

The presence of biofilm structures in atherosclerotic plaques of arteries from legs amputated as a complication of diabetic foot ulcers

- **Objective:** Atherosclerosis, rather than microcirculatory impairment caused by endothelial cell dysfunction, is the main driver of circulatory compromise in patients with diabetic limbs. The presence of atherosclerotic plaque at the trifurcation is a significant contributor to amputation of diabetic legs. The presence of bacteria and other microorganisms in atherosclerotic plaque has long been known, however, the cause of chronic inflammation and the role of bacteria/viruses in atherosclerosis have not been studied in detail. The objective of this study was to clarify the cause of the chronic inflammation within atherosclerotic plaques, and determine if any bacteria and/or viruses are involved in the inflammatory pathway.
- **Method:** This study uses fluorescence microscopy and fluorescence *in-situ* hybridisation (FISH) to identify components of biofilm in atherosclerotic arteries. These tools are also used to identify individual bacteria, and determine the architectural spatial location within the atherosclerotic plaque where the bacteria can be found.
- **Results:** The results indicate that the presence of biofilms in grossly involved arteries may be an important factor in chronic inflammatory pathways of atherosclerotic progression, in the amputated limbs of patients with diabetic foot ulcers and vascular disease.
- **Conclusion:** While the presence of bacterial biofilm structures in atherosclerotic plaque does not prove that biofilm is the proximate cause of atherosclerosis, it could contribute to the persistent inflammation associated with it. Second, the synergistic relationship between the atherosclerotic infection and the diabetic foot ulcer may ultimately contribute to higher amputation rates in diabetics.
- **Declaration of interest:** RAW and RDW have equity interest in PathoGenius, a clinical laboratory using DNA to identify microbes.

biofilm; atherosclerotic plaque; fluorescence in-situ hybridisation (FISH); chronic inflammation; atherosclerosis

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Wound care providers have significantly improved their management of diabetic foot ulcers (DFUs), yet even though there are fewer limb amputations per number of DFUs, the increase in the number of people with diabetes mellitus (and therefore DFUs) is outpacing these improvements. Looking to complicating factors such as atherosclerosis, biofilm and the possible synergy in patients with a DFU, could provide wound care providers with a new treatment strategy.

Biofilm growth is characterised by a group of microorganisms that self-secrete protective matrices, cooperate within a community through quorum sensing molecules and other mechanisms of inter and intracellular signalling, and pursue a parasitic strategy for causing host infection. The biofilm infection strategy is one of attaching to host tissue surfaces, producing senescence of host cells in the area of infection, and providing nutrition to the biofilm constituents through inflammatory pathways which lead to the production of plasma exudate that can be used by the

community as a nutrient source.^{1,2} The infection manifests clinically as a slow undulating pattern of chronic inflammation that can be improved with antibiotics, only to return when the antibiotics are withdrawn. Chronic infections are difficult or even impossible to eradicate even with very high doses of antibiotics for many months.³

Biofilms can cause chronic infections and are associated with a number of chronic disease states including cystic fibrosis, infectious endocarditis, and chronic wounds.⁴⁻⁶ In the majority of chronic wounds (including DFUs), it has been demonstrated that biofilm architecture is present on the wound surface. In fact most chronic infections have the inflammatory biochemistry (elevated pro-inflammatory cytokines) and excessive neutrophils commonly associated with biofilm infections.

