

Atieh, M. A. (2008). Accuracy of Real-Time Polymerase Chain Reaction Versus Anaerobic Culture in Detection of Aggregatibacter actinomycetemcomitan s and Porphyromonas gingivalis : A Meta-Analysis . *Journal of Periodontology*, *79*(9), 1620–1629. https://doi.org/10.1902/jop.2008.070668

Balanescu, S., Calmac, L., Constantinescu, D., Marinescu, M., Onut, R., & Dorobantu, M. (2010). Systemic Inflammation and Early Atheroma Formation: Are They Related? *Mædica*, *5*(4), 292. /pmc/articles/PMC3152836/

Bartova, J., Sommerova, P., Lyuya-Mi, Y., Mysak, J., Prochazkova, J., Duskova, J., Janatova, T., & Podzimek, S. (2014). Periodontitis as a Risk Factor of Atherosclerosis. *Journal of Immunology Research*, *2014*, 1–9. https://doi.org/10.1155/2014/636893

Basaranoglu, S. T., Ozsurekci, Y., Aykac, K., Aycan, A. E., Bıcakcigil, A., Altun, B., Sancak, B., Cengiz, A. B., Kara, A., & Ceyhan, M. (2019). *Streptococcus mitis/oralis* Causing Blood Stream Infections in Pediatric Patients. *Japanese Journal of Infectious Diseases*, *72*(1), 1–6. https://doi.org/10.7883/yoken.JJID.2018.074

Beck, J., Garcia, R., Heiss, G., Vokonas, P. S., & Offenbacher, S. (1996). Periodontal Disease and Cardiovascular Disease The Relation of Periodontal Infections to Systemic Diseases. *Journal Of Periodontology*, *67(10/SUP)*, 1123–1137. https://doi.org/10.1038/sj.ebd.6400272

Beheshti, S., Madsen, C. M., Varbo, A., Benn, M., & Nordestgaard, B. G. (2018). Relationship of familial hypercholesterolemia and high low-density lipoprotein cholesterol to ischemic stroke: Copenhagen general population study. *Circulation*, *138*(6), 578–589. https://doi.org/10.1161/CIRCULATIONAHA.118.033470

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., Chiuve, S. E., Cushman, M., Delling, F. N., Deo, R., De Ferranti, S. D., Ferguson, J. F., Fornage, M., Gillespie, C., Isasi, C. R., Jiménez, M. C., Jordan, L. C., Judd, S. E., Lackland, D., … Muntner, P. (2018). Heart disease and stroke statistics - 2018 update: A report from the American Heart Association. *Circulation*, *137*(12), E67–E492. https://doi.org/10.1161/CIR.0000000000000558

Bennett, M. R., Sinha, S., & Owens, G. K. (2016). Vascular smooth muscle cells in atherosclerosis. *Circulation Research*, *118*(4), 692. https://doi.org/10.1161/CIRCRESAHA.115.306361

Bensing, B. A., Rubens, C. E., & Sullam, P. M. (2001). Genetic loci of Streptococcus mitis that mediate binding to human platelets. *Infection and Immunity*, *69*(3), 1373–1380. https://doi.org/10.1128/IAI.69.3.1373-1380.2001

Berenson, G. S., Srinivasan, S. R., Bao, W., Newman, W. P., Tracy, R. E., & Wattigney, W. A. (1998). Association between Multiple Cardiovascular Risk Factors and Atherosclerosis in Children and Young Adults. *New England Journal of Medicine*, *338*(23), 1650–1656. https://doi.org/10.1056/nejm199806043382302

Berkhemer, O. A., Fransen, P. S. S., Beumer, D., Van Den Berg, L. A., Lingsma, H. F., Yoo, A. J., Schonewille, W. J., Vos, J. A., Nederkoorn, P. J., Wermer, M. J. H., Van Walderveen, M. A. A., Staals, J., Hofmeijer, J., Van Oostayen, J. A., Lycklama Nijeholt, G. J., Boiten, J., Brouwer, P. A., Emmer, B. J., De Bruijn, S. F., … Dippel, D. W. J. (2015). A randomized trial of intraarterial treatment for acute ischemic stroke. *New England Journal of Medicine*, *372*(1), 11–20. https://doi.org/10.1056/NEJMoa1411587

Bhat, V. M., Cole, J. W., Sorkin, J. D., Wozniak, M. A., Malarcher, A. M., Giles, W. H., Stern, B. J., & Kittner, S. J. (2008). Dose-response relationship between cigarette smoking and risk of ischemic stroke in young women. *Stroke*, *39*(9), 2439–2443. https://doi.org/10.1161/STROKEAHA.107.510073

Binder, C. J., Chang, M. K., Shaw, P. X., Miller, Y. I., Hartvigsen, K., Dewan, A., & Witztum, J. L. (2002). Innate and acquired immunity in atherogenesis. *Nature Medicine*, *8*(11), 1218–1226. https://doi.org/10.1038/nm1102-1218

Binder, C. J., Hörkkö, S., Dewan, A., Chang, M. K., Kieu, E. P., Goodyear, C. S., Shaw, P. X., Palinski, W., Witztum, J. L., & Silverman, G. J. (2003). Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nature Medicine*, *9*(6), 736–743. https://doi.org/10.1038/NM876

Blake, G. J., & Ridker, P. M. (2000). Are statins anti-inflammatory? *Current Controlled Trials in Cardiovascular Medicine*, *1*(3), 161–165. https://doi.org/10.1186/CVM-1-3-161/COMMENTS

Blann, A. D. (2003). How a Damaged Blood Vessel Wall Contibutes to Thrombosis and Hypertenasion. *Pathophysiology of Haemostasis and Thrombosis*, *33*(5–6), 445–448. https://doi.org/10.1159/000083843

Bonati, L. H., Kakkos, S., Berkefeld, J., de Borst, G. J., Bulbulia, R., Halliday, A., van Herzeele, I., Koncar, I., McCabe, D. J. H., Lal, A., Ricco, J. B., Ringleb, P., Taylor-Rowan, M., & Eckstein, H. H. (2021). European Stroke Organisation guideline on endarterectomy and stenting for carotid artery stenosis. *European Stroke Journal*, *6*(2), I–XLVII. https://doi.org/10.1177/23969873211012121

Bos, D., Portegies, M. L. P. P., Van Der Lugt, A., Bos, M. J., Koudstaal, P. J., Hofman, A., Krestin, G. P., Franco, O. H., Vernooij, M. W., & Ikram, M. A. (2014). Intracranial

carotid artery atherosclerosis and the risk of stroke in whites: The Rotterdam study. *JAMA Neurology*, *71*(4), 405–411. https://doi.org/10.1001/jamaneurol.2013.6223

Bruce L Pihlstrom, Bryan S Michalowicz, N. W. J. (2018). Periodontal diseases. *Nutritional Aspects of Aging: Volume 2*, *366*(9499), 159–174. https://doi.org/10.1201/9781351075145

Bubner, B., Gase, K., & Baldwin, I. T. (2004). Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *BMC Biotechnology*, *4*. https://doi.org/10.1186/1472-6750-4-14

Burchfiel, C. M., David Curb, J., Rodriguez, B. L., Abbott, R. D., Chiu, D., & Yano, K. (1994). Glucose intolerance and 22-year stroke incidence: The honolulu heart program. *Stroke*, *25*(5), 951–957. https://doi.org/10.1161/01.STR.25.5.951

Byrnes, J. R., & Wolberg, A. S. (2017). Red blood cells in thrombosis. *Blood*, *130*(16), 1795. https://doi.org/10.1182/BLOOD-2017-03-745349

Cahill, T. J., & Prendergast, B. D. (2016). Infective endocarditis. *Lancet (London, England)*, *387*(10021), 882–893. https://doi.org/10.1016/S0140-6736(15)00067-7

Camaré, C., Pucelle, M., Nègre-Salvayre, A., & Salvayre, R. (2017). Angiogenesis in the atherosclerotic plaque. *Redox Biology*, *12*, 18. https://doi.org/10.1016/J.REDOX.2017.01.007

Campbell, B. C. V., Meretoja, A., Donnan, G. A., & Davis, S. M. (2015). Twenty-Year History of the Evolution of Stroke Thrombolysis With Intravenous Alteplase to Reduce Long-Term Disability. *Stroke; a Journal of Cerebral Circulation*, *46*(8), 2341–2346. https://doi.org/10.1161/STROKEAHA.114.007564

Campbell, L. A., & Kuo, C. C. (2004). Chlamydia pneumoniae - An infectious risk factor for atherosclerosis? In *Nature Reviews Microbiology* (Vol. 2, Issue 1, pp. 23–32). Nature Publishing Group. https://doi.org/10.1038/nrmicro796

Chang, S. N., Chen, J. J., Huang, P. S., Wu, C. K., Wang, Y. C., Hwang, J. J., & Tsai, C. T. (2023). Sodium-Glucose Cotransporter-2 Inhibitor Prevents Stroke in Patients With Diabetes and Atrial Fibrillation. *Journal of the American Heart Association*, *12*(10), 27764. https://doi.org/10.1161/JAHA.122.027764

Charo, I. F., & Taubman, M. B. (2004). Chemokines in the pathogenesis of vascular disease. *Circulation Research*, *95*(9), 858–866. https://doi.org/10.1161/01.RES.0000146672.10582.17

Chen, R., Ovbiagele, B., & Feng, W. (2016). Diabetes and Stroke: Epidemiology, Pathophysiology, Pharmaceuticals and Outcomes. *American Journal of the Medical Sciences*, *351*(4), 380–386. https://doi.org/10.1016/j.amjms.2016.01.011

Chhibber-Goel, J., Singhal, V., Bhowmik, D., Vivek, R., Parakh, N., Bhargava, B., & Sharma, A. (2016). Linkages between oral commensal bacteria and atherosclerotic plaques in coronary artery disease patients. *Npj Biofilms and Microbiomes 2017 2:1*, *2*(1), 1–13. https://doi.org/10.1038/s41522-016-0009-7

Chia, J. S., Lien, H. T., Hsueh, P. R., Chen, P. M., Sun, A., & Chen, J. Y. (2002). Induction of cytokines by glucosyltransferases of Streptococcus mutans. *Clinical and Diagnostic*

Laboratory Immunology, *9*(4), 892–897. https://doi.org/10.1128/CDLI.9.4.892-897.2002

Chiu, B. (1999). Multiple infections in carotid atherosclerotic plaques. *American Heart Journal*, *138*(5 II), S534-6. https://doi.org/10.1016/s0002-8703(99)70294-2

Choi, S. E., Sagris, D., Hill, A., Lip, G. Y. H., & Abdul-Rahim, A. H. (2023). Atrial fibrillation and stroke. *Expert Review of Cardiovascular Therapy*, *21*(1), 35–56. https://doi.org/10.1080/14779072.2023.2160319

Cohen, D. J., Malave, D., Ghidoni, J. J., Iakovidis, P., Everett, M. M., You, S., Liu, Y., & Boyan, B. D. (2004). Role of oral bacterial flora in calcific aortic stenosis: An animal model. *Annals of Thoracic Surgery*, *77*(2), 537–543. https://doi.org/10.1016/S0003-4975(03)01454-1

Collins, R., Armitage, J., Parish, S., Sleight, P., & Peto, R. (2002). MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: A randomised placebo-controlled trial. *Lancet*, *360*(9326), 7–22. https://doi.org/10.1016/S0140-6736(02)09327-3

Contractor, T., & Khasnis, A. (2011). Left Atrial Appendage Closure in Atrial Fibrillation: A World without Anticoagulation? *Cardiology Research and Practice*, *2011*(1). https://doi.org/10.4061/2011/752808

Corvol, J. C., Bouzamondo, A., Sirol, M., Hulot, J. S., Sanchez, P., & Lechat, P. (2003). Differential Effects of Lipid-Lowering Therapies on Stroke Prevention: A Meta-analysis of Randomized Trials. *Archives of Internal Medicine*, *163*(6), 669–676. https://doi.org/10.1001/ARCHINTE.163.6.669

Cotti, E., Dessì, C., Piras, A., & Mercuro, G. (2010). Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature. *International Journal of Cardiology*. https://doi.org/10.1016/j.ijcard.2010.08.011

Cuadra-Saenz, G., Rao, D. L., Underwood, A. J., Belapure, S. A., Campagna, S. R., Sun, Z., Tammariello, S., & Rickard, A. H. (2012). Autoinducer-2 influences interactions amongst pioneer colonizing streptococci in oral biofilms. *Microbiology (United Kingdom)*, *158*(7), 1783–1795. https://doi.org/10.1099/mic.0.057182-0

Curtiss, L. K., & Tobias, P. S. (2009). Emerging role of Toll-like receptors in atherosclerosis. In *Journal of Lipid Research* (Vol. 50, Issue SUPPL.). J Lipid Res. https://doi.org/10.1194/jlr.R800056-JLR200

Cushman, M. (2007). Epidemiology and Risk Factors for Venous Thrombosis. *Seminars in Hematology*, *44*(2), 62. https://doi.org/10.1053/J.SEMINHEMATOL.2007.02.004

D. Wolcott, R., J. Wolcott, J., & Palacio, C. (2012). A Possible Role of Bacterial Biofilm in the Pathogenesis of Atherosclerosis. *Journal of Bacteriology & Parasitology*, *03*(01), 1–7. https://doi.org/10.4172/2155-9597.1000127

D'Aiuto, F., Nibali, L., Parkar, M., Suvan, J., & Tonetti, M. S. (2005). Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. *Journal of Dental Research*, *84*(3), 269–273. https://doi.org/10.1177/154405910508400312

D'Aiuto, F., Parkar, M., & Tonetti, M. S. (2007). Acute effects of periodontal therapy on biomarkers of vascular health. *Journal of Clinical Periodontology*, *34*(2), 124–129. https://doi.org/10.1111/J.1600-051X.2006.01037.X

Danesh, J. (2005). Antibiotics in the prevention of heart attacks. *Lancet (London, England)*, *365*(9457), 365–367. https://doi.org/10.1016/S0140-6736(05)17842-8

Danesh, J., Whincup, P., Walker, M., Lennon, L., Thomson, A., Appleby, P., Gallimore, J. R., & Pepys, M. B. (2000). Low grade inflammation and coronary heart disease: Prospective study and updated meta-analyses. *British Medical Journal*, *321*(7255), 199–204. https://doi.org/10.1136/bmj.321.7255.199

Danesh, J., Whincup, P., Walker, M., Lennon, L., Thomson, A., Appleby, P., Wong, Y. K., Bernardes-Silva, M., Ward, M., & West, R. (2000). Chlamydia pneumoniae IgG titres and coronary heart disease: Prospective study and meta-analysis. *BMJ*, *321*(7255), 208–213. https://doi.org/10.1136/bmj.321.7255.208

Darveau, R. P. (2009). The oral microbial consortium's interaction with the periodontal innate defense system. *DNA and Cell Biology*, *28*(8), 389–395. https://doi.org/10.1089/dna.2009.0864

De Meyer, S. F., Andersson, T., Baxter, B., Bendszus, M., Brouwer, P., Brinjikji, W., Campbell, B. C., Costalat, V., Dávalos, A., Demchuk, A., Dippel, D., Fiehler, J., Fischer, U., Gilvarry, M., Gounis, M. J., Gralla, J., Jansen, O., Jovin, T., Kallmes, D., … Clot Summit Group. (2017). Analyses of thrombi in acute ischemic stroke: A consensus statement on current knowledge and future directions. *International Journal of Stroke : Official Journal of the International Stroke Society*, *12*(6), 606–614. https://doi.org/10.1177/1747493017709671

Debelian, G. J., Olsen, I., & Tronstad, L. (1994). Systemic diseases caused by oral microorganisms. In *Dental Traumatology* (Vol. 10, Issue 2, pp. 57–65). https://doi.org/10.1111/j.1600-9657.1994.tb00061.x

Deloukas, P., Kanoni, S., Willenborg, C., Farrall, M., Assimes, T. L., Thompson, J. R., Ingelsson, E., Saleheen, D., Erdmann, J., Goldstein, B. A., Stirrups, K., König, I. R., Cazier, J. B., Johansson, Å., Hall, A. S., Lee, J. Y., Willer, C. J., Chambers, J. C., Esko, T., … Samani, N. J. (2013). Large-scale association analysis identifies new risk loci for coronary artery disease. *Nature Genetics*, *45*(1), 25. https://doi.org/10.1038/NG.2480

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of Oral and Maxillofacial Pathology : JOMFP*, *23*(1), 122. https://doi.org/10.4103/JOMFP.JOMFP_304_18

DeStefano, F., Anda, R. F., Kahn, H. S., Williamson, D. F., & Russell, C. M. (1993). Dental disease and risk of coronary heart disease and mortality. *British Medical Journal*, *306*(6879), 688–691. https://doi.org/10.1136/bmj.306.6879.688

Desvarieux, M., Demmer, R. T., Rundek, T., Boden-Albala, B., Jacobs, D. R., Papapanou, P. N., & Sacco, R. L. (2003). Relationship between periodontal disease, tooth loss, and carotid artery plaque: The oral infections and vascular disease epidemiology study (INVEST). *Stroke*, *34*(9), 2120–2125. https://doi.org/10.1161/01.STR.0000085086.50957.22

Di Stefano, M., Polizzi, A., Santonocito, S., Romano, A., Lombardi, T., & Isola, G. (2022). Impact of Oral Microbiome in Periodontal Health and Periodontitis: A Critical Review on Prevention and Treatment. *International Journal of Molecular Sciences*, *23*(9). https://doi.org/10.3390/IJMS23095142

Di Tullio, M. R., & Homma, S. (2002). Mechanisms of cardioembolic stroke. *Current Cardiology Reports*, *4*(2), 141–148. https://doi.org/10.1007/S11886-002-0027-3

Diamantis, E., Kyriakos, G., Quiles-Sanchez, L. V., Farmaki, P., & Troupis, T. (2017). The Anti-Inflammatory Effects of Statins on Coronary Artery Disease: An Updated Review of the Literature. *Current Cardiology Reviews*, *13*(3), 209. https://doi.org/10.2174/1573403X13666170426104611

Dunton, A. D., Göpel, T., Ho, D. H., & Burggren, W. (2021). Form and Function of the Vertebrate and Invertebrate Blood-Brain Barriers. *International Journal of Molecular Sciences*, *22*(22), 12111. https://doi.org/10.3390/IJMS222212111

El Tawil, S., & Muir, K. W. (2017). Thrombolysis and thrombectomy for acute ischaemic stroke. *Clinical Medicine*, *17*(2), 161. https://doi.org/10.7861/CLINMEDICINE.17-2-161

Elkind, M. S. V., Luna, J. M., Moon, Y. P., Boden-Albala, B., Liu, K. M., Spitalnik, S., Rundek, T., Sacco, R. L., & Paik, M. C. (2010). Infectious burden and carotid plaque thickness: the northern Manhattan study. *Stroke*, *41*(3). https://doi.org/10.1161/STROKEAHA.109.571299

Elson, G., Dunn-Siegrist, I., Daubeuf, B., & Pugin, J. (2007). Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. *Blood*, *109*(4), 1574–1583. https://doi.org/10.1182/BLOOD-2006-06-032961

Fagundes, N. C. F., Couto, R. S. D. A., Brandão, A. P. T., Lima, L. A. de O., Bittencourt, L. de O., Souza-Rodrigues, R. D. de, Freire, M. A. M., Maia, L. C., & Lima, R. R. (2020). Association between Tooth Loss and Stroke: A Systematic Review. *Journal of Stroke and Cerebrovascular Diseases : The Official Journal of National Stroke Association*, *29*(8). https://doi.org/10.1016/J.JSTROKECEREBROVASDIS.2020.104873

Farsak, B., Yildirir, A., Akyön, Y., Pinar, A., Öç, M., Böke, E., Kes, S., & Tokgözoğlu, L. (2000). Detection of chlamydia pneumoniae and Helicobacter pylori DNA in human atherosclerotic plaques by PCR. *Journal of Clinical Microbiology*, *38*(12), 4408–4411. https://doi.org/10.1128/jcm.38.12.4408-4411.2000

Feigin, V. L., Krishnamurthi, R. V., Theadom, A. M., Abajobir, A. A., Mishra, S. R., Ahmed, M. B., Abate, K. H., Mengistie, M. A., Wakayo, T., Abd-Allah, F., Abdulle, A. M., Abera, S. F., Mohammed, K. E., Abyu, G. Y., Asgedom, S. W., Atey, T. M., Betsu, B. D., Mezgebe, H. B., Tuem, K. B., … Zaki, M. E. (2017). Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet Neurology*, *16*(11), 877–897. https://doi.org/10.1016/S1474-4422(17)30299-5

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., Hegele, R. A., Krauss, R. M., Raal, F. J., Schunkert, H., Watts, G. F., Borén, J., Fazio, S.,

Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., Sluis, B. van de, Taskinen, M.-R., … Catapano, A. L. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *European Heart Journal*, *38*(32), 2459. https://doi.org/10.1093/EURHEARTJ/EHX144

Fernandes, C. P., Oliveira, F. A. F., Silva, P. G. D. B., Alves, A. P. N. N., Mota, M. R. L., Montenegro, R. C., Burbano, R. M. R., Seabra, A. D., Lobo Filho, J. G., Lima, D. L. F., Soares Filho, A. W. E., & Sousa, F. B. (2014). Molecular analysis of oral bacteria in dental biofilm and atherosclerotic plaques of patients with vascular disease. *International Journal of Cardiology*, *174*(3), 710–712. https://doi.org/10.1016/J.IJCARD.2014.04.201

Fiorillo, L., Cervino, G., Laino, L., D'Amico, C., Mauceri, R., Tozum, T. F., Gaeta, M., & Cicciù, M. (2019). Porphyromonas gingivalis, Periodontal and Systemic Implications: A Systematic Review. *Dentistry Journal*, *7*(4). https://doi.org/10.3390/DJ7040114

Flaherty, M. L., Kissela, B., Khoury, J. C., Alwell, K., Moomaw, C. J., Woo, D., Khatri, P., Ferioli, S., Adeoye, O., Broderick, J. P., & Kleindorfer, D. (2012). Carotid artery stenosis as a cause of stroke. *Neuroepidemiology*, *40*(1), 36–41. https://doi.org/10.1159/000341410

Frieri, M., & Stampfl, H. (2016). Systemic lupus erythematosus and atherosclerosis: Review of the literature. *Autoimmunity Reviews*, *15*(1), 16–21. https://doi.org/10.1016/J.AUTREV.2015.08.007

Frostegård, J. (2005). Atherosclerosis in Patients With Autoimmune Disorders. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(9), 1776–1785. https://doi.org/10.1161/01.ATV.0000174800.78362.EC

Fujihara, K., Suzuki, H., Sato, A., Kodama, S., Heianza, Y., Saito, K., Iwasaki, H., Kobayashi, K., Yatoh, S., Takahashi, A., Yamada, N., Sone, H., & Shimano, H. (2013). Carotid artery plaque and LDL-to-HDL cholesterol ratio predict atherosclerotic status in coronary arteries in asymptomatic patients with type 2 diabetes mellitus. *Journal of Atherosclerosis and Thrombosis*, *20*(5), 452–464. https://doi.org/10.5551/JAT.14977

Fure, B., Wyller, T. B., & Thommessen, B. (2005). TOAST criteria applied in acute ischemic stroke. *Acta Neurologica Scandinavica*, *112*(4), 254–258. https://doi.org/10.1111/J.1600-0404.2005.00470.X

Furlan, J. C., Vergouwen, M. D. I., Fang, J., & Silver, F. L. (2014). White blood cell count is an independent predictor of outcomes after acute ischaemic stroke. *European Journal of Neurology*, *21*(2), 215–222. https://doi.org/10.1111/ENE.12233

Gaetti-Jardim, E., Marcelino, S. L., Feitosa, A. C. R., Romito, G. A., & Avila-Campos, M. J. (2009). Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. *Journal of Medical Microbiology*, *58*(12), 1568–1575. https://doi.org/10.1099/JMM.0.013383-0/CITE/REFWORKS

Gelfand, E. V., & Cannon, C. P. (2004). Antibiotics for secondary prevention of coronary artery disease: an ACES hypothesis but we need to PROVE IT. *American Heart Journal*, *147*(2), 202–209. https://doi.org/10.1016/J.AHJ.2003.09.011

Gerace, E., Mancuso, G., Midiri, A., Poidomani, S., Zummo, S., & Biondo, C. (2022). Recent Advances in the Use of Molecular Methods for the Diagnosis of Bacterial Infections. *Pathogens*, *11*(6). https://doi.org/10.3390/PATHOGENS11060663/S1

Gomes, M. S., Blattner, T. C., Sant'Ana Filho, M., Grecca, F. S., Hugo, F. N., Fouad, A. F., & Reynolds, M. A. (2013). Can apical periodontitis modify systemic levels of inflammatory markers? A systematic review and meta-analysis. In *Journal of Endodontics* (Vol. 39, Issue 10, pp. 1205–1217). https://doi.org/10.1016/j.joen.2013.06.014

Grad, E., Pachino, R. M., & Danenberg, H. D. (2011). Endothelial C-reactive protein increases platelet adhesion under flow conditions. *American Journal of Physiology - Heart and Circulatory Physiology*, *301*(3). https://doi.org/10.1152/ajpheart.00067.2011

Grau, A. J., Becher, H., Ziegler, C. M., Lichy, C., Buggle, F., Kaiser, C., Lutz, R., Bültmann, S., Preusch, M., & Dörfer, C. E. (2004). Periodontal Disease as a Risk Factor for Ischemic Stroke. *Stroke*, *35*(2), 496–501. https://doi.org/10.1161/01.STR.0000110789.20526.9D

Grau, A. J., Boddy, A. W., Dukovic, D. A., Buggle, F., Lichy, C., Brandt, T., & Hacke, W. (2004). Leukocyte count as an independent predictor of recurrent ischemic events. *Stroke*, *35*(5), 1147–1152. https://doi.org/10.1161/01.STR.0000124122.71702.64

Grau, A. J., Urbanek, C., & Palm, F. (2010). Common infections and the risk of stroke. *Nature Reviews. Neurology*, *6*(12), 681–694. https://doi.org/10.1038/NRNEUROL.2010.163

Guarner, J., Sumner, J., Paddock, C. D., Shieh, W.-J., Greer, P. W., Reagan, S., Fischer, M., Beneden, C. A. Van, & Zaki, S. R. (2006). Diagnosis of invasive group a streptococcal infections by using immunohistochemical and molecular assays. *American Journal of Clinical Pathology*, *126*(1), 148–155. https://doi.org/10.1309/KHGV-R72C-BRM4-FQ58

