RESEARCH ARTICLE

Exposure to Actinobacteria resident in water-damaged buildings and resultant immune injury in Chronic Inflammatory Response Syndrome

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ACRONYMS

AI	Actinobacteria Index
ANCA	Anti-neutrophilic cytoplasmic autoantibody
CIRS	Chronic Inflammatory Response Syndrome
Ct	Corynebacterium tuberculostearicum
DI	Dominance Index
EMT	Epithelial to mesenchymal transformation
GENIE	Commercial transcriptomic assay: Gene expression, inflammation explained
HH	Human habitat, skin *Actinobacteria*
MAPK	Mitogen associated protein kinase
MARCoNS	Multiple antibiotic-resistant coagulase-negative Staphylococci
MHM	Molecular hypometabolism
MSH	Melanocyte stimulating hormone
NGS	Next Generation Sequencing
PI	Prevalence Index
SH	Soil habitat *Actinobacteria*
Smad	"Small Mothers Against Decapentaplegic," signal transducers for TGFBR
TGF beta-1	Transforming growth factor beta-1
TGFBR	Transforming growth factor-beta receptor
TLR2	Toll receptor two
WDB	Water damaged building

Abstract

The indoor air quality literature has expanded to include a wider variety of contaminants responsible for adverse human health effects. The increased use of Next Generation Sequencing (NGS), combined with the advent of transcriptomic assays, has defined specific causation of innate immune activation within this growing list of pathogenic microbes. Here we report the correlation of specific *Actinobacteria* as shown by NGS, with specific differential gene activation, using a transcriptomic assay (GENIE; Progene Dx, LLC, Bedford, Massachusetts).

The study provides newly described indices of dominance (DI) and prevalence (PI) for exposure to *Actinobacteri*a that have enabled clinicians to develop targeted treatment protocols to improve health issues as shown by reduction of symptoms, restoration of normal proteomics and transcriptomics, and correcting molecular hypometabolism (MHM), upregulation of MAPKs and TGFBR, as part of a chronic inflammatory response syndrome (CIRS), acquired following exposure to the interior environment of a water-damaged building (WDB).

NGS has shown differences in populations of *Actinobacteria* found in WDB that are paralleled by differences in innate immune responses. *Actinobacteria* carried on human skin (HH, human habitat), as opposed to dwelling in soils (SH, soil habitat), consistent with prior publications, can now be represented by DI and PI pinpointing of specific areas of WDB requiring remediation.

A review of the literature shows the capability of HH organisms to induce inflammatory processes in skin and systemically. In addition, the rapid expansion of research on extracellular vesicles suggests a reasonable basis for a mechanism by which resident HH initiate a cascade of inflammatory and metabolic events leading to adverse health effects following exposure to Gram-positive organisms, particularly lipophilic *Corynebacteria*, *Cutibacteria* and *Mycobacteria*, all of which contain mycolic acids in their cell walls.

The role of unique structural differences in specific mycolic acids stratifying those *Actinobacteria* that are inflammatory may become fertile ground for the treatment of HH-associated illnesses, including CIRS. A review of the biology of receptors for TGF beta-1 adds to the importance of preventing the downstream signaling from upregulated TGFBR in illness associated with elevated PI.

The aim of the paper is to identify distinguishing clinical features, including inflammation and immunoreactivity involving MAPK and TGFBR that are seen in illness associated with indoor exposure to *Actinobacteria*.

BACKGROUND

Two recent publications have focused attention on exposure to *Actinobacteria* in WDB[1, 2]. Further, the expanding database on specific causation in patients sickened by exposure to WDB adds weight to an under-used part of the case definition of CIRS. The case definition for chronic inflammatory response syndrome (CIRS) has included exposure to *Actinobacteria* since introduced in 2003[3] and then codified by the US GAO report of 2008[4].

CIRS is a chronic inflammatory response syndrome acquired following exposure to the interior environment of a WDB with resident microbes which include filamentous fungi, *Actinobacteria,* and other bacteria, including inflammagens and toxigens[5]. Little is known about the pathogenesis of *Actinobacteria* exposure for those with CIRS. While we understand that the "inner sancta" of WDB are usually colonized by HH *Actinobacteria*, as opposed to SH[6, 7], the potential for such skin-dwelling organisms to create specific immune injury has not been confirmed prospectively.

By analyzing immunoreactivity and concurrent exposure to *Actinobacteria* in CIRS cases without significant exposure to filamentous fungi or other bacteria, we have identified biomarkers[1, 2] that give specific causation for *Actinobacteria*. For the first time, we can see a clinical progression following exposure as confirmed by NGS to development of elevated levels of mitogen-activated protein kinases (MAPKs) for those treatment naïve patients with elevated DI, followed by enhanced levels of transforming growth factor beta receptors (TGFBR) receptors 1, 2 or 3 in those treatment naïve patients with elevated PI. Thus, treatment using a long-standing protocol[5] corrects the transcriptomic biomarkers and brings a salutary clinical response. The question that remains, however, is what is the mechanism of injury from HH *Actinobacteria*?

ACTINOBACTERIA INDICES - DI AND PI - MAY PLAY A ROLE IN DEFINING PATHOGENESIS

A recent application using laboratory data from EnviroBiomics, Inc., of San Antonio, Texas, has led to the development of indices for exposure to *Actinobacteria* showing differences in subsequent immunoreactivity for *Actinobacteria* from human skin carriage, HH, as opposed to *Actinobacteria* from soil-based habitat, SH. These indices, named a Dominance Index and a Prevalence Index, correlate with MAPK activation and TGFBR activation, respectively. Furthermore, treatment corrects the immunoreactivity; and the corrected immunoreactivity restores the indices to be less than 2.0.

We are left with the question of whether there is a transmission of inflammatory elements of HH *Actinobacteria* that directly affect host innate immune responses. This immunoreactivity is separate from molecular hypometabolism due to exposure to small molecular weight ribotoxins[8]. Given the recent finding that *Actinobacteria* can make compounds that activate TGFBR receptors[9], we are left with the tantalizing questions of (i) what is the source in WDB that makes people sick from *Actinobacteria*; and (ii) what will correct the injury?

A viable possibility is the known ability of *Actinobacteria* to make extracellular vesicles that carry potentially inflammatory

molecular signaling compounds from inside the cell wall, a proteoglycan matrix, to the outside world[10]. These vesicles, of 20-150 nanometer size, are identified following ultracentrifugation at 100,000 G but are otherwise undetectable in serum[11].

It is only recently that Gram-positive organisms, including *Actinobacteria* and *Staphylococcus*, have been identified as manufacturing extracellular vesicles. The bulk of research is focused on the ability of Gram-negative rods to make similar extracellular vesicles but without the barrier of a proteoglycan cell wall[11].

Recent research[12, 13] has focused on *Actinobacteria* with growing filaments where there are defects in the proteoglycan wall at the tips, theoretically permitting more rapid egress of extracellular vesicles from intracellular to extracellular spaces, diffusing into tissue and blood.