Atherosclerotic plaque contain bacteria and other microorganisms.⁷⁻⁹ However, early efforts to determine the clinical significance of the presence of these microorganisms in plaque proved inconclusive, mainly due to the failure of poorly designed antibiotic trials.¹⁰ Many epidemiological studies have

established positive associations between cardiovascular disease risk factors, morbidity, mortality, and markers of infection. Specific pathogens along with their potential contribution by direct or indirect mechanisms to atherosclerosis pathogenesis have been recently reviewed.¹⁰ The cause of the chronic inflammation within atherosclerotic plaques that leads to disease progression, and what role, if any, bacteria and/or viruses play in the inflammatory pathway remains unclear. With well-established technologies, such as DNA sequencing, polymerase chain reaction (PCR) and fluorescent in situ hybridisation (FISH), for identifying and quantifying microbial DNA from human sample sources, it is now possible to determine some characteristics of the microorganisms in atherosclerosis. First, the most common source of microorganisms within atherosclerotic plaques most closely correlates with the oral microbiome, rather than bacteria from any other niche, such as gut, skin, or sinus.^{11–14} Secondly, the arrangement of the microorganisms within the plaques is heterogeneous,¹⁵ in that the samples followed a pattern of regions of high microbial density directly adjacent to an area which was almost void of microbial DNA. Finally, it has been shown that the regions of high microbial density were polymicrobial.^{15,16} These features suggested microorganisms at the arterial wall are in biofilm mode of growth.

Biofilm has been well defined to show a heterogeneous arrangement of the microbial cells with specific regions of high cell numbers, or clusters, and regions void of microorganisms.^{17,18} Furthermore, most biofilm infections, regardless of the site, have been shown to have significant diversity.^{1,19,20}

Atherosclerosis also demonstrates a persistent undulating pattern of inflammation, which is a classic characteristic of biofilm infection.^{21,22} Studies to examine the effects of antibiotics have usually been designed to target select bacteria instead of a polymicrobial mix of bacteria and have often comprised low doses of various durations.²³ It is now an important clinical finding that when inflammatory markers are increased, atherosclerosis is more dangerous.²¹

This study uses a host of technologies to identify components of biofilm in grossly involved atherosclerotic anterior tibial arteries and its association with the several host tissues in the amputated legs of patients with diabetes. Furthermore, to differentiate bacteria from host cells, and determine their architectural spatial structure within the atherosclerotic plaque.

Methods

Ethical approval

This project was approved by the Southwest Regional Wound Care Center and Texas Tech University Medical Center Review Board. Patients were consulted at University Medical Center (Lubbock, Texas) or the

Southwest Regional Wound Care Center (Lubbock, Texas) by a member of the study team after they had consented to a major limb amputation, and were prospectively enrolled in this study.

Participants

This project entailed identifying legs that were amputated for vascular disease and/or unhealed DFU that were placing the patient at an unacceptable risk. The patients underwent the consenting process as per the institutional review board (IRB; Texas Tech University Health Science Center IRB #L08-085) approved protocol. Subject inclusion criteria was leg or legs that were amputated for vascular disease and/or unhealed DFU that were placing the patient at an unacceptable risk and were at least 18 years of age or older. Subjects were excluded if they had a traumatic amputation, which did not involve vascular disease and/or diabetes.

Tissue collection

Amputated legs were dissected and then bloc removal of atherosclerotic arteries was performed within the pathology department at University Medical Center. Arteries that had palpable atherosclerotic plaque of 5–10cm in length were preferred. The specimens were flash frozen in a sterile porous tissue collection block in liquid nitrogen and stored at -80°C until required. Frozen tissue was transferred to the Research and Testing Laboratory at Lubbock.

Plaque analysis

The analysis of the plaques involved taking a cross-section of a plaque for PCR and sequencing to identify the microorganisms present. Small probes were developed for the universal detection of bacteria and adapted with Alexa Red for FISH evaluation of the remaining sample. These were subjected to microscopy and/or confocal microscopy to further define the three dimensional arrangement of bacteria within the plaque.

Tissue preparation and sectioning

Frozen artery samples were cut into 40–50mm cross-sections using a sterile scalpel and placed in a form with cryomatrix material using aseptic techniques, frozen in a -80°C freezer for 5–10 minutes until solidified, and finally attached to a sectioning block with the same cryomatrix material. Tissue cross-sections, 6–8 μm thick, were cut using a cryostat and mounted on microscope slides. Tissue sections used for FISH underwent an additional step and were immediately fixed in 100% ethanol (1 minute), allowed to air dry (3 minutes), before being placed in a freezer slide box and stored at -80°C until required. Each microscope slide contained two tissue cross-sections, one was used for negative dye, or the negative FISH probe control, and the other received dye or FISH probe.