Gui, Y., Zheng, H., & Cao, R. Y. (2022). Foam Cells in Atherosclerosis: Novel Insights Into Its Origins, Consequences, and Molecular Mechanisms. *Frontiers in Cardiovascular Medicine*, *9*. https://doi.org/10.3389/FCVM.2022.845942

Hahn, C. Lo, Best, A. M., & Tew, J. G. (2000). Cytokine induction by Streptococcus mutans and pulpal pathogenesis. *Infection and Immunity*, *68*(12), 6785–6789. https://doi.org/10.1128/IAI.68.12.6785-6789.2000

Hajishengallis, G., Sharma, A., Russell, M. W., & Genco, R. J. (2002). Interactions of oral pathogens with toll-like receptors: possible role in atherosclerosis. In *Annals of periodontology / the American Academy of Periodontology* (Vol. 7, Issue 1, pp. 72–78). Ann Periodontol. https://doi.org/10.1902/annals.2002.7.1.72

Haraszthy, V. I., Zambon, J. J., Trevisan, M., Zeid, M., & Genco, R. J. (2000). Identification of Periodontal Pathogens in Atheromatous Plaques. *Journal of Periodontology*, *71*(10), 1554–1560. https://onlinelibrary.wiley.com/doi/full/10.1902/jop.2000.71.10.1554

Hayashi, C., Viereck, J., Hua, N., Phinikaridou, A., Madrigal, A. G., Gibson, F. C., III, Hamilton, J. A., & Genco, C. A. (2011). Porphyromonas gingivalis Accelerates Inflammatory Atherosclerosis in the Innominate Artery of ApoE Deficient Mice. *Atherosclerosis*, *215*(1), 52. https://doi.org/10.1016/J.ATHEROSCLEROSIS.2010.12.009

Heitmann, B. L., & Gamborg, M. (2008). Remaining teeth, cardiovascular morbidity and death among adult Danes. *Preventive Medicine*, *47*(2), 156–160. https://doi.org/10.1016/J.YPMED.2008.04.007

Herzberg, M. C., Nobbs, A., Tao, L., Kilic, A., Beckman, E., Khammanivong, A., & Zhang, Y. (2005). Oral Streptococci and Cardiovascular Disease: Searching for the Platelet Aggregation-Associated Protein Gene and Mechanisms of Streptococcus sanguis - Induced Thrombosis . *Journal of Periodontology*, *76*(11-s), 2101–2105. https://doi.org/10.1902/jop.2005.76.11-s.2101

Holmes, E., Loo, R. L., Stamler, J., Bictash, M., Yap, I. K. S., Chan, Q., Ebbels, T., De Iorio, M., Brown, I. J., Veselkov, K. A., Daviglus, M. L., Kesteloot, H., Ueshima, H., Zhao, L., Nicholson, J. K., & Elliott, P. (2008). Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature*, *453*(7193), 396–400. https://doi.org/10.1038/nature06882

Hovland, A., Mundal, L. J., Igland, J., Veierød, M. B., Holven, K. B., Bogsrud, M. P., Tell, G. S., Leren, T. P., & Retterstøl, K. (2019). Risk of Ischemic Stroke and Total Cerebrovascular Disease in Familial Hypercholesterolemia: A Register Study from Norway. *Stroke*, *50*(1), 172–174. https://doi.org/10.1161/STROKEAHA.118.023456

Huang, R., Li, M., & Gregory, R. L. (2011). Bacterial interactions in dental biofilm. *Virulence*, *2*(5), 435–444. /pmc/articles/PMC3322631/

Inzitari, D., Eliasziw, M., Gates, P., Sharpe, B. L., Chan, R. K. T., Meldrum, H. E., & Barnett, H. J. M. (2000). The Causes and Risk of Stroke in Patients with Asymptomatic Internal-Carotid-Artery Stenosis. *New England Journal of Medicine*, *342*(2st), 1693–1700. https://doi.org/10.1056/nejm200006083422302

Ionita, M. G., Van Den Borne, P., Catanzariti, L. M., Moll, F. L., De Vries, J. P. P. M., Pasterkamp, G., Vink, A., & De Kleijn, D. P. V. (2010). High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *30*(9), 1842–1848. https://doi.org/10.1161/ATVBAHA.110.209296

Iso, H., Jacobs, D. R. J., Wentworth, D., Neaton, J. D., Cohen, J. D., & Group*, for the M. R. (2010). Serum Cholesterol Levels and Six-Year Mortality from Stroke in 350,977 Men Screened for the Multiple Risk Factor Intervention Trial. *Http://Dx.Doi.Org/10.1056/NEJM198904063201405*, *320*(14), 904–910. https://doi.org/10.1056/NEJM198904063201405

Janket, S. J., Qvarnström, M., Meurman, J. H., Baird, A. E., Nuutinen, P., & Jones, J. A. (2004). Asymptotic Dental Score and Prevalent Coronary Heart Disease. *Circulation*, *109*(9), 1095–1100. https://doi.org/10.1161/01.CIR.0000118497.44961.1E

Johansson, B. B. (1999). Hypertension mechanisms causing stroke. *Clinical and Experimental Pharmacology and Physiology*, *26*(7), 563–565. https://doi.org/10.1046/j.1440-1681.1999.03081.x

Johansson, I., Tidehag, P., Lundberg, V., & Hallmans, G. (1994). Dental status, diet and cardiovascular risk factors in middle-aged people in northern Sweden. *Community Dentistry and Oral Epidemiology*, *22*(6), 431–436. https://doi.org/10.1111/j.1600-0528.1994.tb00792.x

Joshipura, K. J., Douglass, C. W., & Willett, W. C. (1998). Possible explanations for the tooth loss and cardiovascular disease relationship. In *Annals of periodontology / the American Academy of Periodontology* (Vol. 3, Issue 1, pp. 175–183). https://doi.org/10.1902/annals.1998.3.1.175

Joshipura, K. J., Hung, H. C., Rimm, E. B., Willett, W. C., & Ascherio, A. (2003). Periodontal disease, tooth loss, and incidence of ischemic stroke. *Stroke*, *34*(1), 47–52. https://doi.org/10.1161/01.STR.0000052974.79428.0C

Kaipio, J., Näyhä, S., & Valtonen, V. (2004). Fluoride in the drinking water and the geographical variation of coronary heart disease in Finland. *European Journal of Cardiovascular Prevention and Rehabilitation : Official Journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology*, *11*(1), 56–62. https://doi.org/10.1097/01.HJR.0000114969.39211.48

Kallio, K. A. E., Hyvärinen, K., Kovanen, P. T., Jauhiainen, M., & Pussinen, P. J. (2013). Very low density lipoproteins derived from periodontitis patients facilitate macrophage activation via lipopolysaccharide function. *Metabolism: Clinical and Experimental*, *62*(5), 661–668. https://doi.org/10.1016/J.METABOL.2012.09.015

Kamak, G., Yildirim, E., & Rencber, E. (2015). Evaluation of the relationship between periodontal risk and carotid artery calcifications on panoramic radiographs. *European Journal of Dentistry*, *9*(4), 483–489. https://doi.org/10.4103/1305-7456.172614

Kamal, N., Holodinsky, J. K., Stephenson, C., Kashayp, D., Demchuk, A. M., Hill, M. D., Vilneff, R. L., Bugbee, E., Zerna, C., Newcommon, N., Lang, E., Knox, D., & Smith, E. E. (2017). Improving Door-to-Needle Times for Acute Ischemic Stroke: Effect of Rapid Patient Registration, Moving Directly to Computed Tomography, and Giving Alteplase at the Computed Tomography Scanner. *Circulation: Cardiovascular Quality and Outcomes*, *10*(1). https://doi.org/10.1161/CIRCOUTCOMES.116.003242

Kamel, H., & Healey, J. S. (2017). Cardioembolic Stroke. *Circulation Research*, *120*(3), 514. https://doi.org/10.1161/CIRCRESAHA.116.308407

Kankaanpää, J., Sämpi, M., Bloigu, R., Wang, C., Akhi, R., Kesäniemi, Y. A., Remes, A. M., Ukkola, O., & Hörkkö, S. (2018). IgA antibodies to phosphocholine associate with long-term cardiovascular disease risk. *Atherosclerosis*, *269*, 294–300. https://doi.org/10.1016/J.ATHEROSCLEROSIS.2017.12.010

Kapoor, K., Kak, V. K., & Singh, B. (2003). Morphology and Comparative Anatomy of Circulus Arteriosus Cerebri in Mammals. *Anatomia, Histologia, Embryologia*, *32*(6), 347–355. https://doi.org/10.1111/J.1439-0264.2003.00492.X

Karhunen, V., Forss, H., Goebeler, S., Huhtala, H., Ilveskoski, E., Kajander, O., Mikkelsson, J., Penttilä, A., Perola, M., Ranta, H., Meurman, J. H., & Karhunen, P. J. (2006). Radiographic assessment of dental health in middle-aged men following sudden cardiac death. *Journal of Dental Research*, *85*(1), 89–93. https://doi.org/10.1177/154405910608500116

Katan, M., & Luft, A. (2018). Global Burden of Stroke. *Seminars in Neurology*, *38*(2), 208–211. https://doi.org/10.1055/s-0038-1649503

Keene, D., Price, C., Shun-Shin, M. J., & Francis, D. P. (2014). Effect on cardiovascular risk of high density lipoprotein targeted drug treatments niacin, fibrates, and CETP inhibitors: meta-analysis of randomised controlled trials including 117 411 patients. *The BMJ*, *349*. https://doi.org/10.1136/BMJ.G4379

Keene, K. L., Hyacinth, H. I., Bis, J. C., Kittner, S. J., Mitchell, B. D., Cheng, Y. C., Pare, G., Chong, M., O'Donnell, M., Meschia, J. F., Chen, W. M., Sale, M. M., Rich, S. S., Nalls, M. A., Zonderman, A. B., Evans, M. K., Wilson, J. G., Correa, A., Markus, H. S., … Fornage, M. (2020). A GWAS Meta-Analysis of Stroke in 22,000 individuals of African descent identifies novel associations with stroke. *Stroke*, *51*(8), 2454. https://doi.org/10.1161/STROKEAHA.120.029123

Kelly, P. J., Lemmens, R., & Tsivgoulis, G. (2021). Inflammation and Stroke Risk: A New Target for Prevention. *Stroke*, 2697–2706. https://doi.org/10.1161/STROKEAHA.121.034388

Kerekes, K., & Olsen, I. (1990). Similarities in the microfloras of root canals and deep periodontal pockets. *Endodontics & Dental Traumatology*, *6*(1), 1–5. http://www.ncbi.nlm.nih.gov/pubmed/2202588

Kerrigan, S. W., & Cox, D. (2009). The thrombotic potential of oral pathogens. In *Journal of Oral Microbiology* (Vol. 1, Issue 2009). https://doi.org/10.3402/jom.v1i0.1999

Kinane, D. F., Stathopoulou, P. G., & Papapanou, P. N. (2017). Periodontal diseases. *Nature Reviews. Disease Primers*, *3*. https://doi.org/10.1038/NRDP.2017.38

Klarström Engström, K., Khalaf, H., Kälvegren, H., & Bengtsson, T. (2015). The role of Porphyromonas gingivalis gingipains in platelet activation and innate immune modulation. *Molecular Oral Microbiology*, *30*(1), 62–73. https://doi.org/10.1111/omi.12067

Klotz, O., & Manning, M. (1912). Fatty streaks in intima of arteries. *J Pathol Bacteriol*, *16*, 211–220.

Kobiyama, K., Saigusa, R., & Ley, K. (2019). Vaccination against Atherosclerosis. *Current Opinion in Immunology*, *59*, 15. https://doi.org/10.1016/J.COI.2019.02.008

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S., & Robert J. Palmer, J. (2002). Communication among Oral Bacteria. *Microbiology and Molecular Biology Reviews*, *66*(3), 486. https://doi.org/10.1128/MMBR.66.3.486-505.2002

Kolenbrander, P. E., Palmer, R. J., Periasamy, S., & Jakubovics, N. S. (2010). Oral multispecies biofilm development and the key role of cell-cell distance. *Nature Reviews Microbiology*, *8*(7), 471–480. https://doi.org/10.1038/nrmicro2381

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., Behre, C. J., Knight, R., Fagerberg, B., Ley, R. E., & Bäckhed, F. (2010). Microbes and Health Sackler Colloquium: Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, 1–7. https://doi.org/10.1073/pnas.1011383107

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., Behre, C. J., Knight, R., Fagerberg, B., Ley, R. E., & Bäckhed, F. (2011). Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(SUPPL. 1), 4592–4598. https://doi.org/10.1073/pnas.1011383107

Koskinen, S., Lundqvist, A., & Ristiluoma, N. (2012). *Health, functional capacity and welfare in Finland in 2011*. https://www.julkari.fi/bitstream/handle/10024/90832/Rap068_2012_netti.pdf?sequence=1&isAllowed=y

Kovanen, P. T. (2019). Mast Cells as Potential Accelerators of Human Atherosclerosis—From Early to Late Lesions. *International Journal of Molecular Sciences 2019, Vol. 20, Page 4479*, *20*(18), 4479. https://doi.org/10.3390/IJMS20184479

Kozarov, E., Sweier, D., Shelburne, C., Progulske-Fox, A., & Lopatin, D. (2006). Detection of bacterial DNA in atheromatous plaques by quantitative PCR. *Microbes and Infection*, *8*(3), 687–693. https://doi.org/10.1016/J.MICINF.2005.09.004

Kreth, J., Merritt, J., Shi, W., & Qi, F. (2005). Competition and coexistence between Streptococcus mutans and Streptococcus sanguinis in the dental biofilm. *Journal of Bacteriology*, *187*(21), 7193–7203. https://doi.org/10.1128/JB.187.21.7193-7203.2005

Kumar, D. R., Hanlin, E. R., Glurich, I., Mazza, J. J., & Yale, S. H. (2010). Virchow's contribution to the understanding of thrombosis and cellular biology. *Clinical Medicine and Research*, *8*(3–4), 168–172. https://doi.org/10.3121/cmr.2009.866

Kumar, V., & Sharma, A. (2010). Neutrophils: Cinderella of innate immune system. *International Immunopharmacology*, *10*(11), 1325–1334. https://doi.org/10.1016/J.INTIMP.2010.08.012

Kunjathoor, V. V., Febbraio, M., Podrez, E. A., Moore, K. J., Andersson, L., Koehn, S., Rhee, J. S., Silverstein, R., Hoff, H. F., & Freeman, M. W. (2002). Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *Journal of Biological Chemistry*, *277*(51), 49982–49988. https://doi.org/10.1074/jbc.M209649200

Kuriakose, D., & Xiao, Z. (2020). Pathophysiology and Treatment of Stroke: Present Status and Future Perspectives. *International Journal of Molecular Sciences*, *21*(20), 1–24. https://doi.org/10.3390/IJMS21207609

Kurita-Ochiai, T., Jia, R., Cai, Y., Yamaguchi, Y., & Yamamoto, M. (2015). Periodontal Disease-Induced Atherosclerosis and Oxidative Stress. *Antioxidants*, *4*(3), 577–590. https://doi.org/10.3390/ANTIOX4030577

Kurth, T., Everett, B. M., Buring, J. E., Kase, C. S., Ridker, P. M., & Gaziano, J. M. (2007). Lipid levels and the risk of ischemic stroke in women. *Neurology*, *68*(8), 556–562. https://doi.org/10.1212/01.WNL.0000254472.41810.0D

Lafon, A., Pereira, B., Dufour, T., Rigouby, V., Giroud, M., Béjot, Y., & Tubert-Jeannin, S. (2014). Periodontal disease and stroke: a meta-analysis of cohort studies. *European Journal of Neurology*, *21*(9), 1155-e67. https://doi.org/10.1111/ENE.12415

Lakshmi, S. S. J., & Leela, K. V. (2022). A Review on Updated Species List of Viridans Streptococci causing Infective Endocarditis. *Journal of Pure and Applied Microbiology*, *16*(3), 1590–1594. https://doi.org/10.22207/JPAM.16.3.26

Langer, H. F., & Gawaz, M. (2008). Platelet-vessel wall interactions in atherosclerotic disease. *Thrombosis and Haemostasis*, *99*(3), 480–486. https://doi.org/10.1160/TH07-11-0685

Langlois, M. R., Chapman, M. J., Cobbaert, C., Mora, S., Remaley, A. T., Ros, E., Watts, G. F., Borén, J., Baum, H., Bruckert, E., Catapano, A., Descamps, O. S., Von Eckardstein, A., Kamstrup, P. R., Kolovou, G., Kronenberg, F., Langsted, A., Pulkki, K., Rifai, N., … Nordestgaard, B. G. (2018). Quantifying Atherogenic Lipoproteins: Current and Future Challenges in the Era of Personalized Medicine and Very Low Concentrations of LDL Cholesterol. A Consensus Statement from EAS and EFLM. *Clinical Chemistry*, *64*(7), 1006–1033. https://doi.org/10.1373/CLINCHEM.2018.287037

Lanter, B. B., Sauer, K., & Davies, D. G. (2014). Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture. *MBio*, *5*(3), e01206--14. https://doi.org/10.1128/mBio.01206-14

Lawes, C. M. M., Bennett, D. A., Feigin, V. L., & Rodgers, A. (2004). Blood Pressure and Stroke: An Overview of Published Reviews. In *Stroke* (Vol. 35, Issue 3, pp. 776–785). Lippincott Williams & Wilkins. https://doi.org/10.1161/01.STR.0000116869.64771.5A

Lechner, K., Schacky, C. von, McKenzie, A. L., Worm, N., Nixdorff, U., Lechner, B., Kränkel, N., Halle, M., Krauss, R. M., & Scherr, J. (2020). Lifestyle factors and high-risk atherosclerosis: Pathways and mechanisms beyond traditional risk factors. *European Journal of Preventive Cardiology*, *27*(4), 394. https://doi.org/10.1177/2047487319869400

Lechuga, L., & Weidlich, G. A. (2016). Cone Beam CT vs. Fan Beam CT: A Comparison of Image Quality and Dose Delivered Between Two Differing CT Imaging Modalities. *Cureus*. https://doi.org/10.7759/cureus.778

Lecours, M. P., Letendre, C., Clarke, D., Lemire, P., Galbas, T., Benoit-Biancamano, M. O., Thibodeau, J., Gottschalk, M., & Segura, M. (2016). Immune-responsiveness of CD4+ T cells during Streptococcus suis serotype 2 infection. *Scientific Reports 2016 6:1*, *6*(1), 1–15. https://doi.org/10.1038/srep38061

Lehtiniemi, J., Karhunen, P. J., Goebeler, S., Nikkari, S., & Nikkari, S. T. (2005). Identification of different bacterial DNAs in human coronary arteries. *European Journal of Clinical Investigation*, *35*(1), 13–16. https://doi.org/10.1111/J.1365-2362.2005.01440.X

Li, X., Kolltveit, K. M., Tronstad, L., & Olsen, I. (2000). Systemic diseases caused by oral infection. In *Clinical Microbiology Reviews* (Vol. 13, Issue 4, pp. 547–558). https://doi.org/10.1128/CMR.13.4.547-558.2000

Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and Atherosclerosis. *Circulation*, *105*(9), 1135–1143. https://doi.org/10.1161/hc0902.104353

Lin, H. W., Chen, C. M., Yeh, Y. C., Chen, Y. Y., Guo, R. Y., Lin, Y. P., & Li, Y. C. (2019). Dental treatment procedures for periodontal disease and the subsequent risk of ischaemic stroke: A retrospective population-based cohort study. *Journal of Clinical Periodontology*, *46*(6), 642–649. https://doi.org/10.1111/JCPE.13113

Lindsberg, P. J., & Grau, A. J. (2003). Inflammation and infections as risk factors for ischemic stroke. *Stroke*, *34*(10), 2518–2532. https://doi.org/10.1161/01.STR.0000089015.51603.CC

Lindsberg, P. J., Kantanen, A.-M., Mattila, O. S., Soinne, L., Puolakka, T., Jäkälä, P., Lappalainen, K., & M, K. (2017). Tunnistatko aivoinfarktin trombektomiakandidaatin? *Duodecim*, *133*(12), 1138–1147. http://www.duodecimlehti.fi/api/pdf/duo13762%3Ehttp://hdl.handle.net/10138/297964

Linton, M. F., Yancey, P. G., Davies, S. S., Jerome, W. G., Linton, E. F., Song, W. L., Doran, A. C., & Vickers, K. C. (2019). The Role of Lipids and Lipoproteins in Atherosclerosis. *Science*, *111*(2877), 166–186. https://www.ncbi.nlm.nih.gov/books/NBK343489/

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Ljung, R., Lundgren, F., Appelquist, M., & Cederlund, A. (2019). The Swedish dental health register - Validation study of remaining and intact teeth. *BMC Oral Health*, *19*(1), 116. https://doi.org/10.1186/s12903-019-0804-7

Lockhart, P. B., Bolger, A. F., Papapanou, P. N., Osinbowale, O., Trevisan, M., Levison, M. E., Taubert, K. A., Newburger, J. W., Gornik, H. L., Gewitz, M. H., Wilson, W. R., Smith, S. C., & Baddour, L. M. (2012). Periodontal disease and atherosclerotic vascular disease: Does the evidence support an independent association?: A scientific statement from the American heart association. *Circulation*, *125*(20), 2520–2544. https://doi.org/10.1161/CIR.0b013e31825719f3

Lockhart, P. B., Brennan, M. T., Sasser, H. C., Fox, P. C., Paster, B. J., & Bahrani-Mougeot, F. K. (2008). Bacteremia associated with toothbrushing and dental extraction. *Circulation*, *117*(24), 3118–3125. https://doi.org/10.1161/CIRCULATIONAHA.107.758524

Loesche, W. J. (1997). Association of the oral flora with important medical diseases. *Current Opinion in Periodontology*, *4*, 21–28. http://www.ncbi.nlm.nih.gov/pubmed/9655017

Lourbakos, A., Yuan, Y. P., Jenkins, A. L., Travis, J., Andrade-Gordon, P., Santulli, R., Potempa, J., & Pike, R. N. (2001). Activation of protease-activated receptors by gingipains from Porphyromonas gingivalis leads to platelet aggregation: A new trait

in microbial pathogenicity. *Blood*, *97*(12), 3790–3797. https://doi.org/10.1182/blood.V97.12.3790

MacMahon, S., Duffy, S., Rodgers, A., Tominaga, S., Chambless, L., De Backer, G., De Bacquer, D., Kornitzer, M., Whincup, P., Wannamethee, S. G., Morris, R., Wald, N., Morris, J., Law, M., Knuiman, M., Bartholomew, H., Davey Smith, G., Sweetnam, P., Elwood, P., … Whitlock, G. (2007). Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet (London, England)*, *370*(9602), 1829–1839. https://doi.org/10.1016/S0140-6736(07)61778-4

Markus, H. S. (2011). Stroke genetics. *Human Molecular Genetics*, *20*(R2), 124–131. https://doi.org/10.1093/hmg/ddr345

Martin, S. S., Qasim, A. N., Mehta, N. N., Wolfe, M., Terembula, K., Schwartz, S., Iqbal, N., Schutta, M., Bagheri, R., & Reilly, M. P. (2009). Apolipoprotein B but not LDL Cholesterol Is Associated With Coronary Artery Calcification in Type 2 Diabetic Whites. *Diabetes*, *58*(8), 1887. https://doi.org/10.2337/DB08-1794

Martinelli, I., Bucciarelli, P., & Mannucci, P. M. (2010). Thrombotic risk factors: basic pathophysiology. *Critical Care Medicine*, *38*(2 Suppl). https://doi.org/10.1097/CCM.0B013E3181C9CBD9

Mashima, I., & Nakazawa, F. (2014). The influence of oral Veillonella species on biofilms formed by Streptococcus species. *Anaerobe*, *28*, 54–61. https://doi.org/10.1016/J.ANAEROBE.2014.05.003

Matarín, M., Brown, W. M., Scholz, S., Simón-Sánchez, J., Fung, H. C., Hernandez, D., Gibbs, J. R., De Vrieze, F. W., Crews, C., Britton, A., Langefeld, C. D., Brott, T. G., Brown, R. D., Worrall, B. B., Frankel, M., Silliman, S., Case, L. D., Singleton, A., Hardy, J. A., … Meschia, J. F. (2007). A genome-wide genotyping study in patients with ischaemic stroke: initial analysis and data release. *Lancet Neurology*, *6*(5), 414–420. https://doi.org/10.1016/S1474-4422(07)70081-9

Mattila, K. J., Nieminen, M. S., Valtonen, V. V., Rasi, V. P., Kesaniemi, Y. A., Syrjala, S. L., Jungell, P. S., Isoluoma, M., Hietaniemi, K., Jokinen, M. J., & Huttunen, J. K. (1989). Association between dental health and acute myocardial infarction. *British Medical Journal*, *298*(6676), 779–781. https://doi.org/10.1136/bmj.298.6676.779

McManus, M., & Liebeskind, D. S. (2016). Blood pressure in acute ischemic stroke. In *Journal of Clinical Neurology (Korea)* (Vol. 12, Issue 2, pp. 137–146). Korean Neurological Association. https://doi.org/10.3988/jcn.2016.12.2.137

Mehu, M., Narasimhulu, C. A., & Singla, D. K. (2022). Inflammatory Cells in Atherosclerosis. *Antioxidants*, *11*(2). https://doi.org/10.3390/ANTIOX11020233

Mekke, J. M., Egberts, D. H. J., Waissi, F., Timmerman, N., Bot, I., Kuiper, J., Pasterkamp, G., de Borst, G. J., & de Kleijn, D. P. V. (2021). Mast Cell Distribution in Human Carotid Atherosclerotic Plaque Differs Significantly by Histological Segment. *European Journal of Vascular and Endovascular Surgery*, *62*(5), 808–815. https://doi.org/10.1016/J.EJVS.2021.07.008

Melnick, S. L., Shahar, E., Folsom, A. R., Grayston, J. T., Sorlie, P. D., Wang, S. P., & Szklo, M. (1993). Past infection by Chlamydia pneumoniae strain TWAR and asymptomatic carotid atherosclerosis. *The American Journal of Medicine*, *95*(5), 499–504. https://doi.org/10.1016/0002-9343(93)90332-J

Meschia, J. F., Bushnell, C., Boden-Albala, B., Braun, L. T., Bravata, D. M., Chaturvedi, S., Creager, M. A., Eckel, R. H., Elkind, M. S. V., Fornage, M., Goldstein, L. B., Greenberg, S. M., Horvath, S. E., Iadecola, C., Jauch, E. C., Moore, W. S., & Wilson, J. A. (2014). Guidelines for the primary prevention of stroke: A statement for healthcare professionals from the American heart association/American stroke association. *Stroke*, *45*(12), 3754–3832. https://doi.org/10.1161/STR.0000000000000046

Miller, Y. I. (2005). Toll-like receptors and atherosclerosis: oxidized LDL as an endogenous Toll-like receptor ligand. *Future Cardiology*, *1*(6), 785–792. https://doi.org/10.2217/14796678.1.6.785

Minassian, C., D'Aiuto, F., Hingorani, A. D., & Smeeth, L. (2010). Invasive dental treatment and risk for vascular events: A self-controlled case series. *Annals of Internal Medicine*, *153*(8), 499–506. https://doi.org/10.7326/0003-4819-153-8-201010190-00006

Monack, D. M., Mueller, A., & Falkow, S. (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nature Reviews. Microbiology*, *2*(9), 747–765. https://doi.org/10.1038/NRMICRO955

Montaño, A., Hanley, D. F., & Hemphill, J. C. (2021). Hemorrhagic stroke. *Handbook of Clinical Neurology*, *176*, 229–248. https://doi.org/10.1016/B978-0-444-64034-5.00019-5

Morré, S. A., Stooker, W., Lagrand, W. K., Van Den Brule, A. J. C., & Niessen, H. W. M. (2000). Microorganisms in the aetiology of atherosclerosis. In *Journal of Clinical Pathology* (Vol. 53, Issue 9, pp. 647–654). BMJ Publishing Group. https://doi.org/10.1136/jcp.53.9.647

Muñoz, R., Santamaría, E., Rubio, I., Ausín, K., Ostolaza, A., Labarga, A., Roldán, M., Zandio, B., Mayor, S., Bermejo, R., Mendigaña, M., Herrera, M., Aymerich, N., Olier, J., Gállego, J., Mendioroz, M., & Fernández-Irigoyen, J. (2018). Mass spectrometry-based proteomic profiling of thrombotic material obtained by endovascular thrombectomy in patients with ischemic stroke. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020498

Naik, U. P. (2014). Bacteria exploit platelets. In *Blood* (Vol. 123, Issue 20, pp. 3067–3068). American Society of Hematology. https://doi.org/10.1182/blood-2014-04-565432

Narayanan, Ll., & Vaishnavi, C. (2010). Endodontic microbiology. *Journal of Conservative Dentistry*, *13*(4), 233. https://doi.org/10.4103/0972-0707.73386

Nonnenmacher, C., Dalpke, A., Mutters, R., & Heeg, K. (2004). Quantitative detection of periodontopathogens by real-time PCR. *Journal of Microbiological Methods*, *59*(1), 117–125. https://doi.org/10.1016/j.mimet.2004.06.006

Nyvad, B., & Kilian, M. (1987). Microbiology of the early colonization of human enamel and root surfaces in vivo. *European Journal of Oral Sciences*, *95*(5), 369–380. https://doi.org/10.1111/j.1600-0722.1987.tb01627.x

Okahashi, N., Okinaga, T., Sakurai, A., Terao, Y., Nakata, M., Nakashima, K., Shintani, S., Kawabata, S., Ooshima, T., & Nishihara, T. (2011). Streptococcus sanguinis induces foam cell formation and cell death of macrophages in association with production of reactive oxygen species. *FEMS Microbiology Letters*, *323*(2), 164–170. https://doi.org/10.1111/J.1574-6968.2011.02375.X

Oksala, N., Levula, M., Pelto-Huikko, M., Kytömäki, L., Soini, J. T., Salenius, J., Kähönen, M., Karhunen, P. J., Laaksonen, R., Parkkila, S., & Lehtimäki, T. (2010). Carbonic anhydrases II and XII are up-regulated in osteoclast-like cells in advanced human atherosclerotic plaques-Tampere Vascular Study. *Annals of Medicine*, *42*(5), 360–370. https://doi.org/10.3109/07853890.2010.486408

Osler W. (1908). Diseases of the arteries. *Modern Medicine: Its Practice and Theory. Philadelphia: Lea and Febiger*, 429–447.

