ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

INVESTIGATION OF POSSIBLE MICROORGANISMS AND NANOBACTERIA IN THE ETIOLOGY OF ATHEROSCLEROSIS

FEYZANUR ERDEMİR

Ph.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Prof. Zühtü Tanıl Kocagöz

SECONDARY SUPERVISOR
Prof. Özge Can

ISTANBUL-2024

ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

INVESTIGATION OF POSSIBLE MICROORGANISMS AND NANOBACTERIA IN THE ETIOLOGY OF ATHEROSCLEROSIS

FEYZANUR ERDEMİR

Ph.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Prof. Zühtü Tanıl Kocagöz

SECONDARY SUPERVISOR
Prof. Özge Can

ISTANBUL-2024

DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

25/11/2024
Feyzanur Erdemir

PREFACE AND ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF SYMBOLS

%	Percentage
µg	Microgram
µl	Microliters
Al	Aluminum
As	Arsenic
bp	Base Pairs
C	Carbon
C°	Celsius
Ca	Calcium
CO_2	Carbon Dioxide
kDa	Kilo Daltons
kGy	Kilo Grays
M	Molarity
Mg	Magnesium
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
N	Nitrogen
nm	Nanometer
O	Oxygen
P	Phosphorous
pH	Potential of Hydrogen
S	Sulfur
Si	Silicon

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DAPI	4′,6-Diamidino-2-Phenylindole
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
EDS	Energy-Dispersive X-ray Spectroscopy
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Eosin Methylene Blue Agar
GCF	Gingival Crevicular Fluid
HDL	High Density Protein
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDL	Low Density Protein
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
RNAse	Ribonuclease
SDA	Sabouraud Dextrose Agar
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Investigation of Possible Microorganisms and Nanobacteria in The Etiology of Atherosclerosis

Cardiovascular diseases are one of the leading causes of death worldwide. Since atherosclerotic plaque formation leads to blockage of blood vessels, vital organs such as the heart and brain are affected. Although this is considered a general process, the role of infectious agents has always been an important research topic. This study aimed to examine metagenomic and microbiological investigations of all microorganisms, including nanobacteria, that may cause atheroma plaque formation. Nanobacteria were grown by culturing atheroma plaque homogenates in cell culture medium. As a result of detailed molecular analysis of nanobacteria, it was shown that they consisted of human proteins. Accordingly, it supported the theory that they are not living organisms, but structures formed by biocrystallization. Whole genome sequencing of nucleic acids obtained from two groups of pool samples of atheroma plaques showed that more than 50% of the metagenomic sequences belonged to *Toxoplasma gondii* (*T. gondii*). Examination of *T. gondii* DNA in individual samples of atheroma plaques by PCR (Polymerase Chain Reaction) revealed the presence of *T. gondii* in 8 of 20 atheroma plaques. If more evidence of the role of *T. gondii* in the etiology of atheroma plaque formation becomes available, strategies to treat *T. gondii* infections may become important in preventing atheroma plaque formation in the future.

Keywords: Atherosclerosis, Atheroma plaque, *Toxoplasma gondii*

ÖZET

Aterosklerozun Etiyolojisinde Olası Mikroorganizmaların ve Nanobakterilerin Araştırılması

Dünya çapında kardiyovasküler hastalıklar önde gelen ölüm nedenlerinden biridir. Aterosklerotik plak oluşumu kan damarlarının tıkanmasına yol açtığı için kalp ve beyin gibi hayati organlar etkilenir. Bu genel bir süreç olarak kabul edilse de enfeksiyon etkenlerinin rolü her zaman önemli bir araştırma konusu olmuştur. Bu çalışmada aterom plak oluşumuna sebep olabilecek nanobakteriler dahil bütün mikroorganizmaların metagenomik ve mikrobiyolojik incelemesi hedeflenmiştir. Aterom plak homojenatlarının hücre kültürü ortamında kültürlenmesiyle nanobakteriler yetiştirildi. Nanobakterilerin ayrıntılı moleküler analizi sonucunda bunların insan proteinlerinden oluştuğu gösterildi. Buna göre onların canlı organizmalar değil, biyokristalizasyonla oluşan yapılar olduğu teorisi desteklendi. Aterom plaklardan oluşan iki grup havuz örnekten elde edilen nükleik asitlerin tüm genom dizilimi, metagenomik dizilerin %50'den fazlasının *Toxoplasma gondii* (T. gondii)' ye ait olduğunu gösterdi. Aterom plakların bireysel numunelerinde *T. gondii* DNA'sının PZR (Polimeraz Zincir Reaksiyonu) ile incelenmesi sonucunda 20 aterom plağının 8'inde *T. gondii*'nin varlığını ortaya çıkardı. Aterom plağı oluşumunun etiyolojisinde *T. gondii*' nin rolü daha çok kanıt elde edilebilirse, *T. gondii* enfeksiyonlarını tedavi etme stratejileri gelecekte aterom plağı oluşumunun önlenmesinde önemli hale gelebilir.

Anahtar Sözcükler: Ateroskleroz, Aterom plak, *Toxoplasma gondii*

1 INTRODUCTION

Cardiovascular diseases (CVDs) including coronary heart, rheumatic heart, and cerebrovascular diseases, are the leading cause of death worldwide (1). In 2019, 18.6 million people died of cardiovascular diseases (2). Atherosclerosis has a significant role in the development of all these diseases (3). Atherosclerosis is defined as a thickening of the arterial wall due to the accumulation of cholesterol, cellular waste products, macrophages, calcium salts, and other substances. The progression of atherosclerosis leads to plaque formation, which restricts blood flow (4). Various mechanisms for plaque formation have been proposed. In general, it is considered a chronic inflammatory event (5). One theory suggests that inflammatory events are triggered by autoimmune diseases (6). However, microbial agents are also considered one of the leading causes (7). Although inflammation causing atherosclerosis is regarded as a sterile reaction independent of microbial agents, recent studies have highlighted the possible effects of several microbial agents (8), which have been the subject of a limited number of studies, some showing conflicting results. The potential role of a few organisms such as *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and the Herpes virus family in atherosclerotic disease has been suggested (9), (10), (11), (12). In one study, *Helicobacter pylori* and *C. pneumoniae* DNA were identified by PCR in specific carotid artery atherosclerotic plaques (13). Two other studies identified anti-*Toxoplasma gondii* immunoglobulin G (IgG) antibodies with a higher incidence in patients with atherosclerosis (14), (15). The development of new methodologies, such as epigenetic analysis using next-generation sequencing (NGS), for detecting microbial agents continues to contribute to the identification of unknown pathogens (16). As emerging pathogens or agents of atherosclerosis, nanobacteria have been proposed as candidate agents capable of forming appetite-coating calcium phosphate precipitates (16), (17), (18). Nanobacteria may be responsible for pathological calcifications in kidney and dental pulp stones, along with heart valve and arterial calcifications (19). However, other studies have questioned the microbiological origin of nanobacteria, suggesting that they are formed by the biomineralization of natural molecules.

The main goal of this research was to determine the role of microorganisms, including nanobacteria, in the inflammation process that occurs during the development of atherosclerosis. Many studies have focused on the role of various infectious agents but approaches such as whole genome sequencing, which can cover all possible microorganisms, have remained very limited. This study aimed to investigate the presence of microorganisms and nanobacteria by microbiological culture and genomic examination of atherosclerotic plaques.

2 BACKGROUND

2.1 Atherosclerosis

Atherosclerosis is the restriction of blood flow and thickening of the arterial wall (plaque formation) as a result of the accumulation of cholesterol, cellular waste products, macrophages, calcium and other substances on the arterial wall (20). This is often the result of risk factors such as high cholesterol levels, high blood pressure, obesity, smoking, a sedentary lifestyle and diabetes (21). These blockages can cause serious complications such as heart attacks and stroke. Atherosclerotic vessels tend to enlarge in diameter. In the early stages of the atheroma process, plaques usually grow in the opposite direction of the vessel. In general, the arterial canal is considered to be full when a plaque covers more than 40% of the inner elastic layer of the vessel. In the final process of plaque formation, blood flow appears to be restricted. The first soft lesion of the plaque consists of foam cells, a small number of platelets (platelets), and extracellular fat deposits. It is thought that during the progression of the process, smooth muscle cells proliferate and lead to the formation of fibrous caps (22). Treatment of atherosclerosis usually includes methods such as dietary changes, exercise, medications, and surgery. Patients should be checked regularly to prevent risk factors, and they can slow disease progression by making lifestyle changes.

2.1.1 The molecular mechanism of atherosclerosis

The molecular mechanisms of atherosclerosis occur in three stages: initiation, progression and atheroma formation (23).

The beginning of atherosclerosis:

The artery wall has a three-layered structure. The outermost layer contains mast cells and nerve endings. The middle layer consists of smooth muscle cells, elastin, collagen and other macromolecules. The place where atheroma plaque occurs is the innermost layer. Fat streaks are usually the first sign in the development of

atherosclerosis. The first lesions occur due to the increase in lipoprotein in the intima layer of the artery. (24). Lipoprotein consists of lipids such as phospholipids, proteins, triglycerides and cholesterol. The most important of these is low-density lipoprotein (LDL), which is rich in cholesterol. LDL can adhere to extracellular matrix components (such as proteoglycans), leading to deposition in the vascular intima (25). Adhesion molecules are expressed by the activated endothelial cell. Chemokines stimulate the migration of monocytes to the arterial wall. Monocytes in the bloodstream go to the intima and turn into macrophages. At the same time, T lymphocytes enter the intima and regulate the functions of endothelial and smooth muscle cells. As a result of the increased stimulation of the accumulated leukocytes, smooth muscle cells in the middle layer of the artery migrate to the intima (Figure 1) (23).

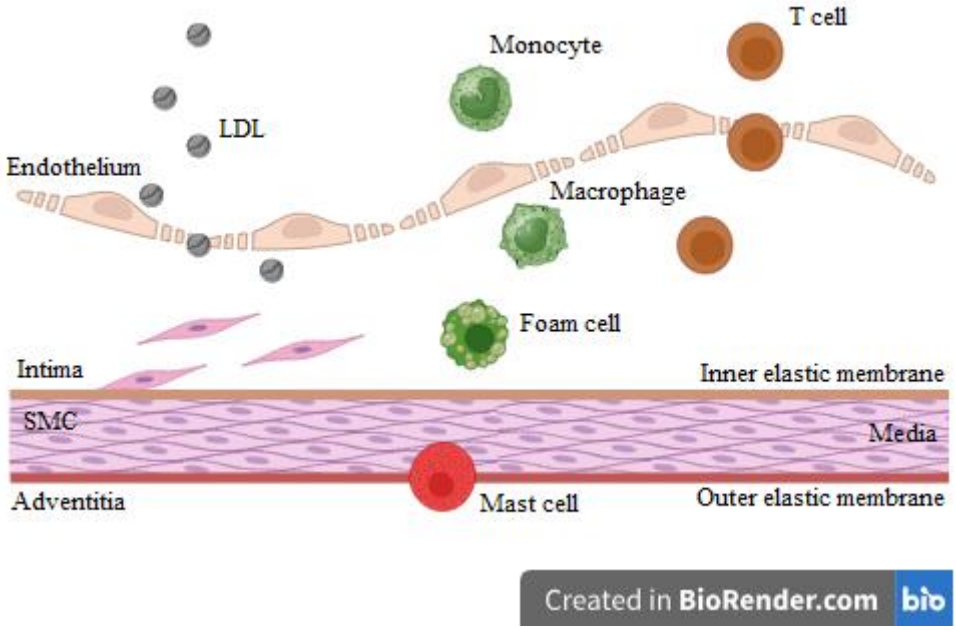

Figure 1. The stimulation of atherosclerosis created in Biorender.com

Development of atherosclerosis:

During atherosclerotic plaque formation, smooth muscle cells produce molecules such as elastin, collagen glycosaminoglycans and proteoglycans that contribute to the thickening of the intimal layer. The production of matrix metalloproteinase enzymes,

which degrade the collagen supporting the fibrous cap, is induced by activated macrophages. Smooth muscle cells and macrophages on the lesion may divide, undergo metaplasia, and undergo cell death. Wastes from dead cells accumulate and form the lipid-rich core of the atheroma (26).