BIOLOGY OF *ACTINOBACTERIA*

Actinobacteria are Gram-positive spore-forming, filamentous bacteria, including over 230 genera. Their genome is enriched with G-C base pairs[14]. They are potentially pathogenic, as shown by toxin formation by the causative organism of diphtheria, *Corynebacterium diphtheriae*, but many species are commensal. Soil is endowed with a diverse number of genera. The *Actinobacteria* are versatile in their metabolic activity, with over 20,000 secondary metabolites known to be made[15]. The presence of *Actinobacteria* has long been recognized as a marker for moisture damage in WDB, and synergism of adverse health effects associated between *Actinobacteria* and both fungi and other bacteria has been reported[16]. Now that we have evidence of specific immunoreactivity of *Actinobacteria* in patients with CIRS added to the known immunotoxic effects of *Mycobacteria* and *Streptomycetes*, it is understood that *Actinobacteria* are important inducers of inflammatory responses[15, 16] in CIRS, with metabolic complications as well[17].

Part of the concern regarding *Actinobacteria* stems from carriage on human skin and mucus membranes that present the potential for additional induction of inflammatory responses in the human host and for shedding bacteria to survive on diverse indoor substrates [6,7]. Following standard remediation, failure to clear *Actinobacteria* suggests that ongoing seeding of indoor environments from moist/oily areas on skin to moist/oily areas on walls, flooring, bathrooms, closets, and bedding is an under-appreciated concern. As expected, *Actinobacteria* can use diverse substrates, including cellulose and lignins, as carbon sources[15]. The characteristic musty smell found in many WDB includes production of geosmin by *Actinobacteria*, creating the logical fallacy that the smell found in WDB buildings is only due to mold growth

Because *Actinobacteria* are small, and fragments of whole cells are often produced, inhalation of particulates can lead to deep deposition in the lung in addition to carriage of viable organisms on the skin. *Actinobacteria* are commonly found in WDB, with 48%-60% of samples positive in a study of 1140 samples[15, 16, 18]. Similarly, *Mycobacteria* are commonly found in WDB, with 23% of materials from WDB having densities of up to 10^7 colony forming units/Gm found[15]. With the expansion of NGS testing, recognition of *Actinobacteria* no longer depends on culture. Sequencing has

shown that 19% of indoor *Actinobacteria* are either *Cutibacteria* (previously named *Proprionobacteria)* or *Corynebacteria*, common skin-dwelling organisms[15].

TGFBR 1, 2, 3 AND SPECIFIC IMMUNOREACTIVITY OF *ACTINOBACTERIA*

We have presented data that support the assignment of the presence of *Actinobacteria* when combined with upregulation of MAPK and TGFBR[2]. Each are non-specific when considered individually but highly significant when taken together, constituting a marker for specific causation for *Actinobacteria* for CIRS. TGFBR activation will lead to increased downstream signaling of the multiple TGF beta pathways[19], including but not limited to fibrosis and epithelial to mesenchymal transformation (EMT). TGFBR signaling further impacts on blood-brain barrier permeability[20]. The best characterization of the TGF beta pathways is through Smad 2 and 3[21].

TGF beta-1 is a well-characterized pluripotent cytokine, dependent on TGFBR receptor signaling, and can be either pro- or anti-inflammatory; pro- or antiapoptotic; with MAPK and Smad activation dependent on TGFBR signaling[19], although CIRS may be an exception to the MAPK linkage.

TGFBR 1 regulates an impressive variety of cellular and intercellular processes, including differentiation, apoptosis, immunosuppression, stimulation of collagen deposition, induction of angiogenesis, through VEGF and HIF, and maintenance of genomics[19]. All these effects occur following the activation of the receptor for TGFBR 1, which is composed of TGFB 1 and TGFBR 2 subunits. This receptor is activated through a sequence beginning with TGFB 2 binding to TGF beta. That combination activates TGFB 1, which then activates downstream targets, including the Smad signaling pathway. Additional non-Smad signaling cascades include P1, -3K/Akt-1, 2, 3, and MAP kinases[19].

The link of TGFB1 to Smad2 and Smad3, once activated, will join Smad4 as a complex that migrates to the nucleus and turns on specific genes[19]. If the transcription complex is less intense or shorter duration, then the effect of TGFB1 is reduced. In other words, the cellular context of conditions inside the cell dramatically changes how the cell responds to TGFB1 and TGFB1 receptors. As always, receptor activity is based on activation factors and deactivation factors. The sum of those two makes the absolute activation state of the pathway[19]. The immune suppression and immune activation of TGFBR 1 is a balance of activation versus deactivation factors. Regulation of expression levels of TGFBR 1 and 2 is a regulatory pathway for TGFBR 1 signaling.

The possibility of a receptor threshold model for determining the specificity of TGFB1 effects has been postulated for years. This idea suggests a critical expression level of the TGFB1 receptor that determines specific responses of a cell. Indeed, regulation of the expression level of TGFB1 can affect the specificity of the TGFBR 1 response[21]. Anything, such as activation of TGFBR 2 by *Actinobacteria*, will be modulated by the expression level of TGFBR 2, which, in turn, impacts the activation state of both Smad and non-Smad signaling pathways. However, the factor of the amount of TGFBR 1 activation

and the differential effects on MAPK pathways suggests that non-Smad signaling predominates at low levels of TGFB1 receptor activation; and higher levels of receptor activation will activate Smad pathways. In this regard, the MAPK signaling pathway is catalytic; Smad is not catalytic[21].

Additional importance[21] is activation of Smad1 and Smad5, separate from Smad2, 3, 4.

These sets of genes, Smad2, 3, 4, and Smad1 and5, are required for epithelial to mesenchymal transformation[21]. Thus, a reasonable prediction is that the receptor interaction and diversity of receptors will be shown to have specific interactions.

The role of TGFBR is vital in the management of patients with acute respiratory stress syndrome (ARDS), COPD, lung fibrosis, and bronchopulmonary dysplasia in infants. None of these four is particularly responsive to steroids. The use of steroids will adversely drive TGFBR 1 not to not express primary lung fibroblasts, but instead to predispose to the expression of myofibroblasts, as manifested by the presence of actin and myosin, which are exclusively Smad 1 dependent processes, at the same time inhibiting Smad 3 and TGFBR 1[22]. Glucocorticoids inhibit classical TGFBR 1 signaling as assessed by the Smad 3 binding element. Further, glucocorticoids alter the expression of TGFBR 3 and shift TGFB1 signaling from Smad2, 3 to Smad 1. Of note, TGFBR3 was the least activated of all the receptors of 1, 2, and 3 in our *Actinomycetes* data set.

When transcriptomic assays for Smad2 and Smad3 and Smad1 become available commercially, we should have more information regarding the mechanisms that affect TGFB1 receptor 3 in pulmonary arteries to reduce Smad1 signaling in the endothelium. This will play a role in vascular remodeling seen in pulmonary hypertension. There is no sustained benefit for glucocorticoids for the treatment of pulmonary hypertension.

Paralleling the lung, fibroblast-specific TGFB1-Smad2/3 signaling remains vitally important in cardiac fibrosis[23]. This finding is of importance in managing heart failure. It results in loss of cardiac myocytes, remodeling of the right ventricle, and the accumulation of interstitial fibrosis, which reduces cardiac output. Inhibition of fibrosis would benefit patients with fibrotic processes ongoing in heart failure. This process is driven by TGFBR 1 effectors, Smad 2 and 3, to promote myoblast differentiation of activation of TGFB 1 and 2[23]. Deletion of TGFB1 and 2 of cardiac myocytes on an experimental basis could protect from cardiac fibrosis and functional decomposition with the remodeling in the pressure overload of cardiac function. These data have been observed in mice but have not been trialed in humans[23].