Paju, S., Sinisalo, J., Pussinen, P. J., Valtonen, V., & Nieminen, M. S. (2007). Is periodontal infection behind the failure of antibiotics to prevent coronary events? *Atherosclerosis*, *193*(1), 193–195. https://doi.org/10.1016/J.ATHEROSCLEROSIS.2006.06.015

Paoletti, R., Gotto, A. M., & Hajjar, D. P. (2004). Inflammation in atherosclerosis and implications for therapy. In *Circulation* (Vol. 109, Issue 23 SUPPL.). Circulation. https://doi.org/10.1161/01.cir.0000131514.71167.2e

Park, B., You, S., Jung, J., Lee, J. A., Yun, K. J., & Lee, M. S. (2015). Korean Studies on Blood Stasis: An Overview. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2015*. https://doi.org/10.1155/2015/316872

Peh, A., O'Donnell, J. A., Broughton, B. R. S., & Marques, F. Z. (2022). Gut Microbiota and Their Metabolites in Stroke: A Double-Edged Sword. *Stroke*, *53*(5), 1788–1801. https://doi.org/10.1161/STROKEAHA.121.036800

Pessi, T., Karhunen, V., Karjalainen, P. P., Ylitalo, A., Airaksinen, J. K., Niemi, M., Pietila, M., Lounatmaa, K., Haapaniemi, T., Lehtimäki, T., Laaksonen, R., Karhunen, P. J., & Mikkelsson, J. (2013). Bacterial signatures in thrombus aspirates of patients with myocardial infarction. *Circulation*, *127*(11), 1219–1228. https://doi.org/10.1161/CIRCULATIONAHA.112.001254

Piechowski-Jóźwiak, B., & Bogousslavsky, J. (2004). Cholesterol as a risk factor for stroke: The fugitive? In *Stroke* (Vol. 35, Issue 6, pp. 1523–1524). Lippincott Williams & Wilkins. https://doi.org/10.1161/01.STR.0000130460.09807.ec

Pillai, R. S., Iyer, K., Spin-Neto, R., Kothari, S. F., Nielsen, J. F., & Kothari, M. (2018). Oral health and brain injury: Causal or casual relation? *Cerebrovascular Diseases Extra*, *8*(1), 1–15. https://doi.org/10.1159/000484989

Potgieter, M., Bester, J., Kell, D. B., & Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiology Reviews*, *39*(4), 567. https://doi.org/10.1093/FEMSRE/FUV013

Pradeep, A. R., Hadge, P., Arjun Raju, P., Shetty, S. R., Shareef, K., & Guruprasad, C. N. (2010). Periodontitis as a risk factor for cerebrovascular accident: A case-control study in the Indian population. *Journal of Periodontal Research*, *45*(2), 223–228. https://doi.org/10.1111/j.1600-0765.2009.01220.x

Pussinen, P. J., Paju, S., Koponen, J., Viikari, J. S. A., Taittonen, L., Laitinen, T., Burgner, D. P., Kähönen, M., Hutri-Kähönen, N., Raitakari, O. T., & Juonala, M. (2019). Association of Childhood Oral Infections With Cardiovascular Risk Factors and Subclinical Atherosclerosis in Adulthood. *JAMA Network Open*, *2*(4), e192523. https://doi.org/10.1001/jamanetworkopen.2019.2523

Ramji, D. P., & Davies, T. S. (2015). Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. In *Cytokine and Growth Factor Reviews* (Vol. 26, Issue 6, pp. 673–685). Elsevier Ltd. https://doi.org/10.1016/j.cytogfr.2015.04.003

Redd, K. T., Phillips, S. T., McMillian, B., Giamberardino, L., Hardin, J., Glover, S., Merchant, A., Susin, C., Beck, J. D., Offenbacher, S., & Sen, S. (2019). PeRiodontal Treatment to Eliminate Minority Inequality and Rural Disparities in Stroke (PREMIERS): A Multicenter, Randomized, Controlled Study. *International Journal of Cerebrovascular Disease and Stroke*, *2*(2). https://doi.org/10.29011/2688-8734.100021

Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C., Fonseca, F., Nicolau, J., Koenig, W., Anker, S. D., Kastelein, J. J. P., Cornel, J. H., Pais, P., Pella, D., Genest, J., Cifkova, R., Lorenzatti, A., Forster, T., Kobalava, Z., … Glynn, R. J. (2017). Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *New England Journal of Medicine*, *377*(12), 1119–1131. https://doi.org/10.1056/NEJMOA1707914/SUPPL_FILE/NEJMOA1707914_DISCLOSURES.PDF

Ridker, P. M., Rifai, N., Rose, L., Buring, J. E., & Cook, N. R. (2002). Comparison of C-Reactive Protein and Low-Density Lipoprotein Cholesterol Levels in the Prediction of First Cardiovascular Events. *New England Journal of Medicine*, *347*(20), 1557–1565. https://doi.org/10.1056/nejmoa021993

Robbins, C. S., Hilgendorf, I., Weber, G. F., Theurl, I., Iwamoto, Y., Figueiredo, J. L., Gorbatov, R., Sukhova, G. K., Gerhardt, L. M. S., Smyth, D., Zavitz, C. C. J., Shikatani, E. A., Parsons, M., Van Rooijen, N., Lin, H. Y., Husain, M., Libby, P., Nahrendorf, M., Weissleder, R., & Swirski, F. K. (2013). Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nature Medicine 2013 19:9*, *19*(9), 1166–1172. https://doi.org/10.1038/nm.3258

Rosendorff, A., & Dorfman, D. M. (2007). Activated protein C resistance and factor V Leiden: A review. *Archives of Pathology and Laboratory Medicine*, *131*(6), 866–871. https://doi.org/10.5858/2007-131-866-APCRAF

Rosenfeld, M. E. (2013). Inflammation and atherosclerosis: Direct versus indirect mechanisms. In *Current Opinion in Pharmacology* (Vol. 13, Issue 2, pp. 154–160). https://doi.org/10.1016/j.coph.2013.01.003

Rosenfeld, M. E., & Campbell, L. A. (2011). Pathogens and atherosclerosis: Update on the potential contribution of multiple infectious organisms to the pathogenesis of

atherosclerosis. *Thrombosis and Haemostasis*, *106*(5), 858–867. https://doi.org/10.1160/TH11-06-0392

Rost, N. S., Wolf, P. A., Kase, C. S., Kelly-Hayes, M., Silbershatz, H., Massaro, J. M., D'Agostino, R. B., Franzblau, C., & Wilson, P. W. F. (2001). Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: The Framingham Study. *Stroke*, *32*(11), 2575–2579. https://doi.org/10.1161/hs1101.098151

Roth, G. A., Mensah, G. A., Johnson, C. O., Addolorato, G., Ammirati, E., Baddour, L. M., Barengo, N. C., Beaton, A., Benjamin, E. J., Benziger, C. P., Bonny, A., Brauer, M., Brodmann, M., Cahill, T. J., Carapetis, J. R., Catapano, A. L., Chugh, S., Cooper, L. T., Coresh, J., … Fuster, V. (2020). Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019: Update From the GBD 2019 Study. *Journal of the American College of Cardiology*, *76*(25), 2982. https://doi.org/10.1016/J.JACC.2020.11.010

Rupf, S., Kannengiesser, S., Merte, K., Pfister, W., Sigusch, B., & Eschrich, K. (2000). Comparison of profiles of key periodontal pathogens in periodontium and endodontium. *Endodontics & Dental Traumatology*, *16*(6), 269–275. http://www.ncbi.nlm.nih.gov/pubmed/11202893

Rymer, M. M. (2011). Hemorrhagic Stroke: Intracerebral Hemorrhage. *Missouri Medicine*, *108*(1), 50. /pmc/articles/PMC6188453/

S Kristiansen, K. M. P. (2000). Health care systems in the Nordic countries--more similarities than differences? *Tidsskr Nor Laegeforen .*, *120*(17), 2023–2029. https://pubmed.ncbi.nlm.nih.gov/11008540/

Sacco, R. L., Benjamin, E. J., Broderick, J. P., Dyken, M., Easton, J. D., Feinberg, W. M., Goldstein, L. B., Gorelick, P. B., Howard, G., Kittner, S. J., Manolio, T. A., Whisnant, J. P., & Wolf, P. A. (1997). Risk factors. *Stroke*, *28*(7), 1507–1517. https://doi.org/10.1161/01.STR.28.7.1507

Saigusa, R., Winkels, H., & Ley, K. (2020). T cell subsets and functions in atherosclerosis. *Nature Reviews Cardiology 2020 17:7*, *17*(7), 387–401. https://doi.org/10.1038/s41569-020-0352-5

Saikku, P., Mattila, K., Nieminen, M. S., Huttunen, J. K., Leinonen, M., Ekman, M. R., Mäkelä, P. H., & Valtonen, V. (1988). SEROLOGICAL EVIDENCE OF AN ASSOCIATION OF A NOVEL CHLAMYDIA, TWAR, WITH CHRONIC CORONARY HEART DISEASE AND ACUTE MYOCARDIAL INFARCTION. *The Lancet*, *332*(8618), 983–986. https://doi.org/10.1016/S0140-6736(88)90741-6

Sakurai, K., Wang, D., Suzuki, J. I., Umeda, M., Nagasawa, T., Izumi, Y., Ishikawa, I., & Isobe, M. (2007). High incidence of actinobacillus actinomycetemcomitans infection in acute coronary syndrome. *International Heart Journal*, *48*(6), 663–675. https://doi.org/10.1536/ihj.48.663

Sanz, M., Marco del Castillo, A., Jepsen, S., Gonzalez-Juanatey, J. R., D'Aiuto, F., Bouchard, P., Chapple, I., Dietrich, T., Gotsman, I., Graziani, F., Herrera, D., Loos, B., Madianos, P., Michel, J. B., Perel, P., Pieske, B., Shapira, L., Shechter, M., Tonetti, M.,

… Wimmer, G. (2020). Periodontitis and cardiovascular diseases: Consensus report. *Journal of Clinical Periodontology*, *47*(3), 268. https://doi.org/10.1111/JCPE.13189

Schillinger, T., Kluger, W., Exner, M., Mlekusch, W., Sabeti, S., Amighi, J., Wagner, O., Minar, E., & Schillinger, M. (2006). Dental and periodontal status and risk for progression of carotid atherosclerosis: The inflammation and carotid artery risk for atherosclerosis study dental substudy. *Stroke*, *37*(9), 2271–2276. https://doi.org/10.1161/01.STR.0000236495.82545.2e

Sen, S., Curtis, J., Hicklin, D., Nichols, C., Glover, S., Merchant, A. T., Hardin, J. W., Logue, M., Meyer, J., Mason, E., Huang, D. Y., Susin, C., Moss, K., & Beck, J. (2023). Periodontal Disease Treatment After Stroke or Transient Ischemic Attack: The PREMIERS Study, a Randomized Clinical Trial. *Stroke*. https://doi.org/10.1161/STROKEAHA.122.042047

Sen, S., Giamberardino, L. D., Moss, K., Morelli, T., Rosamond, W. D., Gottesman, R. F., Beck, J., & Offenbacher, S. (2018). *Periodontal Disease, Regular Dental Care Use, and Incident Ischemic Stroke*. *49*(2), 355–362. https://doi.org/10.1161/STROKEAHA.117.018990

Sessa, R., Pietro, M. Di, Filardo, S., & Turriziani, O. (2014). Infectious burden and atherosclerosis: A clinical issue. *World Journal of Clinical Cases : WJCC*, *2*(7), 240. https://doi.org/10.12998/WJCC.V2.I7.240

Shahi, S., Farhoudi, M., Dizaj, S. M., Sharifi, S., Sadigh-Eteghad, S., Goh, K. W., Ming, L. C., Dhaliwal, J. S., & Salatin, S. (2022). The Link between Stroke Risk and Orodental Status—A Comprehensive Review. *Journal of Clinical Medicine*, *11*(19), 5854. https://doi.org/10.3390/JCM11195854

Singh, R. K., Haka, A. S., Asmal, A., Barbosa-Lorenzi, V. C., Grosheva, I., Chin, H. F., Xiong, Y., Hla, T., & Maxfield, F. R. (2020). TLR4 (Toll-Like Receptor 4)-Dependent Signaling Drives Extracellular Catabolism of LDL (Low-Density Lipoprotein) Aggregates. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *40*(1), 86–102. https://doi.org/10.1161/ATVBAHA.119.313200

Siqueira, J. F., & Rôças, I. N. (2013). Microbiology and Treatment of Acute Apical Abscesses. *Clinical Microbiology Reviews*, *26*(2), 255. https://doi.org/10.1128/CMR.00082-12

Skaggs, B. J., Hahn, B. H., & McMahon, M. (2012). Accelerated atherosclerosis in patients with SLE--mechanisms and management. *Nature Reviews. Rheumatology*, *8*(4), 214–223. https://doi.org/10.1038/NRRHEUM.2012.14

Skeoch, S., & Bruce, I. N. (2015). Atherosclerosis in rheumatoid arthritis: is it all about inflammation? *Nature Reviews Rheumatology 2015 11:7*, *11*(7), 390–400. https://doi.org/10.1038/nrrheum.2015.40

Snow, D. E., Everett, J., Mayer, G., Cox, S. B., Miller, B., Rumbaugh, K., Wolcott, R. A., & Wolcott, R. D. (2016). The presence of biofilm structures in atherosclerotic plaques of arteries from legs amputated as a complication of diabetic foot ulcers. *Journal of Wound Care*, *25*, S16--S22. https://doi.org/10.12968/jowc.2016.25.Sup2.S16

Soehnlein, O. (2012). Multiple roles for neutrophils in atherosclerosis. *Circulation Research*, *110*(6), 875–888. https://doi.org/10.1161/CIRCRESAHA.111.257535

Song, P., Fang, Z., Wang, H., Cai, Y., Rahimi, K., Zhu, Y., Fowkes, F. G. R., Fowkes, F. J. I., & Rudan, I. (2020). Global and regional prevalence, burden, and risk factors for carotid atherosclerosis: a systematic review, meta-analysis, and modelling study. *The Lancet. Global Health*, *8*(5), e721–e729. https://doi.org/10.1016/S2214-109X(20)30117-0

Stary, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., & Wissler, R. W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*, *92*(5), 1355–1374. https://doi.org/10.1161/01.CIR.92.5.1355

Steinklein, J., & Nguyen, V. (2013). Dental anatomy and pathology encountered on routine CT of the head and neck. *AJR. American Journal of Roentgenology*, *201*(6), W843-53. https://doi.org/10.2214/AJR.12.9616

Stingu, C.-S., Schaumann, R., Jentsch, H., Eschrich, K., Brosteanu, O., & Rodloff, A. C. (2013). Association of periodontitis with increased colonization by Prevotella nigrescens. *Journal of Investigative and Clinical Dentistry*, *4*(1), 20–25. https://doi.org/10.1111/J.2041-1626.2012.00129.X

Suk, S.-H., Sacco, R. L., Boden-Albala, B., Cheun, J. F., Pittman, J. G., Elkind, M. S., & Paik, M. C. (2003). Abdominal obesity and risk of ischemic stroke: the Northern Manhattan Stroke Study. *Stroke*, *34*(7), 1586–1592. https://doi.org/10.1161/01.STR.0000075294.98582.2F

Suominen-Taipale, L., Nordblad, A., Vehkalahti, M., & Aromaa, A. (2004). *Suomalaisten aikuisten suunterveys*.

Suzuki, N., Yoshida, A., & Nakano, Y. (2005). Quantitative analysis of multi-species oral biofilms by TaqMan Real-Time PCR. In *Clinical medicine & research* (Vol. 3, Issue 3, pp. 176–185). https://doi.org/10.3121/cmr.3.3.176

Syrjälä, A. M. H., Ylöstalo, P., Hartikainen, S., Sulkava, R., & Knuuttila, M. L. (2009). Number of teeth and myocardial infarction and stroke among elderly never smokers. *Journal of Negative Results in BioMedicine*, *8*(1), 6. https://doi.org/10.1186/1477-5751-8-6

Syrjänen, J. (1993). Infection as a risk factor for cerebral infarction. *European Heart Journal*, *14 Suppl K*(SUPPL. K), 17–19. https://pubmed.ncbi.nlm.nih.gov/8131782/

Syrjänen, J., & Peltola, J. (1989). Dental infections in association with cerebral infarction in young and middle-aged men. *Journal of Internal Medicine*, *225*(3), 179–184. https://doi.org/10.1111/j.1365-2796.1989.tb00060.x

Syrjänen, J., Peltola, J., Valtonen, V., Iivanainen, M., Kaste, M., & Huttunen, J. K. (1989). Dental infections in association with cerebral infarction in young and middle-aged men. *Journal of Internal Medicine*, *225*(3), 179–184. https://doi.org/10.1111/j.1365-2796.1989.tb00060.x

Syrjänen, J., Valtonen, V. V., Livanainen, M., Hovi, T., Malkamäki, M., & Mäkelä, P. H. (1986). Association between cerebral infarction and increased serum bacterial

antibody levels in young adults. *Acta Neurologica Scandinavica*, *73*(3), 273–278. https://doi.org/10.1111/J.1600-0404.1986.TB03275.X

Tampere University Hospital. (2020). *Aivoinfarktin hoidossa nopeus on aivojen pelastus*. https://www.tays.fi/fi-FI/Toimipaikat/Tays_Keskussairaala/Hoitoyksikot/Aivoverenkiertohairioyksikko/Aivoinfarktin_hoidossa_nopeus_on_aivojen(45077)

Thom, D. H., Grayston, J. T., Siscovick, D. S., Wang, S. P., Weiss, N. S., & Daling, J. R. (1992). Association of Prior Infection With Chlamydia pneumoniae and Angiographically Demonstrated Coronary Artery Disease. *JAMA*, *268*(1), 68–72. https://doi.org/10.1001/JAMA.1992.03490010070032

Thompson, R. C., Allam, A. H., Lombardi, G. P., Wann, L. S., Sutherland, M. L., Sutherland, J. D., Soliman, M. A. T., Frohlich, B., Mininberg, D. T., Monge, J. M., Vallodolid, C. M., Cox, S. L., Abd El-Maksoud, G., Badr, I., Miyamoto, M. I., El-Halim Nur El-Din, A., Narula, J., Finch, C. E., & Thomas, G. S. (2013). Atherosclerosis across 4000 years of human history: The Horus study of four ancient populations. *The Lancet*, *381*(9873), 1211–1222. https://doi.org/10.1016/S0140-6736(13)60598-X

Tichopad, A., Bar, T., Pecen, L., Kitchen, R. R., Kubista, M., & Pfaffl, M. W. (2010). Quality control for quantitative PCR based on amplification compatibility test. *Methods*, *50*(4), 308–312. https://doi.org/10.1016/j.ymeth.2010.01.028

Titi, M. A., George, C., Bhattacharya, D., Rahi, A., Woodhead, P. M., Stevenson, W. J., Pillai, A., & Al-Khaffaf, H. (2007). Comparison of carotid Doppler ultrasound and computerised tomographic angiography in the evaluation of carotid artery stenosis. *Surgeon*, *5*(3), 132–136. https://doi.org/10.1016/S1479-666X(07)80039-4

Tomaiuolo, M., Brass, L. F., & Stalker, T. J. (2017). Regulation of Platelet Activation and Coagulation and Its Role in Vascular Injury and Arterial Thrombosis. In *Interventional Cardiology Clinics* (Vol. 6, Issue 1, pp. 1–12). Elsevier Inc. https://doi.org/10.1016/j.iccl.2016.08.001

Tousoulis, Di., Oikonomou, E., Economou, E. K., Crea, F., & Kaski, J. C. (2016). Inflammatory cytokines in atherosclerosis: Current therapeutic approaches. *European Heart Journal*, *37*(22), 1723–1735. https://doi.org/10.1093/eurheartj/ehv759

Traylor, M., Farrall, M., Holliday, E. G., Sudlow, C., Hopewell, J. C., Cheng, Y. C., Fornage, M., Ikram, M. A., Malik, R., Bevan, S., Thorsteinsdottir, U., Nalls, M. A., Longstreth, W. T., Wiggins, K. L., Yadav, S., Parati, E. A., DeStefano, A. L., Worrall, B. B., Kittner, S. J., … Markus, H. S. (2012). Genetic risk factors for ischaemic stroke and its subtypes (the METASTROKE Collaboration): A meta-analysis of genome-wide association studies. *The Lancet Neurology*, *11*(11), 951–962. https://doi.org/10.1016/S1474-4422(12)70234-X

Tsai, W. H., Chuang, S. M., Liu, S. C., Lee, C. C., Chien, M. N., Leung, C. H., Liu, S. J., & Shih, H. M. (2021). Effects of SGLT2 inhibitors on stroke and its subtypes in patients with type 2 diabetes: a systematic review and meta-analysis. *Scientific Reports*, *11*(1), 15364. https://doi.org/10.1038/S41598-021-94945-4

Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Alonso, A., Beaton, A. Z., Bittencourt, M. S., Boehme, A. K., Buxton, A. E., Carson, A. P., Commodore-Mensah, Y., Elkind, M. S. V, Evenson, K. R., Eze-Nliam, C., Ferguson, J. F., Generoso, G., Ho, J. E., Kalani, R., Khan, S. S., Kissela, B. M., … null, null. (2022). Heart Disease and Stroke Statistics—2022 Update: A Report From the American Heart Association. *Circulation*, *145*(8), e153–e639. https://doi.org/10.1161/CIR.0000000000001052

Tuleta, I., Skowasch, D., Aurich, F., Eckstein, N., Schueler, R., Pizarro, C., Schahab, N., Nickenig, G., Schaefer, C., & Pingel, S. (2017). Asthma is associated with atherosclerotic artery changes. *PLoS ONE*, *12*(10). https://doi.org/10.1371/JOURNAL.PONE.0186820

Tuomisto, S., Huhtala, H., Martiskainen, M., Goebeler, S., Lehtimäki, T., & Karhunen, P. J. (2019). Age-dependent association of gut bacteria with coronary atherosclerosis: Tampere sudden death study. *PLoS ONE*, *14*(8). https://doi.org/10.1371/journal.pone.0221345

Tuomisto, S., Karhunen, P. J., & Pessi, T. (2013). Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR. *Gut Pathogens*, *5*(1). https://doi.org/10.1186/1757-4749-5-35

Tuomisto, S., Karhunen, P. J., Vuento, R., Aittoniemi, J., & Pessi, T. (2013). Evaluation of postmortem bacterial migration using culturing and real-time quantitative PCR. *Journal of Forensic Sciences*, *58*(4), 910–916. https://doi.org/10.1111/1556-4029.12124

Tuomisto, S., Pessi, T., Collin, P., Vuento, R., Aittoniemi, J., & Karhunen, P. J. (2014). Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics. *BMC Gastroenterology*, *14*(1). https://doi.org/10.1186/1471-230X-14-40

