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Invasion of chronic lymphocytic leukemia (cll/sll) bloodstream tumor cells by borrelia spirochetes

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Abstract

A sixty-six-year-old woman with a strong family history of breast cancer noted gradual onset of axillary lymph node enlargement in 2019. Axillary lymph node biopsies disclosed Lymph node involvement by Chronic Lymphocytic Leukemia (CLL/SLL). Immunophenotype analysis of nodal tissue and of bloodstream leukemic cells in immunohistochemistry and flow cytometry and next generation molecular diagnostics confirmed CLL/SLL. Replicate absolute peripheral blood lymphocyte counts in excess of 5.0x10e3/microliter confirmed the numerical threshold for diagnosis of Chronic Lymphocytic Leukemia in the bloodstream. FISH studies disclosed an immunoglobulin "mutation negative" CLL/SLL genotype without a light chain restriction pattern. No translocations or germline mutations were present. Borrelia spirochetes in the bloodstream were detected with simultaneous high spirochetemia of both borrelia miyamotoi and borrelia burgdorferi. FISH hybridization of leukemic lymphocytes disclosed extensive borrelia invasion of the cytoplasm and of the nucleus compartments of CLL/SLL tumor cells. Retrospective studies of the patient's archival stored blood smears obtained three years prior in 2016 at age 63 disclosed no evidence of CLL/SLL but confirmed asymptomatic bloodstream borrelia miyamotoi and borrelia burgdorferi borrelia infection. Lyme serology had been reported as negative in 2016. Additionally, retrospective FISH DNA hybridizations focused on preleukemic bloodstream Lymphocytes disclosed rare borrelia spirochetal adherence to benign blood lymphocytes in the year 2016 in the preleukemic blood smears. This case report is the very first to describe precursor chronic asymptomatic Lyme borreliosis and Miyamotoi borrelia bloodstream infections three years prior to the diagnosis of Rai Stage 1 Chronic Lymphocytic leukemia.

Keywords: Chronic Lymphocytic Leukemia, Blood Lymphocyte Counts, Lyme Borreliosis, Miyamotoi Borrelia, Fatigue Syndromes, Neuropsychiatric Illnesses, B Cell Lymphomas, *Borrelia Burgdorferi*, DNA Probes.

Introduction

Lyme borreliosis and miyamotoi borreliosis infections have diverse clinical presentations which exceed the narrow year 2022 Centers for Disease Control and Prevention list of clinical stigmata and symptoms on the CDC website [1]. Patients who are clinically refractory to cure with antibiotic treatment for Lyme disease are estimated to constitute 20% of all physician-diagnosed Lyme borreliosis cases. Chronic infection with *borrelia burgdorferi* and *borrelia miyamotoi* includes both symptom free patients and patients with a wide spectrum of symptomatic cases from chronic pain and fatigue syndromes and neuropsychiatric illnesses to some chronic Lyme patients with suicidal ideation or death. Hematologic diseases including cutaneous small lymphocytic malignant lymphomas in Italian and European patients have been diagnosed in association with patients with concurrent *borrelia burgdorferi* infections [2-10]. Miyamotoi borrelia infections concurrent with nodal B cell lymphomas have been diagnosed in rare patients from Europe and from the USA [11,12].

Dorward and colleagues documented in 1987 with the electron microscope in an in vitro laboratory model that borrelia spirochetes show a tropism for attachment to and penetration of human lymphocytes [13,14]. Lyme meningitis may present as a marked tumor-like excesses of lymphocytes and plasmacytoid cells which resemble malignant lymphoma in the cerebrospinal fluid and in the meninges [15-17]. Borrelia cutis infections may mimic lymphocytic Lymphomas of either the diffuse type or of the follicular type. Borrelia can induce the formation of "Ectopic" germinal centers in cutaneous Borrelia lymphocytoma sites which rarely have been misinterpreted as deposits of follicular pattern lymphoma in the dermis[18,21].

In 2012, a commentary by Stricker and Johnson [21] called attention to the diagnostic shortcomings of current negative blood serology testing techniques for Lyme disease related antibodies and warned that negative Lyme serology test results in physician diagnosed cases of Lyme borreliosis might be augmented by direct microscopic detections of borrelia

infections in blood and in tissue specimens in patients. Such chronic active borrelia infections without a concurrent malignant diagnosis and possibly might guide clinical thinking in select human malignancies which co-exist with chronic borrelia infections. Stricker and Johnson discussed chronic lymphocytic leukemia as a possible disease condition for research scrutiny for associated borrelia infections would be germane [23].

This case report begins with a previously healthy woman with previous asymptomatic mixed species borrelia bloodstream infection three years previously, who in year 2019 palpated enlarged lymph nodes in her axilla. Her family history was positive for breast cancer and remarkable for one brother with a 30 year history of CLL/SLL who is alive and well on Rituxan therapy. Needle biopsies demonstrated increased numbers of small lymphocytes permeating bilateral axillary nodal sites as diffuse infiltrates. Immunohistochemistry confirmed a diagnosis of Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL). FISH studies of the lymph nodes determined a "mutation negative" light chain genotype CLL/SLL. Her peripheral blood in Flow cytometry and in immunohistochemistry (IHC) nodal studies demonstrated that her leukemic cells were positive with the following markers: CD5 (Dim), CD19, CD 23(bright). Kappa and Lambda stains disclosed no light chain restriction pattern. Radiology imaging confirmed enlargement of lymph nodes in bilateral axillary and scapular sites without enlargement of her spleen of liver and without lymphadenopathy in other sites. She was determined to be clinically stable, and she did not receive chemotherapy.