The brain is also involved with TGFB1 signaling[24, 25] as activation of signaling in astrocytes is sufficient to cause neural dysfunction in age-related pathology in rodents. In this experimental model, infusion of albumin into the young rat brain, mimicking blood-
brain barrier leakiness, induced a change in astrocytes following TGFB1 signaling by creating an aged brain phenotype with

abnormal EEG, vulnerability to seizures, and presence of cognitive impairment. In addition, TGFBR 1 signaling reverses these changes in old mice. Thus, this parallel signaling pathway is found in aging human subjects with blood barrier dysfunction[24, 25]. Given the common finding of gray matter nuclear atrophy and cortical gray atrophy seen on NeuroQuant testing in association with elevated TGFB, elevated TGFB1 receptor 1 and 2 and exposure to *Actinobacteria* demonstrates that dysfunction of neurons is one of the earliest triggering of neurologic aging, which maintains considerable plasticity[24]. This duality of TGFB1 activating signaling through TGFBR receptors 1 and 2 with Smad 2 and 3 signaling is also seen in endometrial repair disorders. As shown by experience in rabbits, Yao[26] was able to show that reversal of EMT in rabbits induced by TGFB1 had occurred, retaining plasticity paralleling that seen in the brain.

As expected, the TGFB1/TGFBR interaction remains complex, with new findings showing greater complexity. Specifically, given that the bulk of TGFB1 receptors reside inside cells when Akt activation responds to insulin, or growth factors induce transport of TGFBR to the cell surface increases the cellular response to TGFB1[21, 27]. Further, TGFB1 itself can induce rapid translocation of its receptors and amplify its response. This positive feedback loop, dependent on Akt activation, shows the interaction of TGFB1 receptors with insulin. The TGFB1-induced increased receptor presentation at the cell surface amplifies Smad activation in gene expression, which is further enhanced by Akt activation, thereby increases TGFBR levels in the cell surface and increases activation of TGFB1-responsive Smads[27]. This mechanism of ligand-induced signaling amplification shows promise in the manipulation of TGFB1/TGFBR responsiveness.

As part of the discussion of deactivating factors for TGFB1 signaling, T-cell mediated regulation of TGFBR signaling acts as a crucial criterion for determining T-cell quiescence versus activation[28]. Naïve T-cells in autoimmune patients have reduced TGFBR 1 expression in increased T-cell receptor-driven proliferation compared to healthy subjects. With T-cell receptor engagement, reducing TGFB1 signaling, and downregulating TGFB1 receptor 1, we see another mechanism to control TGFB1 in the ever-expanding complexity in the world of activation and deactivation in TGFB1[28]. Finally, TGFBR signaling also regulates CD103 and CD11B dendritic cells developing in the intestinal wall[29]. The defect of CD103+CD11B+ dendritic cells is accompanied by reduced degeneration of antigen-specific inducible FOXP3 positive regulatory T cells both in experimental laboratories and in vivo and reduced numbers of endogenous TH17 cells in the intestinal mucosa. It appears that TGFBR 1 mediated signaling may explain the specific development of this unique dendritic cell[29].

From the literature: inflammatory effects of exposure to lipophilic HH

The *Actinobacteria* isolated with the greatest frequency in our data from dust sampling in WDB is *Corynebacterium tuberculostearicum (Ct)*. This organism is a ubiquitous colonizer of human skin and is rarely not identified in a roster of pathogenic actinobacteria documented by

EnviroBiomics, Inc (San Antonio, Texas). Using NGS, we showed the presence of *Corynebacteria* colonizers, needed for DI calculations, and which provide bacterial equivalents per mg/substrate dust (BE/mg) needed to calculate PI. We then compared the developing literature on inflammatory effects from organisms such as *Ct* compared to other *Actinobacteria* to determine the role of *Ct* in inflammatory processes of the skin.

Altonsy[30] and co-authors used cell culture, reverse transcription polymerase chain reaction (PCR), ELISA, immunofluorescence microscopy, Western Blot, immunoprecipitation, PCR, small interfering RNA knockdown, and luciferase reporter expression systems to document that *Ct* upregulates messenger RNA and protein levels of inflammatory mediators in two human skin cell lines, HEKs and SCCs. Furthermore, the presence of *Ct* can activate nuclear factor-kB (NF-kB pathway) in response to *Ct* infection, including activation of IkB, the nuclear translocation of NF-kB subunit, and the recruitment of RNA polymerase to the NF-kB response elements in the promoter region of the inflammatory genes[30]. In addition, these sophisticated data confirm that the *Ct*-induced tumor necrosis factor mRNA expression in HEKs is toll-like receptor 2 (TLR2) dependent.

It is well known that human skin, including keratinocytes, provides the first line of defense for exposure to environmental pathogens. There is a symbiotic relationship between the host and the microbial flora on the skin, rendering most skin organisms to be viewed as commensals. In contrast, inflammatory skin problems such as atopic dermatitis, psoriasis, and rosacea, are believed to result from an alteration of a relationship of environmental exposure, host immunity, and skin organisms[30]. The scourge of teenage youth, acne, caused by *Cutibacterium acnes*, is a localized inflammatory condition beginning at the base of pilosebaceous glands. This condition is regulated by the inflammatory and metabolic status of the host interacting with specific microbes. In addition, there are complicating factors on the skin related to genetics[30], resulting in increased colonization with *Staphylococcus aureus* and *Staphylococcus epidermidis*.

In this discussion, we are not including *C. diphtheriae*, a well-known toxin-forming organism that is potentially life-threatening. Organisms such as *Ct* have recently been characterized as being present in a variety of skin environments, including dry, moist, and sebaceous regions[31, 32]. *Ct* has been associated with inflammatory mastitis[33], chronic rhinosinusitis[34], and surgical site infections but without delineated mechanisms of injury.

The data from Altonsy[30] provide valuable insight into the barrier function of keratinocytes, forming tight junctions, in mediating inflammation through secretion of antibacterial peptides, cellular adhesion molecules, and cytokines. This paper provides persuasive evidence that the presence of *Ct* elicits an inflammatory response that is stratified through its effect on different cell lines. Specifically, the mRNA expression profile is time-dependent, and HEKs genes for IL1ra, IL1B, IL6 and, IL17a were rapidly induced (within just 2 hours of exposure), but the early responding genes SCC cells were IL10, IL1B, IL1a, TNF, CXCL10, CSCL1, HBEGF, IL6, and CSF3. The secretion of various inflammatory

proteins, including IL6, IL8, IL1B, CXCL10, and ICAM1, into the culture medium during the in vitro experiments[30].

The mechanism of *Ct* colonization involves activation of NF-kB, which in turn enhances transcription of target genes. Activation by *Ct* of inflammatory genes provides a model for use in other *Actinobacteria*.