Atheroma formation:

With the breakdown of the fibrous cap, blood clot formations settle in the core of the plaque. Procoagulant substances, one of the tissue factors, can cause vascular occlusion and trigger thrombosis. Induced thrombosis can increase extracellular matrix production and stimulate smooth muscle cell migration. All these processes allow the volume to increase in lesion formation and the passage of the lesion into the arterial lumen (27).

2.1.2 The factors affecting formation of atherosclerosis

The causes of atherosclerosis are still unknown. However, some behaviors and environmental factors may increase the risk of disease. If these risks continue to increase, the development of atherosclerosis may also occur. Atherosclerosis is a lipid-induced disease. For this reason, atherosclerosis is likely to occur when low-density lipoprotein (LDL) increases and high-density lipoprotein (HDL) decreases. In addition, high blood pressure of 140/90 mmHg and above is one of the other factors that may play a role in the development of atherosclerosis (28). Another factor is smoking, which can damage blood vessels and initiate atherosclerosis. Long-term smoking will make the vessel wall stiffer, leading to the accumulation of LDL there. This will lead to high blood pressure, and low oxygen transport to the body tissues. Other factors are diabetes, bad diet and obesity (29). The main cause of these factors is lack of physical activity. Low physical activity affects the increase of LDL in the blood and the increase of blood pressure, and as a result, it can cause diabetes and adiposity. Age may be another factor that can cause atherosclerosis. As we get older, plaque build-up in the arteries begins to increase. Symptoms of atherosclerosis begin

to appear at older ages. In addition to this, family history of cardiovascular diseases may also increase the risk of atherosclerosis (30), (31).

2.1.3 The role of microorganisms in the formation of atherosclerosis

Although it has traditionally been said that inflammation leading to atherosclerosis is a sterile event independent of microbial factors, recent studies have brought the possible effects of microorganisms back to the agenda. Studies have mostly suggested that *Chlamydia pneumoniae*, *Helicobacter pylori* and *Mycoplasma pneumoniae* may have a role in the development of atherosclerosis (32), (10), (33), (12). For example, in one study, the presence of *Helicobacter pylori* and *C. pneumoniae* DNA in carotid artery atherosclerotic plaques was investigated using PCR. According to the results of the study, *C. pneumoniae* DNA was detected in 16 of 52 patients and Helicobacter pylori was found in 9 of 52 patients (13).

In recent publications, studies have mostly focused on factors originating from the gastrointestinal tract flora (34). Some factors, especially found in the oral flora, have also been isolated from atherosclerotic plaque (35). With the isolation of some microorganisms in the intestinal flora from atherosclerotic plaque, the role of microbiota in the development of atherosclerosis has begun to be questioned (36), (30). Additionally, studies have emerged showing that the *Toxoplasma gondii* parasite has an effect on atherosclerosis. *T. gondii* is a type of parasite that may affect all nucleated vertebrate. When contact with soil and consumption of unwashed vegetables and fruits, raw meat, contaminated water, its infection may be exposed (37). Although there is little information about the relationship of *Toxoplasma gondii* with atherosclerosis, one study looked at the seroprevalence of anti-*Toxoplasma* antibodies. The higher levels of anti-*T. gondii* IgG was detected with 63.1% in atherosclerotic individuals and low levels were observed with 46.2% in non-atherosclerotic individuals. Although there appears to be a high prevalence of *T. gondii* infection in patients, the effect mechanisms of chronic toxoplasmosis on coronary atherosclerosis need to be elucidated (14). One of the latest studies evaluated the seroprevalence of *T. gondii* in cardiovascular patients in Romania. The seroprevalence of *T. gondii* was

significantly higher in patients with hypertension and unstable angina, and it is thought that individuals with these diagnoses may be more frequently infected with *T. gondii* (15). This and many other studies will be the basis for investigating the effects of microbial agents in patients with cardiovascular diseases.

2.1.3.1 *Toxoplasma gondii*

Toxoplasma gondii was discovered in *Ctenodactylus gundi* that is a rodent, by Nicolle and Manceaux in 1908. The first case was reported as encephalitis in a child in 1941. The definitive and intermediate hosts of *T. gondii* were described and it was proved that felines produce fecal oocysts (38) (39). The first case in Turkey was published in 1953. The first case in the world was reported in 1923 in a baby with congenital hydrocephalus and microphthalmia (40) (41). Today, *T. gondii* is well known morphologically and molecularly, and there is too much progress that has been made in its identification.

2.1.3.1.1 Taxonomy, morphology and patology

With molecular-based classification studies, the Toxoplasma lineage, which was previously in the Sarcocystidae family, was included in the Toxoplasmatidae family, along with the Besnoitia, Hammondia and Neospora lineages (42), (43). There are three infective forms of *T. gondii*. These are tachyzoite, bradyzoite in tissue cysts, and sporozoite in oocysts. While the definitive host of the parasite can be transmitted by oocysts formed by coccidian development in the intestines of felines and ingestion, it can also be transmitted by eating raw or undercooked tissue cysts formed in intermediate hosts. Apart from this, if the intermediate hosts develop acute maternal infection during pregnancy, the parasite can cross the placenta and cause congenital infection in the baby (44).

2.1.3.1.2 Diagnosis techniques and treatments developed against *T. gondii*

Since the disease is usually asymptomatic, differential diagnosis with other diseases must be made (45). ELISA is an important method in acute infection. It is generally recommended to repeat the test at 2–3-week intervals in parallel with a two-fold increase in IgG titers along with positive IgM by ELISA method (46). Also, the diagnosis is corrected by PCR in biopy and biologic liquids. The diagnosis is confirmed by PCR in biopsies or biological fluids. While direct diagnostic methods are used in the laboratory diagnosis of Toxoplasma gondii infection in patients with suppressed immune systems, indirect diagnostic methods are used in patients with intact immune systems (47).

Drugs are used to treat active infections of *T. gondii*. The use of drugs may vary depending on several factors, such as the stage of the disease, the adequacy of the immune system, and where the infection is located. Antibiotics such as Sulfadiazine, Clindamycin (Cleocin), Pyrimethamine (Daraprim), and Azithromax are usually used for infection (48) (49).

2.1.3.2 Nanobacteria

Nanobacteria are called nanoparticles responsible for calcification. Nanobacteria were first discovered by Dr. Folk in travertine hot springs and sedimentary rocks, and it was emphasized that nanobacteria play an important role in the precipitation of carbonate minerals (50). Later, it was observed for the first time in commercial cell culture serum by Kajander and his team. Nanobacteria contain hydroxy apatite crystals in their structures. For this reason, it is seen that it makes them difficult to fix, stain and break for examination, and makes them resistant to antibiotics used in cell culture (51). Although DNA has not yet been isolated, Raolt and her team were able to obtain images with DAPI staining (52). In addition, many studies have observed that they resemble root-shaped bacteria, can divide into two, can pass through filters due to their size (80-500nm), are heat-resistant and can create a specific immune response. In addition, it has been proven that the reproduction of nanobacteria can be prevented by

high doses of EDTA, gamma-radiation and aminoglycoside antibiotics (53) (54). The presence of nanobacteria has been found not only in commercial serum but also in human blood, urine, saliva, dental calculus, kidney cyst fluid, kidney stones, atheroma plaques and meteorites falling to the earth (55) (56) (57) (58).

2.1.3.2.1 The morphology of nanobacteria

Nanobacteria called “Nanobacterium sanguineum” produce a mineralized outer shell-like structure (57). Nanobacteria obtained from geological samples obtained as a result of geological studies petrify with calcium carbonate, iron sulphide, silica and complex silicates and phosphate (59). These structures are morphologically mostly spherical objects that are similar to each other and can exhibit the ability to divide themselves (60). It is considered a gram-negative bacterium because its most important feature in common with known microorganisms is that it can be stained gram negatively (61). Nanoforms have properties that differ from those of identified microorganisms. While Robert L. Folk discovered nanoforms with dimensions of 10-200 nm (62), Romanek, McKay and colleagues working at NASA discovered nanoforms with dimensions of 20 to 200 nm in the ALH84001 meteorite (63) (64). Çiftçioğlu and his team suggested that these forms may show a size difference between 80 and 500 nm. The biggest advantage of their small size is that they can pass through 0.22 mm pore size filters. This advantage provides significant selectivity compared to other bacteria in nanobacteria research (65). It has the ability to change its shape under different culture conditions and has a slow growth characteristic. It is structurally related to carbonate hydroxyapatite (57) (66) (61).

2.1.3.2.2 Methods of growth, imaging and analysis of nanobacteria

For the best growth environment of nanobacteria, 37 °C, 5%–10% CO2, 90%–95% humidity conditions should be provided (61). Under these conditions, the time for division into two is between 1 and 5 days, usually 3 days (67). Nanobacteria are difficult to detect using microbiological methods based on metabolic tests because their growth rate is 10,000 times slower than other bacterial groups such as *Escherichia*

coli (*E coli*) (62). They found that nanobacteria in the culture medium largely used amino acids such as glutamine, asparagine and arginine, but used glucose less. In addition, it was observed that most types of antibiotics could not prevent their growth, but antibiotics such as streptomycin, kanamycin and gentamicin inhibited their growth at high concentrations. Another factor is temperature. It was observed that subcultured nanobacteria in serum-free culture medium were inhibited when exposed to half an hour of heating at 100°C. However, nanobacteria with thick apatite coatings were observed to be more resistant to heat than those with thinner coatings. These forms were not killed even when exposed to one hour of heating at 100°C (51). Nanobacteria can grow on media resistant to agents such as ultraviolet irradiation and heat used in cell culture studies instead of standard microbiological media (55). It can be cultured from tissue, blood and urine. There are some methods for detecting cultured nanobacteria. These are immunological detection with nanobacteria-specific monoclonal antibodies, fluorescent staining techniques and Scanning electron microscope (SEM) and Transmission electron microscope (TEM) (60). Some other methods by which the growth of nanobacteria can be observed are turbidity measurement, enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining method and protein immunoblot techniques (57).

