

# PUTATIVE BIOFILM-FORMING ORGANISMS IN THE HUMAN VASCULATURE: EXPANDED CASE REPORTS AND REVIEW OF THE LITERATURE

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## ABSTRACT

**Objectives:** New technologies, particularly in the field of clinical metagenomics, have opened up new avenues to explore microbial involvement in illnesses where disease initiation is poorly understood. Here we present a series of six patients suffering from diseases with potential microbial involvement: systemic lupus erythematosus, chronic fatigue syndrome, fibromyalgia, multiple sclerosis, amyotrophic lateral sclerosis, and arteriosclerosis.

**Material and methods:** Peripheral blood (first five cases) and suction debris from carotid percutaneous angioplasty (arteriosclerosis) were refrigerated and transported to the site of analysis. DNA was extracted using the standard QIAamp DNA Blood Mini and Protozoal Extraction kits. Bacterial-specific DNA sequences were amplified and subsequently barcoded for DNA sequencing with 16S rRNA-directed primers flanking the variable regions 1 and 2 and variable regions 4 and 5, respectively. Protozoal-specific DNA sequences were amplified and subsequently barcoded for DNA sequencing using low stringency conditions and primers directed to variable regions in the 18S rRNA gene.

**Results:** We found previously unreported mixed bacterial and protozoal biofilm communities in all samples. In all six disease types we revealed protozoa, being close relatives to known aquatic-based protozoa, such as: *Oblongichytrium*, *Chrysocapsa vernalis*, *Ochromonas*, and *Perkinsus qugwadi*. In a blood sample from the patient with lupus erythematosus DNA stains and culture studies revealed an unknown protozoan exhibiting profound biofilm-forming capability: candidatus *Protomyxzoa rheumatica*.

**Conclusions:** Discovery of these potentially pathogenic protozoa in human blood may be of great clinical relevance. However, our preliminary results should be confirmed by independent researchers, and the role of biofilm-forming protozoa in human disease should be elucidated.

**Key words:** protozoa, clinical metagenomics, multiple sclerosis, biofilm, plaque.

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## INTRODUCTION

There are a variety of extensively studied chronic illnesses whereby an initiating aetiological event has yet to be conclusively identified, representing a gap in our current medical framework. The presence of a variety of bacterial and/or viral agents has been frequently observed in association with this class of diseases; however, a single conclusive aetiological agent has yet to be identified. Systemic lupus erythematosus (SLE), chronic fatigue syndrome (CFS), fibromyalgia (FM), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and arteriosclerosis all display a variety of chronic symptoms

and inflammatory features. These diseases suffer from significant treatment limitations that range from palliative measures, immunosuppressive agents, lipid management, and vascular interventions.

Many previously intractable chronic disorders have been shown to be closely associated with polymicrobial biofilm communities [1, 2]. These microbial communities are characterised by adherent, polysaccharide-secreting communal microorganisms. The communal nature of these organisms and the surrounding biofilm matrix results in significant hurdles for treatment as they promote immune system evasion, antimicrobial treatment resistance, and inherent recalcitrance [3]. In the human arterial

system, biofilm bacteria have been documented as contributors to arterial plaque [4, 5]. There are only a handful of protozoal microorganisms reported to have biofilm-forming properties. Among these are *Acanthamoeba* sp., *Trypanosoma brucei*, *Pneumocystis carinii*, and our initial report of protozoan biofilm communities in ALS patients [6-8].

A significant limitation in the study and the involvement of polymicrobial infections and biofilm diseases is the traditional reliance on Koch's postulates. Koch's postulates assume that a single organism will occur in every case of the disease, such an organism occurs in no other disease or in the absence of disease, and once grown in pure culture it can induce disease [9]. Research has demonstrated that a single causal organism may not be easily identified due to its dependence on supportive factors provided from a polymicrobial community. Furthermore, virulence factors may contribute to altering the pathogenic potential of an organism, so the organisms may be more widespread than is acceptable according to Koch's postulates. Lastly, over the last decade molecular technologies have highlighted significant technological deficiencies in our microbial knowledge, with less than an estimated 2% of all bacteria being currently cultivatable [10]. This deficiency is further highlighted in critical medical microbiology, such as sepsis in the intensive care unit, whereby bacteria are unreliably cultivable 10-60% of the time [11, 12]. Taken in total, these limitations have resulted in reconsideration of Koch's postulates [13].

Next Generation Sequencing (NGS) and metagenomic analysis may be used to elucidate and define important trends of microbial populations and disease [14-16]. We hypothesise that by using modern clinical metagenomics and broad-spectrum analysis, previously unreported microbial phenomena may be detected and characterised in these diseases. In this article we document six examples of biofilm communities: five isolated from peripheral venous system samples from SLE, FM, CFS, MS, and ALS, and a single sample from carotid angioplasty. A review of the literature is presented to provide feasibility and context for these findings. If future studies continue to demonstrate similar correlative findings, this may represent a new class of microbial illness.