Turk, A. S., Frei, D., Fiorella, D., Mocco, J., Baxter, B., Siddiqui, A., Spiotta, A., Mokin, M., Dewan, M., Quarfordt, S., Battenhouse, H., Turner, R., & Chaudry, I. (2018). ADAPT FAST study: A direct aspiration first pass technique for acute stroke thrombectomy. *Journal of NeuroInterventional Surgery*, *10*, I4–I7. https://doi.org/10.1136/neurintsurg-2014-011125.rep

Turner, R. (1998). Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet*, *352*(9131), 837–853. https://doi.org/10.1016/S0140-6736(98)07019-6

Vakhitov, D., Tuomisto, S., Martiskainen, M., Korhonen, J., Pessi, T., Salenius, J. P., Suominen, V., Lehtimäki, T., Karhunen, P. J., & Oksala, N. (2018). Bacterial signatures in thrombus aspirates of patients with lower limb arterial and venous thrombosis. *Journal of Vascular Surgery*, *67*(6), 1902–1907. https://doi.org/10.1016/j.jvs.2017.05.090

Valtonen, V. V. (1999). Role of infections in atherosclerosis. *American Heart Journal*, *138*(5 II), S431–S433. https://doi.org/10.1016/s0002-8703(99)70269-3

Virmani, R., Kolodgie, F. D., Burke, A. P., Farb, A., & Schwartz, S. M. (2000). Lessons from sudden coronary death: a comprehensive morphological classification scheme for

atherosclerotic lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(5), 1262–1275. https://doi.org/10.1161/01.ATV.20.5.1262

Virtanen, E., Nurmi, T., Söder, P. Ö., Airila-Månsson, S., Söder, B., & Meurman, J. H. (2017). Apical periodontitis associates with cardiovascular diseases: A cross-sectional study from Sweden. *BMC Oral Health*, *17*(1), 107. https://doi.org/10.1186/s12903-017-0401-6

Vrselja, Z., Brkic, H., Mrdenovic, S., Radic, R., & Curic, G. (2014). Function of circle of Willis. *Journal of Cerebral Blood Flow & Metabolism*, *34*(4), 578. https://doi.org/10.1038/JCBFM.2014.7

Wafa, H. A., Wolfe, C. D. A. A., Emmett, E., Roth, G. A., Johnson, C. O., & Wang, Y. (2020). Burden of Stroke in Europe: Thirty-Year Projections of Incidence, Prevalence, Deaths, and Disability-Adjusted Life Years. *Stroke*, *51*(8), 2418–2427. https://doi.org/10.1161/STROKEAHA.120.029606

Wahlgren, N., Moreira, T., Michel, P., Steiner, T., Jansen, O., Cognard, C., Mattle, H. P., van Zwam, W., Holmin, S., Tatlisumak, T., Petersson, J., Caso, V., Hacke, W., Mazighi, M., Arnold, M., Fischer, U., Szikora, I., Pierot, L., Fiehler, J., … Lees, K. R. (2016). Mechanical thrombectomy in acute ischemic stroke: Consensus statement by ESO-Karolinska Stroke Update 2014/2015, supported by ESO, ESMINT, ESNR and EAN. *International Journal of Stroke*, *11*(1), 134–147. https://doi.org/10.1177/1747493015609778

Weissman, S., Sinh, P., Mehta, T. I., Thaker, R. K., Derman, A., Heiberger, C., Qureshi, N., Amrutiya, V., Atoot, A., Dave, M., & Tabibian, J. H. (2020). Atherosclerotic cardiovascular disease in inflammatory bowel disease: The role of chronic inflammation. *World Journal of Gastrointestinal Pathophysiology*, *11*(5), 104. https://doi.org/10.4291/WJGP.V11.I5.104

Westling, K., Julander, I., Ljungman, P., Vondracek, M., Wretlind, B., & Jalal, S. (2008). Identification of species of viridans group streptococci in clinical blood culture isolates by sequence analysis of the RNase P RNA gene, rnpB. *The Journal of Infection*, *56*(3), 204–210. https://doi.org/10.1016/J.JINF.2007.12.006

Whittle, E., Leonard, M. O., Harrison, R., Gant, T. W., & Tonge, D. P. (2019). Multi-method characterization of the human circulating microbiome. *Frontiers in Microbiology*, *10*(JAN), 1–12. https://doi.org/10.3389/fmicb.2018.03266

Wilck, N., & Ludwig, A. (2014). Targeting the ubiquitin-proteasome system in atherosclerosis: status quo, challenges, and perspectives. *Antioxidants & Redox Signaling*, *21*(17), 2344–2363. https://doi.org/10.1089/ARS.2013.5805

Wilson, H. M. (2010). Macrophages heterogeneity in atherosclerosis – implications for therapy. *Journal of Cellular and Molecular Medicine*, *14*(8), 2055–2065. https://doi.org/10.1111/J.1582-4934.2010.01121.X

Wolf, P. A., Abbott, R. D., & Kannel, W. B. (1991). Atrial fibrillation as an independent risk factor for stroke: The framingham study. *Stroke*, *22*(8), 983–988. https://doi.org/10.1161/01.STR.22.8.983

World Health Organization. (2021). *Cardiovascular diseases*. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

Wu, T., Trevisan, M., Genco, R. J., Dorn, J. P., Falkner, K. L., & Sempos, C. T. (2000). Periodontal disease and risk of cerebrovascular disease: the first national health and nutrition examination survey and its follow-up study. *Archives of Internal Medicine*, *160*(18), 2749–2755. https://doi.org/10.1001/archinte.160.18.2749

Yang, S., Lin, S., Kelen, G. D., Quinn, T. C., Dick, J. D., Gaydos, C. A., & Rothman, R. E. (2002). Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *Journal of Clinical Microbiology*, *40*(9), 3449–3454. https://doi.org/10.1128/JCM.40.9.3449-3454.2002

Yoshida, A., Suzuki, N., Nakano, Y., Oho, T., Kawada, M., & Koga, T. (2003). Development of a 5' fluorogenic nuclease-based real-time PCR assay for quantitative detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. *Journal of Clinical Microbiology*, *41*(2), 863–866. https://doi.org/10.1128/JCM.41.2.863-866.2003

Zheng, W., Tan, T. K., Paterson, I. C., Mutha, N. V. R., Siow, C. C., Tan, S. Y., Old, L. A., Jakubovics, N. S., & Choo, S. W. (2016). StreptoBase: An Oral Streptococcus mitis Group Genomic Resource and Analysis Platform. *PLOS ONE*, *11*(5), e0151908. https://doi.org/10.1371/journal.pone.0151908

Ziu, E., Suheb, M. Z. K., & Mesfin, F. B. (2023). Subarachnoid Hemorrhage. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK441958/

ORIGINAL PUBLICATIONS

PUBLICATION I

Oral Bacterial Signatures in Cerebral Thrombi of Patients with Acute Ischemic Stroke Treated with Thrombectomy

Patrakka O, Pienimäki JP, Tuomisto S, Ollikainen J, Lehtimäki T, Karhunen PJ, Martiskainen M.

Journal of the American Heart Association. 2019;8(11):e012330.

ORIGINAL RESEARCH

Oral Bacterial Signatures in Cerebral Thrombi of Patients With Acute Ischemic Stroke Treated With Thrombectomy

Olli Patrakka, MD; Juha-Pekka Pienimäki, MD; Sari Tuomisto, PhD; Jyrki Ollikainen, MD; Terho Lehtimäki, MD, DDS, PhD; Pekka J. Karhunen, MD, PhD;* Mika Martiskainen, MD*

***Background*—**Chronic infections have been reported to be risk factors for both coronary heart disease and ischemic stroke. DNA of oral bacteria, mainly from the viridans streptococci group, has been detected in coronary thrombus aspirates of myocardial infarction and cerebral aneurysms. Viridans streptococci are known to cause infective endocarditis and possess thrombogenic properties. We studied the presence of oral bacterial DNA in thrombus aspirates of patients with acute ischemic stroke treated with mechanical thrombectomy.

***Methods and Results*—**Thrombus aspirates and arterial blood were taken from 75 patients (69% men; mean age, 67 years) with acute ischemic stroke. The presence of *Streptococcus* species, mainly the *Streptococcus mitis* group, belonging to viridans streptococci as well as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in samples were determined using a quantitative polymerase chain reaction with specific primers and probes. The relative amount of bacterial DNA in a sample was determined with the comparative threshold cycle method. Bacterial DNA was detected in 84% (n=63) of aspired thrombi, and 16% (n=12) of samples were considered bacterial DNA negative. DNA of *Streptococcus* species, mainly the *S mitis* group, was found in 79% (n=59) of samples. The median relative amount of *Streptococcus* species DNA was 5.10-fold higher compared with the control blood samples from the same patients. All thrombi were negative for both *P gingivalis* and *A actinomycetemcomitans.*

***Conclusions*—**This is the first study showing the common presence of bacterial DNA from viridans streptococci in aspired thrombi of patients with acute ischemic stroke. Streptococcal bacteria, mostly of oral origin, may contribute to the progression and thrombotic events of cerebrovascular diseases. **(*J Am Heart Assoc*. 2019;8:e012330. DOI: 10.1161/JAHA.119.012330.)**

Key Words: acute stroke • arterial thrombosis • atherogenesis • atherosclerosis • cerebral ischemia

Cardiovascular and cerebrovascular diseases are major causes of death, and stroke is the leading cause of adult long-term disability in western countries.[1] Stroke can be divided into intracerebral hemorrhage, subarachnoid hemorrhage, and cerebral ischemia. In the Unites States of America, ≈795 000 people experience stroke each year, of which ≈692 000 are acute ischemic strokes (AISs).[2] Treatment of AIS has undergone major developments during the past 2 decades: first, the cerebral computed tomography made it possible to identify patients for medical thrombolysis; second, the intravascular catheter-based revascularization of cerebral vessels began to evolve. The modern intravascular revascularization techniques are stent retriever thrombectomy and direct aspiration of cerebral arterial clots.[3–5]

In addition to traditional causative factors, such as hypertension, hypercholesterolemia, diabetes mellitus, smoking, and obesity, bacterial inflammation has been suggested to contribute directly or indirectly to the development of the atherosclerosis and atherothrombotic events.[6]

Levels of CRP (C-reactive protein) seem to have correlation with the incidence for cardiovascular events and stroke.[7,8] CRP increases the adhesiveness of platelets for endothelium.[9] Inflammation of atherosclerotic plaque may affect its growth and contribute to plaque rupture, leading to thrombosis.[10,11] Inflammation at nonvascular sites may also affect progression

From the Department of Forensic Medicine, Faculty of Medicine and Health Technology, Tampere University and Fimlab Laboratories, Tampere, Finland (O.P., S.T., P.J.K., M.M.); Division of Interventional Radiology, Department of Radiology (J.-P.P.), and Department of Neurology (J.O.), Tampere University Hospital, Tampere, Finland; Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Fimlab Laboratories, and Finnish Cardiovascular Research Center, Tampere, Finland (T.L.); and National Institute for Health and Welfare, Helsinki, Finland (M.M.).

*Dr Karhunen and Dr Martiskainen contributed equally to this work and are co–senior authors.

Correspondence to: Olli Patrakka, MD, Department of Forensic Medicine, Faculty of Medicine and Health Technology, Tampere University, 33014 Tampere, Finland. E-mail: olli.patrakka@tuni.fi

Received February 19, 2019; accepted April 5, 2019.

Clinical Perspective

What Is New?

- We found DNA of viridans streptococci in most aspired thrombi of patients with acute ischemic stroke, which suggests that oral bacteria may have a role in the cause of cerebrovascular disease.

What Are the Clinical Implications?

- Repeated transient bacteremia, caused by poor dental care or bacterial infections, may trap pathogens in atherosclerotic plaques and promote rupture of the plaques; therefore, regular dental care should be emphasized in the primary prevention of acute ischemic stroke.

of atherosclerotic lesions via circulating chemical mediators.[12] Severe periodontitis increased 2-fold the risk of ischemic stroke among middle-aged men but not in women after adjusting for age, sex, number of teeth, vascular risk factors and diseases, childhood and adult socioeconomic conditions, and lifestyle factors.[13] In the Indian population, periodontitis was even a stronger risk factor compared with hypertension and smoking.[14] According to recent studies, periodontitis and tooth loss have an independent direct association with stroke.[15,16]

We have earlier reported that bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombi, and periodontal pathogens were measured in 34.7% of thrombus aspirates from coronary arteries in patients with acute myocardial infarction. The median value for the total amount of bacterial DNA in coronary thrombus was 16 times higher than that found in their blood samples.[17] Furthermore, we were able to detect these bacteria in ruptured and nonruptured cerebral aneurysm samples[18] and from thrombus aspirates of patients with lower limb vascular disorders.[19] The presence of odontogenic bacteria, such as *Porphyromonas gingivalis* and *Streptococcus sanguinis*, has been shown in the atherosclerotic plaque of human carotid artery histologically and by polymerase chain reaction (PCR).[20,21]

In this study, we used a real-time quantitative PCR (qPCR) to detect bacterial DNA from thrombi that have been collected by stent retriever technique from the cerebral arteries of patients with AIS. To our knowledge, there are no earlier studies depicting the role of oral bacteria in the thromboembolic events of cerebral arteries among patients with AIS. We hypothesized that oral bacterial DNA can be found in cerebral arterial thrombi similarly as from coronary or inferior extremity peripheral vessel thrombi.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

The series comprises 75 patients with AIS who were treated by intra-arterial thrombectomy between November 2013 and January 2017 in the Acute Stroke Unit of Tampere University Hospital, Tampere, Finland (Table). This series is a subset from a larger Tampere BMG (Brain Microbes and Genetics) study, focusing on the role of inflammation and genetics in ischemic stroke. A neurologist (J.O.) examined all patients when they arrived to the hospital and evaluated the possibility of revascularization using thrombectomy together with a neurointerventional radiologist (J.-P.P.). The cause of the brain large-vessel occlusions of patients treated with endovascular thrombectomy during the study period in Tampere University Hospital was cardioembolic in 38% and atherosclerotic in 62% of the patients (oral communication, 6th February, J.O.). The only excluding criterion for recruiting patients was that if the thrombus was not retrieved successfully using mechanical thrombectomy. The median delay time between onset of an

Table. Patients' Characteristics

Characteristics	Data for All Patients (N=75)
Age, mean±SD, y	66.9±12.4
Men, n (%)	52 (69.3)
Diabetes mellitus, n (%)	12 (16.0)
Dyslipidemia, n (%)	29 (38.7)
Arterial hypertension, n (%)	40 (53.3)
Coronary heart disease, n (%)	15 (20.0)
Cerebrovascular disease, n (%)*	20 (26.7)
Pulmonary disease, n (%)	4 (5.33)
Renal insufficiency, n (%)	7 (9.33)
Atrial fibrillation, n (%)	48 (64.0)
Heart failure, n (%)	10 (13.3)
Smoking status, %*	34.9
Location of thrombus aspirate, n (%)	
ICA	25 (33.3)
MCA	72 (97.3)
ACA	6 (8.00)
PCA	1 (1.33)
VA	6 (8.00)
AB	1 (1.33)
Arrival time to the hospital (quartile 1, median, quartile 2), h	1.20, 2.30, 3.80

AB indicates basilar artery; ACA, anterior cerebral artery; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; VA, vertebral artery.
*Smoking status was only available from 43 patients.

ischemic stroke and hospital arrival was 2 hours 20 minutes (range, 0-16 hours). Medical history was collected from the Tampere University Hospital digital patient archives. Criteria for dyslipidemia were fP-kol (cholesterol levels measured from plasma after 12 hours fasting (f=fasting, P=plasma)) low-density lipoprotein >3.0 mmol/L, fP-kol high-density lipoprotein <1.0 mmol/L, or fP triglycerides >2.0 mmol/L.

The study was approved by the ethics committee (R13093) of the Tampere University Hospital. The study was explained to the patients, and informed consent was obtained.

Mechanical Thrombectomy and Thrombus Sample Collection

An introducer sheet was placed on a femoral artery, and a control blood sample for bacterial genetic analysis was collected through the sheet. A guiding catheter up to 9F (Merci; Stryker Neurovascular) with a tip balloon was then navigated into the carotid artery proximal to the occluded site. The microcatheter (0.021 inches) with the guide wire was used to navigate through the occluded site and to deploy the stent retriever (Trevo; Stryker Neurovascular) over the thrombus. An additional distal access catheter was used to access the thrombus if needed. Forceful aspiration through proximal catheters was acquired with a 60-mL syringe while retrieving the deployed stent. In a minority of the cases, only direct thrombus aspiration was used. Different device settings were selected by operator case by case. Thrombectomy was repeated until the angiologic result was satisfactory. The gathered thrombus was divided into a 1.5-mL Eppendorf microcentrifuge tube for qPCR analysis, and a histological sample was placed in a formalin container.

Quantitative PCR

Bacterial DNA was extracted from samples using a commercially available QIAamp DNA Mini Kit (Qiagen, Germany), according to the instructions provided.

The presence of bacterial DNA in thrombus and blood samples from the same patients was determined by using published oligonucleotide primers and probes for *Streptococcus* species (mainly the *Streptococcus mitis* group[22]), *P gingivalis*, and *Aggregatibacter actinomycetemcomitans*[23]; and universal bacterial primers and probe[24] were determined with RNaseP (Applied Biosystems, Foster City, CA), as a reference.

qPCR assays were performed using specific TaqMan allele hybridization with the AbiPrism 7900 HT Sequence Detection System (TaqMan; Applied Biosystems, Carlsbad, CA) under standard conditions with the following cycle profile: 50°C for 2 minutes, 95°C for 10 minutes, and 60 cycles of 95°C for 15 seconds and 58°C for 1 minutes. MasterMix was prepared using Maxima Probe/ROX qPCR MasterMix (Thermo Fischer Scientific, Waltham, MA), adding at final concentrations of 900 nmol/L of each primer and 250 nmol/L of each fluorescence-labeled probe. All amplifications and detections were performed as duplicates or quadruples (in uncertain cases), depending on test runs in a MicroAmp optical 384-well reaction plate with optical caps (Sarsted, Nümbrecht, Germany) in a reaction volume of 5 μL, with 1 μL of nondiluted DNA.

Human housekeeping gene, RNaseP, was used as a reference measurement to determine the relative amount of bacterial DNA in the sample. The determination was done by the comparative threshold cycle (Ct) method. The critical Ct is the cycle at which a statistically significant increase in ΔRn (normalized reporter) is first detected and at which the fluorescence becomes detectable above background. Ct is inversely proportional to the logarithm of the initial number of template molecules (ie, the initial amount of sample DNA). Calculation with the Ct method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$) was done with a simplification.[25–28] First, the differences of the Ct values between candidate bacteria and reference gene measurement (candidate bacteria–RNaseP [ΔCt]) for each sample were calculated; then, the comparative Ct (thrombus–patients own blood [ΔΔCt]) was calculated. The samples were marked bacterial positive, if $2^{-\Delta\Delta Ct} \geq 2$,[29,30] or if there was amplified bacterial DNA in the thrombus but not in the control sample. DNA was extracted from the entire thrombus in most of the cases. If the aspired thrombus was large, a small part of it was taken and sent for histological analyses, and DNA was extracted from the rest of the thrombus.

Statistical Analysis

Statistical analysis was done using IBM SPSS Statistics 25 (SPSS, Chicago, IL). Associations between the bacterial findings and nominal parameters were analyzed using Pearson's χ^2 test. Age was treated as normally distributed and, therefore, mean and sample SD values were calculated and the Student *t* test was used. The Mann-Whitney *U*-test was used to analyze the associations between bacterial findings and the median arrival time to the hospital; these values were not normally distributed. Statistical significance was set at $P<0.05$.

Results

Patient Characteristics

Of the study population, 69.3% (n=52) were men and 30.7% (n=23) were women. The mean age of the patients was 66.9 years. None of the patients had been treated with antibiotics or experienced severe infections or septicemia

during the stroke. Characteristics of the study population are presented in the Table.

Presence of Bacterial DNA in Thrombus Aspirates

Of the 75 patients who underwent thrombectomy for treatment of acute stroke, 84.0% (n=63) of aspirated thrombi were positive for bacterial DNA in qPCR compared with 16.0% (n=12) of bacteria-negative thrombi. In addition, 78.7% (n=59) of aspirated thrombi were positive for *Streptococcus* species, mainly the *S mitis* group. Bacterial DNA of *P gingivalis* and *A actinomycetemcomitans* was not found in thrombi. Of the arterial blood samples that were collected during the thrombectomy procedure, 9.33% (n=7) were positive for both bacteria and *Streptococcus* species, mainly the *S mitis* group, and 1.33% (n=1) were positive for both *P gingivalis* and *A actinomycetemcomitans*.

The presence of *Streptococcus* species, mainly the *S mitis* group DNA, was 5.10-fold in median and total bacteria DNA was 7.93-fold in median, compared with the control blood sample from the same patients. N-fold values for qPCR findings are presented in Figure 1. Patients positive for any bacterial DNA were more often men (P=0.067) and more often had diabetes mellitus (P=0.074) and previous cerebrovascular disease (P=0.046) compared with patients negative for any bacterial DNA. However, there were no differences in patients' demographic parameters between those positive or negative for the *S mitis* group bacterial DNA (Figure 2).

Discussion

The present concept is that atherosclerosis is a complex chronic inflammatory disorder driven by oxidized or otherwise modified low-density lipoprotein.[31–33] However, increasing knowledge suggests that the atherosclerotic process may be accelerated by bacterial infection.[34,35]

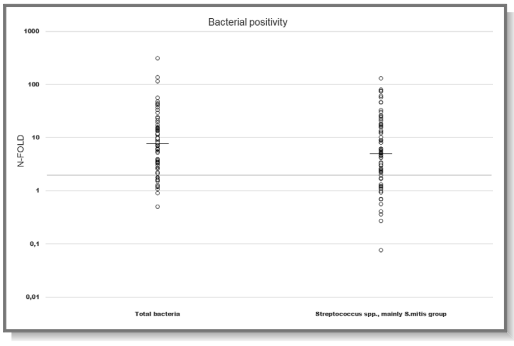


Figure 1. N-fold values >2.0 are significantly positive for bacterial DNA. Line at the 2.0 *y*-axis level indicates that most thrombi collected during thrombectomies of patients with acute stroke are positive for bacterial DNA. Median N-fold values are illustrated with black lines.

In our study, we found that thrombi collected from patients with AIS were 84% positive for bacterial DNA and especially for the *S mitis* group, whereas we could not detect DNA from *P gingivalis* and *A actinomycetemcomitans*. To our knowledge, this is the first study in which bacterial signatures of the thrombi of patients with AIS have been analyzed using qPCR.

Streptococcus species are mostly found in the oral cavity of healthy individuals and in patients with periodontal disease.[36,37] The *S mitis* group comprises 20 species.[38] Oral bacteria can gain access to the bloodstream after trauma or dental procedures (eg, root canal treatment or tooth extraction) and cause transient bacteremia. In tooth extraction, most bacteria translocated into the circulation were viridans streptococci.[39] Viridans streptococci are the most common cause of infective endocarditis and sepsis.[40] It is possible that oral bacteria can be trapped in atrial thrombosis in patients with atrial fibrillation. Moreover, it has been found that bacteria can be found in the circulation without any clinical symptoms.[41]

There are several ways how bacteria can enter the atherosclerotic plaque. Bacteria can be phagocytosed and end up in the atherosclerotic plaque via circulating macrophages. Bacteria can also directly drift in the bloodstream through the vasa vasorum or the neovasculature vessels developing inside the atherosclerotic plaque. Amoxicillin medication in connection with tooth extraction decreased the frequency of positive streptococci findings in peripheral blood samples of the patients.[42] A recent study confirmed that regular dental care lowers the risk for ischemic stroke.[43]

It has been shown that oral pathogens, such as viridans group streptococci, can stimulate endothelial cells to produce various proinflammatory cytokines, such as interleukin 6 and interferon γ.[44,45] These cytokines are involved in the pathogenetic pathway of atherosclerosis and may promote the rupture of the plaque.[46] Oral pathogens seem to activate toll-like receptors, which mediate inflammation responses to pathogens and cause endothelial dysfunction. Endothelial toll-like receptors initiate inflammation and are an important component in the establishment of plaque.[47,48]

In addition to the regular inflammatory pathway, bacteria can straightforwardly interact with a platelet, causing its activation.[49] Bacterial surface proteins of *S mitis* can directly bind to various platelet receptors.[50,51] There can also be indirect activation via plasma immunoglobulin G.[52] Moreover, odontogenic bacteria can secrete gingipains proteinase, which can persuade an increase in platelet intracellular [Ca^{2+}] and lead to the activation and aggregation of platelets.[53,54] Activated platelets induce the recruitment of proatherosclerotic cells and speed up the development of

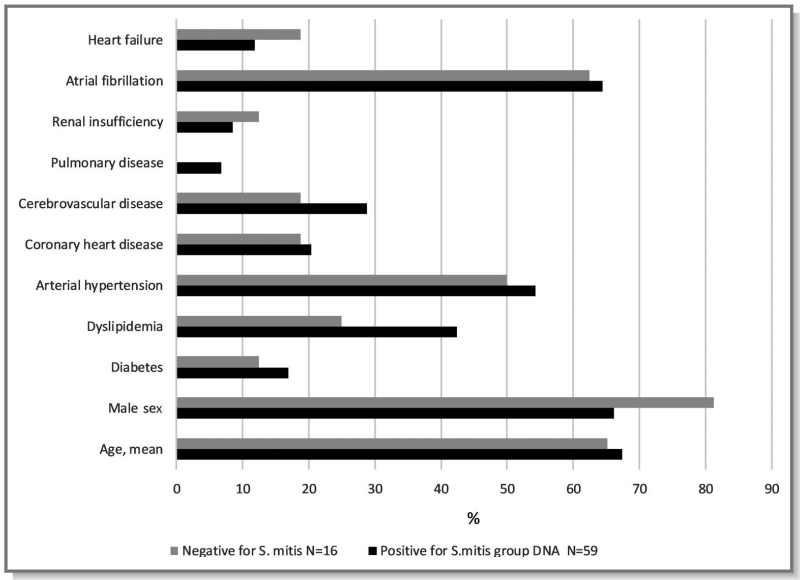


Figure 2. Clinical characteristics of patients with negative or positive thrombus aspirates for the *Streptococcus mitis* group DNA.

atherothrombotic lesions.[55] Activated platelets can take part in the formation of cardioembolic stroke if the hemodynamic conditions are altered, such as in atrial fibrillation.[56,57]

One of the limitations of this study is its small sample size, which prevents further subgroup analyses. The exact impact of living bacteria on thrombosis cannot be declared using PCR. In addition, the PCR method we used detects the presence of bacterial DNA in the examined samples but is unable to separate living bacteria from phagocytized bacterial DNA. Culturing and staining are the most frequently performed techniques when detecting bacterial species. Nonetheless, the PCR method seems to be more accurate and cost-effective in comparison with traditional culturing.[58,59] Although our analysis revealed the absence of *P gingivalis* and *A actinomycetemcomitans* in the thrombi, it does not exclude the possibility of their role in the pathogenesis of AIS. The presence of bacterial DNA was defined by an artificial cutoff value: the sample was considered positive if it contained 2 times more bacterial DNA compared with the control sample from the same patient. In median, the samples contained ≈5 times more *S mitis* DNA than the control samples. However, the validity is also affected by the inhomogeneity of the thrombus material.[60,61]

The findings of this study confirm those of our previous studies,[17,19] suggesting that bacterial infection may be involved in the pathogenesis of coronary and lower limb thrombosis. However, it is not known whether the oral bacteria are one of the causes of atherothrombotic events or whether their role is solely as bystander. The exact role of bacteria in AIS thus remains unclear.