2.1.3.2.3 The pathogenesis of nanobacteria

It is known from the experiment of Kajander and his team that nanobacteria cause cell culture contamination (57). However, there are other cases where it creates a pathological condition. These can cause specific infections by multiplying themselves in certain parts of the human body and causing calcification (68) (69). One of the places where this situation develops is dental plaque, where tartar formation occurs. When a sufficiently alkaline environment is created and the pH increases, the calcium phosphate in the saliva loses its stability and calcium phosphate crystals precipitate on the plaque and mineralization begins (70). The process here is very similar to the process that nanobacteria play in calcification. Another place where nanobacteria can cause calcification is the vascular region. Although the mechanism of vascular

calcification by nanobacteria has not yet been resolved, it is still thought to be the center of calcification at a physiological level in the presence of calcium and phosphate (71). Miller and colleagues cultured calcified femoral arteries, carotid plaques, and aneurysms surgically removed from individuals with bicuspid aortic valves and obtained nanobacteria-like structures. However, they were unable to obtain these structures from noncalcified samples (72). Puskas and his team, who cultured aortic and carotid atherosclerotic plaques obtained from autopsy and performed culture analyses using light microscopy and TEM, detected nanobacteria-like structures according to the analysis results (73). Nanobacteria have also been detected in human kidney stones and have been shown to form apatite and have been defined as root-shaped nano-sized structures with very different cell walls and capsules. Their presence has been detected in urine samples, liver and kidney cystic fluids, and chronic kidney stones by increasing biomineralization (74) (56). Tsurumoto and colleagues investigated nanobacteria in synovial fluids obtained from patients with osteoarthritis and rheumatoid arthritis. They suggested that nanobacteria may be associated with these diseases (75). A similar situation occurred in the study of Eskandary et al. They emphasized that nanobacteria may be associated with intervertebral disc degeneration (76). The presence of nanobacteria was also investigated in HIV-positive individuals. The results showed that these individuals had high levels of nanobacterial infection (77). They suggested that nanobacteria could be transmitted from infected mothers to infants transplacentally or perinatally (78). Although the relationship between nanobacteria, which cause calcification to a significant extent, and many diseases is strengthened with data, it seems not yet possible to make a definitive statement on the diseases.

2.1.3.2.4 Treatments developed for nanobacteria

Nanobacteria can grow and colonize even in conditions of inadequate nutrients. Nanobacteria can resist many factors such as temperature, extreme pH conditions and gamma radiation (15 kGy) and can survive in conditions where most other bacteria cannot survive and can be found in many places due to their size. They can also easily hide from the human immune system (61). For this reason, these features will play a

decisive role in the treatment methods or therapies developed against them. The role of tetracycline in the treatment of nanobacteria, which are frequently encountered in periodontal diseases, has been particularly examined. It is known that tetracycline inhibits metalloproteinases and protein synthesis in bacteria and chelate calcium (79). In the study of Çiftçioğlu and his colleagues, the effect of tetracycline on nanobacteria was examined considering its antibacterial properties. It was argued that tetracycline played an inhibitory role in the proliferation of nanobacteria in human renal cyst fluids and kidney stones treated with tetracycline. In addition, antibacterial drugs other than ampicillin such as trimethoprim, nitrofurantoin and trimethoprim/sulfamethoxazole have lethal effects on nanobacteria (72). In addition to antibiotics, comET ((Nanobak LifeSciences, Tampa, Florida), developed against nanobacteria, is used for the treatment of chronic prostatitis/chronic pelvic pain (80). It contains tetracycline and ethylenediamine-tetraacetic acid (EDTA). comET treatment has been applied to people with coronary artery disease and positive nanobacteria serology and antigen. According to the results, it was found that coronary artery calcium scores and calcified coronary artery plaque volume decreased and regressed in most patients (81). However, despite various studies on nanobacteria, more clinical studies are needed.

3 MATERIALS AND METHODS

3.1. Sample Collection and Study Design

This study included 20 samples (labelled as A1–A20) of atheroma plaques from patients who underwent atherectomy by surgery or percutaneous intervention. All the plaques included in this study were obtained from the carotid artery of patients. Risk factors for atherosclerosis including gender, smoking status, hypertension, coronary artery disease, diabetes mellitus and dyslipidemia were recorded for each patient in this study (Table 1). The samples with a minimum size of 3 mm obtained from each patient were distributed into four tubes. The first tube contained the DNA/RNA shield solution (Zymo Research, USA), the second contained thioglycollate broth together with 1.0-mm-sized zirconium microbeads, the third contained only thioglycollate broth, and the fourth tube was empty. The tubes were transferred to the laboratory at room temperature. Ethical approval for the study was received from University ethics committee. All patients signed an informed consent form to permit using the biological samples and information related to this study.

Table 1. Clinical characteristics of patients with respect to *T. gondii* DNA positivity

	Patients that were *T. gondii* PCR result on atheroma plaques:	
Parameters	Positive (n=8)	Negative (n=12)
Mean Age	69.25	73.0
Mean Age + Std Dev.	69.25 ±3.97	73.0 ±4.95
Sex		
Female	2 (25%)	2 (12%)
Male	6 (75%)	10 (83%)
Smoking Habit	5 (62,5%)	4 (33%)
Hypertension	6 (75%)	7 (58%)
Coronary Artery Disease	3 (37,5%)	8 (66%)
Diabetes Mellitus	5 (62,5%)	7 (58%)
Dyslipidemia	4 (50%)	7 (58%)

3.2. Sample Preparation and Culture Studies

3.2.1. Culture for aerobic and anaerobic microorganisms in atheroma plaques

The atheroma plaque collected in the thioglycollate medium was incubated at 37ºC. Samples were incubated for two weeks until turbidity, indicating growth, was observed. If turbidity was not observed, aliquots of the medium were inoculated in two sheep blood agar (SBA) plates, one to eosin methylene blue agar (EMB), Sabouraud dextrose agar (SDA), Löwenstein-Jensen agar, and TK SLC rapid mycobacterial culture medium (TiBO, Turkiye). One SBA plate was incubated under aerobic and the other under anaerobic conditions. All plates were incubated at 37ºC for one week except SBA which was incubated for two weeks. If growth occurred, smears were prepared from the colonies, Gram-stained, and observed by microscopy. The species of the isolated organisms were determined using MALDI-TOF-MS (Bruker, USA).

3.2.2. Culture for nanobacteria

The tubes that contain atheroma plaque and micro-beads with thioglycolate broth, were placed in the bead homogenizer (Mini-Beadbeater-16) and operated for 1 minute. 400 µl of sterile water was added and mixed by pipetting, samples were transferred into new tubes. The tubes were centrifuged at 5000xg for 2 minutes. The supernatants were filtered through sterile 0.22 µm pore-sized filters. They were inoculated into serum and albumin free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) under mammalian cell culture conditions (37°C; 5% CO_2/90–95% air) and were incubated for 4-8 weeks. Control group contained only DMEM.

3.3. Nucleic Acid Isolation Attempt from Nanobacteria

To determine the presence of nucleic acids in culture-grown nanobacteria, nucleic acid isolation procedure was applied using GeneDireX Genomic DNA Isolation Kit (Vetro Scientifica, Italy). The presence of nucleic acid was investigated by measuring

the UV absorbance of the eluents by spectroscopy (using NanoDrop, Thermo Scientific, USA).

3.4. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) Microanalysis for Nanobacteria

The cultured atheroma plaques were scraped from the flasks, and they were transferred onto a piece of petri dish. (Glass slides were not used since they show silicium which does not belong to the sample, but it is the main component of glass.) After drying the samples, they were analysed both for morphology and chemical composition using Quattro S SEM (Thermo Scientific, USA) with an attached EDAX EDS analyser (AMETEK, USA). In addition, the results of the elemental analysis of *E coli* bacteria and the results of the cultured nanobacteria were compared (Table 2).

3.5. SDS-PAGE and Silver Staining Analysis for Nanobacteria

Sediments from 20 culture flasks inoculated by atheroma plaque homogenates were scraped and suspensions were transferred into microcentrifuge tubes. The samples were mixed with 4x protein loading buffer (0,5 M pH 6.8 Tris-HCI, 0.005% bromophenol blue, 20% SDS, 99% glycerol, 355mM of 2-mercaptoethanol, 1M NaOH) and heated at 95ºC for 3 min. The molecules were separated on 10% polyacrylamide gels using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained by Silver Stain kit (Biorad, USA). ChemiDoc imaging system (Biorad, USA) was used for the imaging polyacrylamide gel.

3.6. Sensibility Analysis to Chemical Agents for Nanobacteria

Nanobacteria grown in culture, was treated with 0,5 mg/ml, 1 mg/ml DNAse I (GeneMark, Chine), 50 μg/ml, 100 μg/ml RNAse A (Bioshop, Canada) and 50 μg/ml, 100 μg/ml Proteinase K (Zymo Research, USA) in separate tubes and they were incubated at 37°C in a heating block, for 10 min, 30 min, and 1 h respectively. The enzymes were inactivated by heating 5 min at 75°C, 30 min at 60°C and 15 min at

65°C, respectively. Then the samples were separated by 10% SDS-PAGE and they were analysed after silver staining.

3.7. Metabolite Profiling of the Cultured Atheroma Plaque for Nanobacteria by LC-QTOF-MS

Qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) was applied to identify the extracts from nanobacteria.

3.8. Whole Genome Sequencing Analysis for Atheroma Plaques

For isolating genetic material, a 5-mm tissue slice was placed in 2 mL of DNA/RNA shield reagent and was ground with the help of a pestle and mortar. The suspension was collected into a 2 mL centrifuge tube and homogenized using a syringe. DNA was isolated from each sample using a DNA purification kit (GeneMark, China). DNA from samples A1, A2, A3, A6, A8, and A9; and from samples A7, A11, A12, A14, A15, A18, and A19 were pooled into two tubes as Group 1 (G1) and Group 2 (G2). Whole genome sequencing of these two samples was performed (Eurofins Genomics, Germany). Raw sequencing data is processed using fastp (version 0.20.0) software to remove poor quality bases (below Phred Quality 20 were removed) using the sliding window approach. Adapters and reads shorter than 30bp were also removed by using the same software. MetaPhlAn (version 3.0.7) which relies on unique clade-specific marker genes identified from ~17,000 reference genomes (~13,500 bacterial and archaeal, ~3,500 viral, and ~110 eukaryotic) were used for the taxonomic profiling. Unclassified reads were subjected to KrakenUniq (version 0.5.8). Kraken classifies reads by breaking each read into overlapping k-mers. Each k-mer is mapped to the lowest common ancestor (LCA) of the genomes containing that k-mer in a precomputed reference database. For each read, a classification tree is found by pruning the taxonomy and only retaining taxa (including ancestors) associated with k-mers in that read. Each node is weighted by the number of k-mers mapped to the node, and the path from root to leaf with the highest sum of weights is used to classify the read. KrakenUniq computes the number of unique k-mers observed for each taxon, which allows it to filter more false positives. Read counts of input samples observed

at various taxa levels (Phylum, Genus, and Species) are collected and normalized by using the rarefy function implemented in the vegan bioconductor package (version 2.5.7) to compare species abundances. Rarefied read counts enable better comparisons of operational taxonomic unit (OTU) profiles between samples with different sample sizes. Abundance measured by the percentage of OTU assigned reads from various taxonomic levels is determined. As metagenomic analysis indicated the presence of *T. gondii* DNA, further evidence was obtained by nested PCR specific to this organism.

3.9. Nested PCR for Detecting *T. gondii* in Atheroma Plaque Samples

Based on human whole genome sequencing, a high proportion of *T. gondii* DNA sequences were identified in the G1 and G2 sample pools. To obtain direct evidence for the presence of these organisms, two specific primers, 5`-CGCTGCAGGGAGGAAGACGAAAGTTG-3` as forward and 5`-CGCTGCAGACACAGTGCATCTGGATT-3,` as reverse primers, were used to amplify DNA from the individual samples in the first round of PCR. For nested PCR, 5`-GTGCTTGGAGCCACAGAAGG-3' as forward and 5`-CTCCTCCTCCCTTCGTCCAA-3` as reverse primers were designed and used to amplify a 323-bp product. A ready-to-use PCR Master Mix II Kit (GeneMark, China) was used. The program was initial denaturation at 95 °C for 1 min; 45 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s; 72 °C for 2 min as final extension. PCR products were analyzed on 1.2% agarose gel. *T. gondii* DNA grown in mouse peritoneum was used as a positive control, and sterile deionized water was used as a negative control.