Activation of NF-kB itself is non-specific in that many compounds and transcription factors interact with NF-kB. We cannot say with specificity that activation of IL1B, for example, is solely due to *Ct* but does provide the basis to look for *Ct* and related organisms to identify the source of inflammation in patients in WDB exposed to *Actinobacteria*. An important feature of microbial infection/colonization is the ability to trigger the activation of Toll receptors by NF-kB. *Ct* upregulates the expression of TLR2, potentially amplifying inflammatory responses in keratinocytes. Toll 2 activation was also induced by lipoproteins produced by periodontal-resident *Actinobacteria viscosus*, thereby inducing inflammatory responses[35] associated with periodontal disease. The authors do not comment on the seeding of bathroom surfaces by toothbrushes of those with periodontal disease or mouth-to-mouth contact.

Hinic[31] discusses *Ct* as a lipophilic *Corynebacterium* characterized in 2004. In a sample of 18 hospitalized patients who had been on prolonged treatment with broad-spectrum antibiotics, the authors could not provide reliable *Ct* identification through routine microbiological or biochemical tests. The use of matrix-assisted laser desorption ionization-time of flight mass spectrometry was practical but a less cumbersome and more efficient method was 16S rRNA gene sequencing, which is now the standard for identification of *Ct*. Some of the delays in recognizing the pathogenic effects of *Ct* may well be due to the absence of the use of NGS. Therefore, the clinical relevance with being underestimated. In this group of 18 patients, 87.5% of the 16 strains analyzed showed multi-drug resistance. An additional problem with recognizing *Ct* and other *Actinobacteria* pathogenicity could be ascribed to failure to use transcriptomic testing.

Brown[32], in 1984, discussed *Ct* as a novel leprosy-derived *Corynebacterium*. The relationship of *Ct* to *Mycobacteria* is demonstrated by typing cell walls, identifying major primary cell wall polysaccharides, major glycolipids, and major cell wall polysaccharides, major glycolipids, and characteristic mycolic acids.

In another older paper[36], elements of lepra bacilli were found in *C. acnes*, *Ct*, and *Mycobacterium leprae.* Forensic analysis of bacterial communities in legal cases[37] also supports the commonality of skin carriage of *Ct.*

In the era of widespread antibiotic resistance, we rarely consider skin carriage of *Ct* and other lipophilic *Corynebacteria* as carriers of resistance genes. Nevertheless, resistance to macrolides, lincosamide, and streptogramin B[38] is frequently found on healthy human skin in the presence of *Ct* and *C. jeikeium*. This finding has implications for those who wish to stop seeding indoor areas of WDB with HH species: topical antibiotics are not the answer.

There may be additional reservoirs of HH *Actinobacteria* other than skin, as shown by

analysis of middle ear fluid of children and adults undergoing cochlear implants[39]. In adults, the predominant bacteria found in middle ear fluid were *C. acnes* and *Ct*.

In terms of seeding, however, a novel study looked at dust in animal sheds on farms, comparing bacteria found in sheds to bacteria found in mattresses of farm families[40]. The investigators found HH *Ct*, *C. mucifaciens*, and SH organisms *Brevibacteria* and *Arthrobacter* together with MARCoNS.

The lipophilic *Corynebacteria* found on human skin are primarily biofilm producers[41], rendering eradication even more complicated, with 94% having a hydrophobic cell surface and 75% can form biofilms. In addition, these attributes were present in all nosocomial infections from these *Corynebacteria*, suggesting that biofilm formation can be contributory to opportunistic infections.

Abreu[34] presents the argument that (i) chronic rhinosinusitis (CRS) is characterized by an upregulation of the mucin gene Muc5A in goblet cells found in the surface epithelium; and that (ii) there is no difference in numbers of bacteria found in biopsies of CRS patients compared to controls, but that (iii) the microbial composition of the determined microbial burden is disturbed; with a (iv) depleted number of lactic acid bacteria and a concomitant increase of a single species, that being *Ct*.

The role of *Ct* revealed in a literature search[42] of the microbiology of normal, non-inflamed sinuses also supported the concept that *Ct* is potentially pathogenic and that modulation of sinus flora with topical probiotics could be used to treat sinus infection.

A study of 190 CRS patients from Belgium with endoscopic sampling done on anterior nares, nasopharynx, plus maxillary and ethmoid sinuses were compared to nasopharyngeal samples from 100 controls[43]. NGS was performed; risk factors for CIRS included a decreased microbiome diversity and a potential pathogenic role for *Ct*.

A curious finding in a study of 19 patients with granulomatosis with polyangiitis (Wegener's granulomatosis)[44] studied with NGS and followed for a total of 78 visits was published 3/21. *Corynebacteria* and *Staphylococci* were the most identified bacteria. The C/S ratio was calculated with inactive disease maintaining stability. The ratio dipped before relapse in those with relapse, rising to a higher level during and post relapse. Levels of ANCA also rose in relapse. The authors conclude that increased levels of *Staphylococci* are an initiating factor in inflammatory disease exacerbation, with *Corynebacteria* sustaining the ANCA elevation. One can only speculate what would have followed the use of *Lactobacilli* topically in the face of rising *Corynebacteria* dominance.

METHODS:

ACTINOBACTERIA INDICES

Pathogenic *Actinobacteria* are now reported by Envirobiomics, Inc., for each *Actinobacteria* assay performed, making replication of our data straightforward, especially when an additional factor called an *Actinobacteria* Index (AI) is included with each set of NGS reports on settled dust taken from WDB. These indices were developed following a review of 120 consecutive

Actinobacteria tests performed by EBI on patients seeking *Actinobacteria* testing as part of an evaluation of buildings that had water damage. No buildings without known water intrusion were subjected to testing. Further, GENIE testing was done to evaluate simultaneous transcriptomic findings in patients with CIRS and Indices. No control patients were evaluated.

Habitats for *Actinobacteria* as human skin (HH) and soil (SH) were identified by microbiological reference. EBI identified 60 pathogenic *Actinobacteria*. From this roster, species with reporting incidence of greater than 95% were selected for Index assignment, finding 33 species from HH and 13 species for SH, listed below.

A correlation was found from the building NGS data, including a deidentified lab number with a match to deidentified transcriptomic results obtained from a proprietary assay, GENIE (ProgeneDx, LLC, Milford, Massachusetts). This correlation was present in all 66 patients' data when dust samples NSG and GENIE were analyzed and sent to the principal investigator (RS) with permission obtained from the patients of their attending physicians. Each study has a calculated DI and a PI, making for 132 data points. Patients were treated (N=101) or untreated (N= 31) at entry into the observational study, with age and gender known. The study provided no treatment. 37 patients were female, mean age 48.9. 29 patients were male, mean age 51.4.

RESULTS:

The purpose of the Indices, called a Dominance Index (DI) and a Prevalence Index (PI), was to assist physicians in making clinical decisions regarding treatment. Dominance is the ratio of HH to SH, using the percentage appearance in the group of 33 species of HH divided by the percentage of species found in the 13 species of SH. For the DI, the number of Bacterial Equivalents/mg (BE) is not factored into the ratio. DI is then correlated with the upregulation of genes that code for MAPKs to ascribe where dysfunctional immunoregulation caused by environmental exposure begins. The DI shows a tight linkage in untreated patients with exposure, as shown by an *Actinobacteria* score as developed by EBI. In the tables, DI and PI are low when the measure is < 2.0; and high when the measure is $\geq$ 2.0.