## MATERIAL AND METHODS

### Patient selection and consent

All patients provided written, informed consent. All patients met inclusion criteria for the classification of the disease of interest. Systemic lupus erythematosus, ALS, and FM cases were obtained through patient consent as part of single case studies. The arterial case is part of an ongoing study that is approved by St. Luke's Hospital IRB (Protocol# 09.072012, Study# 1136445). MS and CFS cases are part of ongoing studies approved by the Western Independent Review Board (WIRB);

(Protocol# 21211127, Study# 1133561). This study was conducted in accordance with the Declaration of Helsinki 1975-2008.

### Sample collection and processing

Peripheral blood (BD Vacutainer K2 EDTA, about 3 ml) and suction debris from elective carotid percutaneous transluminal angioplasty and stenting were collected, refrigerated, and transported via rapid courier to the site of analysis. Bacterial DNA was extracted from 200 µl of sample processed using the standard QIAamp DNA Blood Mini extraction kit (Qiagen) to extract total DNA with a final elution volume of 30 µl. Alternatively, total DNA, including Protozoal DNA, was extracted using the Protozoal Extraction kit and protocol (Fry Laboratories, L.L.C.) with a final elution volume of 30 µl.

### Stains and microscopy

Standard fluorescence microscopy (Nikon Eclipse E600) was used to visualise and capture by photomicrograph Hoechst DNA-stained [17, 18] samples (Fry Laboratories, LLC) using the appropriate filters, NIS Elements D v3.22.14 software, and DS-U2/L2 USB imager (Nikon). Digital image processing and editing was carried out using Photoshop CS 8.0 (Adobe).

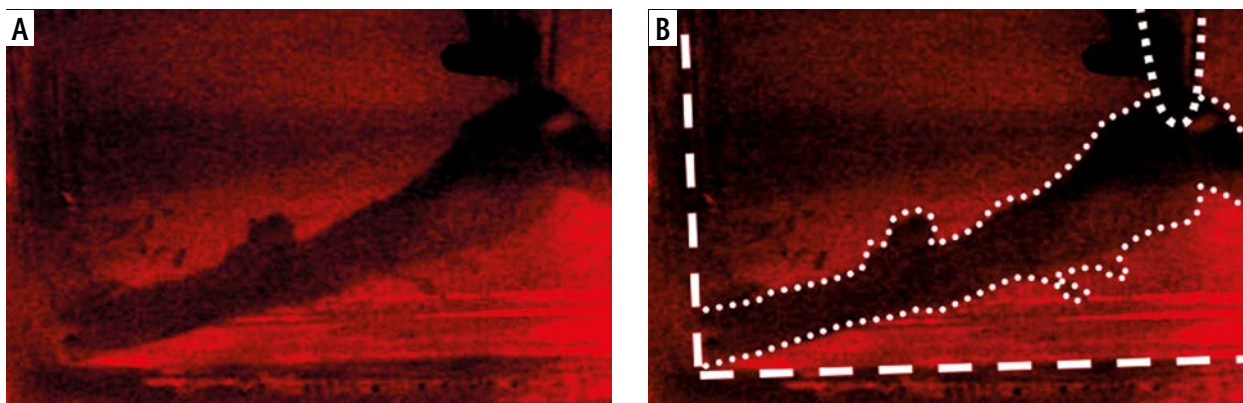
### DNA sequence analysis

Cloned fragment sequencing was performed by Sanger sequencing at the DNA Lab (Arizona State University, Tempe Az, USA) and was deposited in GenBank. Next Generation Sequencing and analysis was performed in-house utilising the RIDI™ system (Fry Laboratories, LLC), which was developed to complement the IonTorrent™ PGM sequencer. Bacterial-specific DNA sequences were amplified and subsequently barcoded for DNA sequencing with 16S rRNA-directed primers flanking the variable regions 1 and 2, and variable regions 4 and 5, respectively. Protozoal-specific DNA sequences were amplified and subsequently barcoded for DNA sequencing using low stringency conditions and primers directed to variable regions in the 18S rRNA gene. All processed metagenomic data were deposited at the Sequence Read Archive (SRA) at the National Centre for Biotechnology Information.