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Achieving a high-quality tissue cross-section of 6–8 μm is challenging, maintaining the proper temperatures within the cryostat is extremely important. Additionally, the hard plaques will very quickly dull a cryostat blade. For this reason, it is necessary to start sectioning with a new blade, prepare the sample surface by trimming the tissue block carefully, and finally adjust to an unused portion of the blade just before cutting sample sections for the microscope slides.

Biofilm component staining and epifluorescence microscopy

Haematoxylin and eosin stain (H&E) staining was performed using the following protocol. Briefly, frozen sections on microscope slides were air-dried (3–5 minutes) to remove moisture, then stained with filtered 0.1% Mayer's Hematoxylin (Sigma, MHS-16) (10 minutes), then rinsed in a Coplin jar in cool running distilled water (5 minutes). The samples were then dipped in 0.5% eosin 12 times, followed by several rinses in distilled water until the eosin stopped streaking. The samples were sequentially dipped in 50% ethanol 10 times, 70% ethanol 10 times, equilibrated in 95% ethanol for 30 seconds, then 100% ethanol (1 minute), and finally dipped in xylene several times and allowed to dry. Finally, anti-fade reagent (Life Technologies, Cat #S36940) was applied to the sample and a coverslip placed over the top.

Thin atherosclerotic artery cross-sections mounted on microscope slides were dyed with multiple fluorescently labelled probes (Life Technologies, Molecular Probes) with different excitation and emission spectra in order to visualise the distribution of cells, polysaccharides, and proteins in samples. Before the application of any dyes, the frozen atherosclerotic artery tissue cross-sections on microscope slides were allowed to dry at room temperature (3–5 minutes), washed three times in 1X PBS (2 minutes), fixed in 4% paraformaldehyde (10 minutes) at room temperature, and rinsed three more times in 1X PBS for 2 minutes each time.

FITC FM 1-43, a biofilm cell body stain, is a fluorescein-5-isothiocyanate derivative dye that fluoresces green upon binding. Isothiocyanates are part of a group of active esters, which form stable carboxamide bonds and these amine-reactive reagents will conjugate with virtually any protein or peptide. Amine-reactive reagents react with non-protonated aliphatic amine groups, including the amine terminus of proteins and the ϵ -amino group of lysines. The FM dyes have little or no fluorescence in aqueous media and are thought to insert into the surface membrane where they become intensely fluorescent.²⁴

FilmTracer FM 1-43 (Molecular Probes) was diluted to a working concentration of 1 $\mu\text{g}/\text{ml}$ according to instructions, and a 100 μl aliquot was pipetted on

top of the tissue and incubated in the dark (20 minutes). The sample was rinsed gently in distilled water, and allowed to air dry at room temperature (3 minutes).

Texas Red Con A (Molecular Probes) is a widely used lectin in cell biology and selectively binds to α -mannopyranosyl and α -glucopyranosyl residues.²⁵ Dye solutions were prepared according to manufacturer recommendations to a final concentration of 50 $\mu\text{g}/\text{ml}$, 50–100 μl of dye solution was added to samples following the addition of FM 1-43.

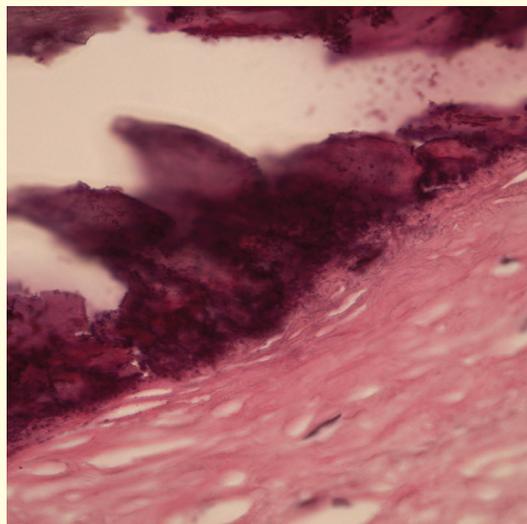
Finally, DAPI (4',6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that exhibits approximately 20-fold enhancement of fluorescence upon binding to A-T rich regions of dsDNA. The SlowFade Gold Antifade Reagent (Life Technologies) with or without DAPI was used as received before adding a coverslip and microscopy.