Table 1 DI, UNTREATED N = 15

DI	LOW	LOW	HIGH	HIGH
Z SCORE MAPK	0	1	0	1
N =	6	0	2	7
%	40	0	13	47

TABLE 2 DI, TREATED N = 51

DI	LOW	LOW	HIGH	HIGH
Z SCORE MAPK	0	1	0	1
N=	23	8	15	5
%	45	16	29	10

We did not find a significant correlation of DI > 1.9 and positive MAPKs with SH organisms (LOW DI). However, we did find a significant association with DI > 1.9 with HH organisms (HIGH DI, see Table 1) and positive MAPKs found in 47% of untreated patients. For treated patients (see Table 2), the incidence of high DI and + MAPK 47% to 10%; the ratio of untreated with high MAPK to low fell over 90% from 3.6 to 0.34 for the same measure in treated patients.

PI is the ratio of the average BE in the 5 HH organisms when sorted by individual mean BE counts for the 5 highest mean BE for SH organisms. PI > 1.9 is significantly correlated with genes for TGF beta-1 signaling (TGFBR 1, 2, 3) in untreated patients. As published, here is where precise, specific causation of immunoreactivity caused by exposure to *Actinobacteria,* is based (see Table 3, 4). Both indices were analyzed for specific abnormalities of immunoreactivity seen on GENIE.

TABLE 3, PI UNTREATED N = 16

PI	LOW	LOW	HIGH	HIGH
Z SCORE TGFBR	0	1	0	1
N=	10	0	0	6
%	62	0	0	38

TABLE 4, PI TREATED N = 50

PI	LOW	LOW	HIGH	HIGH
Z SCORE TGFBR	0	1	9	1
N=	9	1	30	10
%	18	2	60	20

Treatment effects are again noted, with a high z score, high TGFBR in untreated falling from 38/0 to 0.33% in those treated. Low PI, i.e., not with excess HH, was not associated with high TGFBR, but high PI was found to be associated.

If the AI is determined simultaneously with GENIE, then causation of *Actinobacteria* immunoreactivity in human illness is unveiled. This concept of direct linkage of exposure to gene abnormalities is (i) not novel and is (ii) based on observed transcriptomic responses to environmental cues.

If the DI in untreated patient's dust is more significant than 1.9, then there is a higher percentage of HH species present than SH. If the PI in the untreated patient's dust is more than 1.9, the mean of HH organisms will be greater than the mean number of SH organisms.

We hope that the indices will eventually lead to greater sophistication in remediation efforts, focusing more on HH organisms and not simply total pathogenic *Actinobacteria*, as remediation using just fungal measurements alone has been well established in peer-reviewed literature not to be reliable, as shown by many publications[1]. Unfortunately, we do not know yet that the use of Indices will make remediation more effective, more accurate, and less expensive. However, we are hopeful that there will be the expansion of the use of precipitation techniques and filtration, adding removal of human-seeded HH where they are found, based on pre-remediation and post-remediation Indices.

To calculate the indices, the study used the following formula:

Dominance Index

Take the sum of HH *Actinobacteria* species and divide by 33.
Now take the sum of SH *Actinobacteria* species and divide by 13.
Now divide HH/33 by SH/13.
The result is the DI.

Prevalence Index

Take the average of the BE for the first five species listed in HH and divide that number by the mean of the BE for the first five SH. That result is the PI.

NOTE: the first five HH and SH species are identified below by an * for HH and SH.

Human Habitat (HH) derived *Actinobacteria*

*Cutibacterium (Propionibacterium) acnes**

*Corynebacterium tuberculostearicum **
*C. xerosis**
*C. amycolatum**
*C. simulans**
*Used to calculate PI

Actinomadura chibensis
Actinomyces canis
Actinomyces europaeus
Actinomyces meyeri
Actinomyces neuii
Actinomyces odontolyticus
Actinomyces turicensis
Corynebacterium accolens
C. argentoratense
C. coyleae

C. falsenii
C. glucuronolyticum
C. hansenii
C. imitans
C. jeikeium
C. kroppenstedtii
C. matruchotii
C. minutissimum
C. propinquum
C. resistens
C. riegelii
C. striatum
C. sundsvallense
C. ureicelerivorans
Dermatophilus congolensis
Cutibacterium (Propionibacterium) avidum
Cutibacterium (Propionibacterium) granulosum
Rothia mucilaginosa

Soil Habitat-derived (SH) *Actinobacteria*

*Rathayibacter tritici**
*Brevibacterium mcbrelneri**
*Curtobacterium flaccumfaciens**
*Rhodococcus fascians**
*Saccharopolyspora rectivirgula**
*Used to calculate PI

Arthrobacter creatinolyticus
Arthobacter crystallopoietes
Brevibacterium paucivorans
Clavibacter michiganensis
Gordonae terrae
Nocardia higoensis
Rhodococcus equi
Sanguibacter suarezii

DISCUSSION:

We are faced with a new syndrome, which devolves from data-gathering and responds to safe and effective treatment. However, it has not yet confirmed the role of extracellular vesicles, TGFBR signaling, and inflammatory responses to mycolic acids in the pathogenesis of HH-related CIRS, all of which require further study. However, the following open discussion will include these elements, beginning with a targeted review of the literature.

EXTRACELLULAR VESICLES

A rapidly expanding approach to the role of *Actinobacteria* in CIRS involves extracellular vesicles. These lipid-bilayers form blebs, or bubbles, 20-150 nanometers in diameter[45]. They are produced by all living organisms, including eukaryotes, bacteria, and Archaea. Vesicles are known to contain a variety of charges including nucleic acids, lipoproteins, enzymes, and toxins. Thus, they have important roles in physiology and microbial pathogenesis. Since the 1960's we have known that extracellular vesicles in Gram-negative bacteria are produced by pinching off the outer membrane and then released into the local environment. This mechanism does not apply to fungi, Gram-positive bacteria, and *Actinobacteria* because they have a cell wall in addition to a cell membrane. The cell wall, technically a proteoglycan matrix, serves as an impermeable barrier thought to prevent vesicles from leaving or entering the cell[45].

We know more about extracellular vesicles in *Mycobacteria* than any other *Actinobacteria* genera. Their cell walls have unusually low permeability [11, 46] in part due to the significant content of mycolic acids linked to arabinogalactan. This structure creates an

asymmetric hydrophobic bilayer. While there is a fluidity of this structure, the innermost part of the bilayer has less fluidity compared to the outer surfaces [11,46]. Differences in the structure of mycolic acids affect the fluidity and permeability of the bilayer and may explain the differences in sensitivity of various mycobacteria to inhibitors of lipophilic *Actinobacteria*. Passage of hydrophilic nutrients and inhibitors in contrast to the vesicles themselves can traverse cell walls through channels recently discovered called porins [11, 46].

The structure of the proteoglycan matrix of a Gram-positive bacteria and *Mycobacteria* parallels that of fungi which also has a thick wall outside of the cellular membrane. The fungal cell wall is constructed of chitin, beta-glucans, and mannans. However, without a signal stimulating secretion, and because very few genes have been shown to influence the vesiculation in bacteria, no studies remain to explain the purpose of secretion systems versus the overall energy costs of export of cellular materials[45].