## CASE REPORTS

### Systemic lupus erythematosus

After informed consent a peripheral venous blood sample was obtained from a 55-year-old Caucasian male who developed chronic fatigue in the late 1980s, which progressed to a diagnosis of SLE, meeting four of fourteen



**Fig. 1.** Biofilm-like structures from primary culture. **A)** Biofilm-like material formed in primary culture from blood from an SLE patient, raised by a micropipette. **B)** Annotated view showing culture flask perimeter via long rectangle hash marks. Biofilm-like material indicated by circular hash marks. Pipette tip is indicated by square hash marks. Scale bar in panel A represents 1 cm

American College of Rheumatology criteria: elevated ANA, anti-dsDNA, pulmonary interstitial fibrosis, and non-erosive arthritis [19]. Höecht DNA staining of peripheral blood exhibited evidence of a biofilm-like structure with putative indwelling organisms (Fig. 1A). Thirty milliliters of enriched eukaryotic cell culture media was spiked with 200 µl of peripheral blood containing these putative biofilm communities and incubated at 37°C. A visible biofilm-like layer evolved over seven days (Fig. 2). Due to the suspected protozoal elements in the patient sample, broad-spectrum DNA amplification from total genomic DNA harvested from blood, subsequent cloning, sequencing using semi-pan-protozoal primers, and read assembly revealed protozoal DNA fragments, which were deposited in GenBank (KJ914572). This includes a characteristic sequence of a proposed organism of particular interest tentatively named *candidatus Protomyxzoa rheumatica*.

### Chronic fatigue syndrome

After informed consent, a peripheral venous blood sample was obtained from a 49-year-old Caucasian male who met the Centres for Disease Control and Prevention and Fukuda 1994 criteria [20, 21]. Upon microscopy with Höecht staining, biofilm-like structures were observed (Fig. 1B). Subsequent metagenomic analysis revealed significant fungoid-like protist sequences consistent with divergent *Oblongichytrium* sp., *Schizochytrium minutum*, and *Schizochytrium aggregatum* (SRR1301144). No sequences were identified exceeding 95% identity, indicating these sequences may potentially represent a novel species (Table 1). Of note, the highest sequence contributor (80.6%), *Oblongichytrium* sp., is related to other thraustochytrids, some of which are shellfish pathogens [22, 23].