3.10 Statistical Analysis

The study data were statistically analyzed using R programming language (version 4.3.3.) was used for all statistical analysis. Fisher’s exact test was performed to observe a significance between metadata variables and *T. gondii*.

4 RESULTS

4.1 Microorganism Culture Analysis

Among all cultured samples, growth of specimen A14 was observed in only one SBA plate under anaerobic conditions. Gram staining of the isolate showed Gram-negative coccobacilli (Figure 2) and was identified as *Cutibacterium acnes* by MALDI-TOF analysis.

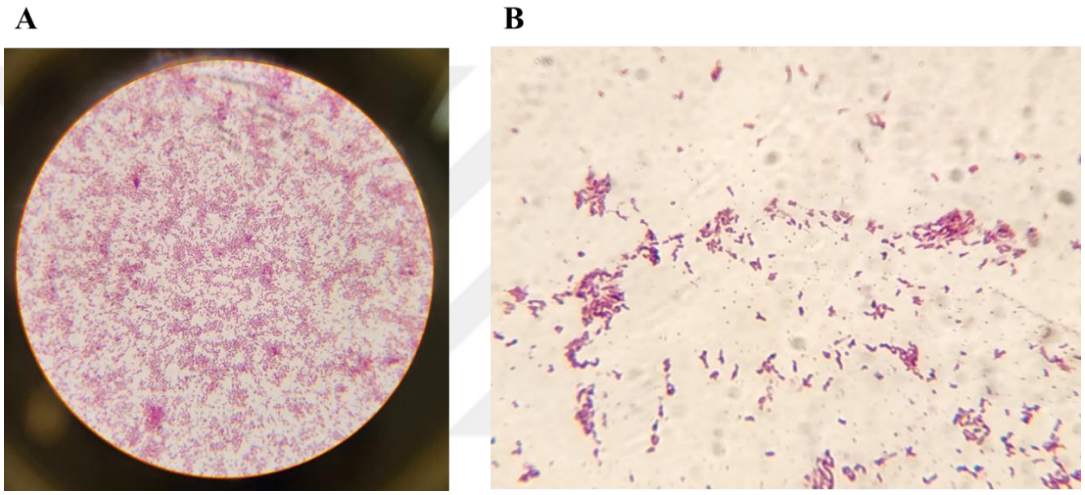

Figure 2. Light microscope images of A14 sample which inoculated on blood agar in anaerobic condition. (Figure 1A. *Cutibacterium acnes* magnified by 4x objection; Figure 1B. *Cutibacterium acnes* magnified by 100x objection)

4.2 Nucleic Acid Content Analysis for Nanobacteria

The spectrophotometric absorbance measurement at 260nm wavelength of the samples indicated absence of nucleic acids.

4.3 SEM and EDS Analysis

4.3.1 Evidence for the presence of nanobacteria in cultured atheroma plaques

In cell culture, approximately 2 months after culturing all patient samples with DMEM, spherical structures with sizes ranging from 80 to 900 nm began to be

observed (Figure 3). After about 3 to 3.5 months, it was observed that these structures merged to form larger structures (Figure 3C). In addition, elemental analyses (C, N, O, Mg, As, P, S, Ca, Si, AI) of the samples were made (Figure 4). Compared to other elements, a high percentage of carbon (C: 45.5%), nitrogen (N: 14.3%) and oxygen (O: 38.4%) was detected. In addition, the results of the elemental analysis of *E coli* bacteria and the results of the cultured A19 patient sample were compared (Table 1). The amount of carbon in the A19 patient sample was approximately 1.5 times higher than in the *E coli* bacteria. Nitrogen was about 2.5 times less and oxygen was 1.2 times more.

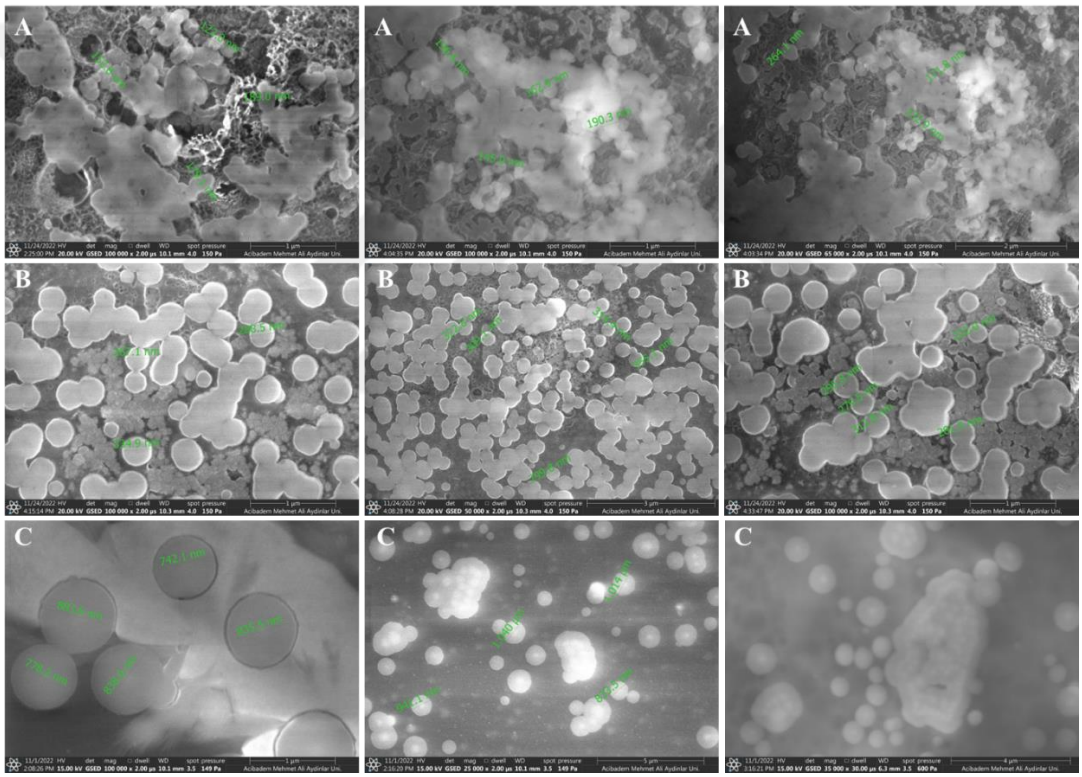

Figure 3. SEM images of the cultured atheroma plaque samples A) A12; B) A18; C) A2. Bars: A12: 1;1;2 µm; A18: 1;3;1µm; A2: 1;5;4 µm.

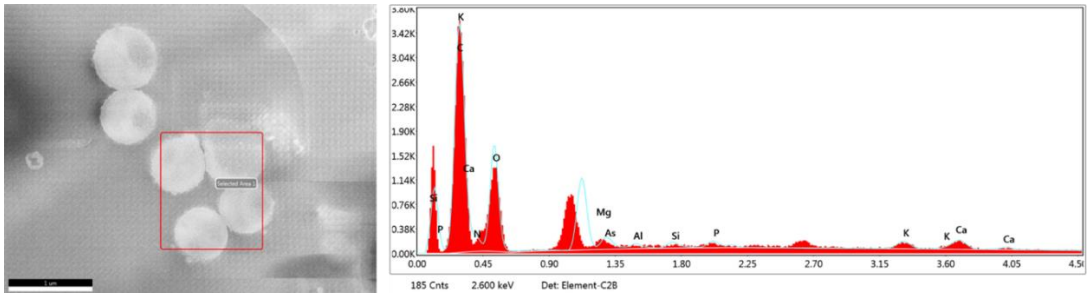

Figure 4. EDS analysis of A19 sample

Table 2. EDS analysis for sample A19 and E coli

Samples	C	N	O	Mg	As	P	S	Ca	Si	AI
Sample A19	45.5	14.3	38.4	0	0.2	0.3	0.4	0.5	0.2	0.2
E coli	25.8	34.6	30.5	0	0.5	4.6	0.7	0.3	0.2	0.2

4.3.2 Evidence for the presence of albumin in nanobacteria

Based on the table shown in Table 2, we predicted that nanobacteria are formed of human proteins, mostly albumin. To test this hypothesis, we incubated bovine serum albumin (BSA) with DMEM medium free of FBS. The same spherical structures as in atheroma plaque samples were observed by SEM. Also, elemental analysis showed similar proportions for carbon, nitrogen, oxygen and the other atoms. Figure 6 shows the difference between the analysis of nanobacteria from atheroma plaque and bovine serum albumin.

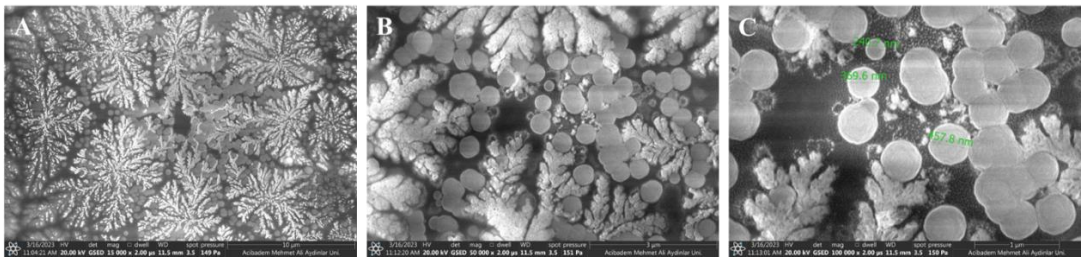

Figure 5. SEM images of albumin with DMEM medium Bars: A.10 µm; B.3 µm; C.1 µm.

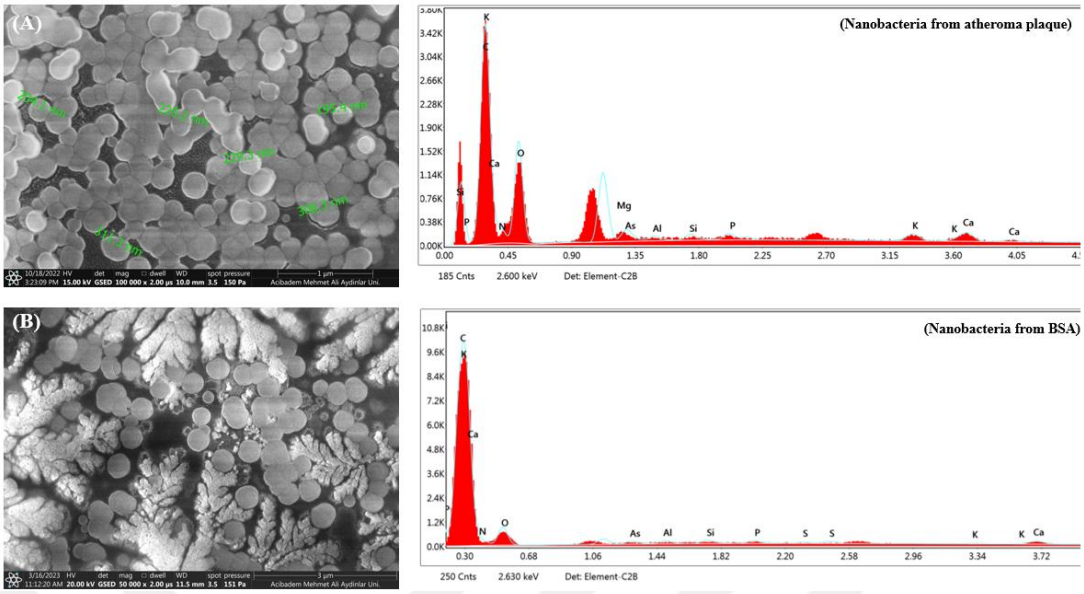

Figure 6. (A) SEM images and EDS analysis of sediment from a culture flask showing nanobacteria-like structures. (B) SEM images and EDS analysis of incubated BSA show the same structures. Bars: A: 1 μm; B: 3 μm.