Three years prior to the discovery of her CLL/SLL diagnosis, in 2016 the patient had volunteered to participate in a pro bono research study directed by this author to evaluate the use of microscopy to search for borrelia burgdorferi in her peripheral blood. She had not received a Lyme vaccine previously and was ELISA seronegative for Lyme related antibodies in 2016. Bloodstream borrelia was identified with acridine orange staining and her absolute counts of bloodstream lymphocytes were normal in number and in morphology. She did not receive antibiotic therapy. In 2022, after being notified of the year 2019 diagnosis of CLL/SLL, the author retrieved from storage archival blood smears from 2016. DNa probes in FISH method confirmed positive hybridizations for Borrelia gene sequences in single separate borrelia in the bloodstream and biofilm communities diagnostic of chronic blood stream infection. Leukemic phase blood smears in 2022 also showed persistence of chronic biofilm positive double borrelia species infection. The author then commenced a search for possible invasion by borrelia of leukemic lymphocytes in the year 2022 blood films motivated by the Italian patient reports of concurrent B cell lymphomas in skin sites and concurrent Borrelia burgdorferi infections.

Methods

Blood Smears

EDTA anticoagulated peripheral blood was smeared onto clean glass slides, and the slides were air dried, and heat fixed, followed by post fixation in 99% isopropyl alcohol, and again air dried and stored for use in FISH studies.

Wright Giemsa Stains

Blood smears were stained with Wright Giemsa stain according to the manufacturer's recommendation (Volusol Inc).

Immunohistochemistry

Monoclonal antibody CB10 [24], which uniquely binds to borrelia burgdorferi Outer surface protein A (OSPA) conjugated to Horseradish peroxidase was layered over blood smears previously fixed in absolute alcohol and incubated at room temperature for 30 minutes followed by triplicate washes with PBS pH 7.0. Diaminobenzidine chromogen (Vector Labs catalog SK 4105 ImmPACT DAB substrate kit, peroxidase) was applied according to the manufacturer's recommendations. All positive and negative controls produced expected results.

FISH

Fluorescence in situ hybridization was completed as previously described. Briefly, single DNA probes were individually dropped onto the blood films and the slides were then flooded with 100% DMF reagent. The flooded slides were then individually heated (Not cover slipped) to 115 Degrees F for 30 minutes and carefully observed to assure that the DNA probes in solution did not evaporate. The slides were then removed from the heating block and were cover slipped over RPMI tissue culture medium and examined under oil immersion 1000x final magnification with an LED monochromatic light source delivered through a substage darkfield condenser to detect tissue bound sites of FISH DNA probe hybridizations. The photography was completed with a 16 MP digital camera mounted on a trinocular microscope.

FISH DNA Probe Reagents for Borrelia Species Gene Target Sites

Borrelia burgdorferi B31 strain DNA probe for **gene bbo 0147 for the flagellin B gene of *borrelia burgdorferi:***
5'-(FITC)-cacggt-**TAATCTTACCAGAAACTCCC**-accgtg–(Dabcyl)-3' {green color)
DNA probe for gene **bb00740 inner cell membrane** (common to thirty *borrelia burgdorferi* strains (GenBank) sequences:
(5"-(Cy5) -cgcgag**ATATATTCAAGCAAATTCGATGACATC**-ctcgcg-(Dabcyl -)3'). (red color)

Borrelia Miyamotoi strain LB2001 DNA probe for **gene Miyamotoi Flagellin B** (BA1_FlaB_MIY_mb3)
5'(-Cy5)-cgtccg-**CGTCAGCCATAAATGCTTC-CAGAAATAA**-ccgagc
–(BBQ650)-3' (red color)

Borrelia miyamotoi Strain LB2001: DNA probe for **gene GlpQ of *borrelia miyamotoi,*** (BA2_glpQ_MIY_MB3) (yellow color)
5'–(Cy3)-cgtcgg-**AGAACATACCTTAGAAGCTAAAG-**CATATGC-ccgagc- (BHQ_2)-3' (yellow color)

Because the patient resided in Wisconsin, which is hyperendemic for ticks carrying *borrelia burgdorferi*, *borrelia miyamotoi*, and *babesia Duncani*, special manual microscopy of Giemsa-stained

blood films was undertaken to search for babesia co-infection in peripheral blood smears. This required microscopy at 1000x oil immersion magnification to search for ring trophozoite babesia inclusions inside erythrocytes in one hundred consecutive microscopic fields of view.

Coombs method for detection antibody coated borrelia spirochetes and antibody coated borrelia biofilm communities was deployed as previously published. Coombs antiglobulin reagent labeled with a blue fluorochrome Biotium mix N stain 403 was layered over thin blood smears and incubated at room Temperature for 30 minutes, and then the slides were washed three times in PBS ph7.4. and examined in Immunofluorescence microscopy [25].

Controls

Positive Control for borrelia burgdorferi used pure cultures strain B31 borrelia burgdorferi of American Type Culture Collection ATCC 35210 in log phase culture thin smears on clean glass slides fixed in Absolute isopropyl alcohol and post alcohol heat fixed.