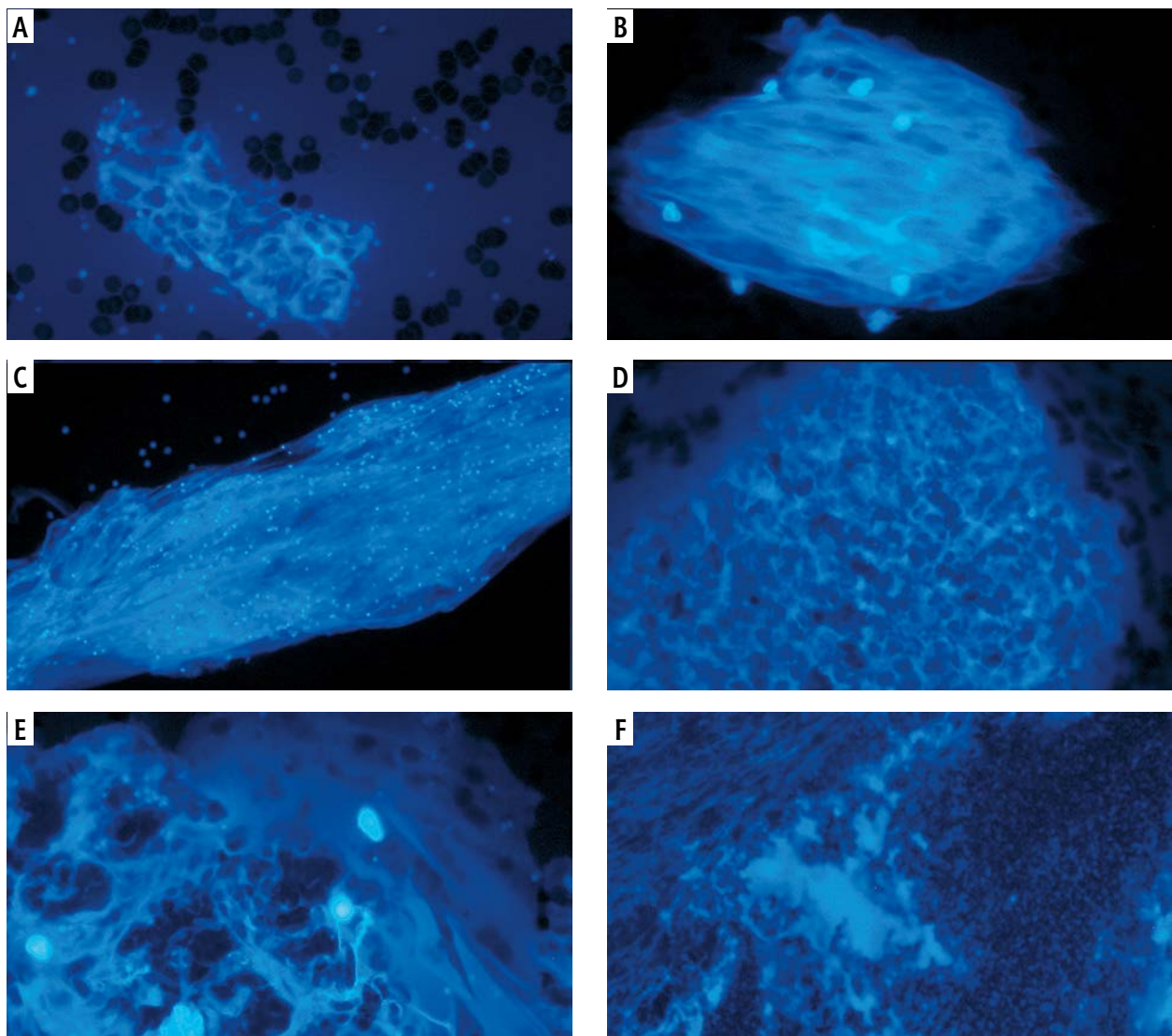
### Fibromyalgia

After informed consent, peripheral venous blood was obtained from a 55-year-old Caucasian female

meeting the American College of Rheumatology criteria for fibromyalgia. A large thrombotic-like strand was recovered from a peripheral blood Vacutainer tube draw that measured approximately 28 mm in length and 5 mm in diameter when flattened on a standard microscope slide with cover glass placed on top (Fig. 1C). The large size of the putative biofilm-like strand allowed for direct manipulation via sterile glass needles and revealed a firm, gelatinous consistency. Putative bacteria were visualised in addition to larger eukaryotic-like cellular structures. The strand was collected, total DNA was harvested, and metagenomic analysis revealed a mixed population of bacteria and a potentially novel protozoal contributor. Bacteria consisting of 5% or more of the total sequences included a divergent *Polaromonas naphthalenivorans*-like species, *Rubrivivax gelatinosus*, *Pelomonas saccharophila*, and a divergent *Smithella propionica*-like organism (Table 2) (SRR1301142). Protozoal sequence analysis yielded a divergent *Chrysocapsa vernalis*-like organism (64% of the total sequences) (Table 1) (SRR1301143).

### Multiple sclerosis

After informed consent, peripheral venous blood was obtained from a 57-year-old female Caucasian with a history of MS meeting the McDonald Criteria and with an EDSS severity scale of 7 [24]. Höecht DNA staining of the peripheral blood showed additional biofilm-like communities (Fig. 1D). Next Generation Sequencing and clinical metagenomic analysis revealed a low sequence count of potentially incidental bacterial findings of a high percentage identity to *Propionibacterium acnes* and *Ralstonia solanacearum*: 53.8% and 46.2%, respectively (Table 2) (SRR1301145). Protozoal sequence analysis yielded slightly higher sequence counts for *Ochromonas danica*, with 84% having 95% or greater sequence identity and the remaining 9.9% being more divergent (Table 1) (SRR1301146).



**Fig. 2.** Biofilm-like structures by fluorescent microscopy. **A)** Tight DNA positive cluster found in the blood from an SLE patient. **B)** Filamentous DNA positive clustering with adherent neutrophils found in the blood from a CFS patient. **C)** Large filamentous strand found containing many DNA-positive cells. **D)** Large cluster of densely packed cells with interspersed DNA-positive material found in blood from an MS patient. **E)** Sheet and filamentous DNA-positive material with imbedded and adherent neutrophils found in the blood of an ALS patient. **F)** Dense DNA-positive material found among the captured debris from a carotid angioplasty. Scale bars in A, B, D, and E correspond to approximately 10  $\mu\text{m}$ , while bars in C and F correspond to 50  $\mu\text{m}$

## Amyotrophic lateral sclerosis

After informed consent, peripheral venous blood was obtained from a 49-year-old Caucasian male with a suspected diagnosis of ALS meeting the Awaji criteria from an independent neurologist [25]. This patient had complicating degenerative cervical disease that was not alleviated after surgical correction. Suspect microbial elements were observed by H $\ddot{o}$ chst DNA staining (Fig. 1E). Clinical metagenomics using NGS revealed a detectable population of protozoa (SSR1301141) (Table 1). Of note, the primary organism detected by DNA BLAST analysis (69.4%) was most closely related to *Perkinsus qugwadi*,

a known aggressive oyster pathogen [26]. In retrospect, this patient acknowledged lifelong consumption of raw shellfish.