FISH was used to demonstrate the location of bacteria in the same sections of atherosclerotic arteries highly occluded with plaque. A broad range bacterial 16S generic ribosomal probe previously described was obtained (Integrated DNA Technologies) with sequence 5'-F-ACT GCT GCC TCC CGT AGG AGT TTA TTC CTT-3', where F is a 5' Alexa Fluor 546 (NHS Ester) modification.^{26–28}

The same procedures were followed as described for tissue preparation and sectioning. Before the incubation with FISH probes, the frozen atherosclerotic artery tissue cross-sections on microscope slides were allowed to dry at room temperature (3–5 minutes), washed three times in 1X PBS (2 minutes), fixed in 4% paraformaldehyde (10 minutes) at room temperature, and rinsed three more times in 1X PBS for 2 minutes each time. Epitopes were exposed using hot 95°C 0.01M sodium citrate buffer (pH 6) in Coplin staining jars for 15 minutes, and rinsed in room temperature 1X PBS (10 minutes). The slides were then treated with proteinase K (working solution approximately 1–2 $\mu\text{g}/\text{ml}$) for 15 minutes at room temperature, and rinsed for 4 minutes at room temperature in 1X PBS. Slides were then hybridised with a working probe solution at 50 $\text{ng}/\mu\text{l}$ in a humidity chamber at 45°C overnight. This step destabilises the protein-RNA interactions and enables the probe to access the mRNA and also decreases nonspecific probe binding with non-target mRNAs and the cellular background. This probe was selected in part because it has previously been well characterised.^{26,27} The negative control tissue samples received a hybridisation buffer without a probe.

The slides were washed in a hybridisation wash buffer for 20 minutes at 45°C, and rinsed briefly in distilled water and 1X PBS. The slowfade gold antifade reagent (Life Technologies) with or without DAPI was used as received before adding a coverslip and microscopy on the probe applied or negative

Fig 1. Hematoxylin and eosin stained cross section of a highly occluded atherosclerotic artery at 40X magnification. Haemalum primarily stains the nuclei of cells blue. Eosin counterstains basic structures various shades of red



Fluorescence microscopy

Tissue sections mounted on slides were evaluated under the appropriate UV wavelength. Digital images were captured using three wavelengths for three different colour channels. For non-specific biofilm component dyes, the cell body fluorophore is green, the α -mannopyranosyl and α -glucopyranosyl sugar residues are red, and the nuclei fluoresce blue. For FISH, the probe is red, the non-specific nuclei is blue (DAPI), and the green is normal tissue autofluorescence. Composite images were generated by overlaying the three colour channels to visualise either biofilm matrix components, or with the FISH probe, bacteria appearing red, nuclei appearing blue, and background tissue autofluorescence appearing green or yellow.

Results

We recruited 10 patients who consented to the use of their amputated leg for the research project. All had DFU's with and without osteomyelitis. Most had critical limb ischaemia which could not be corrected. We did not correlate the imaging results to the patient condition.

Atherosclerotic plaques are commonly visualised using H&E staining. Fig 1 shows a highly occluded atherosclerotic artery, with haemalum (oxidation product of haematoxylin) colouring the nuclei of cells, and a few other objects, such as keratohyalin granules and calcified material blue. The nuclear

control samples, respectively. Although there were no samples completely sterilised or cleansed to guarantee no potential bacteria and to ensure no non-specific probe binding, artery samples that were not highly occluded exhibited no non-specific probe binding.