4.4 Protein Analysis for Nanobacteria by SDS PAGE and Silver Staining

The analysis of homogenates of cultured nanobacteria from 20 patient samples by silver-stained macromolecules, separated by SDS PAGE, revealed molecules ranging 20 to 250 kDa (Figure 7). Molecular patterns of all samples were similar. Four bands with highest intensity belonging to molecules with approximately molecular weights of 75, 65, 50 and 25 kDa, were observed.

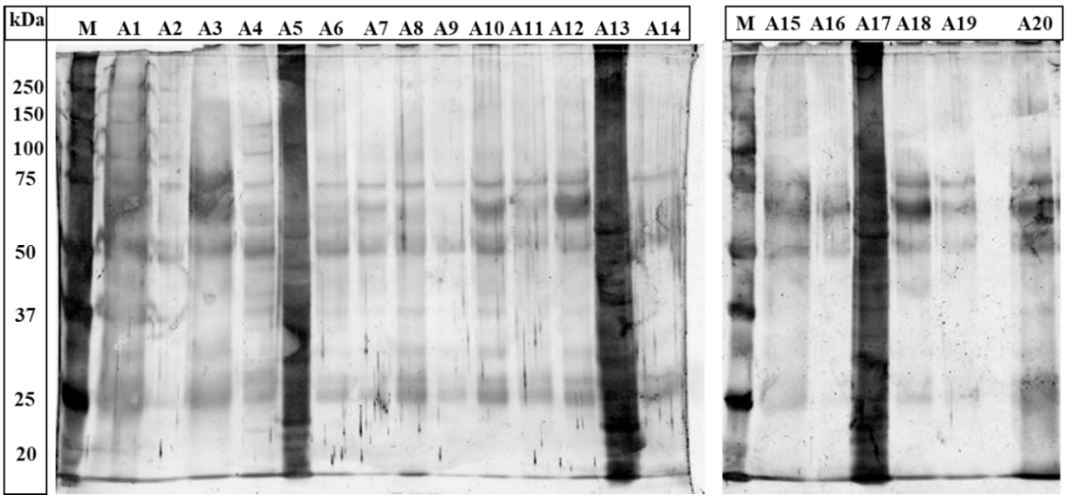

Figure 7. Silver stained 12% SDS PAGE of the cultured atheroma plaque, A1 to A20. M: Protein marker.

4.4.1 Protease and nuclease susceptibility of nanobacterial molecules

Treatment of nanobacterial molecules by proteinase K digested completely all molecules indicating that these are proteins. Treatment with DNAse or RNAse did not affect any of the molecules (Figure 8).

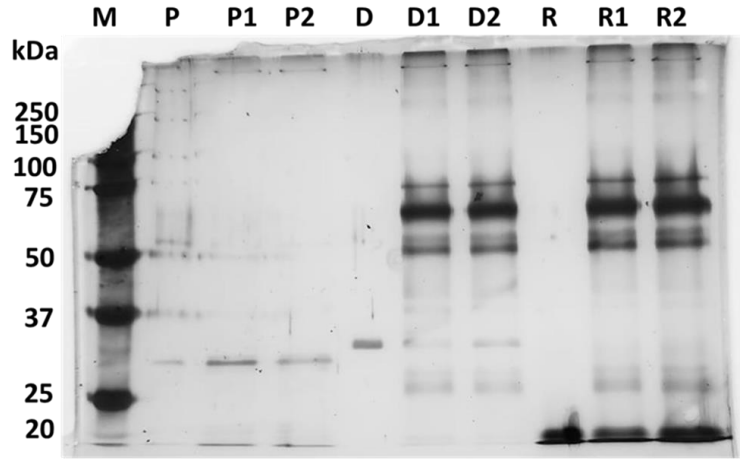

Figure 8. The effect of proteinase K, DNAse and RNAse to the molecules of nanobacteria. M: protein marker, P: Proteinase K, P1: A12 sample with 50 μg Proteinase K, P2: A12 sample with 100 μg Proteinase K, D: DNAse I, D1: A12 sample with 0.5 mg DNAse I, D2: A12 sample with 1 mg DNAse I, R: RNAse, R1: A12 sample with 50 μg RNAse, R2: A12 sample with 100 μg RNAse

4.4.2 The analysis of cultured albumin in SDS-PAGE

Due to the high detection of albumin, albumin was added to the DMEM media used as a negative control and left to culture for 1 month. When we analyzed BSA used in this experiment by SDS PAGE we observed that BSA was not pure but contained several other proteins (Figure 9).

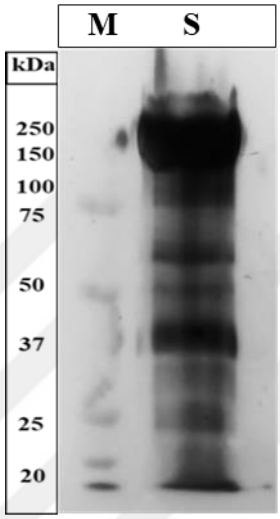

Figure 9. Silver stained SDS PAGE of incubated albumin in DMEM medium. (Lane M: protein marker, Lane S: Sample incubated albumin)

4.5 The Results of Metabolic Profiling of Nanobacteria

The data processing detected the presence of 21 human proteins (forming at least 2% of the whole protein content) in the extract from nanobacteria cultured from atheroma plaque. This data article contains the unique peptide values and corresponding database search hit identities of the 21 compounds and the comprehensive list of m/z values detected during the LC-QTOF-MS analysis (Table 3).

Table 3. The list of proteins identified from nanobacteria

Percentage in total proteins	Mass (kDa)	Description
14	71	ALBU_HUMAN Albumin
7	66	K2C1_HUMAN Keratin_ type II cytoskeletal 1
6	46	A1AT_HUMAN Alpha-1-antitrypsin
5	52	6-aminohexanoate-cyclic-dimer hydrolase
5	59	K1C10_HUMAN Keratin_ type I cytoskeletal 10
4	15	Hemoglobin alpha chain
4	15	Hemoglobin beta chain
4	14	FABPL_HUMAN Fatty acid-binding protein_ liver
3	45	HPT_HUMAN Haptoglobin
3	38	IGHA1_HUMAN Immunoglobulin heavy constant alpha 1
3	79	TRFE_HUMAN Serotransferrin
3	13	S10A9_HUMAN Protein S100-A9
2	13	H2B1K_HUMAN Histone H2B type 1-K
2	16	CALM1_HUMAN Calmodulin-1
2	28	CAH1_HUMAN Carbonic anhydrase 1
2	11	IGKC_HUMAN Immunoglobulin kappa constant
2	36	IGHG1_HUMAN Immunoglobulin heavy constant gamma 1
2	30	APOA1_HUMAN Apolipoprotein A-I
2	71	Serum albumin precursor (Allergen Bos d 6).
2	70	Heat shock cognate 70 kDa protein (HSP70).
2	65	K22E_HUMAN Keratin type II cytoskeletal 2 epidermal

The sample (A2) was run at SDS-PAGE and the gel was stained by silver technique. The resulting bands were compared by LC-QTOF MS data analysis (Figure 10). According to results, metabolites detected in LC-QTOF MS have molecular weights (70, 55, 42, 30 and 29 kDa) corresponding to the bands in SDS PAGE. The SDS PAGE gel was analysed by Gel Analyzer Program.

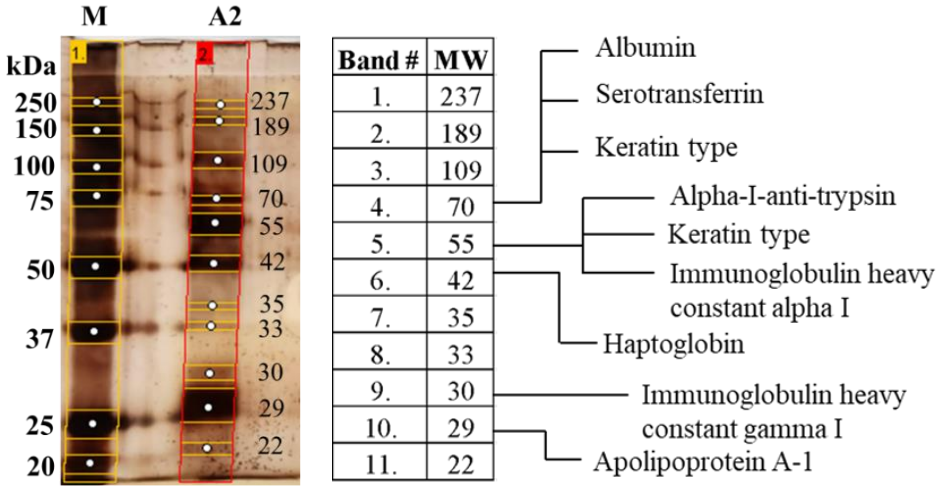

Figure 10. SDS-PAGE separated proteins obtained from sample A2. The molecular weights of a few corresponding to human proteins determined by LC-QTOF-MS are indicated next to the electrophoresis gel. (Lane M: protein marker, A2: nanobacterial proteins cultured from atheroma plaque, MW: molecular weight)

4.6 The Result of Whole Genome Sequencing Analysis

G1 and G2 were subjected to whole genome sequencing. Sequences belonging to the human genome were eliminated, and those belonging to the metagenome were revealed. In the analysis run, the heatmap and bar graphs representing taxonomic abundance at the species level were formed to compare species richness from all samples (Figure 11). The results showed that more than 50% of sequences belonged to *T. gondii.*

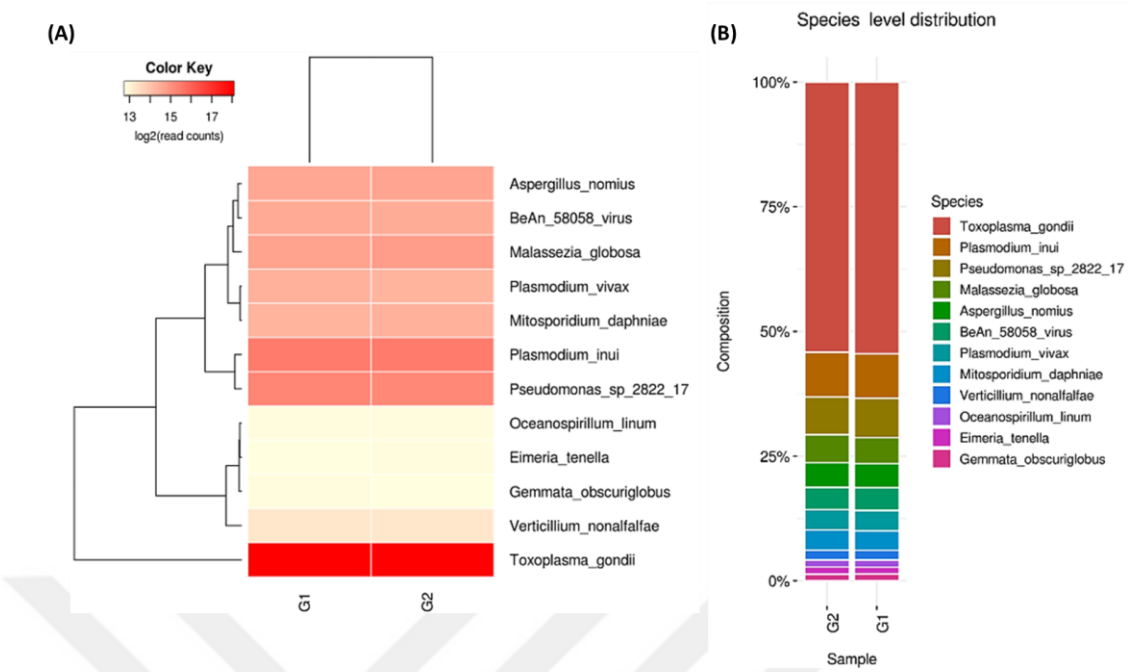

Figure 11. (A) The various taxonomic levels of group 1 and group 2. (B) Bar plot(s) showing the taxonomic abundance corresponding to G1 and G2.