## Atherosclerosis angioplasty debris

A sterile collection cup was received containing debris obtained from an elective percutaneous transluminal angioplasty of the internal carotid artery (ICA) performed at St. Luke's Catheterisation Unit, Phoenix, Arizona, USA. This sample was obtained from a 72-year-old Caucasian male with obstruction of the left ICA. Analysis of the angioplasty debris derived from the procedure by

**Table 1.** Protozoal sequence findings per sample

Sample	Type	Total sequences	Identified species	Sequence counts	% Contribution*
CFS	B	29857	<i>Oblongichytrium species*</i>	15738	80.6
			<i>Schizochytrium minutum*</i>	2961	15.2
			<i>Schizochytrium aggregatum*</i>	465	2.4
FM	F	2671	<i>Chrysocapsa vernalis*</i>	9	64.3
MS	B	7705	<i>Ochromonas danica</i>	136	84.0
			<i>Ochromonas danica*</i>	16	9.9
ALS	B	130353	<i>Perkinsus qugwadi*</i>	38706	69.4
			<i>Aristerostoma marinum*</i>	2602	4.7
			<i>Perkinsus species*</i>	2576	4.6
			<i>Colponema edaphicum*</i>	1761	3.2
			<i>Allantion species</i>	1698	3.0
			<i>Aureococcus anophagefferens*</i>	1487	2.7
			<i>Dinophyceae species*</i>	1353	2.4
			<i>Spumella species</i>	855	1.5
			<i>Perkinsus mediterraneus*</i>	736	1.3
			<i>Vorticella convallaria</i>	627	1.1
AP	A	4825	<i>Perkinsus qugwadi*</i>	510	68.3
			<i>Ochromonas species</i>	87	11.6
			<i>Ochromonas species*</i>	75	10.0
			<i>Allantion species</i>	38	5.1
			<i>Chromophyton vischeri*</i>	11	1.5

A – angioplasty debris; B – blood sample; F – filament; NSS – no significant sequences; \*BLAST hit has < 97% identity and < 10-75 eValue or matches another 100% identity call, but has < 95% identity and < 100bp; \*does not total 100% as low probability sequence calls (< 5 or < 1%) are not reported

fluorescence microscopy revealed a complex amorphous group of prokaryotic-like and eukaryotic-like organisms (Fig. 1F). A mixed population of potentially opportunistic bacteria (SSR1300599) and two different types of protozoa were identified (SSR1300602) through NGS and subsequent metagenomic analysis. The primary identified bacteria were *Blastobacter denitrificans* (22.6%), *Rubrivivax gelatinosus* (17.8%), and *Streptococcus mitis* (7.9%) (Table 2). The primary protozoal contributors were identified as another divergent *Perkinsus qugwadi*-like species (68.3%) and an *Ochromonas* sp. (11.6%) (Table 1).

## LITERATURE REVIEW

### Systemic lupus erythematosus

It appears that for the provocation and progression of the SLE, Epstein-Barr virus (EBV) may play a role, although it is not the primary factor responsible [27, 28]. A current model of this disease is that molecular mimic-

ry by EBV could be responsible for the cross-reactivity, ultimately giving rise to the production of autoantibodies [29]. The epitopic mimicry hypothesis has also been examined for links with *Burkholderia* sp. because dsDNA reactive antibodies exhibit a high affinity for antigens from this class of organisms [30]. In the mouse model of SLE an infection by *Toxoplasma* sp. is protective against disease progression, probably through immunomodulatory effects [31]. A series of genetic risk factors have been identified as being associated with SLE. Nonetheless, they do not seem to be responsible for the development of the disease but rather for exhibiting immunomodulatory functions [32]. Mutations in the FcγRIIb receptor are known SLE risk factors that appear to result in a hyperactive immune response, increased risk of SLE, and increased resistance to infection [33-36]. In the malaria FcγRIIb-deficient mouse model, an enhanced resistance and clearance of malarial parasites has been noted [37]. It is possible that continual induction of the immune response via a chronic infection in the context of immune