Positive Control for *borrelia miyamotoi* used pure cultures of Borrelia miyamotoi strain LB2001 thin smears fixed on clean glass slides (a gift from Catherine Brissette PhD, School of Medicine, University of North Dakota)

Negative tissue controls used normal autopsy liver tissue sections and peripheral blood smears from normal patient volunteers.

Negative IHC controls used Antibodies to Alpha Synuclein (StressMarq Inc) and antibodies to Cytokeratin Ae1/Ae3 (SantaCruz Biotechnology Inc).

Negative Controls for FISH used A Nonsense DNa probe Gn2, which by design functioned as a negative control in FISH studies was designed such that its structure does not correspond to any known DNa sequence in GenBank deposits at the NCBI NIH site.
5' (-Marina Blue NHS AmMC6)-cgcgat- **TCTCCGAACGTGTCACGC**-gtcgcg-(BHQ_1)-3' (Nonsense probe)

Results

Blood smears from 2016 showed normal lymphocyte morphology in Wright Giemsa stains in peripheral blood lymphocytes. Rare benign blood lymphocytes demonstrated questionable borrelia like forms in adjacent to lymphocyte cell membrane sites. No evidence of spirochetes compatible with borrelia inside lymphocyte nucleus compartments was found (Figure 1).

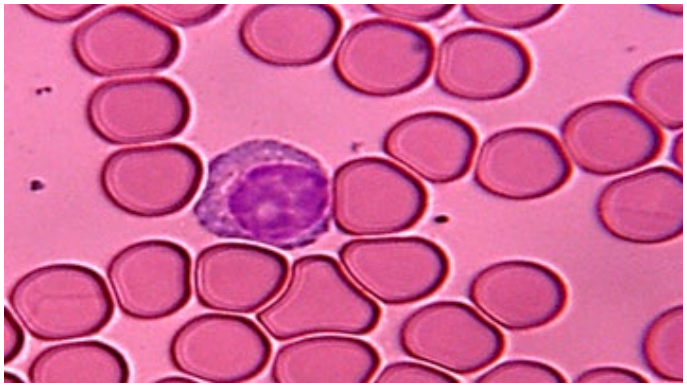

Figure 1: Giemsa stain of a benign mature small lymphocyte in preleukemic blood smear from 2016.

A normal chromatin pattern is present. No borrelia spirocheteare present in this field of view.
Magnification 1000x original.

Blood smears from year 2022 blood films demonstrated leukemic nuclei of larger caliber lymphocytes than those seen in the 2016 blood lymphocytes. Giemsa stains disclosed spirochetes compatible with borrelia inside leukemic cells. The full hematopathology repertoire of Immunohistochemical staining CD markers confirmed chronic lymphocytic leukemia/ small lymphocytic lymphoma (Figure 2).

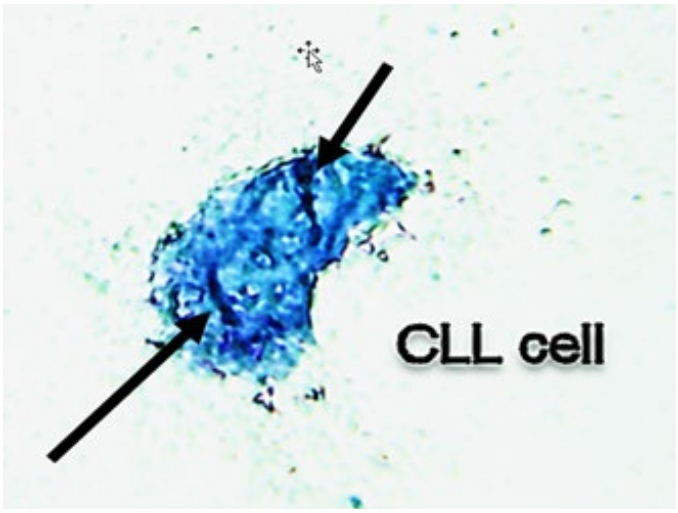

Figure 2: Giemsa stain of Leukemic Lymphocyte which contains centrally positioned borrelia spirochetes (2) black arrows which appear to reside inside the nucleus in the same optical plane of focus. Note: The enlarged nucleus shows an immature open uncondensed chromatin pattern with several prominent white color nucleoli. Magnification 1000x original.

Immunohistochemistry studies in 2016 blood smears demonstrated borrelia specific protein OSP A borrelia spirochetes attached to the cell membrane of rare blood benign lymphocytes (Figure 3).

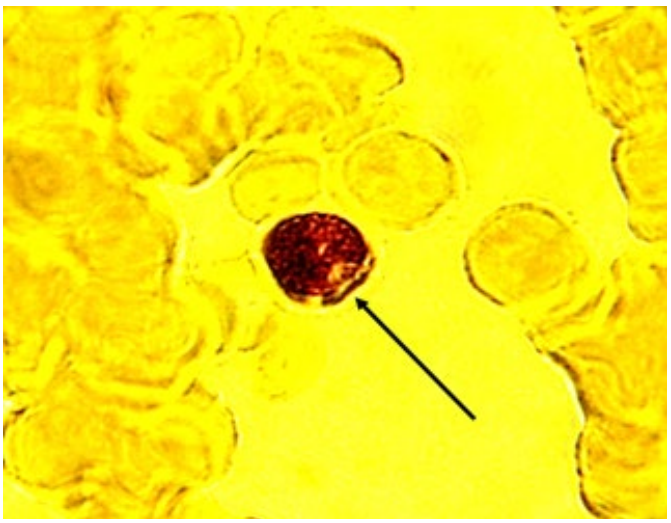

Figure 3: Immunohistochemistry with Monoclonal antibody CB10 specific for protein OSP A of borrelia burgdorferi group sl spirochetes. This stain shows a benign mature small caliber lymphocyte with a DAB stained borrelia spirochete with black color attached to the cell membrane at 3 o'clock to 6 o'clock. Magnification1000x original.