Conclusion

We found DNA of *Streptococcus* species, mainly the *S mitis* group, belonging to viridans streptococci, in most aspirated thrombi of the patients with AIS. This suggests that viridans streptococci may have a role in the cause of cerebrovascular disease. Regular dental care should be emphasized in the primary prevention of AIS.

Sources of Funding

This study was supported with grants from the Competitive Research Funding of the Tampere University Hospital (grant X51001 for Lehtimäki and Karhunen), the Emil Aaltonen Foundation (Lehtimäki), and the Academy of Finland (grant 286284 for Lehtimäki), the Tampere Tuberculosis Foundation, the Finnish Foundation for Cardiovascular Research, the Yrjö Jahnsson Foundation, the Pirkanmaa Cultural Foundation (Tuomisto), the European Union 7th Framework Program (grant 201668 for AtheroRemo (European Collaborative Project on Inflammation and Vascular Wall Remodelling in

Atherosclerosis), and EU (European Union) Horizon 2020 (grant 755320 for TAXINOMISIS Project: A multidisciplinary approach for the stratification of patients with carotid artery disease).

Disclosures

None.

References

1. Katan M, Luft A. Global burden of stroke. *Semin Neurol*. 2018;38:208–211.
2. Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, Chiuve SE, Cushman M, Delling FN, Deo R, de Ferranti SD, Ferguson JF, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Lutsey PL, Mackey JS, Matchar DB, Matsushita K, Mussolino ME, Nasir K, O'Flaherty M, Palaniappan LP, Pandey A, Pandey DK, Reeves MJ, Ritchey MD, Rodriguez CJ, Roth GA, Rosamond WD, Sampson UKA, Satou GM, Shah SH, Spartano NL, Tirschwell DL, Tsao CW, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P. Heart disease and stroke statistics-2018 update: a report from the American Heart Association. *Circulation*. 2018;137:e67–e492.
3. Wahlgren N, Moreira T, Michel P, Steiner T, Jansen O, Cognard C, Mattle HP, van Zwam W, Holmin S, Tatlisumak T, Petersson J, Caso V, Hacke W, Mazighi M, Arnold M, Fischer U, Szikora I, Pierot L, Fiehler J, Gralla J, Fazekas F, Lees KR. Mechanical thrombectomy in acute ischemic stroke: consensus statement by ESO-Karolinska Stroke Update 2014/2015, supported by ESO, ESMINT, ESNR and EAN. *Int J Stroke*. 2016;11:134–147.
4. Turk AS, Frei D, Fiorella D, Mocco J, Baxter B, Siddiqui A, Spiotta A, Mokin M, Dewan M, Quarfordt S, Battenhouse H, Turner R, Chaudry I. ADAPT FAST study: a direct aspiration first pass technique for acute stroke thrombectomy. *J NeuroIntervent Surg*. 2014;6:260–264.
5. Berkhemer OA, Fransen PS, Beumer D, van den Berg LA, Lingsma HF, Yoo A, Schonewille WJ, Vos JA, Nederkoorn PJ, Wermer MJH, van Walderveen MAA, Staals J, Hofmeijer J, van Oostayen JA, Lycklama à Nijeholt GJ, Boiten J, Brouwer PA, Emmer BJ, de Bruijn SF, van Dijk LC, Kappelle LJ, Lo RH, van Dijk EJ, de Vries J, de Kort PLM, van Rooij WJJ, van den Berg JSP, van Hasselt BAAM, Aerden LAM, Dallinga RJ, Visser MC, Bot JCJ, Vroomen PC, Eshghi O, Schreuder THCML, Heijboer RJJ, Keizer K, Tielbeek AV, den Hertog HM, Gerrits DG, van den Berg-Vos RM, Karas GB, Steyerberg EW, Flach HZ, Marquering HA, Sprengers MES, Jenniskens SFM, Beenen LFM, van den Berg R, Koudstaal PJ, van Zwam WH, Roos YBWEM, van der Lugt A, van Oostenbrugge RJ, Majoie CBLM, Dippel DWJ. A randomized trial of intraarterial treatment for acute ischemic stroke. *N Engl J Med*. 2015;372:11–20.
6. Rosenfeld ME. Inflammation and atherosclerosis: direct versus indirect mechanism. *Curr Opin Pharmacol*. 2013;13:154–160.
7. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347:1557–1565.
8. Rost NS, Wolf PA, Kase CS, Kelly-Hayes M, Silbershatz H, Massaro JM, D'Agostino RB, Franzblau C, Wilson PWF. Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: the Framingham study. *Stroke*. 2001;32:2575–2579.
9. Grad E, Pachino RM, Danenberg HD. Endothelial C-reactive protein increases platelet adhesion under flow conditions. *Am J Physiol Heart Circ Physiol*. 2011;301:H730–H736.
10. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, Behre CJ, Knight R, Fagerberg B, Ley RE, Bäckhed F. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci USA*. 2011;108(suppl 1):4592–4598.
11. Lanter BB, Sauer K, Davies DG. Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture. *MBio*. 2014;5:e01206–e01214.
12. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res*. 2004;95:858–866.
13. Grau AJ, Becher H, Ziegler CM, Lichy C, Buggle F, Kaiser C, Lutz R, Bültmann S, Preusch M, Dörfer CE. Periodontal disease as a risk factor for ischemic stroke. *Stroke*. 2004;35:496–501.
14. Pradeep AR, Hadge P, Arjun Raju P, Shetty SR, Shareef K, Guruprasad CN. Periodontitis as a risk factor for cerebrovascular accident: a case-control study in the Indian population. *J Periodontal Res*. 2010;45:223–228.
15. Pillai RS, Iyer K, Spin-Neto R, Kothari SF, Nielsen JF, Kothari M. Oral health and brain injury: causal or casual relation? *Cerebrovasc Dis Extra*. 2018;8:1–15.
16. Virtanen E, Nurmi T, Söder PÖ, Airila-Månsson S, Söder B, Meurman JH. Apical periodontitis associates with cardiovascular diseases: a cross-sectional study from Sweden. *BMC Oral Health*. 2017;17:107.
17. Pessi T, Karhunen V, Karjalainen PP, Ylitalo A, Airaksinen JK, Niemi M, Pietila M, Lounatmaa K, Haapaniemi T, Lehtimäki T, Laaksonen R, Karhunen PJ, Mikkelsson J. Bacterial signatures in thrombus aspirates of patients with myocardial infarction. *Circulation*. 2013;127:1219–1228.
18. Pyysalo MJ, Pyysalo LM, Pessi T, Karhunen PJ, Lehtimäki T, Oksala N, Öhman JE. Bacterial DNA findings in ruptured and unruptured intracranial aneurysms. *Acta Odontol Scand*. 2016;74:315–320.
19. Vakhitov D, Tuomisto S, Martiskainen M, Korhonen J, Pessi T, Salenius JP, Suominen V, Lehtimäki T, Karhunen PJ, Oksala N. Bacterial signatures in thrombus aspirates of patients with lower limb arterial and venous thrombosis. *J Vasc Surg*. 2018;67:1902–1907.
20. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J*. 1999;138:S534–S536.
21. Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. *J Periodontol*. 2000;71:1554–1560.
22. Tuomisto S, Karhunen PJ, Vuento R, Aittoniemi J, Pessi T. Evaluation of post mortem bacterial migration using culturing and real-time quantitative PCR. *J Forensic Sci*. 2013;58:910–916.
23. Nonnenmacher C, Dalpke A, Mutters R, Heeg K. Quantitative detection of periodontopathogens by real time PCR. *J Microbiol Methods*. 2004;59:117–125.
24. Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J Clin Microbiol*. 2002;40:3449–3454.
25. Suzuki N, Yoshida A, Nakano Y. Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR. *Clin Med Res*. 2005;3:176–185.
26. Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5' fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J Clin Microbiol*. 2003;41:863–866.
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–408.
28. Tuomisto S, Pessi T, Collin P, Vuento R, Aittoniemi J, Karhunen PJ. Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics. *BMC Gastroenterol*. 2014;14:40.
29. Bubner B, Gase K, Baldwin IT. Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *BMC Biotechnol*. 2004;4:14.
30. Tichopad A, Bar T, Pecen L, Kitchen RR, Kubista M, Pfaffl MW. Quality control for quantitative PCR based on amplification compatibility test. *Methods*. 2010;50:308–312.
31. Tuttolomondo A, Di Raimondo D, Pecoraro R, Arnao V, Pinto A, Licata G. Atherosclerosis as an inflammatory disease. *Curr Pharm Des*. 2012;18:4266–4288.
32. Tousoulis D, Oikonomou E, Economou EK, Crea F, Kaski JC. Inflammatory cytokines in atherosclerosis: current therapeutic approaches. *Eur Heart J*. 2016;37:1723–1732.
33. Paoletti R, Gotto AM Jr, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. *Circulation*. 2004;109:III-20–III-26.
34. Morré SA, Stooker W, Lagrand WK, van den Brule AJC, Niessen HWM. Microorganisms in the aetiology of atherosclerosis. *J Clin Pathol*. 2000;53:647–654.
35. Valtonen VV. Role of infections in atherosclerosis. *Am Heart J*. 1999;138:S431–S433.
36. Zheng W, Tan TK, Paterson IC, Mutha NV, Siow CC, Tan SY, Old LA, Jakubovics NS, Choo SW. StreptoBase: an oral *Streptococcus mitis* group genomic resource and analysis platform. *PLoS One*. 2016;11:e0151908.
37. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*. 2005;43:5721–5732.
38. Jensen A, Scholz CFP, Kilian M. Re-evaluation of the taxonomy of the Mitis group of the genus *Streptococcus* based on whole genome phylogenetic analyses, and proposed reclassification of *Streptococcus dentisani* as *Streptococcus oralis* subsp. *dentisani* comb. nov., *Streptococcus tigurinus* as *Streptococcus oralis* subsp. *tigurinus* comb. nov., and *Streptococcus oligofermentans* as a later synonym of *Streptococcus cristatus*. *Int J Syst Evol Microbiol*. 2016;66:4803–4820.

39. Narayanan LL, Vaishnavi C. Endodontic microbiology. *J Conserv Dent*. 2010;13:233–239.
40. Cahill TJ, Prendergast BD. Infective endocarditis. *Lancet*. 2016;387:882–893.
41. Whittle E, Leonard MO, Harrison R, Gant TW, Tonge DP. Multi-method characterization of the human circulating microbiome. *Front Microbiol*. 2019;9:3266.
42. Lockhart PB, Brennan MT, Sasser HC, Fox PC, Paster BJ, Bahrani-Mougeot FK. Bacteremia associated with tooth brushing and dental extraction. *Circulation*. 2008;117:3118–3125.
43. Sen S, Giamberardino LD, Moss K, Morelli T, Rosamond WD, Gottesman RF, Beck J, Offenbacher S. Periodontal disease, regular dental care use, and incident ischemic stroke. *Stroke*. 2018;49:355–362.
44. Chia JS, Lien HT, Hsueh PR, Chen PM, Sun A, Chen JY. Induction of cytokines by glucosyltransferases of *Streptococcus mutans*. *Clin Diagn Lab Immunol*. 2002;9:892–897.
45. Hahn CL, Best AM, Tew JG. Cytokine induction by *Streptococcus mutans* and pulpal pathogenesis. *Infect Immun*. 2000;68:6785–6789.
46. Ramji DP, Davies TS. Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev*. 2015;26:673–685.
47. Hajishengallis G, Sharma A, Russell MW, Genco RJ. Interactions of oral pathogens with toll-like receptors: possible role in atherosclerosis. *Ann Periodontol*. 2002;7:72–78.
48. Curtiss LK, Tobias PS. Emerging role of toll-like receptors in atherosclerosis. *J Lipid Res*. 2009;50:S340–S345.
49. Arman M, Krauel K, Tilley DO, Weber C, Cox D, Greinacher A, Kerrigan SW, Watson SP. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood*. 2014;123:3166–3174.
50. Bensing BA, Rubens CE, Sullam PM. Genetic loci of *Streptococcus mitis* that mediate binding to human platelets. *Infect Immun*. 2001;69:1373–1380.
51. Kerrigan SW, Cox D. The thrombotic potential of oral pathogens. *J Oral Microbiol*. 2009;1:1–10.
52. Naik UP. Bacteria exploit platelets. *Blood*. 2014;123:3067–3068.
53. Lourbakos A, Yuan YP, Jenkins AL, Travis J, Andrade-Gordon P, Santulli R, Potempa J, Pike RN. Activation of protease-activated receptors by gingipains from *Porphyromonas gingivalis* leads to platelet aggregation: a new trait in microbial pathogenicity. *Blood*. 2001;97:3790–3797.
54. Engström KK, Khalaf H, Kälvegren H, Bengtsson T. The role of *Porphyromonas gingivalis* gingipains in platelet activation and innate immune modulation. *Mol Oral Microbiol*. 2015;30:62–73.
55. Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. *Thromb Haemost*. 2008;99:480–486.
56. Herzberg MC, Nobbs A, Tao L, Kilic A, Beckman E, Khammanivong A, Zhang Y. Oral streptococci and cardiovascular disease: searching for the platelet aggregation-associated protein gene and mechanisms of *Streptococcus sanguis*-induced thrombosis. *J Periodontol*. 2005;76:2101–2105.
57. Tomaiuolo M, Brass LF, Stalker TJ. Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis. *Interv Cardiol Clin*. 2017;6:1–12.
58. Atieh MA. Accuracy of real-time polymerase chain reaction versus anaerobic culture in detection of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*: a meta-analysis. *J Periodontol*. 2008;79:1620–1629.
59. Aly BH, Hamad MS, Mohey M, Amen S. Polymerase chain reaction (PCR) versus bacterial culture in detection of organisms in otitis media with effusion (OME) in children. *Indian J Otolaryngol Head Neck Surg*. 2012;64:51–55.
60. Muñoz R, Santamaría E, Rubio I, Ausín K, Ostolaza A, Labarga A, Roldán M, Zandio B, Mayor S, Bermejo R, Mendigaña M, Herrera M, Aymerich N, Olier J, Gállego J, Mendioroz M, Fernández-Irigoyen J. Mass spectrometry-based proteomic profiling of thrombotic material obtained by endovascular thrombectomy in patients with ischemic stroke. *Int J Mol Sci*. 2018;19:498.
61. De Meyer SF, Andersson T, Baxter B, Bendszus M, Brouwer P, Brinjikji W, Campbell BC, Costalat V, Dávalos A, Demchuk A, Dippel D, Fiehler J, Fischer U, Gilvarry M, Gounis MJ, Gralla J, Jansen O, Jovin T, Kallmes D, Khatri P, Lees KR, López-Cancio E, Majoie C, Marquering H, Narata AP, Nogueira R, Ringleb P, Siddiqui A, Szikora I, Vale D, von Kummer R, Yoo AJ, Hacke W, Liebeskind DS. Analyses of thrombi in acute ischemic stroke: a consensus statement on current knowledge and future directions. *Int J Stroke*. 2017;12:606–614.

PUBLICATION II

Association between Oral Pathology, Carotid Stenosis, and Oral Bacterial DNA in Cerebral Thrombi of Patients with Stroke

Patrakka O, Mehtonen H, Tuomisto S, Pienimäki JP, Ollikainen J, Huhtala H, Pessi T, Oksala N, Lehtimäki T, Järnstedt J, Martiskainen M, Karhunen PJ.

Stroke Research and Treatment. 2021;2021.

Hindawi
Stroke Research and Treatment
Volume 2021, Article ID 5402764, 10 pages
https://doi.org/10.1155/2021/5402764

Research Article

Association between Oral Pathology, Carotid Stenosis, and Oral Bacterial DNA in Cerebral Thrombi of Patients with Stroke

Olli Patrakka,[1] **Helena Mehtonen**,[2] **Sari Tuomisto**,[1] **Juha-Pekka Pienimäki**,[3] **Jyrki Ollikainen**,[4] **Heini Huhtala**,[5] **Tanja Pessi**,[6] **Niku Oksala**,[3] **Terho Lehtimäki**,[7] **Jorma Järnstedt**,[2] **Mika Martiskainen**,[1,8] **and Pekka J. Karhunen**[1]

[1]*Department of Forensic Medicine, Faculty of Medicine and Health Technology, Tampere University and Fimlab Laboratories, Tampere, Finland*
[2]*Medical Imaging Center, Department of Radiology, Tampere University Hospital, Tampere, Finland*
[3]*Vascular Centre, Tampere University Hospital, Tampere, Finland*
[4]*Department of Neurology, Tampere University Hospital, Tampere, Finland*
[5]*Faculty of Social Sciences, Tampere University, Tampere, Finland*
[6]*Hemorrhagic Brain Pathology Research Group, Tampere University, Tampere, Finland*
[7]*Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Fimlab Laboratories and Finnish Cardiovascular Research Center Tampere, Tampere, Finland*
[8]*National Institute for Health and Welfare, Helsinki, Finland*

Correspondence should be addressed to Olli Patrakka; olli.patrakka@tuni.fi

Received 29 May 2021; Accepted 17 August 2021; Published 7 September 2021

Academic Editor: Taskin Duman

Background and purpose. Risk of acute ischemic stroke has been associated with carotid artery atherosclerosis as well as with periodontal disease. We studied whether oral pathology or carotid atherosclerosis was associated with the presence and quantity of bacterial DNA in their aspirated thrombi. *Methods*. Thrombus aspirates and control arterial blood were taken from 71 patients (70.4% male; mean age, 67.4 years) with acute ischemic stroke. Tooth pathology was registered using CT scans. Carotid stenosis was estimated with CTA and ultrasonography. The presence of bacterial DNA from aspirated thrombi was determined using quantitative PCR. We also analyzed the presence of these bacterial DNAs in carotid endarterectomies from patients with peripheral arterial disease. *Results*. Bacterial DNA was found in 59 (83.1%) of the thrombus aspirates (median, 8.6-fold). Oral streptococcal DNA was found in 56 (78.9%) of the thrombus aspirates (median, 5.1-fold). DNA from *A. actinomycetemcomitans* and *P. gingivalis* was not found. Most patients suffered from poor oral health and had in median 19.0 teeth left. Paradoxically, patients with better oral health had more oral streptococcal DNA in their thrombus than the group with the worst pathology ($p = 0.028$). There was a trend (OR 7.122; $p = 0.083$) in the association of ≥50% carotid artery stenosis with more severe dental pathology. Oral streptococcal DNA was detected in 2/6 of carotid endarterectomies. *Conclusions*. Stroke patients had poor oral health which tended to associate with their carotid artery stenosis. Although oral streptococcal DNA was found in thrombus aspirates and carotid endarterectomy samples, the amount of oral streptococcal DNA in thrombus aspirates was the lowest among those with the most severe oral pathology. These results suggest that the association between poor oral health and acute ischemic stroke is linked to carotid artery atherosclerosis.

1. Introduction

Cardiovascular and cerebrovascular diseases are one of the leading causes of death and disability worldwide, with acute ischemic stroke alone resulting in over 5 million deaths annually [1, 2]. Carotid artery atherosclerosis has been found to be an important cause of large vessel disease and ischemic stroke [3, 4]. Traditional stroke risk factors are

hypertension, hypercholesterolemia, diabetes mellitus, smoking, and obesity [1]. Periodontal disease has been found to be a new risk factor for stroke, with the direct mechanism still remaining unclear [5, 6]. In Finland, dental infections are common with the prevalence of caries being almost 100%,and severe periodontitis 15-20% and periapical lesions up to 27% [7]. It has been suggested that oral infections contribute to carotid artery intimamedia thickness leading to carotid artery stenosis and subclinical atherosclerosis [8]. A significant association between tooth loss levels and carotid artery plaque prevalence has been reported [9].

In our recent research [10], we found that DNA typical for bacteria of oral origin can be found in cerebral thrombi of acute ischemic stroke patients. DNA of bacteria typical for both endodontic and periodontal infections have earlier been found in cerebral aneurysms [11], in thrombus aspirates of patients with acute myocardial infarction [12] and in lower limb arterial and deep venous thrombosis [13]. In all these studies, the most frequently found bacterial DNA belonged to the *Streptococcus mitis* group, which belongs to viridans group streptococci. Viridans streptococci species are a known cause of infective endocarditis [14]. However, we do not know what the origin of streptococci in thrombus aspirates is. Bacteria may gain access to the systemic circulation via the root canal of infected teeth or through periodontal pocket and may end up in a thrombus because viridans streptococcal species possess thrombogenic properties [15, 16]. As odontogenic infection has been associated with the development of carotid artery disease and rupture of the atherosclerotic plaque, it may be possible that streptococci originate from complicated carotid plaques [17].

In this study, we applied dental radiography as well as carotid radiological imaging to examine whether oral health and carotid artery stenosis are related to the amount of oral bacterial DNA found in cerebral thrombi using radiologic data of acute ischemic stroke patients. We also analyzed the presence of oral bacterial DNA in an unrelated series of carotid endarterectomies from symptomatic patients.

2. Materials and Methods

2.1. Subjects. The series comprises 71 acute ischemic stroke patients who were treated by intra-arterial thrombectomy between November 2013 and January 2017 in Acute Stroke Unit of Tampere University Hospital (Table 1). A neurologist (J.O.) examined all patients when they arrived to the hospital and evaluated the possibility of revascularization using thrombectomy together with a neurointerventional radiologist (J.-P.P.) based on clinical symptoms and computed tomography angiography (CTA). All patients have been treated in Acute Stroke Unit of Tampere University Hospital according to modern medical standards, and all methods were carried out in accordance with relevant guidelines and regulations. The degree of carotid stenosis was estimated using CTA or ultrasonography which was performed to 58 (81.7%) of the patients. The etiology of the brain large vessel occlusion of patients treated with endovascular thrombectomy during the study period in Tampere University Hospital was cardioembolic in 38% and atherosclerotic in 62% of the patients (personal communication, Dr. Jyrki Ollikainen). There were no patients with coagulation disorders or undetermined stroke etiology. Thrombus was aspirated from M1-segment of the middle cerebral artery in most of the patients ($n = 70$; 98.6%). The only excluding criteria for recruiting patients were unsuccessful retrieval of the thrombus using mechanical thrombectomy. The median delay time between onset of an ischemic stroke and hospital arrival was 2 h 30 min (range, 0-16 h). Medical history was collected from the Tampere University Hospital digital patient archives. Criteria for dyslipidemia were fP – kol – LDL > 3.0 mmol/L, fP – kol – HDL < 1.0 mmol/L, or fP – Trigly > 2.0 mmol/L.

Table 1: Patients' characteristics.

All patients $N = 71$	
Age (mean ± SD)	67.4 ± 12.5
Male gender, *n* (%)	50 (70.4)
Diabetes, *n* (%)	12 (16.9)
Dyslipidemia, *n* (%)	29 (45.3)
Arterial hypertension, *n* (%)	38 (53.5)
Coronary heart disease, *n* (%)	14 (20.0)
Cerebrovascular disease[a], *n* (%)	18 (40.9)
Pulmonary disease, *n* (%)	4 (5.71)
Renal insufficiency, *n* (%)	6 (8.57)
Atrial fibrillation, *n* (%)	47 (66.2)
Heart failure, *n* (%)	9 (12.7)
Arrival time to the hospital, hours (median)	2.20

[a]Data for previous cerebrovascular disease was available only for 44 patients.

Carotid arteries from 6 patients of the Tampere Vascular Study (TVS) were obtained (N.O.) aseptically during open carotid endarterectomy in 2005–2009 from symptomatic patients with hemodynamically significant (≥50%) carotid stenosis. Control samples from two patients were obtained from the left internal thoracic artery (LITA) during coronary artery bypass due to symptomatic coronary artery disease. All open vascular surgical procedures were performed at the Division of Vascular Surgery and the Heart Center at Tampere University Hospital.

2.2. Imaging and Assessment of Dental Pathology Using Brain Computed Tomography. Noncontrast computed tomography scans (Lightspeed VCT, GE Medical Systems, United States) were taken of all patients when arrived to the hospital to exclude intracranial hemorrhage, which were used for the assessment of oral health. The parameters of those CT scans were as follows: slice thickness of 0.63 mm, field of view 320.0 mm, voltage of 120 kV, and current of 649 mA. With Philips Brilliant™ Workspace (Philips Healthcare, The Netherlands), CT images were reconstructed into 1 mm multislice axial, sagittal, and coronal planes. Additionally, synthetic panorama and 1 mm multislice reconstructions of the right

and left oblique sagittal and slice series in accordance with dental arches were reconstructed.

CT reconstructions were inter and intraobserved by two evaluators, an experienced (J.J.) and a trainee oral and maxillofacial radiologist (H.M.) using Carestream Vue PACS software (Carestream Health, United States) and diagnostic monitors (Barco, Belgium). The assessments were performed in dim lighting. Scores that differed between the observers were jointly assessed. In total number of teeth, number of missing teeth, and per each tooth, the following parameters were registered: periapical condition, horizontal and vertical alveolar bone loss, furcation lesions, and condition of pericoronal spaces and caries.

The condition of a tooth was first registered as sound, filled, caries with pulp exposed or teeth being decayed as residual root, but due to the resolution and artefacts deteriorating diagnostic accuracy, we ended up combining the groups as sound/filled and caries with pulp exposed/residual roots. Periapical infection was registered if an osteolytic lesion was found surrounding the root apex. Vertical/angular defect ≥ 1/3 of the root length was considered as vertical bone loss. Scores for horizontal bone loss were difficult to assess from CT scans and because scores differed between evaluators, they were left out from the analyses. Furcation lesion was registered if an osteolytic finding at the furcation was found. Pericoronal space was defined to be infected if the pericoronal space was ≥3 mm, and/or surrounding bone showed signs of infection.