Fig 2. Cross section of a control biofilm at 40X magnification. The same sample is imaged through the progression of transmitted light (a), Concanavalin A selectively binds and stains α -mannopyranosyl and α -glucopyranosyl sugar residues red b) FM 1-43 is a green biofilm cell body stain; c), and DAPI labels DNA blue (d). Finally, the four images are merged in to visualize the separate areas of biofilm components, as well as the saturated areas (white) indicating the presence of all three dyes within that space (e).

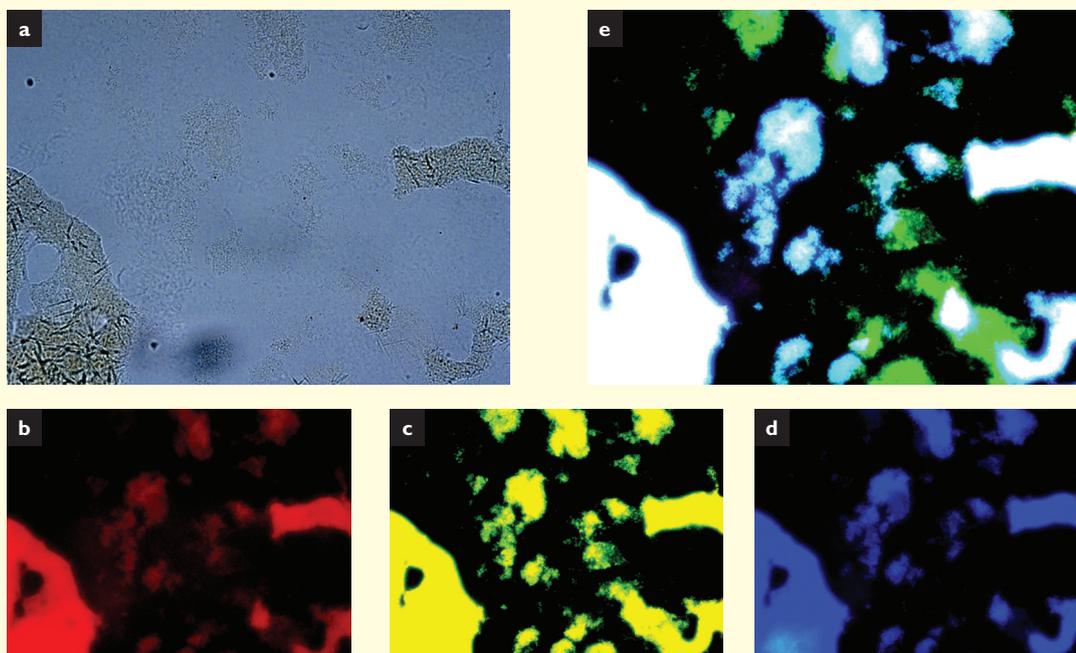
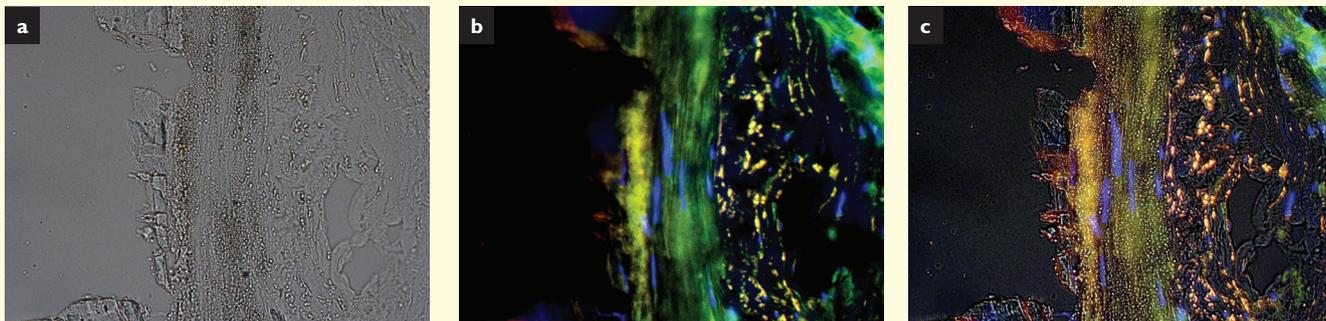