Extensive invasion of leukemic blood lymphocytes as found in 2022 smears in immunohistochemistry detection of Outer surface protein A of borrelia burgdorferi resident inside leukemic cells. IHC studies for borrelia miyamotoi specific proteins were not undertaken due to the lack of available commercially available antibodies to borrelia miyamotoi protein GLPQ and due to the availability of FISH DNa hybridization results for the GLPQ gene expression in the borrelia resident inside the patient's leukemic lymphocytes (Figure 4).

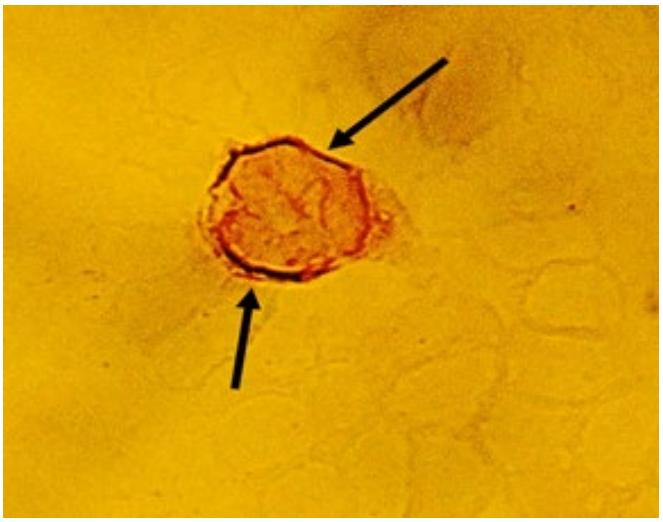

Figure 4: Immunohistochemistry with Monoclonal antibody CB10 specific for protein OSP A of borrelia burgdorferi group sl spirochetes. Two borrelia spirochetes (Black arrows) are attached to a large caliber leukemic lymphocyte. Magnification 1000x original.

FISH studies of smears from 2022 demonstrated borrelia DNA content inside live *borrelia burgdorferi* and *borrelia miyamotoi* spirochetes which have invaded leukemic lymphocytes in blood. Leukemic cells contained both *borrelia burgdorferi* (sl) group spirochetes and *borrelia miyamotoi* spirochetes. Cytoplasmic and nuclear compartments of the leukemic cells contained intact borrelia spirochetes (Figure 5, 6).

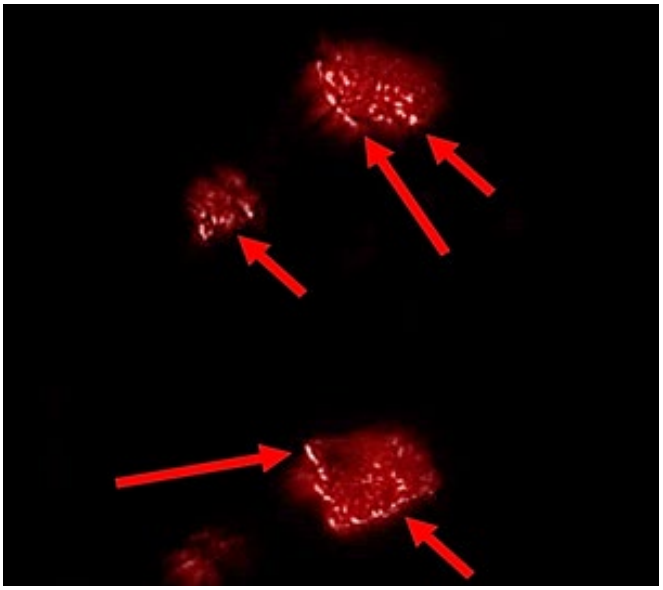

Figure 5: Red arrows point to strong signal hybridizations of DNA probe bbo0740-Cy5 inside the nucleus of three leukemic cells. Magnification 400x original.

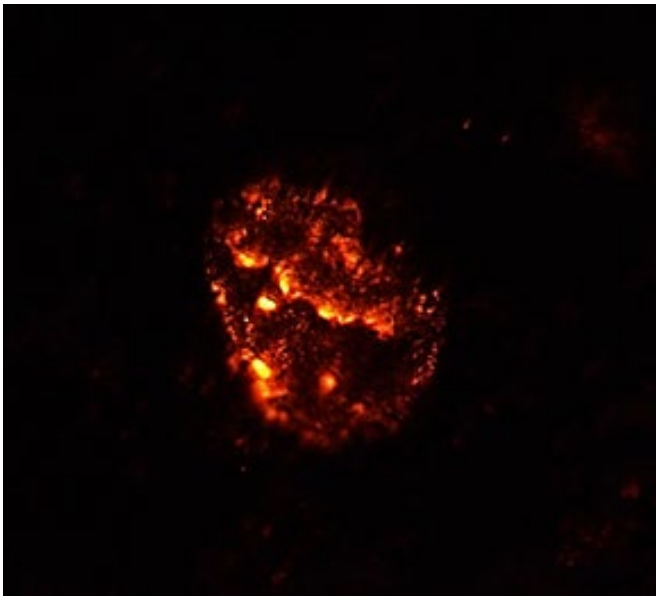

Figure 6: Yellow fluorescence signals inside a leukemic cell designate sites of Hybridization of DNA probe GLPQ-Cy3 in several spirochetes and spirochetal fragments inside a tumor cell. Magnification 1000x original.