**Table 2.** Bacterial sequence findings per sample

Sample	Type	Total Sequences	Identified Species	Sequence Counts	% Contribution <sup>‡</sup>
<b>CFS</b>	<b>B</b>	<b>13383</b>	<b>NSS</b>	<b>NA</b>	<b>NA</b>
FM	F	86834	<i>Polaromonas naphthalenivorans</i> *	92	27.6
			<i>Rubrivivax gelatinosus</i>	43	12.9
			<i>Pelomonas saccharophila</i>	38	11.4
			<i>Smithella propionica</i> *	17	5.1
			<i>Bdellovibrio bacteriovorus</i> *	14	4.2
			<i>Sinomonas atrocyanea</i> *	11	3.3
			<i>Mucilaginibacter daejeonensis</i> *	10	3.0
			<i>Acinetobacter baumannii</i> *	8	2.4
			<i>Polynucleobacter necessarius</i> *	6	1.8
			<i>Mucilaginibacter daejeonensis</i>	5	1.5
MS	B	27998	<i>Propionibacterium acnes</i>	7	53.8
			<i>Ralstonia solanacearum</i>	6	46.2
<b>ALS</b>	<b>B</b>	<b>41555</b>	<b>NSS</b>	<b>NA</b>	<b>NA</b>
AP	A	55973	<i>Blastobacter denitrificans</i>	339	22.6
			<i>Rubrivivax gelatinosus</i>	267	17.8
			<i>Streptococcus mitis</i>	119	7.9
			<i>Xylophilus ampelinus</i>	96	6.4
			<i>Streptococcus pseudopneumoniae</i>	77	5.1
			<i>Streptococcus marimammalium</i>	76	5.1
			<i>Curvibacter delicatus</i>	71	4.7
			<i>Leptothrix mobilis</i>	69	4.6
			<i>Rhodopseudomonas palustris</i>	43	2.9
			<i>Roseateles depolymerans</i>	26	1.7
			<i>Blastomonas natatoria</i> *	25	1.7
			<i>Methylotenera mobilis</i>	25	1.7
			<i>Paucibacter toxinivorans</i>	23	1.5
			<i>Streptococcus pneumoniae</i>	22	1.5
			<i>Rhizobium lupini</i>	19	1.3
			<i>Diaphorobacter nitroreducens</i>	15	1.0

A – angioplasty debris; B – blood sample; F – filament; NSS – no significant sequences; \*BLAST hit has < 97% identity and < 10<sup>-75</sup> eValue or matches another 100% identity call, but has < 95% identity and < 100 bp; ‡does not total 100% as low probability sequence calls (< 5 or < 1%) are not reported

hyperactivity may contribute to the total SLE risk. It is interesting to note that a major accepted therapy for SLE includes anti-malarial medications, specifically chloroquine and hydroxychloroquine [38-42]. Thus, there are several lines of evidence supporting the idea that a genetic predisposition for immunohyperactivity in conjunction with molecular mimicry may be associated with the initiation of SLE.

## Chronic fatigue syndrome

Chronic fatigue syndrome is closely related to FM and Gulf War Veterans' Illness by several overlapping clinical features [43]. Several infectious aetiologies have been scrutinised for association with CFS. Acute infections by viral and non-viral pathogens have been associated with the subsequent development of CFS in a subset of

patients [44]. Persistent enteroviruses have been reported in patients with CFS, and there is some symptomology overlap with irritable bowel syndrome [45, 46]. Most recently, 142 patients with CFS and herpesvirus infections were treated with some reported degree of success [47]. A number of studies cite the role of *Mycoplasma* sp. and *Coxiella burnetti* in CFS [48-54]. Currently there are few publications regarding a potential role of protozoa in CFS. Infection by intestinal parasite *Giardia* sp. has also been correlated with CFS [55, 56]. It has also been considered, based on a case whereby *Acanthamoeba* sp. was detected in a previously diagnosed CFS patient [57], that features of CFS may be due to an immunocompromised status. It is also of note that CFS patients present with significantly increased antibody titres to various *Apicomplexan* protozoa, such as *Toxoplasma gondii* and *Sarcocystis* sp. [58]. Then, CFS appears to have an infectious component. However, it is unclear if the direct activity of an infection or if an immunological failure is responsible for the disease presentation.

## Fibromyalgia

Bacteria have been studied to some extent, with various *Mycoplasma* species and viral organisms showing some level of non-trivial prevalence. However, neither significant clinical impact nor adoption of new treatment modalities has resulted from this research [52]. Doxycycline, an adjunctive medication in the treatment of malaria, has been utilised in the treatment of FM patients [53, 59], and this drug has been shown to be effective, although the mechanism was proposed to be due to a global anti-inflammatory effect [60, 61].

## Multiple sclerosis

A number of studies have been published on the potential protozoal role in MS. Reports dating back to the late 1800s support and suggest that a malaria-like organism might be associated with MS [62, 63]. It is interesting to note that seasonal tick emergence, and potentially other disease vectors, have geographic and timing associations with the prevalence of MS [64]. Furthermore, many studies have shown efficacy of doxycycline and minocycline therapy in the treatment of MS [65-73]. It has been speculated that the mechanism of these antibiotics in the treatment of MS is related to their anti-inflammatory effects, but such an effect has not been conclusively demonstrated. Of note, doxycycline is used for the prophylaxis and treatment of malaria [74-81].