Fig 3. Cross section from the same highly occluded atherosclerotic artery sample visualised in Fig 1 at 40X magnification. Transmitted light image is shown (a), the merged image file of the three fluorescent dyes (b), and the merged image file of the dyes superimposed over the transmitted light (c)



staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colours other eosinophilic structures (basic) in various shades of red, pink, and orange. Though these stains are very useful, it is clearly not possible to distinguish any bacteria from host cells. There are some small structures of interest that seem to be concentrated at the interface of the plaque and the arterial wall.

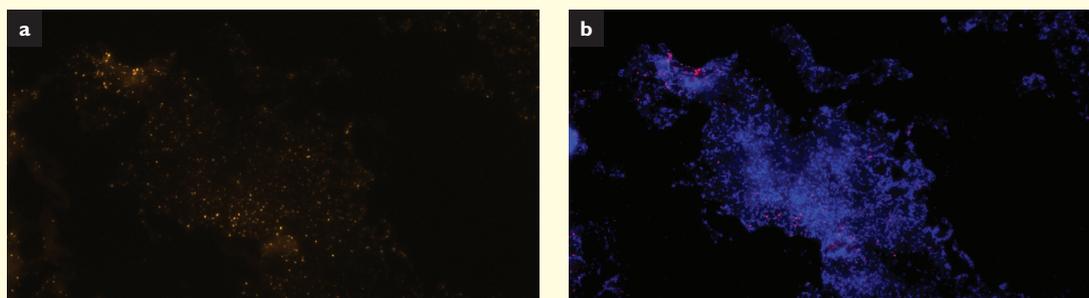
The primary components of biofilm extracellular matrices (ECM) have been shown to be polysaccharides, proteins, nucleic acids in the form of extracellular DNA, and lipids.²⁹ control biofilms, as previously described,³⁰ were used to optimise each stain protocol using three (FITC FM 1-43, Texas Red Con A, DAPI) well-characterised, non-specific dyes to identify the components and structures of mature biofilm in atherosclerotic arteries. The procedure was optimised so that control biofilm samples could be simultaneously dyed with tissue samples. It should be noted that the sequence of dye addition is important in order not to destroy the sample. The sequence began with Concanavalin A, followed by FM 1-43 (biofilm cell body stain), and ended with DAPI. Fig 2 represents the progression of images collected through transmitted light (Fig 2a), b) GFP (Fig 2b), c) FITC (Fig 2c), and DAPI (Fig 2d) filters. Finally, the four images are merged in Fig 2e to visualise

the separate areas of biofilm components, as well as the saturated areas (white) indicating the presence of all three dyes within that space.

Fig 3 is a cross section from the same atherosclerotic plaque sample visualised in Fig 1, following the same image progression of Fig 2. For the sake of brevity, Fig 3a the transmitted light image is shown, Fig 3b the merged image file of the three fluorescent dyes, and Fig 3c the merged image file of the dyes superimposed over the transmitted light.