Wright Giemsa-stained blood smears from 2022 disclosed Babesia ring trophozoites in red blood cells. The species of babesiosis was not determined. The estimated percentage of parasitized RBC was between 1% and 5% in serial counts of 1000 erythrocytes in oil immersion magnification examinations (Figure 7).

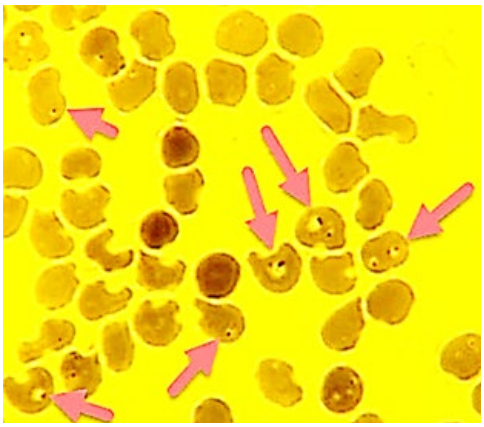

Figure 7: Arrows point to Giemsa stained babesia species ring trophozoites (species compatible with either Babesia Duncani or Babesia microti) which have invaded erythrocytes. Magnification 1000x original.

Immunofluorescence microscopy detected blue fluorescent staining of single borrelia spirochetes coated with Coombs antibody and of biofilms of borrelia coated with Coombs antibody (Figure 8).

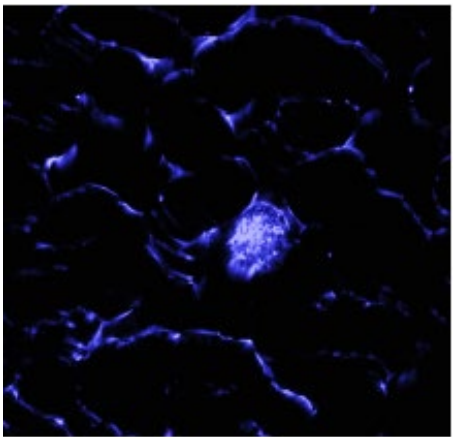

Figure 8: Leukemic blood (2022) with Antibody coated borrelia spirochetes in Coombs method.

All positive and negative controls in Immunohistochemistry with *borrelia burgdorferi* and with *Borrelia miyamotoi* DNA probes returned expected results. *Borrelia burgdorferi* and *borrelia miyamotoi* FISH specific DNa probes returned expected positive and negative results with the cultured reference glass slide control material. The nonsense DNa probe returned negative hybridization results in FISH studies.

Discussion: Asymptomatic bloodstream Borrelia infections with dual species burgdorferi and miyamotoi borrelia were found in preleukemic blood smears from year 2016. Lymphocytes in blood smears from 2016 were normal in appearance and in total lymphocyte absolute counts in blood in 2016. Retrospective FISH studies of 2016 blood smears with DNa specific gene probes disclosed parasitic borrelia spirochetal hybridization signals adherent to the cell membranes of rare benign lymphocytes.

Leukemic phase asymptomatic Borrelia infections in year 2022 presented with dual species burgdorferi and miyamotoi borrelia. Double infection of lymphocytes in blood smears from 2022 with burgdorferi and miyamotoi borrelia species were established with FISH studies. The leukemic lymphocyte cytomorphology in 2022 demonstrated enlargement of the nuclei and an uncondensed open chromatin pattern which contrasted with the microscopy from the year 2016 blood lymphocytes whose nuclei were smaller in caliber and showed a more compact condense chromatin pattern.

The differential results from the years 2016 and 2022 FISH focused DNA hybridizations disclosed that outright penetration of the patient's tumoral lymphocytes by live borrelia spirochetes had occurred. These spatial and temporal leukemic and infection events overlap with a biological transformation in lymphocyte biology from a benign to a leukemic status as confirmed with Immunohistochemistry results which confirmed a phenotype of Chronic Lymphocytic leukemia. Mere coincidence of ongoing pre-existing borrelia bloodstream infection and the separate emergence of lymphocytic leukemia/small lymphocytic lymphoma is one possible conclusion here. However, Leukemic cell parasitism by borrelia infection is not a hallmark of usual infection in a neoplastic setting. Further FISH studies with *borrelia burgdorferi* and with *borrelia miyamotoi* specific DNA probes are needed in additional CLL/SLL patients who are residents of endemic *borrelia miyamotoi* and *borrelia burgdorferi* infection to search for evidence of borrelia infectomes inside the nuclei of lymphocytic leukemia/Lymphoma tumor cells.

The microscopic detection of simultaneous *borrelia miyamotoi* and borrelia burgdorferi infection of human lymphocytes is without precedent. Previous reports have described patients with active borrelia infections in CLL patients. These cases have been confirmed with PCR positive confirmation of *borrelia burgdorferi* DNa in biopsies from CLL/SLL patients whose illnesses include cutaneous B cell lymphomatous infiltrates. Previous authors have concluded that cutaneous B cell lymphomas with associated borrelia infections were mere consequences of the immune system impairments of patients by their CLL/SLL illnesses. In this case, host antibodies coated borrelia spirochetes in Coombs reagent testing, and this observation argues that the host serological immune system was not silenced and therefore the spatial co-localization of borrelia inside leukemic tumor cells merits further research study.