4.7 PCR-based Detection of *T. gondii* in Patients

As metagenome analysis of pooled samples indicated the presence of *T. gondii*, to obtain more concrete direct evidence, the presence of the DNA of this parasite in individual atheroma plaque samples was investigated by nucleic acid amplification using specific primers for *T. gondii* by PCR. A 323-bp product belonging to *T. gondii* DNA was obtained from eight samples: A3, A4, A12, A14, A15, A17, A18, and A19 among the 20 atheroma plaques by nested PCR (Figure 12). In conclusion, *T. gondii* DNA was positive in 8 (40%) of 20 patients.

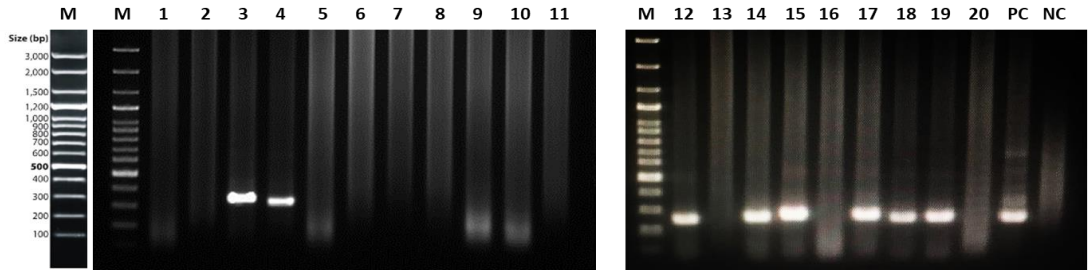

Figure 12. 1.2% agarose gel showing PCR amplification products of *T. gondii* DNA from the atheroma plaque samples, A1–A20. Lane M: DNA marker, PC: positive control (*T. gondii* DNA), NC: negative control (deionized water).

5 DISCUSSION

Cardiovascular pathologies are responsible for several clinical conditions that lead to death due to occlusion of the arteries of vital organs (1), (82). Microorganisms are among the factors that are suspected to play a role in atheroma plaque formations. Although several microorganisms like *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* were claimed to cause atheroma plaque formation, further studies are required to make a strong relation between these organisms and plaque formation.

This study aimed to investigate the possible role of any type of microorganisms in atheroma plaque formation by metagenomic analyses of whole DNA obtained from these plaques. For this purpose, plaque samples from the carotid arteries of 20 patients were inoculated into various culture media and incubated under aerobic and anaerobic conditions to grow any culturable microorganisms. Growth was observed in only one sample identified as *Cutibacterium acnes*, a Gram-positive anaerobic organism. *C. acnes* is an opportunistic pathogen that can infect the damaged endothelium of blood vessels and endocardium. Additionally, *C. acnes* is the second most common pathogen after coagulase-negative Staphylococci isolated from infected internal cerebral ventricular bypasses, which indicates frequent bacteraemia of this organism (83), (84). There is an ongoing debate regarding whether the isolation of *C. acnes* strains from tissues other than the skin is an actual infection or contamination. In this study, *C. acnes* was identified in only one of the 20 patients, and metagenomic analysis indicated no DNA sequences belonging to this organism in atheroma plaque, making it unlikely to be responsible for atheroma plaque formation. From all of the atheroma plaque samples, it was possible to grow nanobacteria. Nanobacteria, which are formations smaller than bacteria, have been the subject of many discussions regarding whether they are living organisms or not, and have also been proposed as candidate agents for atheroma plaque formation (16), (17), (18). It is suggested that nanobacteria may be responsible for pathological calcifications in kidney and dental pulp stones, as well as heart valve calcifications and arterial calcifications (85). However, other studies have questioned the microbiological origin of these particles, suggesting that nanobacteria

are formed by the biomineralization of natural molecules. To investigate if nanobacteria may cause the formation of atheroma plaques, the plaques were cultured in DMEM, where nanobacteria can grow. Growth was observed in all samples within a 1–2-month period, as described in the literature (86). SEM analysis of these formations showed nanobacteria-like structures ranging in size from 80 to 900 nm (Fig. 1), as defined previously (87). The susceptibility of the molecules from these formations to Proteinase K and resistance to DNAse I and RNAse indicated their proteinaceous nature, identified as human proteins by LC-QTOF-MS. The samples were scanned in human, bacterial, and viral databases, and only human proteins were identified. A high albumin level was detected based on the same band patterns and MW as albumin detected using SDS-PAGE (Fig. 2). To test the hypothesis that nanobacteria are made of human proteins, mostly albumin, we incubated BSA with DMEM medium and obtained similar nanobacterial structures. When we analyzed the BSA used in this experiment it turned out to contain several other proteins which also may play a role in the formation of nanobacteria. Accumulation of nanobacteria formation may contribute to atheroma plaque formation, however, it may be proposed that nanobacteria are not the etiological factors that start the plaque formation.

Whole genome sequencing and metagenomic analysis of the pooled plaque samples indicated the presence of *T. gondii* DNA as a large proportion of all DNA. No DNA sequence belonging to *Helicobacter pylori*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* was identified. To have more concrete direct evidence for the presence of *T. gondii*, we did further analysis of individual samples by nested PCR and identified *T. gondii* DNA in 8 out of 20 atheroma plaques. Therefore, we concluded *T. gondii* may be an important etiological factor leading to atheroma plaque formation.

As a limitation of the study, it was not possible to include vascular samples without atheroma plaque formations as negative controls. Although atheroma plaque formation mechanism is not expected to be different in other types of arteries, it will be good to include atheroma plaques from other arteries especially from coronary and cerebral regions, in further studies.

In one study, the seroprevalence of anti-*T. gondii* immunoglobulin G (IgG) antibodies were investigated in atherosclerotic patients by ELISA and showed that anti-*T. gondii* IgG was present in; 63·1% of patients with atherosclerosis compared to 46.2% of non-atherosclerotic individuals [14], (15). We have determined that gender, hypertension, coronary artery disease, diabetes mellitus and dyslipidemia categorical variables had no relationship with *T. gondii* presence (p-value > 0.05). Additionally, the drugs, antiaggregants, statins and beta-blockers that were used by most of the patients did not affect the presence of *T. gondii* in atheroma plaques. The mean age of patients who needed carotid atheroma plaque removal was 71.5 which indicates the chronic nature of the pathology. Although it is hard to conclude because of the low number of patients included in this study, among smokers we identified *T. gondii* in 5 out of 8 patients (62.5%), however in 4 out of 12 patients (33%) in non-smokers. Smoking is known to be a key factor for speeding the development of atherosclerosis which may make vascular endothelium more susceptible to *T. gondii* infection.

T. gondii occurs worldwide. It is a very invasive parasite and can infect cells within seconds when it comes into contact. Once it infects people it can stay lifelong in the body. It is the second most frequent foodborne infection in the United States (88). It causes congenital infections which lead to serious malformations and often death of the fetus. It can cause encephalitis, myocarditis and pneumonitis in immunocompromised patients. It causes asymptomatic chronic infection in healthy people (89), (90). Its probable role in vascular infections and atheroma plaque formation has not been investigated so far. This is the first study with direct evidence of the presence of *T. gondii* in atheroma plaques.

If further evidence is obtained about the role of *T. gondii* in atheroma plaque formation this may change the current classical prevention and treatment strategies of atherosclerosis complications. The presence of *T. gondii* infection in patients with atheroma plaques may be detected by PCR from blood samples if free parasites or their DNA may be found in blood. Although the probability of finding *T. gondii* in blood samples can be expected to be low it may be worth it to do a study to determine the presence of *T. gondii* in patients with atheroma plaques. It is more probable to detect

T. gondii DNA in atheroma plaques. However, it requires an invasive procedure to obtain a biopsy sample from atheroma plaques which cannot be performed because of the high risk of complications. Therefore, further evidence for the relation between *T. gondii* and atheroma plaque formations can be obtained by similar studies on extracted atheroma plaques or autopsy samples. If strong evidence for the relation between *T. gondii* and atheroma plaque formation is identified, this may change the strategies for prevention and treatment of arteriosclerosis.

Prevention measures against *T. gondii* for seronegative pregnant women and patients with weak immune systems are well-established. Since atherosclerosis is a worldwide common health problem it may be important to use all these prevention measures for all communities. The cysts in the meat can be killed by heating the meat to at least over 67°C. Hands, all meat-cutting tools, boards and other materials should be washed with soap and water before and after handling meat. Cats are the main reservoir of *T. gondii* in nature (88), (89). It is important to pay attention to hygiene when cleaning cats' faeces sandbox for the people who have cats at home. When gardening, gloves should definitely be worn. Raw eggs and unpasteurized milk should not be consumed, and vegetables and fruits should be washed thoroughly before eating (91). *T. gondii* infections can be treated with medication using drugs for which their safety is already established. Patients are usually given medications such as pyrimethamine for treatment. In addition, antibiotics such as sulfadiazine, clindamycin, and azithromycin can be used to treat the infection.

In this study among 20 atheroma samples, we detected *T. gondii* DNA in 8 samples. The detection of DNA is strong evidence for infection of this parasite in vascular tissue. Therefore, it may play an important role in the formation of atheroma plaques and vascular occlusion. It is not possible to predict if *T. gondii* invades vascular tissue before or after atheroma plaque formation. Even if it is not the primary etiological cause of plaque formation it may play an important role in creating inflammation and growth of the plaque. If further evidence can be obtained by further studies including a larger number of patients, prevention or treatment of atheroma plaque formation may become possible. Especially in patients diagnosed with

atheroma plaques and vascular occlusion, treatment of *T. gondii* infection may prevent progression of the disease and even ameliorate blood flow by subsiding the inflammation and downsizing of atheroma plaques.

6 CONCLUSION

This study could not detect Gram-positive, Gram-negative, aerobic, and anaerobic bacteria that may cause atherosclerosis. While investigating the effect of nanobacteria-like structures on atherosclerosis, albumin precipitates were detected in all patient samples. The DNA of the parasite, *T. gondii*, was detected at a high rate by sequencing the patient samples. PCR detected *T. gondii* DNA in 8 of 20 patients. This study suggests that *T. gondii* may be one of the causes of atherosclerosis. Further studies confirming the role of *T. gondii* in atheroma plaque formation will be beneficial. Therefore, the next step of the study will be to increase the sample size and detect *Toxoplasma gondii* DNA not only in atheroma plaques but also in blood. In addition, it is thought that *T. gondii* will be detected when samples obtained from atherosclerotic patients are used in mice where *Toxoplasma gondii* can be grown in the mouse peritoneum. As a result, it will be a step towards proving the existence of the *T. gondii* parasite that can cause plaque formation. In future studies, strategies to prevent and treat *T. gondii* infections may be necessary to prevent atheromatous plaque formation. Strategies to prevent and treat *T. gondii* infections may be necessary to prevent atheroma plaque formation.