Combined pathology sum was calculated to assess the overall oral health status in the same way as previously used [18–20]. The number of vertical bone defects as well as periapical, furcation, caries, and pericoronitis lesions was summed per each tooth. The patients were then divided into three equal groups based on the sum per each tooth: "normal to slight pathology" ($n = 21$, sum 0-0.06), "moderate pathology" ($n = 21$, sum 0.07-0.23), and "severe pathology" ($n = 22$, sum 0.24-2.00). Edentulous patients ($n = 5$) were excluded from the analysis.

2.3. Mechanical Thrombectomy and Thrombus Sample Collection. Mechanical thrombectomy and thrombus sample collection were performed by a neurointerventional radiologist as previously described [10]. An introducer sheet was placed into the femoral artery. A blood sample for background analysis was collected through the sheet. Guiding catheter up to 9 Fr (Merci® Concentric medical) with a tip balloon was navigated into the carotid artery proximal to the occluded site. The microcatheter (0.021" Trevo) with the guide wire was used to navigate through the occluded site and to deploy the stent retriever (Trevo®, Stryken neurovascular) over the thrombus. An additional distal access catheter was used to achieve the thrombus if needed. Forceful aspiration through proximal catheter was acquired with a 60 cc syringe while retrieving the deployed stent. Different device settings were selected by the operator selectively case by case. Thrombectomy was repeated until the angiological result of satisfaction. Gathered thrombus was divided into 1.5 cc Eppendorf for quantitative PCR analysis and a histological sample part in a formalin container.

2.4. Quantitative PCR. The presence of bacterial DNA was identified using qPCR with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, Calif) [21] with Maxima Probe/ROX qPCR MasterMix (Thermo Fischer Scientific, Waltham, Mass). Arterial thrombus aspirates were compared with arterial control blood samples, as opposed to venous to venous to reduce any potential bias caused by sampling from different sites and, subsequently, bias resulting from different conditions like flow dynamics and pressure. The presence of bacterial DNA in the thrombus and in control blood samples was determined by using published primers and a probe for *Streptococcus* spp., mainly *S. mitis* group, *A. actinomycetemcomitans*, and *P. gingivalis* using human housekeeping gene, RNAseP (Applied Biosystems), as a reference gene [12]. The positive controls were reference bacteria from ATCC collection (*Streptococcus mitis* ATCC 49456, *A. actinomycetemcomitans* ATCC 700685, *P. gingivalis* ATCC 33277, LCG Standards AB, Borås, Sweden). Each measurement was performed as duplicates or quadruples in uncertain cases. DNA was extracted from the entire thrombus in most of the cases. If the aspired thrombus was large, a small part of it was taken and sent to histological analysesm and DNA was extracted from rest of the thrombus. The relative amounts of bacterial DNA in the samples were calculated by the comparative threshold cycle (Ct) method ($\Delta\Delta$Ct, $\Delta Ct_{sample} - \Delta Ct_{control}$) [12, 22–25], where the sample was a thrombus aspirate. and the control was a blood sample from the same patient. First, the differences of the Ct values (ΔCt) between candidate bacteria and reference gene measurement (Ct from candidate bacteria − Ct from RNAseP) for each sample were calculated; then, the comparative Ct ($\Delta\Delta$Ct) (ΔCt from thrombus−ΔCt from patients own arterial blood) was calculated. The samples were separated into two different groups: samples were marked negative if $2^{-\Delta\Delta Ct} < 2$ and positive if $2^{-\Delta\Delta Ct} \geq 2$ [26, 27]. The presence of bacterial DNA in carotid atherosclerotic plaques and healthy control LITA samples was studied using the same comparative method by keeping a LITA Ct values as a reference.

2.5. Statistical Analysis. Associations between bacterial DNA findings and nominal dental parameters were analyzed using Pearson's chi-square test. Age-adjusted logistic regression analysis was used to estimate the odds ratio (OR) and 95% confidence interval (CI) for associations between bacterial DNA findings, grade of carotid artery stenosis, and combined dental pathology. The number of teeth was not normally distributed, and therefore, median values and quartiles (Q1 and Q3) were calculated. Statistical significance was set at $p \leq 0.05$, and analyses were done using IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.

3. Results

3.1. Patient Characteristics and Bacterial DNA Findings. There were 50 (70.4%) men and 21 (29.6%) women in the study population. The mean age of the patients was 67.4 years. None of the patients had been treated with antibiotics

or experienced severe dental infections or septicemia during the stroke. Using universal bacterial primers, bacterial DNA was found in 59 (83.1%) of the thrombus aspirates with a median of 8.6-fold rate compared to the control peripheral blood sample from the same patient. Viridans streptococcal DNA was found in 56 (78.9%) of the thrombus aspirates (median, 5.1-fold). All thrombi were negative for both *P. gingivalis* and *A. actinomycetemcomitans* bacterial DNA. One arterial blood sample was positive for *P. gingivalis,* and two arterial blood samples were positive for *A. actinomycetemcomitans* bacterial DNA. All characteristics of patients are presented in Table 1. Carotid stenosis was found from 54 (81.2%) patients and carotid dissection from 4 (5.6%) patients. There were 11 (19.0%) patients with ≥50% stenosis and 43 (74.1%) patients with <50% stenosis.

3.2. Association between Dental Pathology and Bacterial DNA Findings in Thrombus Aspirates. Patients had poor oral health. They had in median 19.0 (Q1: 9.0; Q3: 26.0) teeth (range, 0-32 teeth). Of the total 71 patients, periapical lesions were found in 32 (45.1%), vertical bone loss in 20 (28.2%), furcation lesions in 25 (35.2%), caries in 21 (29.6%), and enlarged pericoronal spaces in 3 (4.2%) patients. There was no association between the number of teeth and bacterial DNA counts in thrombus aspirates. Connection between dental pathology and bacterial DNA findings in thrombus aspirates was not statistically significant when dental variables were treated individually (Figure 1). However, when pathologies were summed, a linear correlation between oral streptococcal bacterial DNA findings and tooth condition was found ($p = 0.032$) in age-adjusted analysis, whereas this association was not found for total bacterial DNA amounts ($p = 0.197$). In age-adjusted analysis, patients with better oral health had more oral streptococcal bacterial DNA in their thrombus than the group with the worst pathology ($p = 0.028$). On mean, the group with normal to slight pathology had 25.8-fold difference, the moderate pathology group had 13.0-fold difference, and the severe pathology group had 5.8-fold difference between thrombus and arterial blood oral streptococcal DNA amount (Figure 2).

3.3. Association between Carotid Artery Stenosis and Bacterial DNA Findings in Thrombus Aspirates. There was no association between carotid stenosis and total bacterial DNA amount in thrombus aspirates. However, patients with ≥50% stenosis had slightly more (18.1- v.s. 13.9-fold) oral streptococcal DNA in thrombus aspirates compared to patients with <50% stenosis, but this difference was not statistically significant ($p = 0.578$) in age-adjusted logistic regression analysis, due to high variation in the DNA folds and small number of the cases (Figure 3).

3.4. Association between Carotid Stenosis and Dental Pathology. There was no difference ($p = 0.604$) in the number of teeth between those with ≥50% carotid stenosis (18.7 ± 8.9) compared to those with less than <50% stenosis (17.0 ± 10.1). We found that there were more cases (45.5% v.s. 25.0%) with severe dental pathology among patients with ≥50% carotid stenosis compared to those with less than <50% stenosis, respectively. In logistic regression analysis with age and amount of oral streptococcal DNA as covariates, there was a trend (OR 7.122; 95% CI 0.78-65.5; $p = 0.083$) in the association of ≥50% carotid stenosis with more severe dental pathology, while age (OR 1.044, $p = 0.295$) or amount of streptococcal DNA (OR 1.023, $p = 0.188$) was not significant covariates (Figure 4).

3.5. Oral Bacterial DNA in Carotid Stenosis Samples. Oral streptococcal DNA (viridans group streptococci, mainly *Streptococcus mitis*) was detected in 2 (33%) of the 6 surgically collected sterile atherosclerotic carotid endarterectomy samples showing advanced atherosclerosis. In one of these cases (17%), DNA from *P. gingivalis* was also amplified. Cases negative for oral bacterial DNA also showed severe atherosclerosis.

4. Discussion

It has been reported that carotid artery atherosclerosis is an important cause of large vessel stroke, and a part of acute ischemic stroke may be due to embolism from the carotid arteries [3, 4, 28]. It has also been found that periodontal disease and fewer teeth may be associated with an increased risk of ischemic stroke [5]. We found recently that most thrombus aspirates from acute ischemic stroke patients contained DNA from oral streptococcal bacteria [10]. In the present study, we found that stroke patients had poor oral health. We found an inverse association of the severity of oral pathology but a positive trend of carotid artery stenosis with the amount of streptococcal DNA in thrombus aspirates of stroke patients. Patients showing more severe carotid stenosis tended to have the worst oral health. We found streptococcal DNA in 1/3 of carotid endarterectomy samples from surgical patients suffering peripheral artery disease.

The most frequent bacteria in cerebral thrombus aspirates in our study, viridans group streptococci, are common oral bacteria associated with the development of dental plaque [29]. Oral streptococci may initiate or contribute to platelet aggregation in coronaries [15]. We have earlier reported viridans group streptococcal DNA in thrombus aspirates of patients with acute myocardial infarction [12] and in lower limb arterial and in deep venous thrombosis [13]. Lockhart et al. demonstrated in 2008 that dental operations such as tooth extraction and daily toothbrushing can cause transient bacteremia. Most translocated bacteria into the circulation that could be cultivated were viridans streptococci [30].

In our study, 60.6% of the patients suffered from moderate or severe dental pathology and had 19.0 teeth left on median. The number of teeth is similar to the numbers in a Finnish national survey, where the average amount of teeth was 17.0 in people aged 65-74 [31], while in same aged Swedish population, the average number of remaining teeth is 26.0 [32]. Although the number of dentists does not differ significantly between Sweden and Finland, the practice of preferring tooth extraction instead of treatment of caries applied in Finland may explain the differences in the

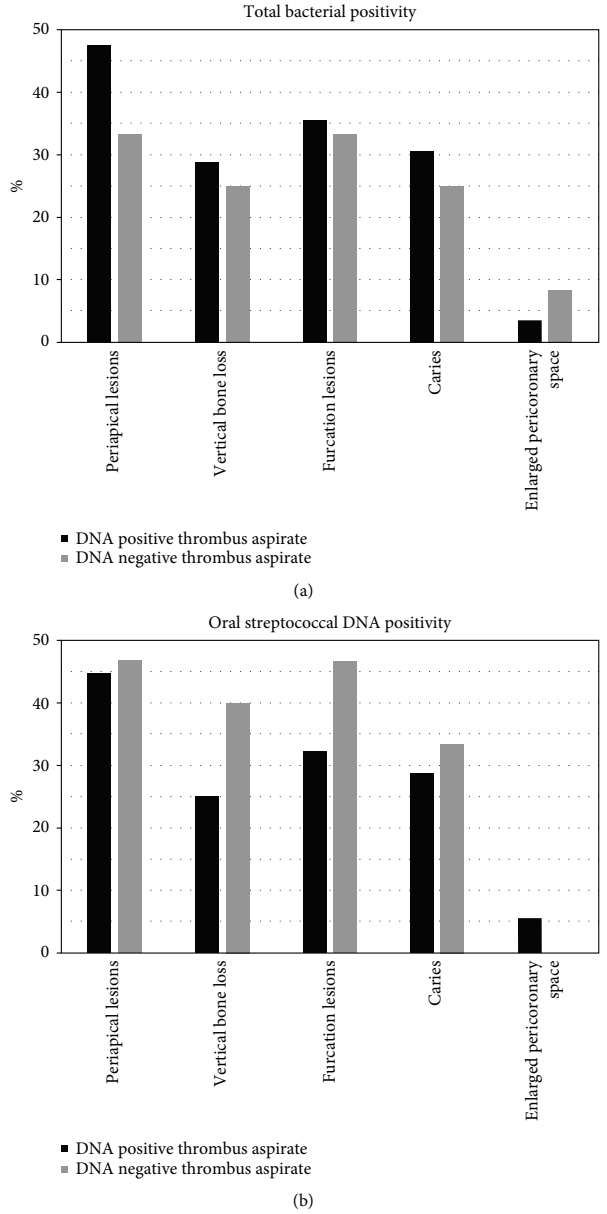


Figure 1: Dental pathological lesions in patients with or without total bacterial DNA (a) and oral streptococcal DNA (b) findings in thrombus aspirates. All pairwise comparisons $p > 0.05$.

number of teeth [7]. Interestingly, stroke prevalence in Finland (395.9 strokes per 100,000 inhabitants) is higher compared to Sweden (368.6 strokes per 100,000 inhabitants) [33], even though the countries share a similar economic and social model as well as a similar health care system [34].

Our findings on the association between bad oral health and low bacterial DNA findings in thrombus aspirates of the same patients might be explained by studies reporting that the longer the periodontal disease exposure, the fewer focal infection focuses there are present. With only few teeth left in the oral cavity as a result of a long-term periodontal disease, the infectious pathway is closing and the continuous flow of oral bacteria through the ulcerated epithelium of gingival periodontal pockets reaching the bloodstream

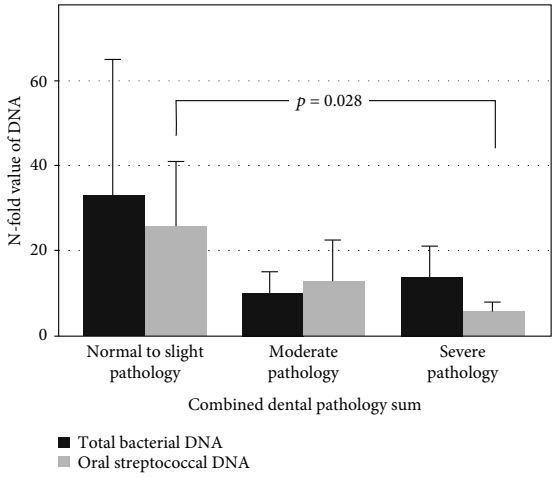


Figure 2: N-fold value of total bacterial ($p = 0.197$) and oral streptococcal ($p = 0.032$) DNA between the thrombus and arterial blood from the same patient and the relationship with combined dental pathology sum. (a) Normal to slight pathology ($n = 21$). (b) Moderate pathology ($n = 21$). (c) Severe pathology ($n = 22$).

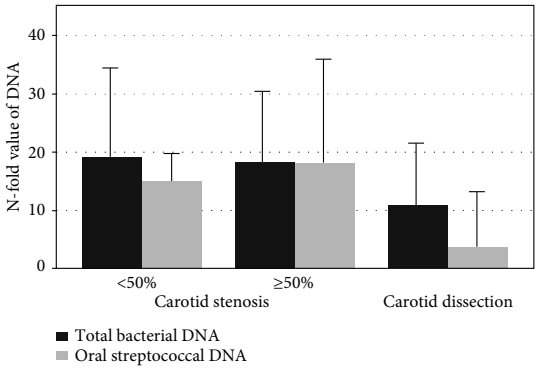


Figure 3: N-fold value of total bacterial DNA ($p = 0.983$) and oral streptococcal DNA ($p = 0.701$) between the thrombus and arterial blood from the same patient and the relationship with <50% ($n = 43$) and ≥50% ($n = 11$) carotid artery stenosis. DNA amounts were lower in patients with carotid dissection ($n = 4$).

decreases [9, 35]. There are several previous studies that have shown the association between ischemic stroke and tooth loss, especially in younger age groups [5, 6, 36–38]. In our study, where the mean age of the patients was 67 years, we found no association between tooth loss and bacterial DNA counts in thrombus aspirates. Among elderly people, tooth loss is not found to be connected with atherosclerotic vascular diseases [6, 39, 40]. Our patients' tooth number did not significantly differ from the same aged Finnish population. In addition, edentulousness is related to lowered oral bacteria (*P. gingivalis*) IgG levels [41].

We found streptococcal DNA in 1/3 of carotid endarterectomy samples from surgical patients suffering peripheral artery disease. Previously, the presence of odontogenic bacteria, such as viridans group streptococci (*S. sanguinis*) and *P. gingivalis*, has been shown in the atherosclerotic plaque of human carotid artery histologically and by polymerase chain reaction [42, 43]. It has been proposed that bacteria present in carotid artery plaques contribute to the enhanced risk of plaque rupture leading to thrombosis [17]. Viridans group streptococci have been found to be the most common gram positive bacteria persisting intracanal disinfection procedures and after root canal treatment [44]. Koren et al. showed that an abundance of *Veillonella* sp. and *Streptococcus* sp. in the oral cavity was linked to their abundance in carotid atherosclerotic plaques [45].

We found that the association of ≥50% carotid stenosis to be related to more severe dental pathology. Our findings are in line with previous studies [9, 46, 47]. Periodontitis has been shown to elevate the overall infectious burden in generally healthy populations [48], apical periodontitis is suggested to be associated with increased levels of systemic inflammation [49], and chronic low-grade oral infection and inflammation have been related to unfavorable systemic cardiovascular effects [50–52]. Thus, bad oral health may contribute to the progression of atherosclerotic lesions via circulating chemical mediators [53–55].

Our patients with ≥50% stenosis had slightly more oral streptococcal DNA in their aspirated thrombi compared to those with <50% stenosis. We may hypothesize that oral bacterial DNA found in thrombi are originated from ruptured carotid artery plaques. There is evidence of oral bacterial inflammation to be related to the development of the atherosclerotic plaque by the inflammatory mechanism in the arterial wall and through cytokine activation [56–59].

There may be other explanations for our findings concerning oral health and bacterial DNA findings in aspirated thrombus. Extraction of teeth can be done for other reasons

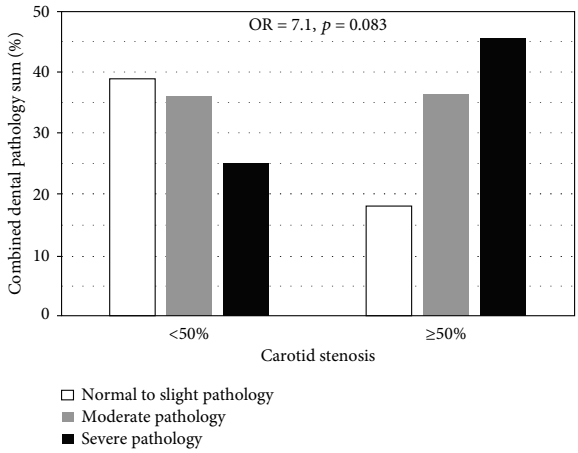


FIGURE 4: Association between severity of carotid artery stenosis and combined dental pathology. In logistic regression analysis with age and amount of oral streptococcal DNA as covariates, there was a trend (OR 7.1; 95% CI 0.78-65.5; $p = 0.083$) in the association of ≥50% carotid stenosis with more severe dental pathology.

than disease related. Temporal sequence is hard to determine. American Heart Association stated in their systematic review that observational studies do not support a causative relationship between periodontal disease and atherosclerotic disease [60]. The relationship has been proposed to be due to different reasons, such as tooth extraction for other reasons than periodontal disease, a change in diet after tooth loss, selection bias, and biological and behavioral factors [61, 62]. Nevertheless, there is a positive linear association between oral health and overall mortality [63–65]. One of the limitations of our study was that we had a small sample size. The grade of carotid artery stenosis was measured using either computerized tomographic angiography or Doppler ultrasound, of which concordance is 79% [66]. We only had two categories of carotid stenosis. Tooth conditions were estimated from CT reconstructions. We did not have the possibility of conducting a clinical examination. Dental and periodontal disease can be recognized with dental CT [67]; yet, it is not as accurate as the golden standard cone-beam computed tomography [68]. In addition, the PCR method we used detects the presence of bacterial DNA in the examined samples but is unable to separate living bacteria from phagocytized bacterial DNA.

5. Conclusions

Stroke patients had poor oral health. We found an inverse association of the severity of oral pathology but a positive trend of carotid artery stenosis with the amount of streptococcal DNA in thrombus aspirates. Our results propose that the association between poor oral health and acute ischemic stroke is linked to carotid atherosclerosis. The question in which way oral bacteria are involved in the pathogenesis of acute ischemic stroke or are they solely bystanders remain still open and should be evaluated in forthcoming studies.

Data Availability

The datasets generated and analyzed during the current study are not publicly available due to the individual person's data that are involved but are available from the corresponding author on reasonable request.

Ethical Approval

The study was approved by the ethics committee (R13093 and R99204) of the Tampere University Hospital, Tampere, Finland. The study was explained to the patients, and informed consent was obtained.

Conflicts of Interest

The authors state no disclosures or conflict of interest.

Authors' Contributions

Patrakka O and Mehtonen H contributed equally to the manuscript. Drs Karhunen P.J. and Martiskainen M contributed equally and share a senior authorship.

Acknowledgments

This study was supported with grants from the Competitive Research Funding of the Tampere University Hospital (Grant X51001 for T.L. and P.K.), the Emil Aaltonen Foundation (T.L.), the Academy of Finland (Grant nos. 286284 and 322098 for T.L), the Tampere Tuberculosis Foundation (T.P. and T.L.), the Finnish Foundation for Cardiovascular Research (grants for T.L. and P.K.), the Yrjö Jahnsson Foundation, Pirkanmaa Cultural Foundation (S.T.), European Union 7th Framework Program (grant 201668 for AtheroRemo), and EU Horizon 2020 (grant 755320 for TAXINOMISIS and grant 848146 for To Aition).

References

[1] L. B. Goldstein, R. Adams, K. Becker et al., "Primary prevention of ischemic stroke: a statement for healthcare professionals from the stroke council of the American Heart Association," *Circulation*, vol. 103, no. 1, pp. 163–182, 2001.

[2] C. C. Beal, "Gender and stroke Symptoms," *The Journal of Neuroscience Nursing*, vol. 42, no. 2, pp. 80–87, 2010.

[3] M. L. Flaherty, B. Kissela, J. C. Khoury et al., "Carotid artery stenosis as a cause of stroke," *Neuroepidemiology*, vol. 40, no. 1, pp. 36–41, 2012.

[4] D. Bos, M. L. P. Portegies, A. van der Lugt et al., "Intracranial carotid artery atherosclerosis and the risk of stroke in whites: the Rotterdam study," *JAMA Neurology*, vol. 71, no. 4, pp. 405–411, 2014.

[5] K. J. Joshipura, H. C. Hung, E. B. Rimm, W. C. Willett, and A. Ascherio, "Periodontal disease, tooth loss, and incidence of ischemic stroke," *Stroke*, vol. 34, no. 1, pp. 47–52, 2003.

[6] A. J. Grau, H. Becher, C. M. Ziegler et al., "Periodontal disease as a risk factor for ischemic stroke," *Stroke*, vol. 35, no. 2, pp. 496–501, 2004.

[7] L. Suominen-Taipale, A. Nordblad, M. Vehkalahti, and A. Aromaa, *Suomalaisten aikuisten suunterveys*, Helsinki, 2004.

[8] P. J. Pussinen, S. Paju, J. Koponen et al., "Association of childhood oral infections with cardiovascular risk factors and subclinical atherosclerosis in adulthood," *JAMA Network Open*, vol. 2, no. 4, article e192523, 2019.

[9] M. Desvarieux, R. T. Demmer, T. Rundek et al., "Relationship between periodontal disease, tooth loss, and carotid artery plaque: the oral infections and vascular disease epidemiology study (INVEST)," *Stroke*, vol. 34, no. 9, pp. 2120–2125, 2003.

[10] O. Patrakka, J. P. Pienimäki, S. Tuomisto et al., "Oral bacterial signatures in cerebral thrombi of patients with acute ischemic stroke treated with thrombectomy," *Journal of the American Heart Association*, vol. 8, no. 11, article e012330, 2019.

[11] M. J. Pyysalo, L. M. Pyysalo, T. Pessi, P. J. Karhunen, and J. E. Öhman, "The connection between ruptured cerebral aneurysms and odontogenic bacteria," *Journal of Neurology, Neurosurgery, and Psychiatry*, vol. 84, no. 11, pp. 1214–1218, 2013.

[12] T. Pessi, V. Karhunen, P. P. Karjalainen et al., "Bacterial signatures in thrombus aspirates of patients with myocardial infarction," *Circulation*, vol. 127, no. 11, pp. 1219–1228, 2013.

[13] D. Vakhitov, S. Tuomisto, M. Martiskainen et al., "Bacterial signatures in thrombus aspirates of patients with lower limb arterial and venous thrombosis," *Journal of Vascular Surgery*, vol. 67, no. 6, pp. 1902–1907, 2018.

[14] T. J. Cahill and B. D. Prendergast, "Infective endocarditis," *The Lancet (London, England)*, vol. 387, no. 10021, pp. 882–893, 2016.

[15] M. C. Herzberg, A. Nobbs, L. Tao et al., "Oral streptococci and cardiovascular disease: searching for the platelet aggregation-associated protein gene and mechanisms ofStreptococcus sanguis-Induced thrombosis," *Journal of Periodontology*, vol. 76, no. 11-s, pp. 2101–2105, 2005.

[16] S. W. Kerrigan and D. Cox, "The thrombotic potential of oral pathogens," *Journal of Oral Microbiology*, vol. 1, no. 1, 2009.

[17] B. B. Lanter, K. Sauer, and D. G. Davies, "Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture," *MBio*, vol. 5, no. 3, pp. e01206–e01214, 2014.

[18] K. J. Mattila, M. S. Nieminen, V. V. Valtonen et al., "Association between dental health and acute myocardial infarction," *British Medical Journal*, vol. 298, no. 6676, pp. 779–781, 1989.

[19] S. J. Janket, M. Qvarnström, J. H. Meurman, A. E. Baird, P. Nuutinen, and J. A. Jones, "Asymptotic dental score and prevalent coronary heart disease," *Circulation*, vol. 109, no. 9, pp. 1095–1100, 2004.

[20] V. Karhunen, H. Forss, S. Goebeler et al., "Radiographic assessment of dental health in middle-aged men following sudden cardiac death," *Journal of Dental Research*, vol. 85, no. 1, pp. 89–93, 2006.

[21] S. Tuomisto, *Intestinal Bacteria Post-Mortem Changes, Migration and Association, [Ph.D. thesis]*, Tampere University, Tampere, 2014.

[22] A. Yoshida, N. Suzuki, Y. Nakano, T. Oho, M. Kawada, and T. Koga, "Development of a 5′ fluorogenic nuclease-based real-time PCR assay for quantitative detection ofActinobacillus actinomycetemcomitansandPorphyromonas gingivalis," *Journal of Clinical Microbiology*, vol. 41, no. 2, pp. 863–866, 2003.

[23] N. Suzuki, A. Yoshida, and Y. Nakano, "Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR," *Clinical Medicine & Research*, vol. 3, no. 3, pp. 176–185, 2005.