## Amyotrophic lateral sclerosis

Arguably one of the most studied and most conflicting series of results is that regarding the potential viral aetiology of ALS [82-84]. Suspected culprits include

polioviruses [85], enteroviruses [85-90], herpesviruses (HHV-6, HHV-7, HHV-8 [91-93]), echoviruses (ECHO6 and ECHO7 [92, 94]), and even human endogenous retroviruses [95]. This line of inquiry is likely spurred by the continued findings of reverse transcriptase activity in patients with ALS [96, 97] and reversible ALS-like symptoms that are sometimes observed in HIV patients [98-101]. However, anti-retroviral therapies have yielded no improvements in patient outcome [102]. Bacteria have also been studied, whereby *Borrelia burgdorferi* (the causative organisms for Lyme disease) [103, 104], *Mycoplasma* sp. [105], and cyanobacteria have all been considered as potential associated factors [106]. It has been reported, as with multiple sclerosis, that the seasonal emergence of ticks is connected with ALS risk [64]. It is clear that patients who suffer from ALS have an increased risk of acute sepsis; however, that risk is elevated post-diagnosis [107]. In addition, there continues to be concern about transplanting organs from diseased ALS patients to healthy individuals as the possibility of transmission of the disease has not been ruled out [108].

## Atherosclerotic arterial plaque

Microbial and viral involvement in atherosclerosis has been previously hypothesised and studied [109]. The presence of *Cytomegalovirus* has been reported in atherosclerotic plaques in 105 patients with acute coronary syndromes [110]. A cell biology potential of macrophages to transition to foam cells has been suggested whereby macrophages have reduced liver X receptor- $\alpha$  in atherosclerotic arteries when infected with *Chlamydia pneumoniae* [111]. In further support, *Chlamydia pneumoniae* antigens have been detected in coronary atheroma [112] as well as early atherosclerotic plaques [113]. However, an analysis of arterial plaques failed to detect *Chlamydia pneumoniae*, HSV1, or *Cytomegalovirus*, but EBV was detected [114]. Additionally, a wide range of oral flora has been detected using PCR amplification techniques in vascular disease samples [115]. It is interesting to note that *Porphyromonas gingivalis* [116], *Streptococcus mutans* [117], and polymicrobial infections [118] have been shown to accelerate inflammatory atherosclerosis in ApoE mutant mice. To further support the dental flora hypothesis, aortic endothelial cells are invaded by viridans group of Streptococci and have been shown to induce an inflammatory response [119]. Invasion by microbes also appears to favour other host changes that influence arterial plaque formation, as in the case where *Porphyromonas gingivalis* accelerated atheroma formation by altering the lipid profile in the host [120]. *Helicobacter* sp. has also been detected in atherosclerotic plaques [121]. Indirectly, bacterial signatures have been found in thrombi derived from patients with myocardial infarction [122]. It has been suggested that statins modify cardiovascular risk via antimicrobial activity rather than through modification

of lipid profiles [123]. Taken in total, no common thread beyond sporadic and inconsistent microbial involvement has been identified, and currently no studies have specifically assessed protozoal involvement in arterial plaque or in the debris resulting from angioplasty.

## DISCUSSION

Here we have demonstrated the presence of mixed putative biofilm communities in six patients with a range of chronic inflammatory and autoimmune diseases. The identified bacteria include both known and potentially novel organisms. All samples have prominent protozoan components as suggested by microscopy and by clinical metagenomic analysis. The sequences identified in SLE are consistent with a novel organism, *candidatus Protomyxzoa rheumatica* (GenBank# KJ914572). In FM, MS, ALS, and angioplasty debris the identified protozoa are potentially of aquatic origin. Generally, the primary identified protozoa are potentially related to known pathogens of animals, thus raising the possibility of zoonotic infection or transmission. It is possible that these organisms may enter a human host via insect vectors, contaminated water, or contaminated food.

It is quite possible that these microorganisms, with additional inquiry, may be eventually considered a benign human flora, in a similar way to how the Human Microbiome Project has expanded the understanding of the diversity of normal intestinal microbiota [124]. Most of the bacteria identified in our samples could be considered incidental, as their opportunistic or non-pathogenic cladistic assignments suggests. However, the potential pathogenic nature of the others, primarily that of detected protozoa, warrants additional inquiry.