Early breakthroughs regarding extracellular vesicles came from studies employing electron microscopy, which documented vesicles observed in the mycobacterial cells[47]. These blebs were similar in size to purified extracellular vesicles, consistent with the idea that the blebs were vesicles in the process of release[48]. As research expanded, extracellular vesicle formation was a consistently observed phenomenon in all of *Mycobacteria*.

Of interest, *Mycobacteria* require iron for growth. Thus, in the face of iron limitation, the production of extracellular vesicles is increased, consistent with cell-to-cell communication in *Mycobacteria*, helping to overcome iron limitation within the host. Furthermore, the isolation of vesicles has been codified in iron-deficient *Mycobacteria* [49, 50].

Seminal work from Prados-Rosales[50] is noted in the field of vesicle immune responses to the benefit of bacteria, including intravesicular content of mycobactin. This iron-donor supports the replication of *Mycobacteria*. Iron deficiency is a sign for pathogens to induce toxins, virulence factors, and iron acquisition systems[50]. The author makes the point that diverse bacteria make vesicles to interact with cells in their environment. "Pathogens (NB: including *Actinobacteria*) frequently release vesicles that contain toxins, adhesins, immunomodulatory molecules to manipulate host-pathogen interactions to the benefit of bacteria[50]." Additional functions of vesicles include transport to other bacteria of antibiotic resistance factors, bacteriolysins, compounds needed for biofilm-formation, and reuptake of iron-laden mycobactin. These secretory pathways also export immunomodulatory factors[50].

In an experiment that foreshadowed the use of mRNA vaccines, Chen used mRNA delivered to extracellular vesicles of alveolar macrophages and analyzed vesicles from the macrophages to assess the effect. The macrophage product caused recipient cells to manufacture a Type 1 interferon and inflammasomes capable of bacterial killing.

Non-tuberculous Mycobacteria, namely M. kansasii, also make vesicles. These vesicles contain IL-10 but no interferon[52].

The most significant interest for pathogenic *Actinobacteria* and extracellular vesicles comes from the location within the cell wall where vesicles are released. Observations of polarly-growing *Streptomyces venezuelae* by time-lapse imaging[12] show the release of extracellular vesicles from tips of vegetative hyphae. Some growth arrest is seen with vesicle extrusion, but the spontaneous formation of new hyphal branches occurs. Treatment with vancomycin works to block peptidoglycan synthesis, stopping new growth. However, removing the antibiotic leads to regrowth of the organism, supporting the concept that the growth zone at the hyphal tips may be necessary for vesicle extrusion[12]. The suggestion is that the cell wall at the hyphal tip is weak and susceptible to rupture, releasing vesicles.

Earlier work[13] showed that maintenance of cell polarity is critical for cell shape and responsiveness to environmental cues for growth. In *Streptomyces*, evolutionarily ancient apical growth is directed by a polarisome-complex. The complex itself is regulated by a eukaryotic protein AfsK which is localized to hyphal tips. AfsK is activated by signals generated by the arrest of cell wall synthesis. The subsequent release of vesicles from hyphal tips appears to be part of the primordial maintenance of cell polarity and growth[13].

Schrempf[54] observes, "Protuberances containing vesicles generate at tips, and alongside of substrate hyphae, and enumerate during late vegetative growth to droplet-like exudates…We conclude that protuberances comprising vesicles arise at sites with enhanced proteoglycan subunits and reduced levels of polymerized and cross-linked peptidoglycan within hyphae."

There are three main research lines regarding the release of extracellular vesicles from the cell wall[45]. The first is that pressure or turgor can force the vesicles through the plasma membrane and then through the cell wall. Here the size of the cell wall pore and the thickness of the vesicles is essential. The second idea is that cell wall-modifying enzymes are released either with or within extracellular vesicles, facilitating a loosening in the wall to enable release[45]. The third consideration is that extracellular vesicles may transit through channels that have become deformed, guided by tubulins[45].

Multiple studies have shown that extracellular vesicles carry various cargoes, including nucleic acids, sugars, and proteins. Genes encoding the alpha-toxin of *Clostridia perfringens* have been isolated from extracellular vesicles[45]. Additional work shows that nucleic acids in extracellular vesicles are protected from the breakdown by exonucleases. Antibiotics that protect microbes have also been isolated from exudates that grow on the surface of *Streptomyces* cultures[45].

Virulence factors also have been isolated from vesicles, including those critical for drug resistance, immune system evasion, host cell invasion, and pathogenesis. For example, pellets of vesicles purified from *Staphylococcus aureus* are enriched in penicillin-binding proteins that bind to beta-lactam antibiotics, providing antibiotic resistance, particularly methicillin. Additional features of *S. aureus* are that their vesicles contain superantigens, lipase, and immune-binding proteins, which help bacteria invade the host immune system[55, 56]. These proteins include staphopain A, a

protein important for cellular invasion, and alpha hemolysins which form pores and may induce apoptosis[45].

Bacteria toxins are important for the pathogenesis of bacterial infection. Some form pores in host membranes, while others result in membrane disruption or entry of other variance factors and suppression of immune responses with the initiation of apoptosis. For intracellular pathogens, toxins have been implicated in invasion as an escape from host cells. In pneumococci, their vesicles contain the toxin pneumolysin, a pore-forming cytolysin that acts as a virulence factor. E. coli, a Gram-negative rod, exports a toxin, ClyA, in vesicles.

Bacillus anthracis also exports toxins in vesicles, including lethal factor, edema factor, and protective antigen, as well as anthrolysin found in the pellet fraction after centrifugation but not in the supernatant, suggesting that most of the toxin release from cells is encapsulated in vesicles.

Purified vesicles from *Staphylococcus aureus* contain pro-inflammatory mediators that elicit a Th17 type response, causing increased production of IgE, which causes inflammation like atopic dermatitis[58]. As expected, mycobacterial extracellular vesicles elicit a profound TLR2 receptor response[59] resulting in an increase in granulomatous inflammation in the lungs and an increase in bacterial load in the spleen[45].

Additional TLR2 data comes from the Netherlands[59]. Immunomodulatory changes are affected by extracellular vesicles from Gram-positive bacteria present in serum via activation of TLR2 and TLR4 and by attaching to molecular pattern receptors. Vesicles can now be shown to be present in diverse bodily fluids.

Vesicles are also associated with biofilm production. Biofilms protect microbial cultures from disruption from exposure from surfaces and antimicrobial molecules. Vesicles are present in the matrix of biofilms and have been visualized protruding from the biofilm. Finding vesicles in planktonic cultures suggests that vesicles play a part in biofilm production, bacterial colonization, and resistance to removal.

Extracellular vesicles and *Cutibacteria acnes*

There is some practical difficulty in the isolation in extracellular vesicles, which demand laboratory isolation following ultracentrifugation at 100,000 G. Crucial to ongoing research is the identification of pathophysiology and an emerging mode of communication in bacteria. *C. acnes* is one such organism now known to be found in the skin but also in the gastrointestinal tract and is being recognized as an underestimated pathogen in a variety of diseases. Jeon[60] identified the isolation of extracellular vesicles from *C. acnes* for the first time, including the identification of 252 different vesicular proteins using LC-MS/MS analyses. The function of these proteins includes biochemical processes, antibiotic resistance, bacterial competition, cell adherence, virulence, and immunogenicity.