Miyamotoi *borrelia infections* concurrent with B cell Lymphomas in published case reports from two patients establish a second category of concurrent borrelia infection and B cell neoplasias. No attempt was made to evaluate the lymphoma cells for possible invasion by borrelia miyamotoi spirochetes.

The patient presented here is distinguished by seven observational findings which have not been previously described in CLL/SLL borrelia infected patients.

First, In this case study, chronic asymptomatic bloodstream *borrelia miyamotoi* and *burgdorferi infections* preceded the diagnosis of CLL/SLL by three years. Second, dual species infection with

miyamotoi borrelia and burgdorferi group sl borrelia in the bloodstream of a CLL/SLL patient is a first of kind observation. Third, outright invasion and cell membrane adherence of benign lymphocytes by borrelia burgdorferi has never previously been observed in vivo but has been documented in laboratory conditions. Fourth, outright invasion of Leukemic lymphocytes by borrelia spirochetes of any species has never previously been observed. Fifth, borrelia miyamotoi infections have been previously identified in patients with B cell lymphoid malignancies but have never been shown to invade neoplastic B cells.

Sixth, biofilm communities of borrelia spirochetes which, confirm a diagnosis of chronicity of infection ,were identified in patient blood smears in her preleukemic blood in year 2016 and were identified in her leukemic blood smears in year 2022. Biofilm infections are a type of chronic infection which has been linked to oncogenic activity in several human malignancies. Seventh, Leukemic blood smears demonstrated Babesia ring trophozoites inside erythrocytes in 2022 which declare emergence of babesia co-infection which was not present in her 2016 blood smears. Eighth, the borrelia spirochetes in preleukemic blood smears from 2016 and in leukemic blood smears were coated with the patient's antibodies in a Coombs method test indicating that the patient's immune system generated a serological host response to borrelia infections (Figure 8).

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Disclaimer Statement

The author has no conflict of interest in connection with this clinical case report.

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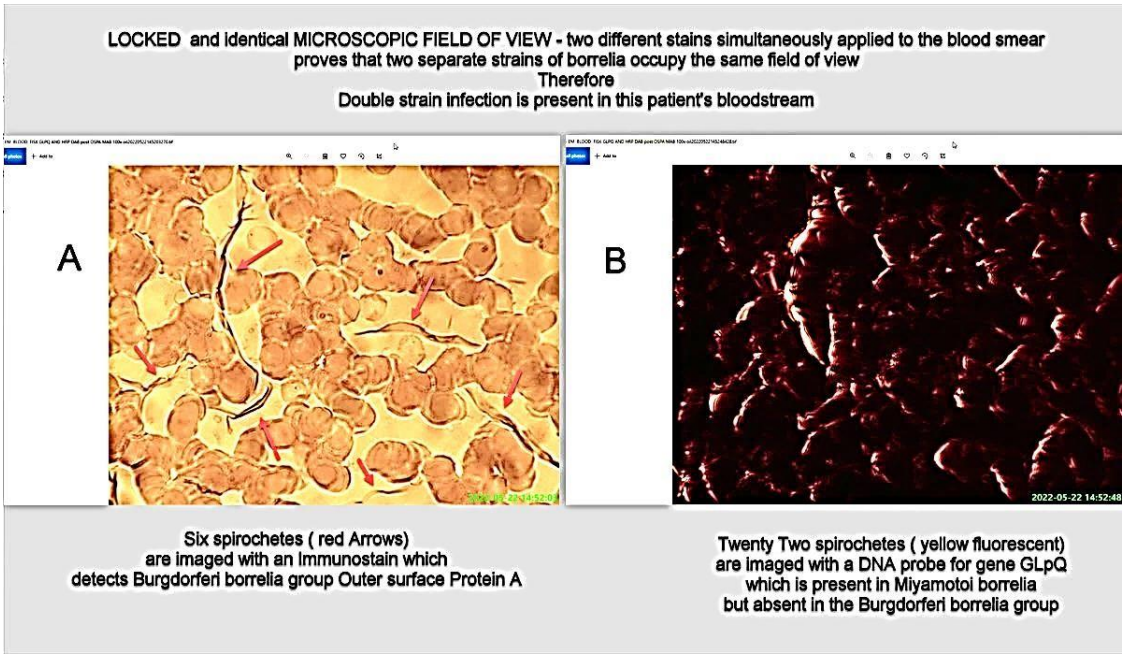
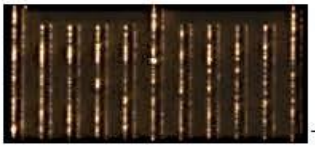
Supplementary File S1:

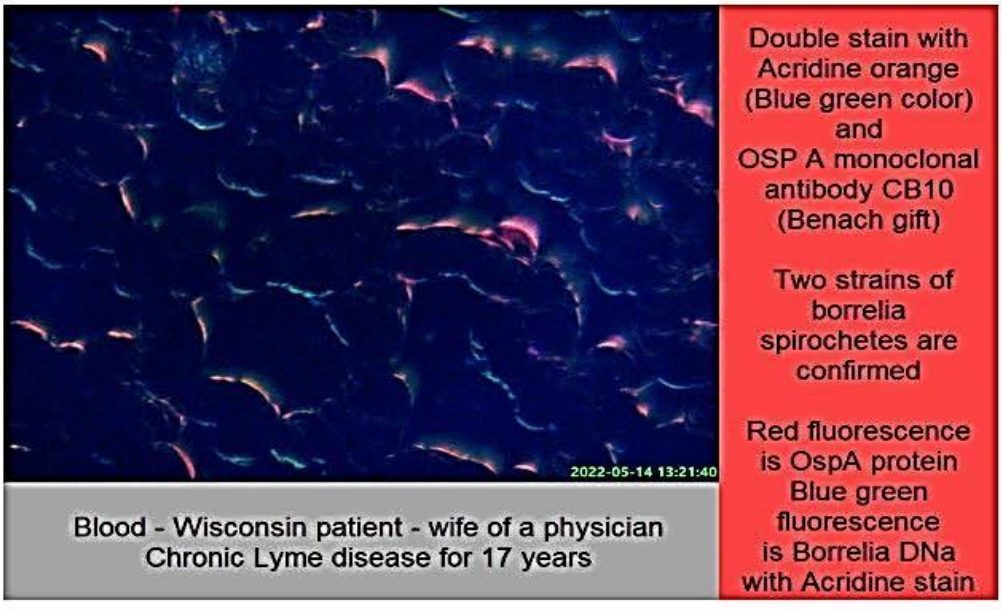
Bloodstream infection with dual species of borrelia (Burgdorferi and Miyamotoi)



Supplementary File S2:



Ten Microns per division



Dual species infection (borrelia burgdorferi (red color – Monoclonal Ab CB10 – OspA specific) and

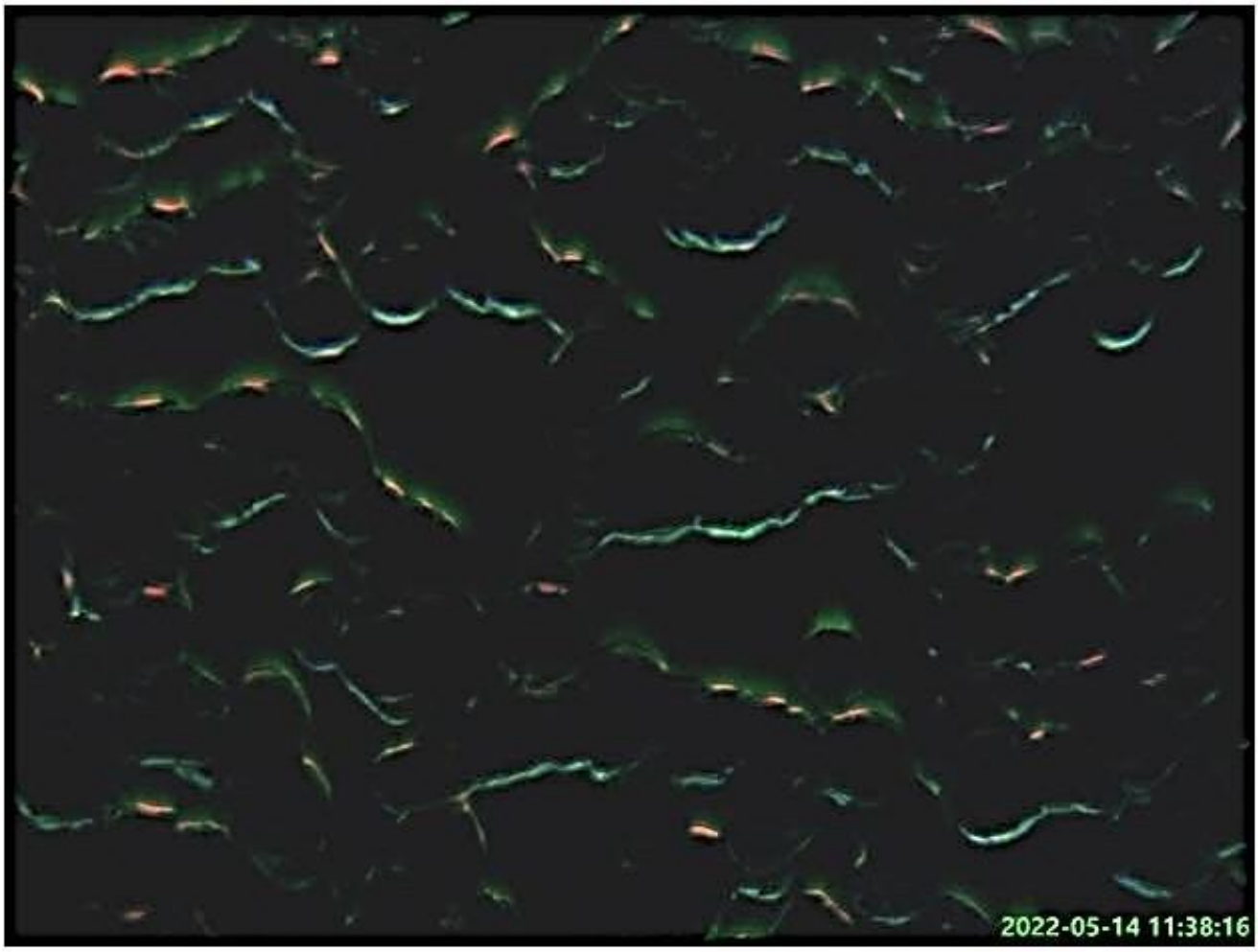
Borrelia miyamotoi – blue color – Miyamotoi borrelia does not contain OspA protein

400 x final magnification – IHC with CB10 monoclonal Ab and mixed with Acridine orange stain – blue color

Note: Protein studies demonstrate two populations of borrelia spirochetes; one population displays

Red color fluorescence signals of Protein OSPA and one population which is negative for red color signals (No content of Protein OSP A). Miyamotoi borrelia lack Protein OSP A. Burgdorferi borrelia group (SL) universally contain protein OSPA.