7 REFERENCES

1. Tsao CW, Aday AW, Almarzooq ZI, Alonso A, Beaton AZ, Bittencourt MS, et al. Heart Disease and Stroke Statistics-2022 Update: A Report from the American Heart Association. Vol. 145, Circulation. 2022.

2. Roth GA, Mensah GA, Johnson CO, Addolorato G, Ammirati E, Baddour LM, et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019. J Am Coll Cardiol. 2020;76(25).

3. Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R, Flores-Ramírez R. Periodontal Disease, Systemic Inflammation and the Risk of Cardiovascular Disease. Vol. 27, Heart Lung and Circulation. 2018.

4. Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. Atherosclerosis: Process, indicators, risk factors and new hopes. Vol. 5, International Journal of Preventive Medicine. 2014.

5. Stoll G, Bendszus M. Inflammation and atherosclerosis: Novel insights into plaque formation and destabilization. Vol. 37, Stroke. 2006.

6. Hansson GK, Hermansson A. The immune system in atherosclerosis. Vol. 12, Nature Immunology. 2011.

7. Campbell LA, Rosenfeld ME. Infection and Atherosclerosis Development. Vol. 46, Archives of Medical Research. 2015.

8. Szulc M, Kustrzycki W, Janczak D, Michalowska D, Baczynska D, Radwan-Oczko M. Presence of Periodontopathic Bacteria DNA in Atheromatous Plaques from Coronary and Carotid Arteries. Biomed Res Int [Internet]. 2015 [cited 2024 Apr 22];2015. Available from: https://pubmed.ncbi.nlm.nih.gov/26504835/

9. Farsak B, Yildirir A, Akyön Y, Pinar A, Öç M, Böke E, et al. Detection of chlamydia pneumoniae and Helicobacter pylori DNA in human atherosclerotic plaques by PCR. J Clin Microbiol. 2000;38(12).

10. Ellis RW. Infection and coronary heart disease. J Med Microbiol [Internet]. 1997 [cited 2024 Apr 22];46(7):535–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9236736/

11. Blasi F, Denti F, Erba M, Cosentini R, Raccanelli R, Rinaldi A, et al. Detection of Chlamydia pneumoniae but not Helicobacter pylori in atherosclerotic plaques of aortic aneurysms. J Clin Microbiol [Internet]. 1996 [cited 2024 Apr 22];34(11):2766–9. Available from: https://pubmed.ncbi.nlm.nih.gov/8897180/

12. Chiu B, Viira E, Tucker W, Fong IW. Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. Circulation [Internet]. 1997 Oct 7 [cited 2024 Apr 22];96(7):2144–8. Available from: https://pubmed.ncbi.nlm.nih.gov/9337182/

13. Kaplan M, Yavuz SS, Cinar B, Koksal V, Kut MS, Yapici F, et al. Detection of Chlamydia pneumoniae and Helicobacter pylori in atherosclerotic plaques of carotid artery by polymerase chain reaction. Int J Infect Dis. 2006;10(2).

14. Alhusseiny SM, Saleh NE, El-Zayady WM, Hussein MS, El-Beshbishi SN. Association between Toxoplasma gondii infection and coronary atherosclerosis. Trans R Soc Trop Med Hyg. 2021;115(10).

15. Dragomir A, Lupu MA, Lighezan R, Paduraru AA, Olariu TR. Toxoplasma gondii Infection in Patients with Cardiovascular Diseases from Western Romania: A Case–Control Study. Life. 2023;13(7).

16. Wainwright M. Nanobacteria and associated “elementary bodies” in human disease and cancer.

Microbiology. 1999;145 (Pt 10).

17. Kajander EO, Çiftçioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc Natl Acad Sci U S A [Internet]. 1998 Jul 7 [cited 2024 May 2];95(14):8274–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9653177/

18. Puskás LG, Tiszlavicz L, Rázga Z, Torday LL, Krenács T, Papp JGY. Detection of nanobacteria-like particles in human atherosclerotic plaques. Acta Biol Hung [Internet]. 2005 [cited 2024 May 2];56(3–4):233–45. Available from: https://pubmed.ncbi.nlm.nih.gov/16196199/

19. Grundy SM, Greenland P, Herd A, Huebsch JA, Jones RJ, Mitchell JH, et al. Cardiovascular and risk factor evaluation of healthy American adults. A statement for physicians by an Ad Hoc Committee appointed by the Steering Committee, American Heart Association. Circulation [Internet]. 1987 Jun [cited 2024 Apr 22];75(6):1340A-1362A. Available from: https://www.scholars.northwestern.edu/en/publications/cardiovascular-and-risk-factor-evaluation-of-healthy-american-adu

20. Atherosclerosis - What Is Atherosclerosis? | NHLBI, NIH [Internet]. [cited 2024 Aug 17]. Available from: https://www.nhlbi.nih.gov/health/atherosclerosis

21. Lechner K, von Schacky C, McKenzie AL, Worm N, Nixdorff U, Lechner B, et al. Lifestyle factors and high-risk atherosclerosis: Pathways and mechanisms beyond traditional risk factors. Eur J Prev Cardiol [Internet]. 2020 Mar 1 [cited 2024 Aug 17];27(4):394. Available from: /pmc/articles/PMC7065445/

22. Nahrendorf M. Myeloid cell contributions to cardiovascular health and disease. Vol. 24, Nature Medicine. 2018.

23. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis (Primer). Nat Rev Dis Prim. 2019;

24. Tavafi M. Complexity of diabetic nephropathy pathogenesis and design of investigations. J Ren Inj Prev. 2013;2(2).

25. Nayer A, Ortega LM. Catastrophic antiphospholipid syndrome: A clinical review. Vol. 3, Journal of Nephropathology. 2014.

26. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis. Nat Rev Dis Prim. 2019;5(1).

27. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. Circ Res [Internet]. 2014 Jun 19 [cited 2024 Jul 10];114(12):1852–66. Available from: https://pubmed.ncbi.nlm.nih.gov/24902970/

28. van den Hoogen PCW, Feskens EJM, Nagelkerke NJD, Menotti A, Nissinen A, Kromhout D. The relation between blood pressure and mortality due to coronary heart disease among men in different parts of the world. Seven Countries Study Research Group. N Engl J Med [Internet]. 2000 Jan 6 [cited 2024 Jul 10];342(1):1–8. Available from: https://pubmed.ncbi.nlm.nih.gov/10620642/

29. Libby P. The changing landscape of atherosclerosis. Nature. 2021 Apr 22;592(7855):524–33.

30. Li DY, Tang WHW. Gut Microbiota and Atherosclerosis. Curr Atheroscler Rep. 2017 Oct 1;19(10).

31. Brandsma E, Kloosterhuis NJ, Koster M, Dekker DC, Gijbels MJJ, Van Der Velden S, et al. A Proinflammatory Gut Microbiota Increases Systemic Inflammation and Accelerates Atherosclerosis. Circ Res [Internet]. 2019 Jan 4 [cited 2024 Jul 11];124(1):94–100. Available from: https://pubmed.ncbi.nlm.nih.gov/30582442/

32. Farsak B, Yildirir A, Akyön Y, Pinar A, Öç M, Böke E, et al. Detection of Chlamydia pneumoniae and Helicobacter pylori DNA in human atherosclerotic plaques by PCR. J Clin

Microbiol [Internet]. 2000 [cited 2024 Apr 22];38(12):4408–11. Available from: https://pubmed.ncbi.nlm.nih.gov/11101572/

33. Blasi F, Denti F, Erba M, Cosentini R, Raccanelli R, Rinaldi A, et al. Detection of Chlamydia pneumoniae but not Helicobacter pylori in atherosclerotic plaques of aortic aneurysms. J Clin Microbiol. 1996;34(11).

34. Ma J, Li H. The Role of Gut Microbiota in Atherosclerosis and Hypertension. Front Pharmacol [Internet]. 2018 Sep 25 [cited 2024 Jul 11];9(SEP). Available from: https://pubmed.ncbi.nlm.nih.gov/30319417/

35. Kramer CD, Simas AM, He X, Ingalls RR, Weinberg EO, Genco CA. Distinct roles for dietary lipids and Porphyromonas gingivalis infection on atherosclerosis progression and the gut microbiota. Anaerobe [Internet]. 2017 Jun 1 [cited 2024 Jul 11];45:19–30. Available from: https://pubmed.ncbi.nlm.nih.gov/28442421/

36. Liu Q, Li Y, Song X, Wang J, He Z, Zhu J, et al. Both gut microbiota and cytokines act to atherosclerosis in ApoE-/- mice. Microb Pathog [Internet]. 2020 Jan 1 [cited 2024 Jul 11];138. Available from: https://pubmed.ncbi.nlm.nih.gov/31682994/

37. Lilly EL, Webster NJ. Detection of Toxoplasma gondii oocysts on organic and conventionally grown produce. Food Microbiol. 2021 Oct 1;99:103798.

38. Weiss LM, Dubey JP. Toxoplasmosis: a history of clinical observations. Int J Parasitol [Internet]. 2009 Jul 7 [cited 2024 Aug 21];39(8):895. Available from: /pmc/articles/PMC2704023/

39. Overdulve JP. The identity of Toxoplasma Nicolle and Manceaux, 1909 with Isospora Schneider, 1881. I. Proc K Ned Akad van Wet - Ser C. 1970;73(1):129–41.

40. Altintas N, Kuman HA, Akisu C, Aksoy U, Atambay M. Toxoplasmosis in last four years in Agean region, Turkey. J Egypt Soc Parasitol [Internet]. 1997 Aug 1 [cited 2024 Aug 21];27(2):439–43. Available from: https://europepmc.org/article/med/9257982

41. Tenter AM, Heckeroth AR, Weiss LM. Toxoplasma gondii: from animals to humans. Int J Parasitol [Internet]. 2000 [cited 2024 Aug 21];30(12–13):1217. Available from: /pmc/articles/PMC3109627/

42. Hill DE, Sreekumar C, Jones J, Dubey JP. Toxoplasma gondii. 2007 [cited 2024 Aug 21];337–53. Available from: https://link.springer.com/chapter/10.1007/978-1-59745-501-5_12

43. Webster JP. Dubey, J.P. Toxoplasmosis of Animals and Humans. Parasites Vectors 2010 31 [Internet]. 2010 Nov 23 [cited 2024 Aug 21];3(1):1–2. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/1756-3305-3-112

44. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet [Internet]. 2004 Jun 12 [cited 2024 Aug 21];363(9425):1965–76. Available from: https://pubmed.ncbi.nlm.nih.gov/15194258/

45. Liesenfeld O, Montoya JG, Kinney S, Press C, Remington JS. Effect of Testing for IgG Avidity in the Diagnosis of Toxoplasma gondii Infection in Pregnant Women: Experience in a US Reference Laboratory. J Infect Dis [Internet]. 2001 Apr 15 [cited 2024 Aug 21];183(8):1248–53. Available from: https://dx.doi.org/10.1086/319672

46. Liesenfeld O, Montoya JG, Tathineni NJ, Davis M, Brown BW, Cobb KL, et al. Confirmatory serologic testing for acute toxoplasmosis and rate of induced abortions among women reported to have positive Toxoplasma immunoglobulin M antibody titers. Am J Obstet Gynecol [Internet]. 2001 [cited 2024 Aug 21];184(2):140–5. Available from: https://pubmed.ncbi.nlm.nih.gov/11174493/

47. Beder D, Taşbent FE. General Features and Laboratory Diagnosis of Toxoplasma gondii Infection. Turkiye parazitolojii Derg [Internet]. 2020 Jun 1 [cited 2024 Aug 21];44(2):94–101. Available from: https://pubmed.ncbi.nlm.nih.gov/32482042/

48. Rajapakse S, Chrishan Shivanthan M, Samaranayake N, Rodrigo C, Deepika Fernando S. Antibiotics for human toxoplasmosis: a systematic review of randomized trials. Pathog Glob Health [Internet]. 2013 Jun [cited 2024 Aug 21];107(4):162. Available from: /pmc/articles/PMC4001466/

49. Konstantinovic N, Guegan H, Stäjner T, Belaz S, Robert-Gangneux F. Treatment of toxoplasmosis: Current options and future perspectives. Food Waterborne Parasitol. 2019 Jun 1;15:e00036.