In an earlier paper by Holland et al.,[61], *C. acnes* was demonstrated in vitro to produce 20 proteins secreted during the growth phase. Included in the biochemical functions were proteases, proteins that degrade compounds, GAPDH, and several proteins unique to *C.*

acnes. These proteins are likely to play a role in host-tissue degradation in inflammation. Despite considerable overlap with the secretomes of various *C. acne* types, distinct differences were identified[61] providing insight into the differential virulence properties of *C. acnes*.

An additional paper on *C. acnes*[62] from Choi et al., showed that the extracellular vesicles induce acne in human epidermal keratinocytes in an in vitro skin model, suggesting that acne is not an infection but an inflammatory response to molecules contained in vesicles. Following treatment of the isolated cells with the extracellular vesicles, the pro-inflammatory cytokines IL8 and GM-CSF induced dysregulated epidermal differentiation. As a control, the extracellular vesicles were shown to be internalized by endocytosis into keratinocytes and that the cellular responses occurred by a TLR-2 dependent signal[62].

In two earlier papers, the role of *C. acnes* acting in vivo via TLR-2 and TLR-4 expression is shown. The first paper by Jugeau[63] from 2005 showed an increase of TLR-2 and TLR-4 expression by keratinocytes followed by an increased expression of keratinocytes of MMP-9, which plays an additive role in inflammation. Confirmation of *C. acnes* acting via Toll expression is critical to understanding the source of systemic inflammation.

In a 2014 paper[64], Bakry defines the role of TLR- 2 and the pathogenesis of acne, focusing on the localization of inflammatory and non-inflammatory lesions in this illness. Skin biopsies of 30 cases with acne and 30 controls show that TLR2 expression was upregulated in the epidermis, pilosebaceous units, and dermal inflammatory infiltrate. There was a significant difference between acne skin and normal skin between acne involved and non-involved skin regarding TLR2 expression with significance in $p < 0.001$ for all modalities[64]. Intense TLR2 expression favored the presence of inflammatory acne lesions as well as dermal inflammatory infiltrate.

Given that TLR2 participates in other actinobacterial interactions with the human skin host, seeing a consistent TLR2 inflammatory and non-inflammatory lesions pattern suggests that the inflammatory picture is heightened by proximity to blood supply in the rete ridge at the base of the sebaceous gland.

This effect of TLR2 activation from *C. acnes* extended to peripheral blood monocytes for patients with non-inflammatory and inflammatory acne[65]. In addition, cell surface expression of CD14 and TLR2 were determined by flow cytometry in 12 patients with severe acne. Not only was TLR2 activation noted in dermal cells and monocytes, with a significant difference seen between patients with non-inflammatory acne versus inflammatory acne in control subjects, but these data[64] suggest that systemic effects are well within the realm of extracellular vesicles produced by *C. acnes*.

The immune phenomenon noticed in *C. acnes* is not limited to TLR2 expression. An additional factor, TNIP1, a negative regulator of the NF-kB signaling pathway (p38, MAPKK, and JNK), is also involved[66]. This paper provides a discussion of a further link of exposure to *C. acne* and then to systemic inflammatory responses, including TNF and

CCL5, as TLR2 sustains increasing CXCL8 and TLR4 expression.

VESICLES AND TGF BETA SIGNALING, Smad AND FIBROSIS

As shown[67] by Liu, vesicles play an essential role in mediating kidney fibrosis upon stimulation of TGFB1. Proximal renal tubular cells increase extracellular production, which induces renal interstitial fibroblast activation. In addition to extracellular secretion, depleted vesicles abolished the ability of TGFB1-treated cells to induce fibroblast activation. Finally, the blockade of vesicle secretion in vivo successfully treated renal fibrosis from ischemic or obstructive injury. The conclusion from this study was that tubule-derived vesicles play an essential role in renal fibrotic injury; treatment strategies targeting vesicles could become a new avenue in developing treatments for renal fibrosis.

Vesicles from glomerular endothelial cells from high glucose treatments triggered EMT and dysfunction of renal podocytes[68]. High glucose induced the endothelial to mesenchymal transition (EMT). Vesicles from endothelial cells are internalized by podocytes undergoing an EMT-like process. TGFB1 mRNA is enriched with vesicles for those treated with glomerular cells mediating the EMT and dysfunction[69].

You and associates[70] investigated the impact of macrophage-derived vesicles on TGFB1-induced EMT in the lung. They found that vesicles derived from lipopolysaccharide primed cells exhibited an ability to promote TGFB1-induced EMT as manifested by a dramatically increased expression of EMT-related proteins. TGFB1 Smad 2/3 signaling proteins were enhanced. Thus, vesicles derived from polysaccharide-stimulating macrophages can activate TGFB1 signaling through Smad 2/3, which in turn increases the expression of EMT-related proteins and EMT itself[70].

The parallel mechanism[71] in myocardial cells injured by diabetes shows that vesicles could improve the injury and reduce fibrosis by inhibiting TGFB1 and Smad signaling pathways[71]. Here we have another example of cellular-derived vesicles improving an organ injury by modulating TGFB1 effects, primarily on Smad signaling pathways and EMT.

TGFB1 also has a role of interaction with the hypoxia-inducible factor. Hypoxia is associated with tissue injury in fibrosis, but fibroblast activation in tissue repair has not been defined. Borges and colleagues[72] demonstrate that epithelial cells injured by hypoxia produce an increased number of vesicles that activate fibroblasts. Specifically, vesicles released by injured epithelial cells promote proliferation, and actin in fibroblast type 1 collagen production. This fibroblast activation is dependent on vesicles delivering TGFB1 mRNA. This study is consistent with the suggestion that TGFB1 mRNA transported by vesicles constitutes the rapid response to initiate tissue repair and activation of fibroblasts from resident parenchyma following injury[72].

In a study from 2010, Ramachandra[73] showed that *Mycobacteria,* when combined with ATP, induced the release of vesicles that contain major histocompatibility complex class 2 molecules (HLA) capable of antigen presentation.

TGFB1 has a role in pulmonary hypertension, particularly if combined with metabolic acidosis and proliferative physiology[17]. In a 2019 paper[74], excessive TGFB1 signaling was confirmed to underly pulmonary hypertension. This pathologic condition was related to the release by pulmonary artery muscle cells of extracellular vesicles. Using RNA Seq, the authors analyzed the RNA cargoes from vesicles at baseline conditions in cases of excessive TGFB1 signaling. Their findings included detection of transcriptomics in 2417 genes in human pulmonary muscle cells. An additional 90 genes were differentially expressed in vesicles in cells treated with TGFB1 compared to basal conditioning, including extracellular matrix remodeling and production[74]. The conclusion was that mRNA translation and uptake was higher in cells in pulmonary hypertension when stimulated with TGFB1.