Efforts were also made to have direct molecular identification of bacteria in the atherosclerotic plaques using FISH. Several species of microorganisms have previously been identified in the literature⁹ and are believed to be associated with atherosclerotic plaque. There is considerable value in demonstrating which organisms, if present, could be responsible for producing components of biofilm ECM and this is the subject of ongoing efforts using species-specific FISH probes and next generation sequencing tools. Additionally, questions of non-specific binding with species-specific FISH probes are of significant importance. Coupled with a lack of knowledge related to any specificity, or even presence, of microorganisms in the collected samples, the authors selected a universal probe rather than an organism specific probe due to the heterogene-

Fig 4. The control biofilm at 40X magnification showing the fluorescence in situ hybridisation probe only (GFP; a) and the same image merged with the DAPI signal (b)



ous nature of biofilms. The universal probe used in this work corresponds with the broad range 16S ribosomal bacterial subunit, is labelled with Alexa red, and has been well characterised.^{26,27} Only DAPI was used as a counter stain in the FISH probe analysis. Tissue samples have natural autofluorescence in the green wavelengths (FITC), while, bacteria fluoresce red when the probe is attached and DAPI (nucleic acids) blue in A-T rich regions in DNA.

Fig 4a is control biofilm at 40X showing the FISH probe only (GFP) and Fig 4b the exact same image merged with the DAPI signal. Thus, the universal 16S probe allows the clear visualisation of bacteria in the control biofilm. Fig 5a is a cross section of a highly occluded atherosclerotic artery, GFP only, and Fig 5b the exact same image merged with FITC and DAPI signals to show the location of the bacteria with respect to the arterial wall and the plaque. The amount of probe detected is quite high, and would seem to suggest overexposure. However, small areas resembling pockets of microorganisms are very densely populated within biofilms. To display the architecture of these areas confocal microscopy was used to generate Fig 6. The three-dimensional construct and high magnification shows the densely red areas of the image are highly concentrated areas of bacteria. This image is representative of observations made for regions of high universal probe fluorescence over the arterial samples examined.

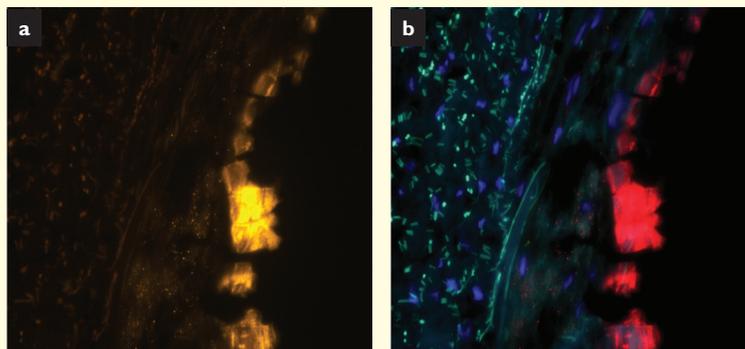
Each of the samples used in this study contained both segments of the artery that were very highly occluded with hard plaque in addition to segments without occlusion. When observing tissue obtained from segments not occluded by plaque, no probe fluorescence was observed in almost all cases outside of the natural auto fluorescence in the green (FITC). In the rare case that fluorescence was observed, it appeared to be consistent with a small number of bacteria, i.e. one to three cells within a viewing window, existing either in a planktonic state, or perhaps introduced as contamination during the preparation process. For the sake of brevity, a figure showing no observable fluorescence is not provided.

Discussion

There is consensus that chronic wounds possess biofilm and that biofilm may contribute to the characteristics of chronic wounds. A chronic wound behaves like a chronic infection involving skin and adjacent tissue.³¹ That is, a chronic wound exhibits persistent inflammation, resistance to antibiotics, and biofilm structures. Atherosclerotic lesions also demonstrate persistent inflammation, resistance to antibiotics and, as demonstrated in this study, biofilm structures.

Although the ability to make an ECM appears to be a universal feature of multicellular communities,