[24] K. J. Livak and T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta\_C_{-}}{}_{T}$ Method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[25] S. Tuomisto, T. Pessi, P. Collin, R. Vuento, J. Aittoniemi, and P. J. Karhunen, "Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics," *BMC Gastroenterology*, vol. 14, no. 1, 2014.

[26] A. Tichopad, T. Bar, L. Pecen, R. R. Kitchen, M. Kubista, and M. W. Pfaffl, "Quality control for quantitative PCR based on amplification compatibility test," *Methods*, vol. 50, no. 4, pp. 308–312, 2010.

[27] B. Bubner, K. Gase, and I. T. Baldwin, "Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR," *BMC Biotechnology*, vol. 4, no. 1, p. 14, 2004.

[28] D. Inzitari, M. Eliasziw, P. Gates et al., "The causes and risk of stroke in patients with asymptomatic internal-carotid-artery stenosis," *The New England Journal of Medicine*, vol. 342, no. 23, pp. 1693–1701, 2000.

[29] P. E. Kolenbrander, R. J. Palmer, S. Periasamy, and N. S. Jakubovics, "Oral multispecies biofilm development and the key role of cell-cell distance," *Nature Reviews. Microbiology*, vol. 8, no. 7, pp. 471–480, 2010.

[30] P. B. Lockhart, M. T. Brennan, H. C. Sasser, P. C. Fox, B. J. Paster, and F. K. Bahrani-Mougeot, "Bacteremia associated with toothbrushing and dental extraction," *Circulation*, vol. 117, pp. 3118–3125, 2008.

[31] S. Koskinen, A. Lundqvist, and N. Ristiluoma, *Health, Functional Capacity and Welfare in Finland in 2011*, Helsinki, 2012.

[32] R. Ljung, F. Lundgren, M. Appelquist, and A. Cederlund, "The Swedish dental health register - validation study of remaining and intact teeth," *BMC Oral Health*, vol. 19, p. 116, 2019.

[33] H. A. Wafa, C. D. A. Wolfe, E. Emmett, G. A. Roth, C. O. Johnson, and Y. Wang, "Burden of stroke in Europe: thirty-year projections of incidence, prevalence, deaths, and disability-adjusted life years," *Stroke*, vol. 51, no. 8, pp. 2418–2427, 2020.

[34] S. Kristiansen, "Health care systems in the Nordic countries–more similarities than differences?," *Tidsskrift for den Norske Lægeforening*, vol. 120, no. 17, pp. 2023–2029, 2000.

[35] A. C. R. T. Horliana, L. Chambrone, A. M. Foz et al., "Dissemination of periodontal pathogens in the bloodstream after periodontal procedures: a systematic review," *PLoS One*, vol. 9, no. 5, p. e98271, 2014.

[36] J. Syrjänen, J. Peltola, V. Valtonen, M. Iivanainen, M. Kaste, and J. K. Huttunen, "Dental infections in association with cerebral infarction in young and middle-aged men," *Journal of Internal Medicine*, vol. 225, no. 3, pp. 179–184, 1989.

[37] T. Wu, M. Trevisan, R. J. Genco, J. P. Dorn, K. L. Falkner, and C. T. Sempos, "Periodontal disease and risk of cerebrovascular disease: the first national health and nutrition examination survey and its follow-up study," *Archives of Internal Medicine*, vol. 160, no. 18, pp. 2749–2755, 2000.

[38] B. L. Pihlstrom and B. S. Michalowicz, "Periodontal diseases," in *Nutritional Aspects of Aging*, vol. 2, pp. 159–174, CRC Press, 2018.

[39] J. Beck, R. Garcia, G. Heiss, P. S. Vokonas, and S. Offenbacher, "Periodontal disease and cardiovascular disease the relation of periodontal infections to systemic diseases," *Journal of Periodontology*, vol. 67, no. 10/SUP, pp. 1123–1137, 2004.

[40] A. M. H. Syrjälä, P. Ylöstalo, S. Hartikainen, R. Sulkava, and M. L. Knuuttila, "Number of teeth and myocardial infarction and stroke among elderly never smokers," *Journal of Negative Results in Biomedicine*, vol. 8, no. 1, p. 6, 2009.

[41] N. Aoyama, J. I. Suzuki, N. Kobayashi et al., "Associations among tooth loss, systemic inflammation and antibody titers to periodontal pathogens in Japanese patients with cardiovascular disease," *Journal of Periodontal Research*, vol. 53, no. 1, pp. 117–122, 2018.

[42] B. Chiu, "Multiple infections in carotid atherosclerotic plaques," *American Heart Journal*, vol. 138, no. 5, pp. S534–S536, 1999.

[43] V. I. Haraszthy, J. J. Zambon, M. Trevisan, M. Zeid, and R. J. Genco, "Identification of periodontal pathogens in atheromatous plaques," *Journal of Periodontology*, vol. 71, no. 10, pp. 1554–1560, 2000.

[44] N. Ll and C. Vaishnavi, "Endodontic microbiology," *Journal of Conservative Dentistry*, vol. 13, no. 4, pp. 233–239, 2010.

[45] O. Koren, A. Spor, J. Felin et al., "Microbes and health Sackler colloquium: human oral, gut, and plaque microbiota in patients with atherosclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, Supplement 1, pp. 4592–4598, 2011.

[46] G. Kamak, E. Yildirim, and E. Rencber, "Evaluation of the relationship between periodontal risk and carotid artery calcifications on panoramic radiographs," *European Journal of Dentistry*, vol. 9, no. 4, pp. 483–489, 2015.

[47] T. Schillinger, W. Kluger, M. Exner et al., "Dental and periodontal status and risk for progression of carotid atherosclerosis: the inflammation and carotid artery risk for atherosclerosis study dental substudy," *Stroke*, vol. 37, no. 9, pp. 2271–2276, 2006.

[48] F. D'Aiuto, L. Nibali, M. Parkar, J. Suvan, and M. S. Tonetti, "Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol," *Journal of Dental Research*, vol. 84, no. 3, pp. 269–273, 2005.

[49] M. S. Gomes, T. C. Blattner, M. Sant'Ana Filho et al., "Can apical periodontitis modify systemic levels of inflammatory markers? A systematic review and meta-analysis," *Journal of Endodontia*, vol. 39, no. 10, pp. 1205–1217, 2013.

[50] F. DeStefano, R. F. Anda, H. S. Kahn, D. F. Williamson, and C. M. Russell, "Dental disease and risk of coronary heart disease and mortality," *British Medical Journal*, vol. 306, no. 6879, pp. 688–691, 1993.

[51] J. Danesh, P. Whincup, M. Walker et al., "Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses," *British Medical Journal*, vol. 321, no. 7255, pp. 199–204, 2000.

[52] E. Cotti, C. Dessì, A. Piras, and G. Mercuro, "Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature," *International Journal of Cardiology*, vol. 148, no. 1, pp. 4–10, 2011.

[53] M. E. Rosenfeld, "Inflammation and atherosclerosis: direct versus indirect mechanisms," *Current Opinion in Pharmacology*, vol. 13, no. 2, pp. 154–160, 2013.

[54] I. F. Charo and M. B. Taubman, "Chemokines in the pathogenesis of vascular disease," *Circulation Research*, vol. 95, no. 9, pp. 858–866, 2004.

[55] E. F. Carrizales-Sepúlveda, A. Ordaz-Farías, R. Vera-Pineda, and R. Flores-Ramírez, "Periodontal disease, systemic inflammation and the risk of cardiovascular disease," *Heart, Lung and Circulation*, vol. 27, no. 11, pp. 1327–1334, 2018.

[56] J. Bartova, P. Sommerova, Y. Lyuya-Mi et al., "Periodontitis as a risk factor of atherosclerosis," *Journal of Immunology Research*, vol. 2014, Article ID 636893, 9 pages, 2014.

[57] D. P. Ramji and T. S. Davies, "Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets," *Cytokine & Growth Factor Reviews*, vol. 26, no. 6, pp. 673–685, 2015.

[58] J. S. Chia, H. T. Lien, P. R. Hsueh, P. M. Chen, A. Sun, and J. Y. Chen, "Induction of cytokines by glucosyltransferases of Streptococcus mutans," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 4, pp. 892–897, 2002.

[59] H. C. Lo, A. M. Best, and J. G. Tew, "Cytokine induction by Streptococcus mutans and pulpal pathogenesis," *Infection and Immunity*, vol. 68, no. 12, pp. 6785–6789, 2000.

[60] P. B. Lockhart, A. F. Bolger, P. N. Papapanou et al., "Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association?: A scientific statement from the American heart association," *Circulation*, vol. 125, no. 20, pp. 2520–2544, 2012.

[61] K. J. Joshipura, C. W. Douglass, and W. C. Willett, "Possible explanations for the tooth loss and cardiovascular disease relationship," *Annals of Periodontology*, vol. 3, no. 1, pp. 175–183, 1998.

[62] I. Johansson, P. Tidehag, V. Lundberg, and G. Hallmans, "Dental status, diet and cardiovascular risk factors in middle-aged people in northern Sweden," *Community Dentistry and Oral Epidemiology*, vol. 22, no. 6, pp. 431–436, 1994.

[63] M. Adolph, C. Darnaud, F. Thomas et al., "Oral health in relation to all-cause mortality: the IPC cohort study," *Scientific Reports*, vol. 7, no. 1, 2017.

[64] L. Jansson, H. Kalkali, and N. F. Mulk, "Mortality rate and oral health–a cohort study over 44 years in the county of Stockholm," *Acta Odontologica Scandinavica*, vol. 76, pp. 299–304, 2018.

[65] A. Holmlund, G. Holm, and L. Lind, "Number of teeth as a predictor of cardiovascular mortality in a cohort of 7, 674

subjects followed for 12 years," *Journal of Periodontology*, vol. 81, pp. 870–876, 2010.

[66] M. A. Titi, C. George, D. Bhattacharya et al., "Comparison of carotid Doppler ultrasound and computerised tomographic angiography in the evaluation of carotid artery stenosis," *The Surgeon*, vol. 5, no. 3, pp. 132–136, 2007.

[67] J. Steinklein and V. Nguyen, "Dental anatomy and pathology encountered on routine CT of the head and neck," *AJR. American Journal of Roentgenology*, vol. 201, no. 6, pp. W843–W853, 2013.

[68] L. Lechuga and G. A. Weidlich, "Cone Beam CT Vs. Fan Beam CT: A Comparison of Image Quality and Dose Delivered Between Two Differing CT Imaging Modalities," *Cureus*, vol. 8, no. 9, article e778, 2016.

PUBLICATION III

Thrombus Aspirates from Acute Ischemic Stroke Patients are Infiltrated by Viridans Streptococci

Patrakka O, Tuomisto S, Pienimäki JP, Ollikainen J, Oksala N, Lampinen V, Ojanen MJT, Huhtala H, Hytönen VP, Lehtimäki T, Martiskainen M, Karhunen PJ.

Journal of the American Heart Association. 2023;12:e030639.

Journal of the American Heart Association

ORIGINAL RESEARCH

Thrombus Aspirates From Patients With Acute Ischemic Stroke Are Infiltrated by Viridans Streptococci

Olli Patrakka, MD; Sari Tuomisto, PhD; Juha-Pekka Pienimäki, MD, PhD; Jyrki Ollikainen, MD; Niku Oksala, MD, PhD, DSc (med.); Vili Lampinen, MSc; Markus J. T. Ojanen, PhD; Heini Huhtala, MSc; Vesa P. Hytönen, PhD; Terho Lehtimäki, MD, DDS, PhD; Mika Martiskainen, MD; Pekka J. Karhunen, MD, PhD

BACKGROUND: Acute ischemic stroke may be due to embolism from ruptured atherosclerotic carotid arteries. DNA of oral bacteria, mainly the viridans streptococci group, has been detected in thrombus aspirates of patients with ischemic stroke as well as in carotid endarterectomy samples. Because viridans streptococci are known to possess thrombogenic properties, we studied whether their presence in thrombus aspirates and in carotid artery specimens can be confirmed using bacterial immunohistochemistry.

METHODS AND RESULTS: Thrombus aspirates from 61 patients with ischemic stroke (70.5% men; mean age, 66.8 years) treated with mechanical thrombectomy, as well as carotid endarterectomy samples from 20 symptomatic patients (65.0% men; mean age, 66.2 years) and 48 carotid artery samples from nonstroke autopsy cases (62.5% men; mean age, 66.4 years), were immunostained with an antibody cocktail against 3 species (*Streptococcus sanguinis*, *Streptococcus mitis,* and *Streptococcus gordonii*) of viridans streptococci. Of the thrombus aspirates, 84.8% were immunopositive for viridans streptococci group bacteria, as were 80.0% of the carotid endarterectomy samples, whereas immunopositivity was observed in 31.3% of the carotid artery samples from nonstroke autopsies. Most streptococci were detected inside neutrophil granulocytes, but there were also remnants of bacterial biofilm as well as free bacterial infiltrates in some samples.

CONCLUSIONS: Oral streptococci were found in aspirated thrombi of patients with acute ischemic stroke as well as in carotid artery samples. Our results suggest that viridans streptococci group bacteria may play a role in the pathophysiology of ischemic stroke.

Key Words: carotid artery stenosis ■ oral health ■ stroke ■ viridans streptococci

Ischemic strokes represent the majority of strokes (85%), and the rest (15%) are hemorrhagic strokes caused by an intracerebral or subarachnoid hemorrhage.[1] Carotid artery atherosclerosis is an important cause of large vessel stroke, and a portion of acute ischemic strokes may be due to embolism from ruptured carotid arteries.[2–4]

Classic risk factors for acute ischemic stroke include age, male sex, hypertension, atrial fibrillation, diabetes, hypercholesterolemia, and smoking, but these do not completely account for the pattern of stroke epidemiology.[5] Stroke is a common complication in severe bacterial infections, such as endocarditis and meningitis, but it also occurs in other, more

Correspondence to: Olli Patrakka, MD, Department of Forensic Medicine, Faculty of Medicine and Health Technology, 33014 Tampere University, Tampere, Finland. Email: olli.patrakka@tuni.fi

This work was presented in part at the World Stroke Congress, October 26 to 29, 2022, in Singapore.

This article was sent to Jose R. Romero, MD, Associate Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.123.030639

For Sources of Funding and Disclosures, see page 8.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- Confirming previous bacterial DNA findings in blood clots of patients with ischemic stroke, oral streptococcal bacteria were found inside neutrophil granulocytes in blood clots and in the wall of symptomatic and in asymptomatic carotid arteries by bacterial immunohistochemistry.

What Question Should be Addressed Next?

- Can the outcome of cerebral infarction be affected with a timely short-term antimicrobial treatment against oral streptococci?
- Could it be possible to develop a safe vaccine against oral streptococci to reduce the risk for an ischemic stroke caused by bacteria-triggered thrombosis?

Nonstandard Abbreviations and Acronyms

ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATCC	American Type Culture Collection
BMG	Brain, Microbes and Genetics
TSDS	Tampere Sudden Death Study
TVS	Tampere Vascular Study

common and milder infections.[5,6] Increased levels of antibodies against several bacteria, such as streptococci, staphylococci, and enterobacteria, were found in 44% of young patients with stroke, but in only 9% of controls.[7] Peripheral blood neutrophil count is an independent predictor of stroke severity on admission, a greater degree of disability at discharge, and 30-day mortality.[8,9] Severe chronic dental infection was found to predispose to stroke in men, possibly by affecting blood coagulation and platelet function.[10] Moreover, previous infections or chronic exposure to common infective agents are also suggested to contribute to carotid atherosclerosis.[11]

Recent developments in molecular microbiological methods have attracted new interest in studying the involvement of pathogens in cardiovascular diseases. We recently amplified viridans group streptococcal DNA using quantitative polymerase chain reaction from the majority of aspirated thrombi of patients with acute ischemic stroke treated with mechanical thrombectomy (Figure 1).[12] Subsequently, we reported that these patients with stroke had poor oral health, which tended to associate with carotid stenosis.[13] Viridans streptococci species belong to the normal oral bacterial microbiome but are also known to cause infective endocarditis and possess thrombogenic properties.[14,15] DNA of viridans group streptococcal bacteria was also detected in thrombus aspirates from patients with myocardial infarction as well as from patients with lower leg thrombosis due to peripheral atherosclerosis.[16,17]

In the current study, we examined whether the presence of viridans streptococci group bacteria can be confirmed in the thrombus aspirates of patients with stroke and in carotid artery specimens using bacterial immunohistochemistry.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The present study is based on a bacterial immunohistochemical study of cerebral thrombus aspirates from patients with acute ischemic stroke in the BMG (Brain, Microbes and Genetics) study, as well as carotid endarterectomy samples from symptomatic patients in TVS (Tampere Vascular Study) and carotid artery samples from nonstroke autopsy cases in TSDS (Tampere Sudden Death Study) (Figure 1).

BMG Study

Thrombus aspirates were collected from 61 consecutive patients with ischemic stroke who had large-vessel occlusion, mainly middle cerebral artery M1 and M2 segment, (mean age, 66.8 years; 70.5% men), treated with mechanical thrombectomy between December 2013 and July 2016 in the Acute Stroke Unit of Tampere University Hospital, Tampere, Finland. The thrombus was aspirated from the middle cerebral artery in 60 of the 61 samples (98.4%) of the cases, with 1 thrombus aspirated from the vertebrobasilar artery. Patients with thrombolysis in cerebral infarction 0 or 1 recanalization grade were excluded as the thrombus was unsuccessfully retrieved. A neurologist examined all patients when they arrived at the hospital and evaluated the possibility of revascularization using thrombectomy together with a neurointerventional radiologist. According to the TOAST classification, the cause of large-vessel occlusions of the brain in patients treated with endovascular thrombectomy during the study period in Tampere University Hospital was cardioembolic in 38% and atherosclerotic in 62% of the cases (personal communication, Dr Jyrki Ollikainen).[18] The median delay between the onset of an ischemic stroke and hospital arrival was 2 hours and 20 minutes (range, 0–16 hours). Medical history was collected from Tampere University Hospital digital patient archives. None of the patients

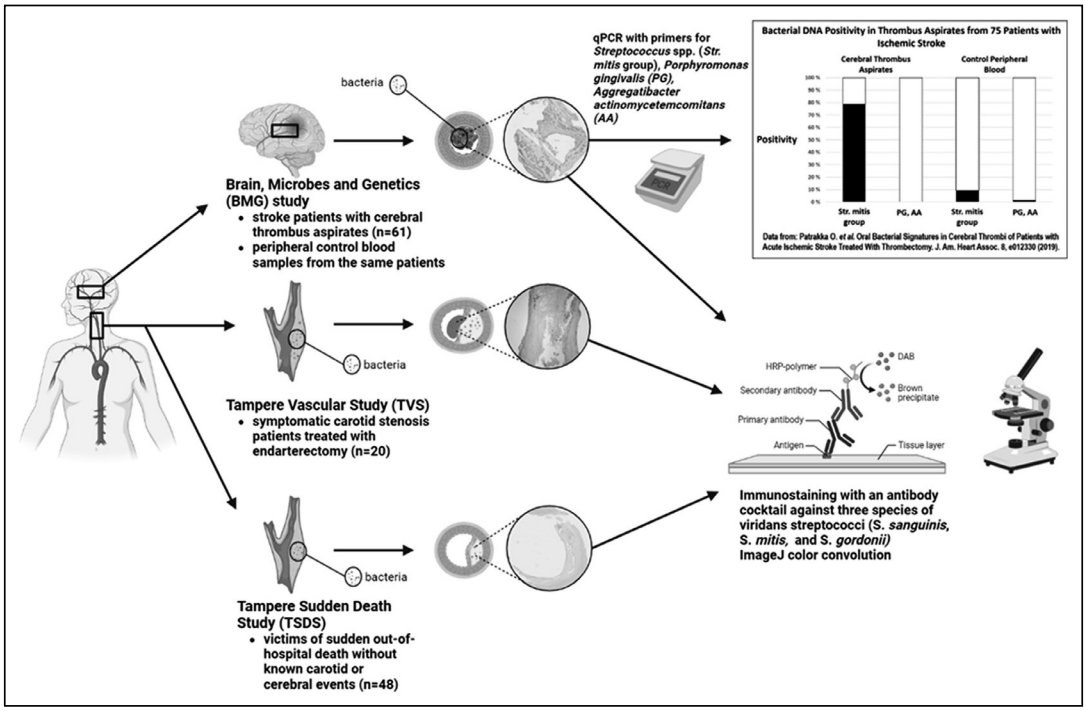


Figure 1. Study protocol.
qPCR data modified from Patrakka et al.[12] Figure created with BioRender.com. DAB indicates 3,3′-Diaminobenzidine; HRP, horseradish peroxidase; and qPCR, quantitative polymerase chain reaction.

had been treated with antibiotics or had experienced severe infections or septicemia during the stroke.

During mechanical thrombectomy, an introducer sheath was placed into a femoral artery, and a control blood sample for bacterial genetic analysis was collected through the sheath. A guiding catheter of up to 9F (Merci, Stryker Neurovascular) with a tip balloon was then navigated into the carotid artery proximally to the occluded site. The microcatheter (0.021") with the guidewire was used to navigate through the occluded site and to deploy the stent retriever (Trevo, Stryker Neurovascular) over the thrombus. An additional distal access catheter was used to reach the thrombus if needed. Forceful aspiration through proximal catheters was achieved with a 60-mL syringe while retrieving the deployed stent. In a minority of the cases, only direct thrombus aspiration was used. The device settings were selected by the operator case by case. Thrombectomy was repeated until the angiologic result was satisfactory. The gathered thrombus was divided into a segment placed in a 1.5-mL microcentrifuge tube for quantitative polymerase chain reaction analysis and a histological sample placed in a formalin container.

Tampere Vascular Study

Endarterectomy samples were collected from 20 symptomatic patients (mean age, 66.2 years; 65% men) diagnosed with ipsilateral carotid artery stenosis. All open vascular surgical procedures were performed at the Division of Vascular Surgery and the Heart Center at Tampere University Hospital between 2006 and 2009. The patients had experienced amaurosis fugax, a transient ischemic attack, or a stroke. Eight (40%) patients had an acute ischemic stroke. The severity of the carotid stenosis was histologically classified, the average being 82% (range, 69%–99%). All samples were classified as either fibrotic or calcified atheroma (American Heart Association type V) or complicated atheroma with rupture and thrombosis/hemorrhage (American Heart Association type VI) according to the American Heart Association classification.[19,20] Medical history was collected from Tampere University Hospital digital patient archives.

In the study, a small, 2×2- to 3-mm endarterectomy sample that could be extracted without endangering the patient was immediately placed in sterile 10% buffered formalin. From the initial 96 samples obtained for

histology, 20 (20.8%) were selected because they were large enough for immunohistochemical studies.

Tampere Sudden Death Study

Postmortem carotid artery samples were collected from 48 autopsy cases (mean age, 66.4 years; 62.5% men) during medicolegal autopsies at the Department of Forensic Medicine at the University of Tampere between 2010 and 2015, representing a cross-section of the population. The selection criteria for the cases were out-of-hospital death, time elapsed postmortem of <6 days, intact middle torso and bowel, no signs of bacterial infections or drug addiction, and no visible wounds or necrosis. None of the patients had died of stroke.

The time interval between death and storage of the body in the mortuary was <24 hours in all cases. In the mortuary, the bodies were kept at 4 °C. Low temperatures prevent bacterial growth, and bacterial populations are unlikely to alter during such storage conditions.[21] Based on hospital records, incident police reports with data on drugs found in the home and possible treatments, as well as physician's admission notes, none of the patients with a sudden out-of-hospital death had used antibiotics within 2 weeks prior to their death. The relatives of the deceased were sent a structured interview concerning risk factors and other background information.

Ethics

All participating patients gave their written consent to the study. TVS was approved by the ethics committee of Pirkanmaa Hospital District (R99204). All clinical investigations were conducted according to the principles of the Declaration of Helsinki. The BMG study was approved by the ethics committee of Pirkanmaa Hospital District (R13093), and the study was explained to the patients. In TSDS, informed consent was given by relatives. TSDS was approved by the ethics committee of Pirkanmaa Hospital District (R09097). Methods were reported according Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[22]

Quantitative Polymerase Chain Reaction

Bacterial DNA was extracted from the BMG samples using a commercially available QIAamp DNA Mini Kit (Qiagen) according to the instructions provided. Quantitative polymerase chain reaction was used to detect the presence of bacterial DNA in the thrombus and blood samples from the same patients by using published oligonucleotide primers and probes for *Streptococcus* spp., mainly the *Streptococcus mitis* group, as well as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Universal bacterial primers and probe were determined with RNaseP (Applied Biosystems) as a reference. These methods are described in detail elsewhere.[12]

Immunohistochemistry

Antibodies against 3 major viridans streptococcal species—*Streptococcus sanguinis* (American Type Culture Collection [ATCC] 10556), *S mitis* (ATCC 49456), and *Streptococcus gordonii* (ATCC 10558)[12]—were produced by ThermoFisher using 90-day rabbit immunization protocol. The performance of the resulting antibodies was confirmed with Western blot by staining the respective streptococcal lysates (data not shown). All histological samples were immunostained with an antibody cocktail containing antibodies raised against the 3 species of viridans streptococci. The immunostaining intensity was assessed with a semiquantitative score (+/++/+++). ImageJ software (github.com/fiji/fiji) was used to create color convolution in order to increase the contrast in the immunostained samples for better visualization of the results. Control stainings were performed with an IgG isotype control antibody (ab37415, Abcam) as well as with an IgG anti–*Escherichia coli* antibody (ab137967, Abcam) raised in rabbits. The specificities of the antibodies were tested in a histological tissue section by inoculating them with a suspension of streptococcal bacteria species (obtained from ATCC) and performing an immunohistochemical study using an antibody against the same bacteria (Figure S1). Gut and liver samples from the TSDS autopsy cases were stained with a streptococcal cocktail to show the ability of the antibodies to detect streptococci in feces as well as in liver Kupffer cells (Figure S2).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 28. Associations between bacterial immunohistochemistry and sample frequencies were analyzed using χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS

The mean age and sex of the participants did not differ between the 3 study groups (Table). Cerebrovascular disease was less frequent, and coronary heart disease was more common in the TSDS series. Patients with symptomatic carotid stenosis in the TVS series were more often smokers compared with the other groups. Hypertension was reported more frequently as a risk factor in symptomatic BMG and TVS series patients compared with the TSDS autopsy series.