The greatest cause of death in the modern world is cardiovascular disease. Various microbes have been demonstrated in atherosclerotic plaques. However, no targeted metagenomic analysis of angioplasty debris has been previously performed. Previous research has linked the dependence of parasitic apicomplexans, such as *Babesia* sp., *Toxoplasma gondii*, and *Plasmodium* sp., to host lipid and cholesterol profiles [125-129]. Additionally, some aspects of host immunological response appear to be-in part-affected by serum lipoproteins and lipid content [130, 131]. Taken into consideration with our arterial angioplasty findings, a shared mechanism between host lipid levels, microbial dependence, and immune system modulation suggests that atherosclerosis may be a multifactorial process that includes a previously unrecognised polymicrobial component. To date, no studies have extensively targeted protozoa in atherosclerosis; although previous reports have indicated the presence of aberrant eukaryotic cells [132] in addition to well-studied inflammatory processes. These results may be consistent with a complex community of bacteria and protozoa in the developing atherosclerotic plaque.

Detection of potentially pathogenic protozoa may be of great clinical significance. Fungal and archaea contribution were not specifically targeted. However, our primer pairs have been shown to amplify a significant portion of these organisms. In the future we plan to specifically target fungi, archaea, and viral contribution using a similar metagenomic approach.

Intravascular abnormalities, such as webs and septa, as well as other unusual findings observed via ultrasound (so-called chronic cerebrospinal venous insufficiency – CCSVI) have been reported in MS patients [133-137]. It may be that similar structures to those observed in the FM case (large thrombotic-like strands) may also be present in MS patients. However, endovascular attempts to remove these blockages have had varying degrees of success in reversing symptoms of MS [134, 138-154]. The formation of streamers by bacterial biofilms in a cardiovascular stent model has been shown to produce a dramatic reduction in flow rate similar to that seen in CCSVI [155]. We hypothesise that the haemodynamic flow change observed in CCSVI may be due to such a polymicrobial biofilm phenomenon.

Additional corroborating data that indicates involvement of microbes in these diseases include treatments that have been demonstrated to be efficacious in the absence of a clear mechanism of action. Therapy for SLE includes chloroquine hydrochloride, which is also an anti-malarial drug used worldwide [38-42, 156]. Anti-malarial treatments including quinine derivatives, doxycycline, and minocycline have also been shown to be efficacious in the management of MS [65-67, 69-73, 157]. In CFS, the use of doxycycline has been shown to be efficacious and produce symptom improvement [59, 158]. There have been anecdotal reports of ALS patients utilising antimicrobials and experiencing extended survival rates; however, no comprehensive study exists to date. It is also important to note that antimicrobial therapies are not curative in these diseases. It is unclear if this is most consistent with these organisms being involved in the disease progression if these organisms are inherently recalcitrant as observed in polymicrobial biofilm communities, or if their presence is a consequence of dysfunction of the immune system in the patient.

In light of the reported results, clinical observations, and current literature we propose the following scenario in these polymicrobial biofilm infections. An initial insult is the protozoan entry into the bloodstream, probably vector mediated (mosquito, tick, or another arthropod), although parenteral routes are also possible. An incubation period of days to weeks ensues, and then there is a time of illness with malaise and flulike symptoms. In the majority of affected individuals there is a remission, and some may experience persistent malaise and progressive clinical symptoms. If such a microorganism persists in a biofilm community, it may become protected from immune and inflammatory responses by polysaccharides,



nucleic acids, and peptides building the biofilm scaffold. In periods of emotional stress, illness, trauma, or dietary excess these biofilm communities may resurge and spread as immune surveillance is curtailed. In addition, biofilm can be permanently attached to the vascular wall. Due to quorum sensing and other mechanisms, excessive growth of such a microbial community and parasitic burden is diminished. However, possible cracks in the biofilm could expose the underlying organisms to the immune system, initiating response of the human host. Such disruptions in the biofilm matrix and immune system recognition may be intermittent, which may explain the relapsing and remitting nature of MS.

## SUMMARY

Published evidence does not exclude, and in some instances supports, the involvement of protozoa in many of the above-presented diseases. The role of protozoa in human disease is well known. Malaria, for example, infected more than 219 million and killed 660,000 worldwide in 2010 alone. It is well known that biofilm-dwelling bacteria play a significant role in chronic human disease. Here we suggest that chronic, biofilm-based protozoal infections may be at the heart of a variety of chronic human diseases. Interestingly, in all our patients, except for the patient with SLE (where DNA stains and culture studies revealed a novel microorganism: *candidatus Protomyxzoa rheumatica*), we demonstrated protozoa being close relatives or already known aquatic-based protozoa.

Based on our results, we hypothesise that biofilm-forming protozoa may represent a non-trivial component in human disease and that the human vascular system may be a significant site of colonisation. It was through a number of technological innovations: DNA extraction technology, NGS, and clinical metagenomic analysis, that this work and future projects are possible. It is plausible that there may be previously unrecognised vascular polymicrobial biofilm phenomena that could trigger, potentiate, or contribute to vascular and immunological dysfunction.

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