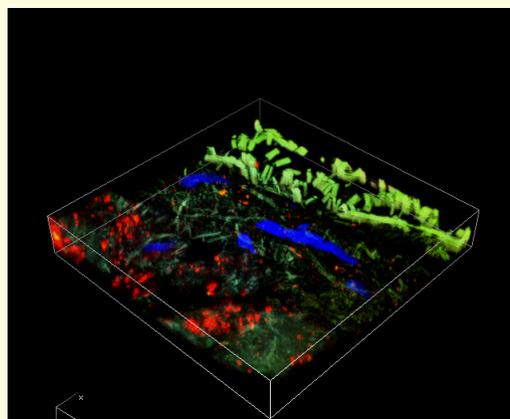
Fig 5. A cross section of highly occluded atherosclerotic artery sample bound with the 16S Universal bacteria probe only (a) and the exact same image merged with GFP and DAPI signals to show the location of the bacteria with respect to the arterial wall and the plaque (b)



there is a remarkable diversity in the means by which these matrices are constructed.²⁹⁻³² The structure, physiology, and chemistry of biofilms vary substantially according to two key factors: the nature and composition of the microbes residing in the biofilm, and the local environment where the biofilm is attached. As a result, the structural integrity of the biofilm is critically dependent upon the ECM produced by constituent microbes. The matrices themselves are diverse microenvironments, and contribute to the organisation of the community.^{32,33} The ECM, and thus the biofilm architecture, can be dramatically altered by very slight changes in environmental conditions.³²

The primary components of biofilm ECM have been shown to be polysaccharides, proteins, nucleic acids in the form of extracellular DNA, and lipids.²⁹ The first aim of this work was to explore using well-

Fig 6. Confocal microscopy was used to construct a three-dimensional image of a cross section of a highly occluded atherosclerotic artery to visualise the concentrated areas of FISH probe-bacteria (red) with respect to the auto-fluorescence of the sample (green) and DNA stained by DAPI (blue). Calibrated Width: 127.28µm, Height: 127.28µm, and Depth: 19.80µm



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characterised, non-specific dyes to identify components and structures of mature biofilm in atherosclerotic arteries. If, in fact, microbial biofilms might be contributing to the persistent activation of inflammatory pathways evident in atherosclerotic lesions,²¹ there should be a biofilm ‘footprint’ in or around the plaque and arterial wall. The microscopy performed in this work indeed indicates the presence of biofilm matrix components. Researchers in other areas have also used the same stains to indicate the presence of biofilm components.³⁴ However, since these stains are non-specific and cannot differentiate between substances generated either by the human host or microbes, a more precise technique was also used to selectively identify bacterial DNA and provide insight into the location of the bacteria within the atherosclerotic plaque complex.

Lanter et al. have been able to image biofilm structures in carotid artery plaques and even hypothesise that biofilm stimulated by human host signals may lead to plaque rupture.³⁵ The biofilm growth mode could explain a number of other behaviours of atherosclerosis, such as persistent inflammation, patchy localisation, and poor response to low dose systemic antibiotics.

FISH of bacterial DNA was used in this work to illuminate the architecture and location of bacteria within the atherosclerotic artery/plaque complex. FISH, along with other staining techniques, identified large amounts of bacteria (16S universal probe) organised in dense clusters and voids classic of biofilm in a host infection. If these were contami-

nants, we would expect the distribution of bacteria to be in a random pattern throughout the plaque and tissue cross-section. However, three different plaques from three different patients demonstrated the presence of biofilm structures and all were located between the vascular smooth muscle and the plaque in the lumen.

Conclusion

The presence of bacterial biofilm structures in atherosclerotic plaque does not prove that biofilm is the proximate cause of atherosclerosis. However, biofilm structures in atherosclerotic plaque are interesting for several reasons. First, the presence of biofilm and components of biofilm matrices could contribute to the persistent inflammation associated with atherosclerosis. Second, microorganisms growing in biofilms are associated with chronic infections. This fact raises the possibility that a chronic infection in the arteries of the lower extremity (producing atherosclerosis) may be working synergistically with a chronic infection of the diabetic foot (DFU). This synergistic relationship may ultimately contribute to higher amputation rates in diabetics. The molecular mechanisms of both the microbes and the host response for both of these areas of biofilm disease still needs to be elucidated. However, the presence of biofilm and components of biofilm matrices suggest an important area of inquiry for the understanding of atherosclerosis, management of chronic wounds, and the salvaging of diabetic limbs. ■

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