Shelke showed that extracellular vesicles conveyed biological messages between cells either by surface-to-surface interaction or by shuttling bioactive molecules into recipient cells cytoplasm. Their research showed that vesicles released by mast cells harbor both active and latent TGFB on their surfaces, which were associated with vesicles trafficking into the endocytic compartment of recipient mesenchymal stem cells within 60-minutes of in vitro exposure**.**

These vesicles associated with TGFB1 are retained within the intracellular components at the time of signaling, resulting in prolonged cellular signaling compared to free TGFB1. Therefore, the vesicles are inducing the migratory phenotype of mesenchymal cells involving Smad-dependent pathways[75]**.**

INFLAMMATORY RESPONSE TO MYCOLIC ACIDS IN HH *CORYNEBACTERIA*

Since we postulate a role for HH-dwelling *Actinobacteria*, especially *Corynebacteria* in CIRS, we suggest a mechanism to link systemic immunity to inflammation caused by these skin organisms. The reader is referred to the work of Yasmine Belkaid[76, 77, 78, 79, 80, 81] from NIH, for additional in-depth references. Her paper[81] from 2018 demonstrates that *Corynebacteria* are the predominant skin microbes that promote a dramatic increase in the activation of a defined subset of gamma delta T cells. These T cells are enriched in mucosal and epithelial tissues. They mount a heightened cytokine response to transformed or infected cells (ref, Bite-Sized Immunology, Matthew Ebert, Cardiff University UK, accessed 8/27/21). The remarkable feature is that this effect (i) is long-lasting, (ii) is independent of other microbes colonizing the skin, and (iii) is mediated by interleukin-23. The potential links among interleukin-23, TGFB1, and Th17 T reg imbalance are apparent.

Corynebacteria effects can be discrete and non-inflammatory under controlled steady-state conditions. However, when the host is fed a high-fat diet, *Corynebacteria accolens* promotes inflammation that is IL-23-dependent. This effect is conserved among *Corynebacteria* species and depends on the expression in the cell envelope of mycolic acids.

These data then support a mode of communication between the immune system and skin organisms showing that the skin

controls the bacterial determinants. Despite this advance in this paper, little is known about the mechanisms in which microbes influence the skin and immune system in a steady state. The discovery of the role of mycolic acids is striking when comparing HH organisms, which are lipophilic and routinely have mycolic acids, to absence of mycolic acids in SH organisms. We find that among SH organisms[82], only *Rhodococcus* and *Gordonae* routinely express mycolic acids.

This effect of *C. accolens* on gamma delta T17 cells was not confined to *C. accolens* alone. Belkaid reports that 8 of 9 species of *Corynebacteria* induced a robust gamma delta T17 response, supporting the idea that the response was a conserved feature among species tested[81]. One characteristic that distinguishes *Corynebacteria* from other Gram-positive bacteria is an external lipid bilayer of branched fatty acids, mycolic acids, which form a structure like size and location to the Gram-negative outer membrane[82]. The only species of *Corynebacteria* that did not make the gamma delta T-cell effect was C. *amycolatum*, an organism that does not contain mycolic acids.

To evaluate the hypothesis that mycolic acids played a role in inducing T-cell responses, a mutant strain of *C. accolens* was developed to deleting the mycolic acid synthase gene. When the mutant was applied to the skin, there was no gamma delta T-cell response within the skin. A series of experiments assessing the contribution of the *Corynebacteria* cell envelope shows that the ability of *Corynebacteria* to activate gamma delta T-cells in vitro is dependent on a cell envelope containing mycolic acid but not the lipoarabinomannans bound to mycolic acids[81].

In *Mycobacteria tuberculosis and* non-tubercular bacteria, mycolic acids are linked to lipoarabinomannans. To further support the idea that mycolic acid plays a crucial role in the ability of *Corynebacteria* to active gamma delta T-cells, additional experiments were reported[81] that showed that MINCLE receptor, previously shown to be involved in recognition of bacteria fatty acids derived from *M. tuberculosis*, CARD9 a downstream signaling mediator for several C-type lectin receptors and MyD88 signaling transducer downstream of most Toll-like receptors, were not required to mount a response to *C. accolens*. Thus, while there may be an unidentified receptor or alternative mode of antigen presentation, the research published leaves us with *Corynebacteria* alone influencing the cytokine environment that promoted gamma delta T-cell responses.

Further work showed[81] that the ability to induce gamma delta TH17 cells is evolutionarily conserved in the *Corynebacteria* genus with minor exceptions such as *C. amycolatum*. The conclusion is that mycolic acid expressions in HH organisms are associated with the pathogenic effect of *Corynebacteria*.

We find these results striking as they support the idea that predominant skin microbes evoked both profound skin immunity and inflammation in response to its cell envelope compounds which may represent a microbial skin signal that differentiates HH from SH *Actinobacteria*.

Of note are the differences in mycolic acid contents of cell walls between *Mycobacteria*

and *Corynebacteria*, which have a thick wall of proteoglycan linked to arabinogalactan, linked to long-chain fatty acids, the mycolic acids. *Corynebacteria* have a mixture of saturated and unsaturated fatty acids up to thirty-six carbons long[82]. In contrast, *Mycobacteria* have longer fatty acid chains, up to ninety carbons long, with oxygen molecules as part of their chemical structure[82]. *Streptomyces*, common soil-dwelling *Actinobacteria*, have a cell wall surrounded by a proteoglycan mesh, like Gram-negative organisms. Mycolic acids play a significant role in the formation of a secondary permeability barrier analogous to the outer cell membrane of Gram-negative[82]. The high proportion of mycolic acid residues in *M. tuberculosis* cell walls contribute to pathogenicity and antibiotic resistance.

CONCLUSIONS:

As transcriptomics and NGS lead the way to define the inflammatory and metabolic role of exposure to pathogenic HH *Actinobacteri*a, the mechanisms suggested herein will require replication from other clinicians. Our data is consistent with adverse effects of exposure to HH, but prospective trials are yet unpublished. DI and PI show tremendous promise as clinical and remediation tools, and their use is expanding rapidly, but the molecular biology underlying these Indices requires additional study. Thus, while extracellular vesicles could be the "missing link" when combined with increased expression of MAPK, TGF beta-1 and TGFBR tying local inflammation to adverse systemic and immune abnormalities in CIRS, we must solve the laboratory demands (i) to isolate intact extracellular vesicles and (ii) to confirm the prospective acquisition of adverse health effects following exposure to Actinobacterial vesicles.

We feel that clinical delineation of molecular pathways is less important than recognizing and removing HH *Actinobacteria* from the inner sancta of WDB. Now that we know the role of TGFBR and the treatment of high PI sites, we are better prepared to intervene quickly and efficiently to correct unwanted pulmonary fibrosis, for example.

In the interim, transcriptomics can reliably show what selected immune perturbations are present and are associated with the acquisition of adverse health effects, even though the detailed aspects of pathophysiology may still be obscure. Given that the treatment protocol used to correct the specific immunoreactivity correlated with DI and PI has been in long-standing use, marked by safety and efficacy, accumulation of data from patient-centered research can proceed as the molecular biology of extracellular vesicles, TGFBR, and adverse organ function from lung to the brain, to systemic illness, is unveiled.

A more fundamental change is for attitudes of health care providers may be in order as well. Discussion regarding specific immunoreactivity and causation of CIRS seems quite different from the reputation of organisms such as *Ct* or *C. acnes*, as either unheard of or is thought to be just a benign source of teenage pimples. We will need to develop a respect for *Actinobacteria* on the human skin, nose, axilla, and pilosebaceous glands. Fortunately, we now have the tools to shed light on previously ignored skin-dwelling immunopathogenic bacteria.

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