Table. Clinical Characteristics of the Study Series

Characteristics	BMG study (N=61)	TVS (N=20)	TSDS (N=48)
Age, mean±SD, y	66.8±10.9	66.2±8.42	66.4±15.6
Men, n (%)	43 (70.5)	13 (65.0)	30 (62.5)
Diabetes, n (%)	11 (18.0)	4 (20.0)	7 (14.6)
Dyslipidemia, n (%)	24 (39.3)	17 (85.0)	N/A
Arterial hypertension, n (%)	31 (50.8)	15 (75.0)	15 (31.3)
Coronary heart disease, n (%)	13 (21.3)	6 (30.0)	26 (54.2)*
Cerebrovascular disease, n (%)	61 (100)	19 (95.0)	2 (4.2)*
Atrial fibrillation, n (%)	39 (63.9)	N/A	N/A
Heart failure, n (%)	9 (14.8)	1 (5.00)	N/A
Smoking status, n (%)	13 (36.1)†	15 (75.0)	18 (37.5)

BMG indicates Brain, Microbes and Genetics; N/A, data not available; TSDS, Tampere Sudden Death Study; and TVS, Tampere Vascular Study.
*Cause of death.
†Data available from 36 patients.

As regards the aspirated cerebral artery thrombi of patients with stroke, DNA from *Streptococcus* spp., mainly the *S mitis* group, was found in 78.7% of the cases participating in the BMG study (Figure 1), whereas DNA from *P gingivalis* and *A actinomycetemcomitans* was not detected. Of the arterial blood control samples collected during the thrombectomy procedure, 9.33% were positive for *Streptococcus* spp. and 1.33% for both *P gingivalis* and *A actinomycetemcomitans*. These findings have been described in detail in a previous article.[12]

Of the 61 thrombus aspirate samples from the patients with stroke in the BMG study, 39 (84.8%) were histologically positive (+/++/+++) for viridans streptococci group bacteria (Figure 2). Most streptococci were detected inside neutrophil granulocytes, but there were also remnants of bacterial biofilm as well as free bacterial infiltrates in some samples (Figure 3). High numbers of neutrophilic granulocytes were present in the samples.

Most (80.0%) carotid endarterectomy samples obtained from symptomatic patients included in TVS were positive for oral streptococci (Figure 2). In contrast, the carotid artery samples from the TSDS study comprising out-of-hospital deaths, mainly due to coronary heart disease, showed considerably less (31.3%) immunopositivity, with 16.7% of the samples being clearly immunopositive (++/+++). In the TVS endarterectomies and TSDS carotid artery sample series, oral streptococci were found in clusters of streptococcal antibody-positive neutrophilic granulocytes as well as remnants of bacterial biofilm (Figure 3). The immunopositivity scores were highest in the BMG thrombus aspirates and TVS carotid endarterectomies ($P<0.001$) (Figure 2).

Staining of the samples with an IgG isotype antibody or *E coli* antibody resulted in a negligible signal, suggesting the reliability of the viridans streptococcal antibody cocktail staining (Figure 3).

DISCUSSION

The decline in strokes during the 20th century can only incompletely be explained by conventional risk factors and their temporal trends.[5,23] It has been long suspected that bacterial infection may play a role in the pathogenesis of stroke.[6] Using a molecular microbiological approach, we have found that thrombus aspirates from patients with stroke contained viridans streptococcal DNA fragments.[12] In the present study, we confirmed the presence of viridans streptococcal

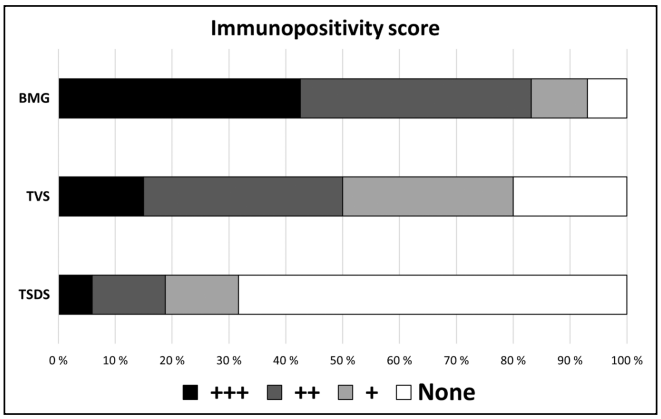


Figure 2. Sample frequencies and a 3-step classification of intensity of immunopositivity ($P<0.001$).
BMG indicates Brain, Microbes and Genetics; TSDS, Tampere Sudden Death Study; and TVS, Tampere Vascular Study.

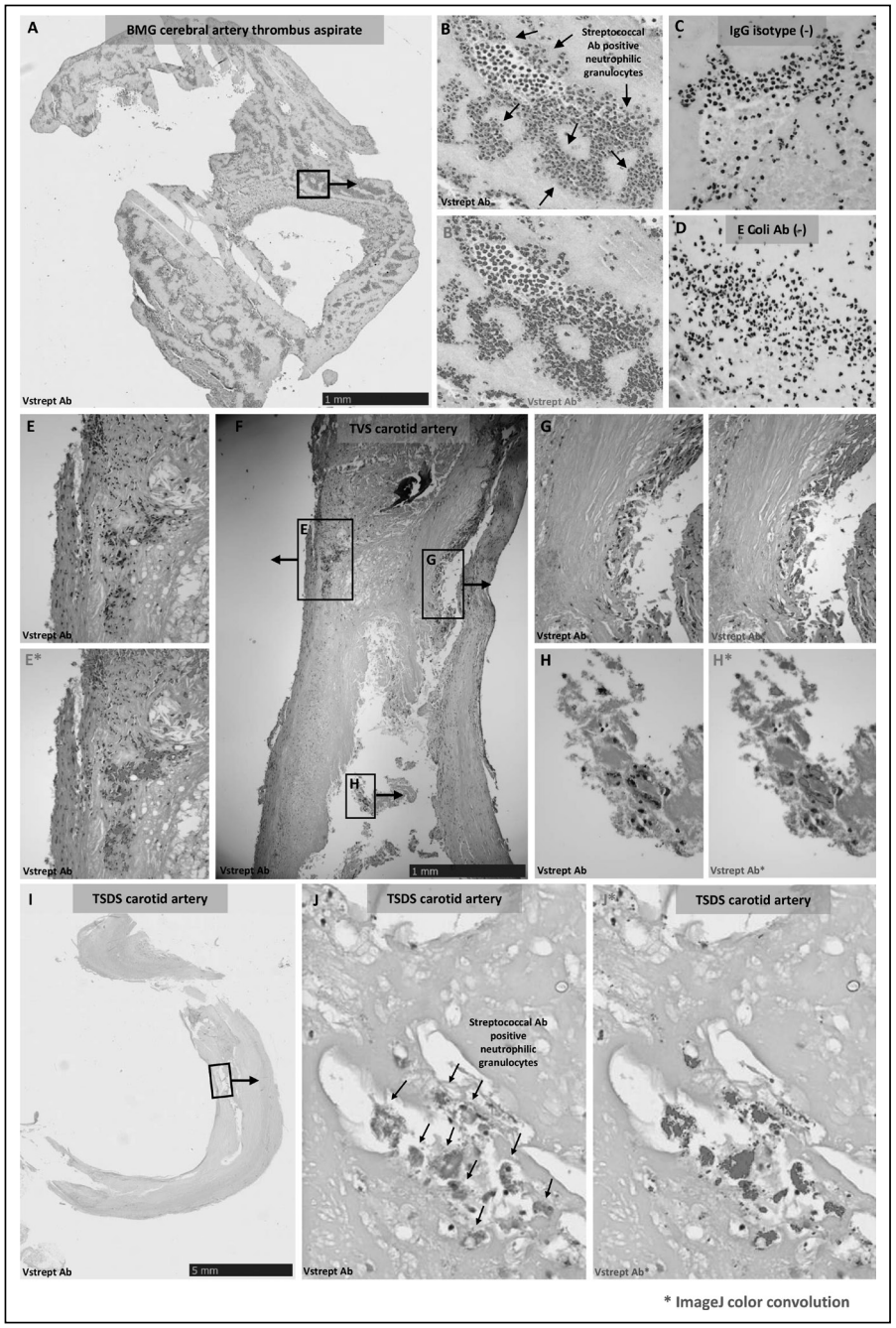


Figure 3. Bacterial immunostaining of the samples.
Viridans streptococcal immunostaining showing diaminobenzidine (brown) and color convoluted (red) microscopical photographs of bacterial infiltrates in thrombus aspirates from the BMG series (**A** and **B**), ruptured carotid atheroma samples from TVS (**E** through **H**), and carotid artery samples from the TSDS series (**I–J**). Thrombus aspirates were also stained with IgG isotype (**C**) and *Escherichia coli* (**D**) antibodies to show the specificity of the bacterial immunostaining. BMG indicates Brain, Microbes and Genetics; N/A, data not available; TSDS, Tampere Sudden Death Study; and TVS, Tampere Vascular Study.

bacteria using immunohistochemistry. Almost all of the thrombus aspirates contained oral streptococci, located mainly inside neutrophilic granulocytes. In symptomatic patients subjected to carotid endarterectomy, most carotid samples were strongly immunopositive for viridans group streptococci. We also found clear streptococcal immunopositivity in 17% of carotid artery samples from autopsy cases representing a cross-section of the population. To our knowledge, only 1 study has previously reported the presence of viridans group streptococci in carotid artery endarterectomies using immunolocalization,[24] but the presence of viridans group streptococci in cerebral thrombi has not been previously shown. Our results suggest that there may be a connection between symptomatic inflammation in carotid arteries and cerebral thrombus.

The present concept is that atherosclerosis is a complex chronic inflammatory disorder in the arterial intima, driven by oxidized or otherwise modified low-density lipoprotein.[25–27] Increasing knowledge suggests that the atherosclerotic process may also be accelerated by a bacterial infection, with infections of oral origin being extensively studied.[28–32] Both oxidized low-density lipoprotein and bacteria activate Toll-like receptor–mediated pathways, leading to the elevation of cytokine levels and systemic inflammation, which are related to the initiation, development, and rupture of an atherosclerotic plaque.[33–36] The immune responses that have evolved to combat bacterial infections are thus shared with those involved in the immune response to the inflammatory components of atherogenesis.[37] Oral infections have also been suggested to contribute to carotid artery intima-media thickness, leading to carotid artery stenosis and subclinical atherosclerosis,[38] and, after adjusting for conventional risk factors, a significant association has been reported between the level of tooth loss (as a marker of past periodontal disease) and the prevalence of carotid artery plaques.[39]

Oral bacteria can enter the bloodstream after dental procedures (eg, root canal treatment or tooth extraction) causing transient bacteremia. In tooth extraction, most bacteria translocated into the circulation have been found to be viridans streptococci.[40,41] Koren et al found that the abundance of viridans streptococcal DNA in atherosclerotic plaques correlated with their abundance in the oral cavity.[42] After accessing systemic circulation, bacteria might end up in an atherosclerotic lesion via a circulating macrophage or directly through neovasculature developing inside the carotid atherosclerotic plaque.[43] Viridans streptococci can directly interact with platelets, causing their activation.[15] Activated platelets (ie, platelet-derived growth factor) induce the recruitment of proatherosclerotic cells, which speeds up the atherosclerotic processes.[44–46] Following bacteremia, bacteria-induced thrombi might thus also be trapped in the left atrial appendage, causing a cardioembolic stroke in patients with altered hemodynamics, such as in atrial fibrillation.[45,47] In our BMG series, 63.9% of the patients had atrial fibrillation. However, despite that, 62% of the cases were considered to be due to atherosclerosis, suggesting uncertainty in determining the cause.

Oral bacteria are known to form biofilms on hard tooth surfaces but also on soft epithelial tissue.[48] Viridans streptococci are the initial colonizers in the development of a dental plaque.[49] It has been suggested that bacteria form biofilm-like structures in the atherosclerotic plaque.[50,51] This could contribute to the persistent inflammation associated with atherosclerosis. Biofilm-phenotype bacteria are much more resistant to antibiotics than the same bacteria in planktonic form.

To validate the specificity of the immunohistochemical stainings, we used several controls. The rabbit IgG isotype control is a primary antibody that lacks specificity to the target. Isotype controls are used as negative controls to differentiate a nonspecific background signal from a specific antibody signal. An *E coli*–specific antibody was used to show the specificity of the viridans streptococci antibody cocktail. All IgG isotype control–stained samples as well as the *E coli* immunostainings were negative.

The limitations of our study include the fact that we used 3 different sets of patient samples and that the endarterectomy samples and thrombus aspirate samples were taken from different patients. This cross-sectional study is a hypothesis-generating study and cannot demonstrate any etiological relation. In the future, it would be interesting to collect the samples from the same patients. We only used an antibody cocktail against viridans streptococci species, and it is possible that other bacteria may also be involved in the pathogenesis of thrombosis. To demonstrate the cause, a prospective multicenter study with larger patient series should be designed.

CONCLUSIONS

We verified using immunohistochemistry that streptococci can be found in the aspirated thrombi of patients with acute ischemic stroke as well as in atherothrombotic symptomatic carotid artery plaque samples, suggesting their possible involvement in the pathogenesis of ischemic stroke.

ARTICLE INFORMATION

Received July 9, 2023; accepted October 27, 2023.

Affiliations

Department of Forensic Medicine, Faculty of Medicine and Health Technology, Tampere University and Fimlab Laboratories, Tampere, Finland (O.P., S.T., V.L., M.J.O., V.P.H., M.M., P.J.K.); Vascular Centre (J-P.P., N.O.) and Department of Neurology (J.O.), Tampere University Hospital, Tampere, FinlandSurgery, Faculty of Medicine and Health Technology (N.O.)Laboratory

of Protein Dynamics, Faculty of Medicine and Health Technology (M.J.O., V.P.H.) and Faculty of Social Sciences (H.H.), Tampere University, Tampere, FinlandDepartment of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Fimlab Laboratories and Finnish Cardiovascular Research Center Tampere, Tampere, Finland (T.L.); and National Institute for Health and Welfare, Helsinki, Finland (M.M.).

Sources of Funding

This study was supported by grants from the Ida Montin Foundation (O.P.); The Finnish Medical Foundation (O.P.); the Competitive Research Funding of Tampere University Hospital (grant X51001 for T.L. and P.K.); the Emil Aaltonen Foundation (T.L.); the Academy of Finland (grants 286284 and 322098 for T.L. as well as 331946 for V.P.H.); the Tampere Tuberculosis Foundation (T.P. and T.L.); the Finnish Foundation for Cardiovascular Research (grants for T.L., MJ.T.O., and P.K.); the Sigrid Juselius Foundation (grant for V.P.H.); the Yrjö Jahnsson Foundation, Pirkanmaa Regional Fund of the Finnish Cultural Foundation (S.T.); the Elli and Elvi Oksanen Foundation, Pirkanmaa Regional Fund of the Finnish Cultural Foundation (M.J.T.O and V.L.); the European Union 7th Framework Programme (grant 201668 for AtheroRemo); and the European Union Horizon 2020 (grant 755320 for TAXINOMISIS and grant 848146 for To Aition).

Disclosures

None.

Supplemental Material

Figures S1–S2
References[52–54]

REFERENCES

1. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet*. 2008;371:1612–1623. doi: 10.1016/S0140-6736(08)60694-7
2. Inzitari D, Eliasziw M, Gates P, Sharpe BL, Chan RKT, Meldrum HE, Barnett HJM. The causes and risk of stroke in patients with asymptomatic internal-carotid-artery stenosis. *N Engl J Med*. 2000;342:1693–1700. doi: 10.1056/NEJM200006083422302
3. Flaherty ML, Kissela B, Khoury JC, Alwell K, Moomaw CJ, Woo D, Khatri P, Ferioli S, Adeoye O, Broderick JP, et al. Carotid artery stenosis as a cause of stroke. *Neuroepidemiology*. 2012;40:36–41. doi: 10.1159/000341410
4. Bos D, Portegies MLPP, Van Der Lugt A, Bos MJ, Koudstaal PJ, Hofman A, Krestin GP, Franco OH, Vernooij MW, Ikram MA. Intracranial carotid artery atherosclerosis and the risk of stroke in whites: the Rotterdam study. *JAMA Neurol*. 2014;71:405–411. doi: 10.1001/jamaneurol.2013.6223
5. Grau AJ, Urbanek C, Palm F. Common infections and the risk of stroke. *Nat Rev Neurol*. 2010;6:681–694. doi: 10.1038/nrneurol.2010.163
6. Syrjanen J. Infection as a risk factor for cerebral infarction. *Eur Heart J*. 1993;14:17–19.
7. Syrjänen J, Valtonen VV, Livanainen M, Hovi T, Malkamäki M, Mäkelä PH. Association between cerebral infarction and increased serum bacterial antibody levels in young adults. *Acta Neurol Scand*. 1986;73:273–278. doi: 10.1111/j.1600-0404.1986.tb03275.x
8. Furlan JC, Vergouwen MDI, Fang J, Silver FL. White blood cell count is an independent predictor of outcomes after acute ischaemic stroke. *Eur J Neurol*. 2014;21:215–222. doi: 10.1111/ene.12233
9. Grau AJ, Boddy AW, Dukovic DA, Buggle F, Lichy C, Brandt T, Hacke W. Leukocyte count as an independent predictor of recurrent ischemic events. *Stroke*. 2004;35:1147–1152. doi: 10.1161/01.STR.0000124122.71702.64
10. Syrjänen J, Peltola J. Dental infections in association with cerebral infarction in young and middle-aged men. *J Intern Med*. 1989;225:179–184. doi: 10.1111/j.1365-2796.1989.tb00060.x
11. Elkind MSV, Luna JM, Moon YP, Boden-Albala B, Liu KM, Spitalnik S, Rundek T, Sacco RL, Paik MC. Infectious burden and carotid plaque thickness: the northern Manhattan study. *Stroke*. 2010;41:e117–e122. doi: 10.1161/STROKEAHA.109.571299
12. Patrakka O, Pienimäki JP, Tuomisto S, Ollikainen J, Lehtimäki T, Karhunen PJ, Martiskainen M. Oral bacterial signatures in cerebral thrombi of patients with acute ischemic stroke treated with thrombectomy. *J Am Heart Assoc*. 2019;8:e012330. doi: 10.1161/JAHA.119.012330
13. Patrakka O, Mehtonen H, Tuomisto S, Pienimäki JP, Ollikainen J, Huhtala H, Pessi T, Oksala N, Lehtimäki T, Järnstedt J, et al. Association between oral pathology, carotid stenosis, and oral bacterial DNA in cerebral thrombi of patients with stroke. *Stroke Res Treat*. 2021;2021:1–10. doi: 10.1155/2021/5402764
14. Cahill TJ, Prendergast BD. Infective endocarditis. *Lancet (London, England)*. 2016;387:882–893. doi: 10.1016/S0140-6736(15)00067-7
15. Kerrigan SW, Cox D. The thrombotic potential of oral pathogens. *J Oral Microbiol*. 2009;1:1. doi: 10.3402/jom.v1i0.1999
16. Pessi T, Karhunen V, Karjalainen PP, Ylitalo A, Airaksinen JK, Niemi M, Pietila M, Lounatmaa K, Haapaniemi T, Lehtimäki T, et al. Bacterial signatures in thrombus aspirates of patients with myocardial infarction. *Circulation*. 2013;127:1219–1228. doi: 10.1161/CIRCULATIONAHA.112.001254
17. Vakhitov D, Tuomisto S, Martiskainen M, Korhonen J, Pessi T, Salenius JP, Suominen V, Lehtimäki T, Karhunen PJ, Oksala N. Bacterial signatures in thrombus aspirates of patients with lower limb arterial and venous thrombosis. *J Vasc Surg*. 2018;67:1902–1907. doi: 10.1016/j.jvs.2017.05.090
18. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE III. Classification of subtype of acute ischemic stroke. *Stroke*. 1993;23:35–41.
19. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, American Heart Association. *Circulation*. 1995;92:1355–1374. doi: 10.1161/01.CIR.92.5.1355
20. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2000;20:1262–1275. doi: 10.1161/01.ATV.20.5.1262
21. Tuomisto S, Karhunen PJ, Pessi T. Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR. *Gut Pathogens*. 2013;5:5. doi: 10.1186/1757-4749-5-35
22. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol*. 2020;18:e3000410. doi: 10.1371/journal.pbio.3000410
23. Lindsberg PJ, Grau AJ. Inflammation and infections as risk factors for ischemic stroke. *Stroke*. 2003;34:2518–2532. doi: 10.1161/01.STR.0000089015.51603.CC
24. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J*. 1999;138:S534–S536. doi: 10.1016/S0002-8703(99)70294-2
25. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115–126. doi: 10.1056/NEJM199901143400207
26. Tousoulis D, Oikonomou E, Economou EK, Crea F, Kaski JC. Inflammatory cytokines in atherosclerosis: current therapeutic approaches. *Eur Heart J*. 2016;37:1723–1735. doi: 10.1093/eurheartj/ehv759
27. Paoletti R, Gotto AM, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. *Circulation*. 2004;109:III20–III26. doi: 10.1161/01.CIR.0000131514.71167.2e
28. Morré SA, Stooker W, Lagrand WK, Van Den Brule AJC, Niessen HWM. Microorganisms in the aetiology of atherosclerosis. *J Clin Pathol*. 2000;53:647–654. doi: 10.1136/jcp.53.9.647
29. Valtonen VV. Role of infections in atherosclerosis. *Am Heart J*. 1999;138:S431–S433. doi: 10.1016/S0002-8703(99)70269-3
30. Grau AJ, Becher H, Ziegler CM, Lichy C, Buggle F, Kaiser C, Lutz R, Bültmann S, Preusch M, Dörfer CE. Periodontal disease as a risk factor for ischemic stroke. *Stroke*. 2004;35:496–501. doi: 10.1161/01.STR.0000110789.20526.9D
31. Joshipura KJ, Hung HC, Rimm EB, Willett WC, Ascherio A. Periodontal disease, tooth loss, and incidence of ischemic stroke. *Stroke*. 2003;34:47–52. doi: 10.1161/01.STR.0000052974.79428.0C
32. Sen S, Giamberardino LD, Moss K, Morelli T, Rosamond WD, Gottesman RF, Beck J, Offenbacher S. Periodontal disease, regular dental care use, and incident ischemic. *Stroke*. 2018;49:355–362. doi: 10.1161/STROKEAHA.117.018990
33. Hahn CL, Best AM, Tew JG. Cytokine induction by streptococcus mutans and pulpal pathogenesis. *Infect Immun*. 2000;68:6785–6789. doi: 10.1128/IAI.68.12.6785-6789.2000
34. Chia JS, Lien HT, Hsueh PR, Chen PM, Sun A, Chen JY. Induction of cytokines by glucosyltransferases of *Streptococcus mutans*. *Clin Diagn Lab Immunol*. 2002;9:892–897.

35. Ramji DP, Davies TS. Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev*. 2015;26:673–685. doi: 10.1016/j.cytogfr.2015.04.003
36. Kurita-Ochiai T, Jia R, Cai Y, Yamaguchi Y, Yamamoto M. Periodontal disease-induced atherosclerosis and oxidative stress. *Antioxidants*. 2015;4:577–590. doi: 10.3390/antiox4030577
37. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. *Nat Med*. 2002;8:1218–1226. doi: 10.1038/nm1102-1218
38. Pussinen PJ, Paju S, Koponen J, Viikari JSA, Taittonen L, Laitinen T, Burgner DP, Kähönen M, Hutri-Kähönen N, Raitakari OT, et al. Association of childhood oral infections with cardiovascular risk factors and subclinical atherosclerosis in adulthood. *JAMA Netw Open*. 2019;2:e192523. doi: 10.1001/jamanetworkopen.2019.2523
39. Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Papapanou PN, Sacco RL. Relationship between periodontal disease, tooth loss, and carotid artery plaque: the oral infections and vascular disease epidemiology study (INVEST). *Stroke*. 2003;34:2120–2125. doi: 10.1161/01.STR.0000085086.50957.22
40. Narayanan L, Vaishnavi C. Endodontic microbiology. *J Conserv Dent*. 2010;13:233. doi: 10.4103/0972-0707.73386
41. Lockhart PB, Brennan MT, Sasser HC, Fox PC, Paster BJ, Bahrani-Mougeot FK. Bacteremia associated with toothbrushing and dental extraction. *Circulation*. 2008;117:3118–3125. doi: 10.1161/CIRCULATIONAHA.107.758524
42. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, Behre CJ, Knight R, Fagerberg B, Ley RE, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci USA*. 2011;108:4592–4598. doi: 10.1073/pnas.1011383107
43. Camaré C, Pucelle M, Nègre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. *Redox Biol*. 2017;12:18–34. doi: 10.1016/j.redox.2017.01.007
44. Arman M, Krauel K, Tilley DO, Weber C, Cox D, Greinacher A, Kerrigan SW, Watson SP. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood*. 2014;123:3166–3174. doi: 10.1182/blood-2013-11-540526
45. Herzberg MC, Nobbs A, Tao L, Kilic A, Beckman E, Khammanivong A, Zhang Y. Oral streptococci and cardiovascular disease: searching for the platelet aggregation-associated protein gene and Mechanisms of streptococcus sanguis–induced thrombosis. *J Periodontol*. 2005;76:2101–2105. doi: 10.1902/jop.2005.76.11-S.2101
46. Tomaiuolo M, Brass LF, Stalker TJ. Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis. *Int Cardiol Clin*. 2017;6:1–12. doi: 10.1016/j.iccl.2016.08.001
47. Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. *Thromb Haemost*. 2008;99:480–486. doi: 10.1160/TH07-11-0685
48. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. *Virulence*. 2011;2:435–444. doi: 10.4161/viru.2.5.16140
49. Kolenbrander PE, Palmer RJ, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol*. 2010;8:471–480. doi: 10.1038/nrmicro2381
50. Snow DE, Everett J, Mayer G, Cox SB, Miller B, Rumbaugh K, Wolcott RA, Wolcott RD. The presence of biofilm structures in atherosclerotic plaques of arteries from legs amputated as a complication of diabetic foot ulcers. *J Wound Care*. 2016;25:S16–S22. doi: 10.12968/jowc.2016.25.Sup2.S16
51. Wolcott RD, Wolcott JJ, Palacio C, Rodriguez S. Possible role of bacterial biofilm in the pathogenesis of atherosclerosis. *J Bacteriol Parasitol*. 2012;3:1–7. doi: 10.4172/2155-9597.1000127
52. Doern CD, Burnham CAD. It's not easy being green: the Viridans group streptococci, with a focus on pediatric clinical manifestations. *J Clin Microbiol*. 2010;48:3829–3835. doi: 10.1128/JCM.01563-10
53. Hines IN, Son G, Kremer M. Contribution of gut bacteria to liver pathobiology. *Gastroenterol Res Pract*. 2010;2010:1–13. doi: 10.1155/2010/453563
54. Tuomisto S, Pessi T, Collin P, Vuento R, Aittoniemi J, Karhunen PJ. Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics. *BMC Gastroenterol*. 2014;14:40. doi: 10.1186/1471-230X-14-40