50. Folk RL. SEM imaging of bacteria and nannobacteria in carbonate sediments and rocks. J Sediment Res. 1993 Sep 1;63(5):990–9.

51. Kajander EO, Kuronen I, Akerman KK, Pelttari A, Ciftcioglu N, Kajander EO, et al. Nanobacteria from blood: the smallest culturable autonomously replicating agent on Earth. SPIE [Internet]. 1997 Jul 11 [cited 2024 May 2];3111:420–8. Available from: https://ui.adsabs.harvard.edu/abs/1997SPIE.3111..420K/abstract

52. Raoult D, Drancourt M, Azza S, Nappez C, Guieu R, Rolain JM, et al. Nanobacteria Are Mineralo Fetuin Complexes. PLoS Pathog [Internet]. 2008 [cited 2024 May 2];4(2). Available from: /pmc/articles/PMC2242841/

53. Ciftcioglu N, Bjorklund M, Kajander EO, Ciftcioglu N, Bjorklund M, Kajander EO. Stone formation and calcification by nanobacteria in the human body. SPIE [Internet]. 1998 Jul 6 [cited 2024 May 2];3441:105–11. Available from: https://ui.adsabs.harvard.edu/abs/1998SPIE.3441..105C/abstract

54. Çiftçioglu N, Björklund M, Kuorikoski K, Bergström K, Kajander EO. Nanobacteria: An infectious cause for kidney stone formation. Kidney Int. 1999;56(5):1893–8.

55. Kajander EO, Kuronen I, Akerman KK, Pelttari A, Ciftcioglu N. Nanobacteria from blood: the smallest culturable autonomously replicating agent on Earth. SPIE [Internet]. 1997 Jul 11 [cited 2024 May 2];3111:420–8. Available from: https://ui.adsabs.harvard.edu/abs/1997SPIE.3111..420K/abstract

56. Hjelle JT, Miller-Hjelle MA, Poxton IR, Kajander EO, Ciftcioglu N, Jones ML, et al. Endotoxin and nanobacteria in polycystic kidney disease. Kidney Int. 2000;57(6):2360–74.

57. Kajander EO, Çiftçioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc Natl Acad Sci U S A [Internet]. 1998 Jul 7 [cited 2024 Apr 22];95(14):8274–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9653177/

58. Miller VM, Rodgers G, Charlesworth JA, Kirkland B, Severson SR, Rasmussen TE, et al. Evidence of nanobacterial-like structures in calcified human arteries and cardiac valves. Am J Physiol Heart Circ Physiol [Internet]. 2004 Sep [cited 2024 May 2];287(3). Available from: https://pubmed.ncbi.nlm.nih.gov/15142839/

59. Quaternaire RFG physique et, 1994 undefined. Interaction between bacteria, nannobacteria, and mineral precipitation in hot springs of central Italy. Erud Folk Phys Quat 1994•erudit.org [Internet]. 1994 [cited 2024 Jun 12];48(3):233–46. Available from: https://www.erudit.org/en/journals/gpq/1994-v48-n3-gpq1907/033005ar/abstract/

60. Ciftcioglu N, McKay DS, Mathew G, Kajander EO. Nanobacteria: Fact or fiction? Characteristics, detection, and medical importance of novel self-replicating, calcifying nanoparticles. J Investig Med. 2006;54(7):385–94.

61. Kajander EO, Ciftcioglu N, Aho K, Garcia-Cuerpo E. Characteristics of nanobacteria and their possible role in stone formation. Urol Res. 2003;31(2):47–54.

62. Dal T, Dal MS. Nanoparticles (nanobacteria) responsible from calcification. J Clin Exp Investig [Internet]. 2011 Dec 12 [cited 2024 May 2];2(4):463–7. Available from: www.clinexpinvest.org

63. Romanek CS, Thomas KL, Gibson EK, McKay DS, Socki RA, Romanek CS, et al. Petrogenesis of Carbon and Sulfur-bearing Minerals in the Martian Meteorite ALH 84001. LPI; 26:1183. Available from: https://ui.adsabs.harvard.edu/abs/1995LPI26.1183R/abstract

64. (PDF) No 'nanofossils' in martian meteorite | Kathie Keprta - Academia.edu [Internet]. https://www.academia.edu/106924619/No_nanofossils_in_martian_meteorite?uc-sb-sw=103371985

65. Çiftçioglu N, Kajander EO. Interaction of nanobacteria with cultured mammalian cells. Pathophysiology. 1998 Jan 1;4(4):259–70.

66. Young JD, Martel J, Young L, Wu CY, Young A, Young D. Putative nanobacteria represent physiological remnants and culture by-products of normal calcium homeostasis. PLoS One. 2009;4(2).

67. Kutikhin AG, Brusina EB, Yuzhalin AE. The role of calcifying nanoparticles in biology and medicine. Int J Nanomedicine. 2012;7:339–50.

68. Kajander EO. Nanobacteria--propagating calcifying nanoparticles. Lett Appl Microbiol. 2006;42(6):549–52. Available from: https://pubmed.ncbi.nlm.nih.gov/16706890/

69. Young JD, Martel J. The rise and fall of nanobacteria. Sci Am;302(1):36–45. Available from: https://pubmed.ncbi.nlm.nih.gov/20063636/

70. Abraham J, Grenón M, Sánchez HJ, Pérez C, Barrea R. A case study of elemental and structural composition of dental calculus during several stages of maturation using SRXRF. J Biomed Mater Res A. 2005;75(3):623–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16116601/

71. Ciftçioğlu N, McKay DS, Kajander EO. Association between nanobacteria and periodontal disease. Circulation. 2003;108(8). Available from: https://pubmed.ncbi.nlm.nih.gov/12939248/

72. Çiftçíoglu N, Miller-Hjelle MA, Hjelle JT, Kajander EO. Inhibition of nanobacteria by antimicrobial drugs as measured by a modified microdilution method. Antimicrob Agents Chemother. 2002;46(7).

73. Puskás LG, Tiszlavicz L, Rázga Z, Torday LL, Krenács T, Papp JGY. Detection of nanobacteria-like particles in human atherosclerotic plaques. Acta Biol Hung. 2005;56(3–4):233–45. Available from: https://pubmed.ncbi.nlm.nih.gov/16196199/

74. Khullar M, Sharma SK, Singh SK, Bajwa P, Sheikh FA, Relan V, et al. Morphological and immunological characteristics of nanobacteria from human renal stones of a north Indian population. Urol Res. 2004; 32(3):190–5. Available from: https://pubmed.ncbi.nlm.nih.gov/15205851/

75. Tsurumoto T, Matsumoto T, Yonekura A, Shindo H. Nanobacteria-like particles in human arthritic synovial fluids. J Proteome Res [Internet]. 2006 May [cited 2024 Aug 25];5(5):1276–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16674119/

76. Eskandary H, Saba M, Yazdi T. Nanobacteria and intervertebral disc degeneration. Med Hypotheses [Internet]. 2005 [cited 2024 Aug 25];65(5):997–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16046079/

77. Sommer AP. Could reduced bone mineral densities in HIV be caused by nanobacteria? J Proteome Res [Internet]. 2004 May [cited 2024 Aug 25];3(3):670–2. Available from: https://pubmed.ncbi.nlm.nih.gov/15253454/

78. Pretorius AM, Sommer AP, Aho KM, Kajander EO. HIV and nanobacteria. HIV Med [Internet]. 2004 Nov [cited 2024 Aug 25];5(6):391–3. Available from: https://pubmed.ncbi.nlm.nih.gov/15544689/

79. Demir T. Is there any relation of nanobacteria with periodontal diseases? Med Hypotheses. 2008 Jan 1;70(1):36–9.

80. Shoskes DA, Thomas KD, Gomez E. Anti-nanobacterial therapy for men with chronic prostatitis/chronic pelvic pain syndrome and prostatic stones: preliminary experience. J Urol [Internet]. 2005 [cited 2024 Aug 25];173(2):474–7. Available from: https://pubmed.ncbi.nlm.nih.gov/15643213/

81. Maniscalco BS, Taylor KA. Calcification in coronary artery disease can be reversed by EDTA-tetracycline long-term chemotherapy. Pathophysiology [Internet]. 2004 Oct [cited 2024 Aug 25];11(2):95–101. Available from: https://pubmed.ncbi.nlm.nih.gov/15364120/

82. Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R, Flores-Ramírez R. Periodontal Disease, Systemic Inflammation and the Risk of Cardiovascular Disease. Hear Lung Circ. 2018 Nov 1;27(11):1327–34.

83. Walti LN, Conen A, Coward J, Jost GF, Trampuz A. Characteristics of infections associated with external ventricular drains of cerebrospinal fluid. J Infect. 2013;66(5).

84. Coenye T, Spittaels KJ, Achermann Y. The role of biofilm formation in the pathogenesis and antimicrobial susceptibility of Cutibacterium acnes. Biofilm [Internet]. 2021 Dec 1 [cited 2024 Apr 22];4. Available from: https://pubmed.ncbi.nlm.nih.gov/34950868/

85. ÇiftÇioğlu N, McKay DS. Pathological calcification and replicating calcifying-nanoparticles: General approach and correlation. Vol. 67, Pediatric Research. 2010.

86. Yaghobee S, Bayani M, Samiei N, Jahedmanesh N. What are the nanobacteria? Vol. 29, Biotechnology and Biotechnological Equipment. 2015.

87. Jelic TM, Roque R, Yasar U, Tomchin SB, Serrato JM, Deem SG, et al. Calcifying nanoparticles associated encrusted urinary bladder cystitis. Int J Nanomedicine [Internet]. 2008 [cited 2024 Apr 22];3(3):385–90. Available from: https://pubmed.ncbi.nlm.nih.gov/18990947/

88. Almeria S, Dubey JP. Foodborne transmission of Toxoplasma gondii infection in the last decade. An overview. Vol. 135, Research in Veterinary Science. 2021.

89. Layton J, Theiopoulou DC, Rutenberg D, Elshereye A, Zhang Y, Sinnott J, et al. Clinical Spectrum, Radiological Findings, and Outcomes of Severe Toxoplasmosis in Immunocompetent Hosts: A Systematic Review. Vol. 12, Pathogens. 2023.

90. Smith NC, Goulart C, Hayward JA, Kupz A, Miller CM, van Dooren GG. Control of human toxoplasmosis. Vol. 51, International Journal for Parasitology. 2021.

91. Khairullah AR, Kurniawan SC, Widodo A, Effendi MH, Hasib A, Silaen OSM, et al. A Comprehensive Review of Toxoplasmosis: Serious Threat to Human Health. Open Public Health J. 2024;17(1).

APPENDIX

APPENDIX 1

APPENDIX 2

7 CURRICULUM VITAE