Supplementary File S3:



Borrelia burgdorferi spirochetes (red color) and borrelia miyamotoi spirochetes (blue color)

Indicate dual species infection in this patient. Final Magnification 1000 x original.

Note: Select spirochetes display both red color and blue color signals. This is suggestive of a chimeric borrelia microbe which contains dual borrelia species gene DNA.

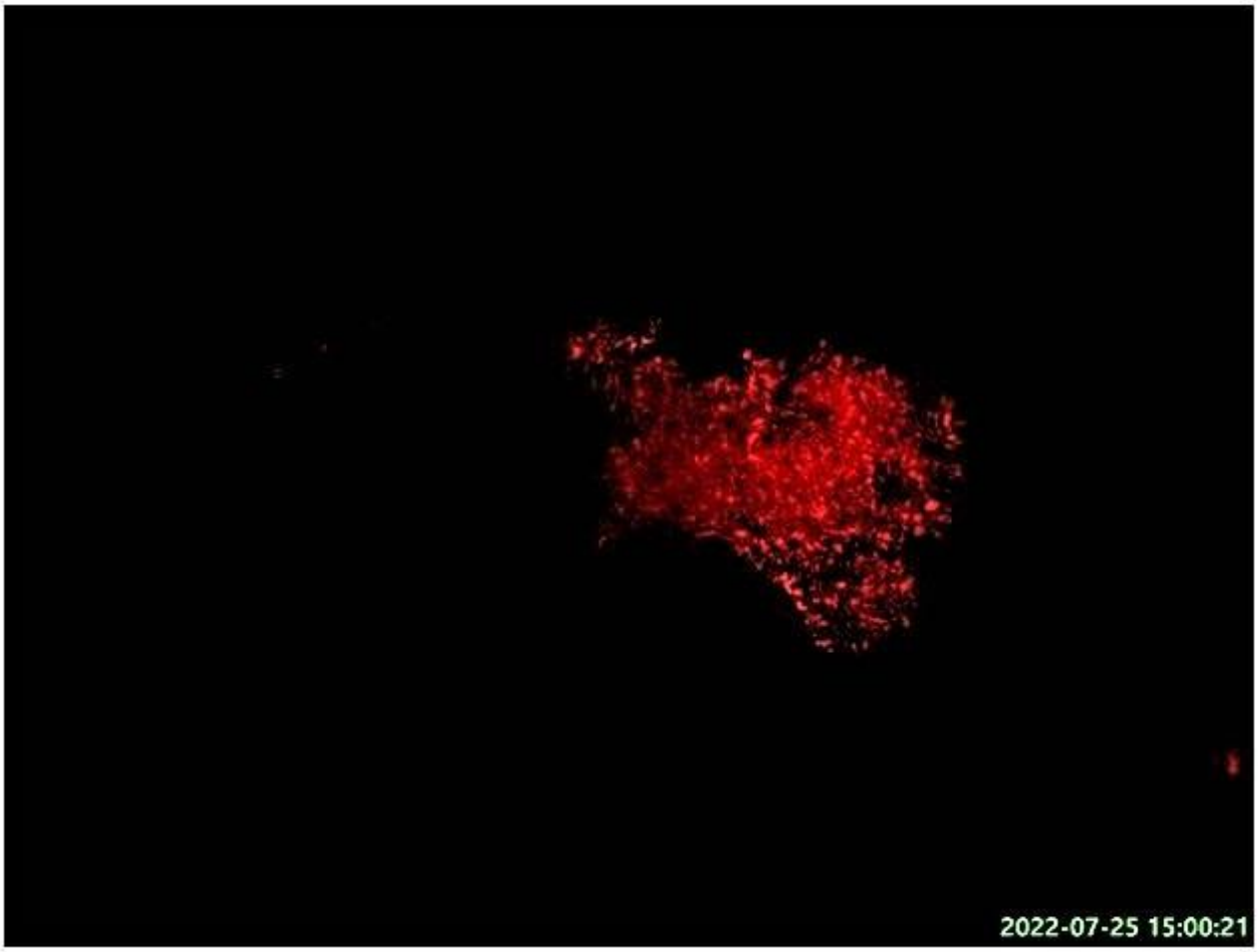
Supplementary File S4:

Borrelia burgdorferi biofilm community (Red color plaque) in peripheral blood patient E.M. IHC with Monoclonal Ab CB10 – specific for OSP A protein of burgdorferi group spirochetes.

Note: a preponderance of granular type borrelia (Red dots) populates the biofilms. Rare cylindrical type borrelia are present at the radial outer margins of each biofilm. Extracellular Protein OspA Deposits are diffusely present inside the amorphous matrix "slime layer" inside the biofilm.

Black spaces inside the biofilm communities are "water channel networks" which provide conduits for Nutrient influxes and for "waste removal" channels for living microbes inside the biofilm.

Final Magnification 1000x original.



Supplementary File S5: