RESEARCH ARTICLE

Newer Molecular Methods Bring New Insights into Human- And Building-Health Risk Assessments from Water-Damaged Buildings: Defining Exposure and Reactivity, the Two Sides of Causation of CIRS-WDB Illness

Authors
Shoemaker R[1], Neil V[2], Heyman A[3], van der Westhuizen M[4], McMahon S[5], Lark D[6].
Affiliations
[1]Center for Research on Biotoxin Associated Illnesses, Pocomoke, MD
[2]Mycotox, Australia
[3]School of Medicine and Health Sciences, George Washington University
[4]Lynne Murfin, MD, Calgary, AB, Canada
[5]Whole World Health Care, Roswell, New Mexico
[6] EnviroBiomics, Inc. San Antonio Tx. & NSJ EnviroSciences Pty Ltd, Newcastle, Au
Corresponding author
R Shoemaker: ritchieshoemaker@msn.com

ABSTRACT:

Scientific disciplines dependent on accurate analytics invariably evolve due to advances in technical aspects of measurement. In disciplines in which adequate measurement is not available for applications to public health policy, the impact of new paradigms in measurement can extend far beyond scientific thought. Both of these concepts apply to the effect of exposure to water-damaged buildings (WDB) on human health. What causes the putative illness and what government should do to make buildings safe for use, have been impacted by development of molecular methods, particularly Next Generation Sequencing (NGS) and transcriptomics.

The impact of human exposure to Actinobacteria, for example, and identification of immune reactivity specific to these bacteria are now revolutionizing: (i) both detection and quantitation of newly recognized pathogenic organisms; and (ii) the approach to the genomic basis of diagnosis and treatment of disease as manifested by differential gene activation. NGS permits quantitation of exposure and confirmation of risk associated with the threshold of exposure, using defined human health biomarkers that in turn has led to advances in the metabolic and inflammatory issues in WDB illness, called CIRS, both from molecular hypometabolism and activation of TGF beta-1 signaling that defines immunoreactivity to Actinobacteria.

Current recommendations for assessment of exposure/reactivity to fungi and methods of remediation based on fungi alone do not support continued use, now that endotoxins and Actinobacteria are found to be the major causes of human illness from exposure to WDB.

Keywords: transcriptomics, molecular hypometabolism, Next-Generation Sequencing, Actinobacteria, endotoxins, Gram-negative rods

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BACKGROUND:

The past 25 years have shown remarkable advances in the diagnosis and treatment of a chronic multisystem, multi-symptom illness, termed chronic inflammatory response syndrome (CIRS), characterized by exposure to the interior environment of a water-damaged building (WDB) with resident toxigenic and inflammagenic microbes, including, but not limited to filamentous fungi, gram-negative bacteria, and Actinobacteria; as well as inflammagens, including, but not limited to hemolysins, mannans, beta-glucans, and spirocyclic drimanes, among others [1].

By established case definition, all adult patients with CIRS caused by or associated with exposure to WDB will also have (i) a multisystem, multi-symptom illness, with symptoms similar to those seen in peer-reviewed, published literature; (ii) laboratory abnormalities similar to those seen in peer-reviewed, published literature; (iii) and will show a salutary response to treatment as shown by the improvement of published objective biomarkers following the use of published treatment protocols (10). Children may also develop CIRS but usually begin with single-system illness that will invariably become multisystem if not treated.

A robust literature supports (i) the existence of WDB; (ii) the existence of CIRS; (iii) the prospective confirmation of acquisition of CIRS following exposure to the interior environment of WDB; (iv) the successful treatment of CIRS, including a double-blinded, placebo-controlled clinical trial; (v) the reproduction of published studies of CIRS; (vi) the publication of at least 15 objective biomarkers found in CIRS cases but statistically found far less commonly in age- and gender-matched controls, including distinctive clusters of symptoms; proteomics; microbiological specimens; pulmonary hypertension; reduced VO2 max; and a distinctive grouping of changes in volumetric MRI findings of (a) forebrain parenchyma; (b) cortical grey matter and (c) caudate; together with (d) atrophy of other grey matter nuclei; and (e) enlargement of superior lateral ventricle; finally, (vii) satisfactory results of therapy have been confirmed by prescribing physicians worldwide when a careful protocol is followed sequentially.

Given this concatenated roster of findings, we are left with the questions, "What offending agents in the complex chemical stew that exist in the ecosystem found in WDB cause CIRS;" and "What are the specific genes that demonstrate immune reactivity to the exposure?"

The answer to the former question requires a detailed review of the current literature (see below). The answer to the latter question comes from the identification of proteomic abnormalities, as well as metabolic complications of proliferative physiology and specific gene reactivity[2] both of which are covered by the use of a patented (US 9,770,170 B2) transcriptomics assay called GENIE that defines the pathways of physiologic gene activation and suppression in CIRS with extraordinary depth and accuracy. When added to sophisticated environmental sampling using molecular methods, including Next Generation Sequencing and transcriptomics, first brought to commercial use by Mould Labs of Australia and Progenedx, respectively, we can now show specific exposures from WDB that result in specific immunologic reactivity.

It is with the link to the immune-based pathophysiology, supported by data from a distinctive group of specific differential

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changes in gene activation, that enables us to call for diagnosis and therapy of CIRS based on validated transcriptomics and proteomics. The days of speculative therapies for CIRS and irreproducible results of unsupported therapies are behind us.

The contribution of Dr. James Ryan connecting CIRS-WDB to the transcriptomic assays cannot be denied. By describing molecular hypometabolism (MHM), found in almost 90% of untreated CIRS patients, we now have a sophisticated molecular test that is being run on RNA samples from patients in the USA, UK, Canada, and Australia. The transcriptomic abnormalities seen on these specimens are indistinguishable when sorted by country of origin and there are no significant racial or gender differences in gene activation or suppression.

Specifically, the transcriptomic changes seen in MHM are:

(i) Defective protein production stemming from ribosomal injury, likely from ribotoxins or ribosomal inhibitory proteins, adversely affecting the sarcin-ricin loop, preventing normal initiation, elongation, and termination of the amino acid chain; and
(ii) Suppression of nuclear-encoded mitochondrial genes which decrease the production of
(a) ATP synthases;
(b) Electron transport chain genes;
(c) Mitoribosomal subunits large and small; and
(d) Translocases[3].

A transcriptomic test, called GENIE, not only will define the adverse effects and potential complications of MHM but when added to by wide open glucose transport channels 1 and 4 due to increased IRS2, there will be proliferative physiology found, an increasing demand for glycolysis to produce pyruvate[2]. Additionally, given the inhibition of translocases in MHM, there will also be a reduction of transport of ions, solutes, ADP and pyruvate across the outer mitochondrial membrane (OMM) into the mitochondrial matrix through the voltage-dependent anion channel (VDAC). VDAC closure results from (i) polycyclic ethers produced by Actinobacteria; (ii) beta tubulins; and (iii) by the use of azole antifungals, particularly itraconazole[2]. Lack of regulation of translocases and VDAC physiology, combined with an increase in cytosolic glucose and pyruvate, leads to aerobic glycolysis and proliferative physiology, which is the cause of many complications of CIRS, including metabolic acidosis, pulmonary hypertension, T regulatory cell deficiency and grey matter nuclear atrophy.

Exposure and reactivity timeline in the United States

A brief look at some of the important events that led to the current status of defining exposure WDB is warranted. Before 2000, and certainly before use of molecular markers while there was some evidence that Actinobacteria and gram-negative bacteria (GNR) could play a role in adverse health effects, the literature was dominated by documentation of exposure, usually by tape lift or culture, to a single filamentous fungus, *Stachybotrys*. “Stachy” become somewhat of a “media darling” as terms like “black mold,” “toxic mold,” and “toxic black mold” came to be widely used in the lay press. This emphasis on *Stachybotrys* was enhanced by a legal case in 2000 from Texas in which the plaintiffs alleged that there was a multisystem illness caused by exposure to Stachybotrys, with injury to cognitive abilities being a prominent feature. When the jury returned a verdict of 32 million dollars (later reduced), the media attention exploded.

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Mold exposures causing respiratory symptoms only:

The role of fungal exposure in litigation grew exponentially, with a California opinion called Geffcken[4] clarifying the defense position in litigation. If a claim for an injury from exposure to fungi in WDB were to be upheld and an award made, there needed to be evidence that mycotoxins found in the patient were the same or similar to those seen in the WDB. This concept then led to an expanded wave of emphasis on mycotoxins.

Meanwhile, the smaller and slower-growing literature on Actinobacteria and GNR bacteria was featured in a few breathless lay articles. To calm the developing public panic about "sick buildings," another opinion on the causation of illness from WDB was put forth from the National Academy of Sciences[5]. In 2004, this opinion acknowledged that respiratory problems were related to exposure to the interior environment of WDB. Shortness of breath, cough, and allergy was noted as represented in the literature published before 2003 cited by NAS. An equally robust literature on extra-respiratory symptoms was not included in the discussion.

WDB recognized to lead to a multi-symptom, multisystem illness:

In 2004, the US EPA weighed in with their summary[6] in which WDB was responsible for a multi-symptom, multisystem illness that included cognitive impairment and neurologic events, including, but not limited to headache.

The gradual switch from respiratory symptoms only to a multisystem, multi-symptom illness was accentuated by the disaster in New Orleans following Hurricane Katrina in 2005. By 2006, the massive number of homeless residents of areas that were still affected by Katrina brought a new wave of publications, some looking at allergy; others confined the analysis to respiratory issues, but an increasing number looked at multisymptom illnesses.

A case/control study performed in 2/2006 looked at homeless residents of the New Orleans area (cases), compared to the crew (controls) of the Scotia Prince, a cruise ship docked at Violet, Louisiana to provide temporary housing for the victims of Katrina. From this study, prepared for St. Bernard Parish, there were clear indications that symptoms and visual contrast sensitivity (VCS) testing, as had been published previously, could separate cases from controls and further stratify illness in cases versus exposed individuals who were not controls but were not sickened. These non-invasive bedside techniques took approximately seven minutes to complete. This study, performed at the direction of the US Senate HEAL Committee, was the first government-associated diagnostic study to suggest treatment as a viable avenue of clinical approach[7].

The symptom rosters correlated well in exposed cases, compared to exposed non-cases which essentially matched to controls. Cognitive and neurologic symptoms were dominant in cases.

Within one day of publication of the findings of this case/control study for the St. Bernard Parish, however, the report was taken off their website.

NIOSH (the National Institute of Occupational Safety and Health), a branch of the US Centers for Disease Control and Prevention (CDC), began a study of two high schools in 2005 (pre-Katrina). The schools[8]

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"were evaluated environmentally by visual inspection and multiple sample types [spore trap, bulk, swab and Environmental Relative Moldiness Index (ERMI)]. The medical team compared 95 occupants of the severely water-damaged school in New Orleans, with 110 occupants of a school that had not experienced significant water damage in Cincinnati, Ohio. The researchers found lower VCS scores in every column in the New Orleans school personnel and statistically significant increases in 18 of the 22 symptoms in every system they evaluated[9] (neurobehavioral, upper and lower respiratory, dermatologic and constitutional systems)".

Multisystem illness and reduced VCS are hallmarks of CIRS.

In 2008, the US GAO[10] published their compiled assessment of the involvement of Federal agencies in WDB evaluations, of issues relating to safety and WDB, in a treatise that also codified the case definition of illness caused by WDB. This report was the first to provide government opinion regarding the status of health and technical aspects stemming from exposure to WDB, noting that in addition to allergy, infection, and respiratory issues, immunologic events could not be ignored. This was the first Federal document that noted the importance of demonstrated host reactivity in addition to exposure.

Unsupported data and the testing status quo:

During this time defense interests in litigation developed an opinion based on a consensus publication from the American College of Occupational Environmental Medicine that the quantity of mycotoxins found in water-damaged buildings was inadequate to cause disease. This position statement, later sun-downed in 2014, is still cited in litigation today even though it ignores the role of individual susceptibility and the role of immune factors in the pathophysiology of the illness. Basing their opinions on an acute one-time exposure in rats and then extrapolating their findings to low-dose, chronic exposure of people, this example of junk science has been accepted over objection countless times in trial.

Inflammation was largely ignored in human health studies aside from several small publications including a double-blinded, placebo-control trial[11] using prospective studies showing consistent changes in these abstruse innate immune inflammatory parameters prospectively, thereby confirming causation of illness[12].

In 2009, the World Health Organization[13] confirmed (i) the causative role of inflammation in multisymptom, multi-system illness; as well as (ii) presented opinions regarding lack of efficacy of air sampling to be used as a diagnostic test. This opinion, while published, did not enjoy wide distribution, as evidenced by air sampling persisting to this day. Specifically, air sampling will only permit the assessment of particulates larger than 3 microns found in the air during the 5 to 10 minutes for the test. Sampling is usually performed in the middle of a room and misses important boundary areas and settled particles.

Air sampling of particulates this size does not permit separation of *Penicillium* species from *Aspergillus*; does not permit the identification of *Wallemia*; and because *Stachybotrys* spores are sticky and heavy, rarely show *Stachybotrys*. The opinion of WHO was stated clearly regarding air sampling that if these findings were to be used there would have to be multiple air samples at a given time in a given room and

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in multiple locations in the room. Further, there needed to be sampling on multiple days per week, multiple weeks per month, and multiple months per year to confirm significance of the use of air sampling.

Categorization of organisms associated with WDB and determination of effects on human health:

By 2006, outstanding work by Dr. Stephen Vesper of the US EPA[14] showed that WDB could be stratified by particular organisms identified with PCR testing (MSQPCR). It was shown that Group One, representing organisms typically found in WDB, could be separated from Group Two, organisms found in buildings without water-damage. Vesper's publication of the Environmental Relative Moldiness Index (ERMI) was the first method to stratify molds of the potential source from a WDB ecosystem separated from a non-water-damaged building ecosystem. Human health effects of elevated ERMI results began to be documented, even though correlation from adverse human health effects were limited in governmental studies to respiratory issues, especially including asthma.

In 2011, a modification of ERMI, called Health Effects Roster of Type Specific (formers) of Mycotoxins and Inflammagens version 2 (HERTSMI-2) was presented at an international congress as a stream-lined method to (i) correlate adverse human health effects with exposure to organisms from Group One found to be accentuated by a factor of 10:1 in cases versus controls; and (ii) whose presence was sorted by the activity of water A(w). Indeed, HERTSMI-2 became the standard exposure tool for physicians actively involved in a treatment protocol to (i) correct the adverse health effects acquired following exposure to WDB and to (ii) assess safety for re-entry into the building after remediation.

Linking exposure to reactivity:

The efforts to correlate exposure with illness were presented in a seminal publication sponsored by Policy Holders of America[15]. The "POA" report looked at all aspects of WDB ecosystems together with a review of the literature of findings of inflammatory response syndrome in patients affected adversely. This publication codified the syndrome as Chronic Inflammatory Response Syndrome (CIRS). As CIRS grew in acceptance by treating physicians, use of HERTSMI-2 led to two publications[16,17] showing (i) superiority of identification of the adverse presence of indicators species found in HERTSMI-2 compared to ERMI for patients with CIRS who were considering re-exposure to WDB; and then to show that (ii) the benefit of use of vasoactive intestinal polypeptide (VIP) was ablated by use in patients with ongoing exposure to WDB.

The use of prospective exposure protocols, namely Sequential Activation of Innate Immune Elements (SAIIE), permitted treating physicians to show that adverse health effects were more likely in patients with elevated HERTSMI-2 scores compared to people with exposure to lower HERTSMI-2 scores.

On the reactivity side, by 2010 works by Petska and colleagues showed the role of ribosomal stress responses to exposure. These ribosomal stress responses focused on particular innate immune response elements, including mitogen associated protein kinases (MAPK). Since testing for MAPK kinases was confined to research laboratories, the ribosomal stress responses, now known to be of vital importance in reactivity, were largely ignored. Pestka was the first prominent

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researcher to focus on molecular methods in CIRS.

NeuroQuant ® (NQ), an advanced technique volumetric brain MRI testing, revealed a reproducible pattern of abnormalities which distinguished CIRS from other neurodegenerative illnesses such as Alzheimer's disease, Parkinson's syndrome, traumatic brain injury and more. NQ differentiated CIRS caused by exposure to WDB from CIRS caused by Lyme disease[18]. NQ documented improvement in forebrain parenchyma and cortical gray matter abnormalities with use of the standardized CIRS treatments protocol that also reduced atrophy found in caudate and multinuclear gray matter to equal controls after the use of intranasal VIP[19].

Insights from Transcriptomics:

By 2016, the new field of transcriptomics was emerging and was applied to CIRS patients. Dr. James Ryan recognized that particular aspects of ribosomal gene suppression were combined with gene suppression of nuclear-encoded mitochondrial genes when analyzed by Next Generation Sequencing using RNA Seq. Dr. Ryan's publication in 2016[3], followed an earlier publication on ciguatera, a field far less controversial but equally important as an inflammatory response syndrome[20]. Next Generation Sequencing looked at 50,000 genes and cost nearly $2000. Clinical application was restricted to research only, but with limited commercial interest, transcriptomics in CIRS showed potential but not widespread adoption by medical providers.

In 2018 Ryan and Shoemaker used a subset of 2000 (of the 50,000) genes shown to be of increased incidence showing abnormalities in CIRS cases versus controls. These 2000 genes were further sorted by signal intensity to generate a panel of 187 genes, with a variety of pathophysiologic processes represented. The resulting gene arrays, stratified by statistical means, were established as a reliable test of differential gene activation/suppression in cases compared to age/gender-matched controls. The test, now called GENIE (Gene Expression, Inflammation Explained), is in wide use by physicians involved in documenting the physiologic basis for adverse human health effects as part of CIRS. GENIE testing, NeuroQuant, and HERTSMI-2 are the three advances using newer methods that have brought us to a new clinical awareness of the role of immunologic reactivity after exposure.

Absence of safety guidelines based on building indices of water-damage:

Part of the problem in finding agreement among indoor environmental experts regarding adverse human health effects acquired following exposure to WDB, is the (i) lack of an effective definition of exposure of occupants. Possibly more important is the (ii) enormous variability of what can be called a WDB. The US GAO case definition[10] currently holds sway as the accepted case definition of WDB, as it focuses on three elements: (i) water intrusion, followed by visible microbial growth, including filamentous fungi; (ii) water intrusion, followed by microbial growth and musty smells; (iii) water intrusion, followed by microbial growth with identification of particular filamentous fungi.

It is rare, however, to find any microbiological ecosystem that does not contain representative fungi, Actinobacteria, and endotoxin-forming Gram-negative rod bacteria (GNR), the so-called "FAB," yet the GAO ignores the crucial roles of

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Actinobacteria and GNR in CIRS. Similarly, the WHO report of 2009, while emphasizing the (i) role of inflammation in the pathogenesis of CIRS, and (ii) is critical of the use of air sampling, fails to acknowledge the vital importance of exposure in WDB to Actinobacteria and GNR.

An additional concern in Federal responses to WDB continues to be the restriction of symptoms considered. The CIRS illness is truly a systemic, gene-driven malady, in which central nervous system and neurological problems are found in over 90% of cases, as is chronic fatigue, musculoskeletal conditions, and non-allergic respiratory issues[21], among 37 individual symptoms, each repetitively found in over 30% of cases.

This omission is not based on the absence of published literature. We maintain that the expanding clinical data stemming from use of molecular markers and the robust scientific literature cited below require a review of accepted policy regarding WDB and CIRS to be revisited, as use of unsupported ideas regarding remediation and novel treatments from mainstream and alternative medicine providers alike pose health risks to patients and consumers.

Water intrusion and Activity of water:

If we look at water intrusion, the variables of duration, location, and specific identification of indoor microbial growth becomes apparent. The lack of use of controlled exposure variables such as time, temperature, salinity, geography and prior injury to the building envelope is concerning. Are we talking about sudden catastrophic events such as a flood in Spring along the Red River or the Mississippi River, for example? Are we talking about a storm surge from an Atlantic coastal hurricane in August? Or a different Atlantic coastal hurricane in October? Or are we talking about indoor flooding inland from a rain event that does not have a massive storm surge but has flooding of rivers and creeks? The duration of flooding and the extent of flooding will also matter in that floodwater levels 8 feet above ground will have a different demand for remediation compared to water levels only 2 feet over the ground. Timing of the acute water event, the temperature of the water, and salinity of the water, all matter.

When the water event is chronic, low grade, like that caused by a pinhole leak in a supply pipe or a faulty seal under a windowsill, the ecological variable becomes the activity of water (A(w)), as available water creates more variables, including stratification of populations of fungi, bacteria (especially GNRs and Actinobacteria), determining the abundance of numbers and species of microbes. We can use indicator species of fungi to tell us whether we have ongoing water intrusion, as seen in A(w) greater than 0.9; or are we dealing with an intrusion/dry out event in which A(w) is lower, perhaps between 0.8 and 0.9; and finally, are we looking at a far more prolonged intrusion/dry out event in which xerophilic fungi dominate compared to *Aspergillus sp, Penicillium* sp., *Stachybotrys sp.* and *Chaetomium* sp.?

The common denominator for water damage is for 48 hours or more. We know that in a weather disaster with wind and flood damage, there are often complicating problems such as loss of electrical power and compromised navigation through areas with damaged bridges and flooded waterways. The 48-hour window of indoor safety from exposure to WDB disappears rapidly in catastrophic, week-long weather events.

A large number of studies of WDBs have stemmed from investigations following

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storm events, with Hurricanes Katrina and Rita of 2005 in the USA a typical example[22,23,24]. Water intrusion will come from interior water leaks or penetration of outside water through the building envelope in a chronic, low-dose water intrusion event. A chronic low dose event is usually marked by the presence of a greater richness of microbial diversity, as there will be a variety of environments typified by different A(w).

Many environments will have a mixture of A(w) depending on the distance away from a point source water intrusion event. In the case of a basement, there is a strong likelihood of a more chronic, more uniform water event but if the basement is a walkout, the water pressure placed against the inground side of a subterranean environment will be greater at one end compared to the downhill side of the same building. In basements and crawl spaces, if an attempt is made to channel intruding water out of the subterranean chamber, a microclimate of wet conditions within the channel and its ecosystem are established, creating a potential for exposure that will have wet conditions, semi-wet conditions, and dry conditions all in the same facility.

Actinobacteria and endotoxin testing:

Failure to account for Actinobacteria and GNR remain an obvious source of error in measuring microbial growth in WDB. Similarly, identification of fungi by "musty smells" will be remiss as many Actinobacteria create compounds that present a musty odor, including geosmin. GNR can also have an odor, so one of the primary members of the triad of identification of amplified mold growth is fundamentally flawed by presence of two non-fungal elements.

An example of faulty testing for Actinobacteria comes from the detection and quantitation of just one species of Actinobacteria, in dust, for example. These organisms have the capability of making trimethylamine (TMA), a microbial volatile organic compound (mVOC), that acts as a pheromone, attracting other species of Actinobacteria to an area of growth conditions that support the additional manufacture of TMA. This response of Actinobacteria to mVOCs is truly remarkable in that the organism can grow a pseudopod that contributes to the ability of Actinobacteria to move[25]. Actinobacteria motility shows the advance of Actinobacteria over obstacles, including vertical obstructions, to get from point A to point B. Once an adequate number of Actinobacteria are in a given environment, the collective production of TMA will alkalinize growth media, including building materials, used by Actinobacteria to grow at the expense of acid-loving fungi. This migration of Actinobacteria will increase species abundance, showing support of amplified growth of Actinobacteria, rather than just mere numbers of one species alone.

Finally, there are species differences in GNRs with the outer membrane of these bacteria surrounding a cell wall which surrounds an inner cell membrane. There is great variation in the endotoxin/ lipopolysaccharide (LPS) composition of the outer cell membrane. This difference in composition accounts for the variable immunogenicity that follows[26].

Complex causation of illness:

Any organized approach to the identification of specific parameters responsible for specific human health effects that do not control for the numerous variables of exposure is inherently compromised. Such

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specific causation is further muddled by the similarity of innate immune responses of the human host to each of the different parameters that could be a part of a complex microbial stew[27]. The GAO report agreed.

There is a dearth of published literature focusing on different sizes of fragments of microbial inflammagens, separate from microbes growing or being present after cell death within the building envelope. Any measures that do not account for the variable size of respirable components will be incomplete, at best.

Next-Generation Sequencing (NGS); Comments by David Lark

Completed in 2003, the Human Genome Project began an era of rapid, affordable, and accurate genomic analysis called Next-Generation Sequencing (NGS), building on "first-generation sequencing'' technologies to yield accurate, fast, and cost-effective sequencing results, which determine the identity of microbial contamination.

Also known as high throughput sequencing, Next Generation Sequencing (NGS or NextGen), it includes several methods that determine the nucleotide sequences of DNA and RNA at a much faster rate and more cheaply than techniques used previously. The application of NGS has revolutionized the study of molecular biology and genomics.

Researchers started to apply these techniques, which enabled them to sequence many other targets more rapidly and at a reduced cost, leading to the extensive expansion of NGS platforms that offer simultaneous sequencing of millions of DNA fragments. Thus, sequencers are now available with throughput high enough to sequence parts, or even a whole genome, in just a single day.

1. The Platform

Several definable steps led to a successful NGS process. The prelude to NGS is template preparation sometimes called library prep, which refers to the building and amplification of a nucleic acid library. The sequencing library is prepared by steps where the nucleic acid sample is fragmented, and the ends of the DNA fragments are tagged synthesized DNA molecules with a known sequence. The library, once built, is then amplified by several rounds of PCR before progression to sequencing.

Further details of this process are shown in **NextGen Methods,** below.

The creation of a new DNA fragment is templated from the fragments in the library. The fragments are washed and flooded sequentially with the known nucleotides. As the nucleotides become incorporated into the new, growing strand of DNA, they are recorded as sequence information.

The technology is now widely used in epidemiology and public health studies, sequencing fungi, bacteria, and viruses. NGS of RNA has replaced other methods such as microarray analysis in gene expression research. Also, such RNA sequencing can provide the entire transcriptome of the targets chosen in a sample in one analysis, without knowing an organism's genetic sequence.

Next Generation Sequencing (NGS) is a robust platform that has enabled the sequencing of vast numbers of nucleotides simultaneously. This powerful tool is revolutionizing fields such as personalized medicine, genetic diseases, and clinical diagnostics by offering a high throughput option with the capability to detect and quantitate multiple targets - be they fungi,

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bacteria,
viruses, or even protozoans, at the same time.

It is a growing field, with the first machines marketed as long ago as 2005. However, in the intervening decade and a half NGS has become a cornerstone of molecular biology and genetics. As such, familiarity with its technical terms will help in gaining a better understanding of the available literature. However, it should be countenanced that due to the likelihood of error-prone sequencing reactions, random errors could occur. Therefore, an allowance of at least 30x coverage is typically required to ensure that each nucleotide sequence is accurate.

NGS is characterized by improved accuracy and speed but also reduced human resources and cost. There has never been a time where it has been as cheap, convenient, or straightforward to sequence a genome. Arguably, the most substantial improvement has been the development of parallel analysis, which increased the sequencing speed. Previously in microbiology and environmental science the conventional characterization of micro-organisms was by morphology, staining properties, and reliance on metabolic criteria – now with NGS this has been supported with genomic definitions derived by NGS.

The genomes of micro-organisms define what they are, so they may contain such information as drug sensitivity and epidemiology to trace sources of infection outbreaks. The key innovation that transforms DNA replication into the DNA-sequencing strategy at the core of both Sanger
and NGS methods is the use of un-extendible, fluorescently labeled modified bases. There are four different colors of modified bases for A, T, G, and C. In Sanger sequencing, only a small percentage of bases are modified, whereas, in NGS, all available bases are modified. In both sequencing techniques, when polymerase incorporates a modified base into the copied strand, extension of the new strand stops, and, critically, this newly terminated strand is uniquely colored to reflect its most recently added base.

Rather than exploit size separation to arrange the fluorescent molecules, NGS uses positional separation: millions of different template DNA strands bind to discrete positions on a glass slide and remain fixed at the same position throughout the entire sequencing reaction. Each template is then extended by a single modified base, and a camera captures an image that resolves both the position of each template on the glass as well as its fluorescent color and intensity. Next, in a step unique to NGS, the modified bases are converted to regular bases, such that they become both extendable and non-fluorescent. This restoration process primes them to undergo subsequent rounds of single-base extension and imaging. At the end of a sequencing run with an imaging cycles, the fluorescence color at each template position in each image is mapped to a base (i.e., A, T, C, or G). The bases form a single template position and are concatenated to yield a DNA sequence of length n, called a "read." Interestingly, although initial NGS read lengths were <100 and trailed behind Sanger's typical 400- to 500-base sequences, newer NGS machines can match or exceed the length of Sanger-generated sequences.

For this study, Illumina sequencing equipment and methodologies are principally utilized, providing a much-broadened approach from the classic Sanger chain-termination method. As previously stated, NGS has had a vast impact on the determination of microbial abundance and diversity in environmental samples.

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Related species can be identified, and more distantly related species can be inferred. Qualitative genomic information is obtained for targeted sections of the genome, and relative abundance of the sequence reads are used to derive quantitative information on individual microbial species.

2. Next Gen Methods

For the studies reported here, methods applied include DNA being extracted from approximately 20 mg of settled dust that was weighed into 2-mL tubes with glass beads 0.1mm size, starting with a bead-milling step for mechanical cell disruption using a Mini Beadbeater-16 for 1 min (Biospec Products Inc., USA).

Once separated from the dust, DNA is extracted from the samples using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine CA) or similar. A known amount of deoxyribonucleic acid sodium salt from Salmon Testes (Sigma Aldrich Co., USA) is added to the samples before extraction as an internal control. DNA is stored at - 20 °C until subsequent analysis. Negative (reagent) and positive (bacterial and fungal mock community) controls are included in the DNA extraction step along with house dust samples.

The bacterial DNA extracted from house dust and control samples was processed for library preparation and sequencing. The gene specific sequences used in this protocol target the 16S V3 and V4 region of the microbial genome. A standard protocol was provided by Illumina (Part # 15044223 Rev. B) to generate the library. Mold DNA was similarly processed but targeted the ITS region of the genome.

Sequencing was performed on an Illumina MiSeq with V3 chemistry resulting in paired-end reads with a length of 500 bp each. One analyzed, using an Illumina MiSeq, the data from the sequencing of the libraries were referenced to the sequence databases via pre-loaded Reporter software to enable bacterial identification.

Focus on exposure and reactivity:
Assessing individual reactivity:

Given that the multiple uncontrolled variables in a WDB prevent logical conclusions regarding the safety of exposure to a given WDB, as well as preventing the calculation of risk of exposure to a given WDB, safety assessments are still possible by shifting emphasis from the building to the occupant. We have the GAO case definition for affected patients that has withstood 12 years of public scrutiny. By applying that case definition, and adding new insights into molecular mechanisms of injury, particularly the transcriptomic test, GENIE, we can finally address specific causation. Now that we have GENIE, we can access specific gene activation (see study below) to assist in defining presence of specific causation.

Determining effectiveness of remediation:

Despite the above well-known confounders that adversely affect the ability to measure elements of specific causation, experts in indoor air quality have codified specific recommendations to remediate indoor environments, including residences, offices, and educational facilities[28]. A newly published expert opinion on remediation guidelines[29] is the first to emphasize the illness status of occupants in the evaluation of remediation needs. Even with these professional opinion papers, however, there still is a paucity of the data needed by policymakers and stakeholders to make recommendations for (i) a threshold needed

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for remediation; and (ii) a reliable method(s) to measure outcomes derived from remediation.

A review of a typical inspection report might shed light on why we do not have thresholds for the safety of occupancy applicable to pre- and post-remediation evaluations. There is no consistency of inspection parameters; no pre-remediation standards for laboratory confirmation of health risk for people and pets exist; and there is no post-remediation, re-exposure trials for patients to use to assure the safety of re-occupancy despite published literature, including double-blinded, placebo-controlled, prospective studies[30,11,12], that give clear protocols on how re-exposure can be performed logically, rigorously and ethically. The medical dictum to "always evaluate action taken," seemingly does not apply to remediation.

Review of the literature on the expanded sampling of WDB

The effect of building and environmental parameters on microbial growth

Moisture available to support microbial growth is measured as the Activity of water - A(w) which is a unitless parameter of the substrate, which has a range 0 to 1, defined as the vapor pressure of the water in the material as a fraction of the surrounding saturation vapor pressure. For dust in vacuum samples, the relative abundances of hydrophilic fungi (growth requirements of $\geq$ 0.90 A(w)) were consistently 2 to 3 times greater in homes with observed mold or other damage than in homes without. Mesophilic fungi ($0.80 \leq$ A(w)<0.90) in vacuum samples unexpectedly showed strong opposite trends: the relative abundances in homes with visible mold were less than in homes without. Xerophilic fungi (<0.80 A(w)) in vacuum samples showed only weak, nonsignificant associations in both directions[31].

The features of WDB that are most strongly associated with a microbial response are not known. Many metrics were considered, summarizing observed water damage and mold, including visible mold, other damage, moisture content of building materials measured through a moisture meter, and window condensation. The strongest relationships were seen between building damage and microbial responses when they were near each other, physically and temporally. Thus, we expect that the better aligned the microbial measurements are to any water damage, in both space and time, the better the ability to detect the damage[31].

Within the habitat of buildings, (i) both fungi selected by known moisture requirements and fungi reported to occur at elevated levels in WDB were increased in certain sample types in damp or moldy buildings, (ii) among time-integrated dust samples, samples with longer but unspecified time-integrated periods showed stronger relationships with moisture conditions than samples of shorter but defined time-integration periods, and (iii) despite issues of taxonomic identification and resolution in amplicon data sets, targeted approaches using the current annotation tools showed stronger relationships with building dampness than untargeted[31].

Adams had previously published a meta-analysis[32] in which she discussed her mechanistic views on processes in the built environment.

"In recent years, the scientific community has begun to recognize the importance of characterizing such human-associated habitats, with increasing numbers of studies seeking to determine the biodiversity, ecology, and public health implications of

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microbial assemblages present in the built environment." Individual efforts to characterize the microbes we encounter in buildings have shown that geography, building design and ventilation, and occupant presence and activity can all contribute as drivers of indoor microbial communities[32].

On the other hand, there are several recognized limitations, with the dominant question being – can we detect true biological differences over the background across studies. High-throughput sequencing has vastly increased the quantity of data resulting from surveys of microbes across many different environments. As the health implications of the indoor microbiome are continually tackled by the research community, understanding the basic factors that govern the potential pool of exposure is fundamental to modulating that environment.

Adams has also published on the airborne fungal and bacterial communities found in non-water damaged residences[33]. She reviewed the importance of outdoor *Streptomycetes* in the outer rooms of a residence compared to skin-sourced organisms found in the inner sections of a residence. The data showed that there is variation in outdoor sources of fungi and bacteria, both in space and time. However, humans provide a strong homogenizing effect of indoor bacterial bioaerosols, which does not occur with fungi. Time-resolved data on both bacterial and fungal diversity in the same outdoor and indoor samples allowed them to show that processes structuring their indoor communities have important differences as well as commonalities.

Actinobacteria increase in abundance as one moves to the more internal rooms of the dwelling.
For example, while the Actinobacteria appear to increase only slightly in the internal rooms, the outdoor *Actinobacteria* are comprised of the *Geodermatophilaceae* and *Nocardiaceae* families which are associated with stone and soil, and the indoors is dominated by the skin associated *Corynebacteriaceae, Propionibacteriaceae,* and *Streptomycetaceae*[33].

Adams enters an experimental mode with her paper from 2015 on human occupation of an environmental chamber[34]. Overall, this study revealed a smaller signature of human body-associated taxa than had been expected based on recent studies of indoor microbiomes, suggesting that occupants may not exert a strong influence on bioaerosol microbial composition in a space that is well ventilated with air that is moderately filtered.

Even in mechanically ventilated buildings, if the air-exchange rate is high, the filter efficiency poor to moderate, and the occupancy light to moderate, the indoor air microbiology can look much like that in outdoor air[24].

Evidence was found that humans can be vectors for microbes to enter the built environment through tracking on clothing and subsequent shedding. The importance of this source contribution to the indoor microbiome may be under appreciated[34].

Using newer sequencing methods Adams showed a surprising fungal richness of indoor carriage, focusing on the skin as a carrier surface[35]. She concluded that while some endogenous fungal growth on typical household surfaces does occur, particularly on drains and skin, all residential surfaces appear to be passive collectors of airborne fungi of outdoor origin, a view of the origins of the indoor microbiome is quite different from bacteria.

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While the presence of these known fungal associates was detected on surfaces, overall, it was found that the influence of outdoor air was dominant. What is perhaps surprising is that there is little evidence of any significant indoor fungal source for airborne assemblages such that the indoors appears to be largely an immigrant assemblage with dispersal and deposition from the outdoors overwhelming indoor growth[35].

Jayaprakash, writing with Adams and Martin Taubel[36] in 2017 shows us how far we have to go if we are to use building health parameters to correlate with human health effects. A better approach is to use molecular methods.

Their analysis was performed with an expanded range of detectable microbial secondary metabolites (*348* fungal and *44* bacterial metabolites), using a more sensitive LC-MS/MS system. Several conclusions can be made. Household environmental characteristics more strongly influenced fungi than bacteria. For bacteria, significant increases in the relative abundance of *Staphylococcus, Streptococcus,* and *Planococcaceae* genera in house dust after families had moved to a new home were noted. For fungi, they found significant decreases for *Phoma, Botrytis,* and *Monographella* genera during moisture damage renovations[36].

Almost all bacteria were reduced in relative abundance during moisture damage renovations and were largely within Actinobacteria. For fungi, they observed mostly decreases in the relative abundance of the selected taxa. For bacteria, most were decreased in relative abundance during moisture damage renovations and were again frequently members of *Actinobacteria*. For fungi, none of the taxa were identified with more than one approach[36].

After renovation activities, the majority of bacterial species associated with moisture damage renovations were allocated to various families within the Actinobacteria. These taxa may have a role in indicating moisture in buildings, as they found dose-response relationships in some taxa.

One of the key findings of this study is that moisture damage interventions neither appear to be linked to major changes in the most abundant bacterial and fungal taxa in house dust, not to mention changes in the overall community structure[36]. In the top 15 most abundant taxa between pre-and post-interventions, constituting close to 60% of bacterial and more than 50% of fungal sequences, little change was noted. However, a reduction in both bacterial and fungal abundance and diversity in house dust after the renovation of moisture damage was observed.

Taken together, these findings suggest that the fungal and bacterial microbiota in house dust of moisture-damaged homes are not marked by a common and striking shift relative to dry homes but rather may experience more subtle changes in some of the less-abundant taxa[36]. The evidence to confirm the involvement of microbial exposure in adverse health effects in damp buildings has been inconclusive in the absence of the use of human health treatment protocols.

Both cultivation and cultivation-independent techniques indicate that representatives of Actinobacteria may be important components of WDB. Sequencing-based findings appear to complement cultivation-based knowledge on the link of certain groups of Actinobacteria within damp buildings. The authors observed a clear concentration of moisture damage renovation

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associated taxa within the Actinobacteria, which may provide a more focused target for future damp-building-related studies.

These data are remarkable when compared to established, "common-knowledge" that (i) filamentous fungi are disproportionately found in WDB compared to dry buildings; and that (ii) remediation restores the indoor ecosystem to dry status. Since newer microbial identification methods support the widely accepted premises upon which building safety is based, we are faced with prophylactic use of mechanical devices to clear the air of particulates as the only potentially effective intervention, especially in areas with boundary layers of reduced air-flow,
to prevent relapse of CIRS patients.

Patovirta[37] showed that when fungi and bacteria grow on building materials, they can produce toxic secondary metabolites and the determining factor of the metabolites that grow is the nature of the building material itself. In the same study, they found unknown and typical fungi on moisture-damaged building materials, which calls for a more detailed investigation of the microbes capable of growing on building materials[37].

The ongoing focus on building health parameters, as opposed to proteomic and transcriptomic assays, is demonstrated by findings from Finland in 2011 from Pitkaranta[39] and Reponen and Vesper USA[38] in 2010.

It is now known that indoor microbial communities are structured by patterns of geography and climate, season, building design, ventilation systems and occupants. Fungal taxa found in house dust from healthy buildings have been shown largely to a subset of outdoor fungi, presumably trafficked inside, and their presence is principally determined by the location of the home[38].

Methods of analysis of microbes:

As if the absence of human health studies by themselves were not high enough of a bar to policy formation, the literature clearly shows that the measures we use to define a WDB are themselves flawed. The use of ERMI only to assess building contamination misses hundreds of species of fungi. Failure to use Next Generation Sequencing (NGS), now commercially available, misses thousands of species of bacteria, particularly Actinobacteria. Granted, the documentation of immune reactivity following exposure to lipopolysaccharide endotoxins and Actinobacteria, using transcriptomic assays, are newly published[2], yet the under-counting of biologically crucial elements found in WDB have been known for at least 15 years.

This limited picture of the overall taxonomic composition was broadened with the advent of DNA sequence-based tools to both identify specific microbes and characterize entire microbiomes.

In a study with NIOSH, Sulyok, writing with Park and Cox-Ganser in 2018[40], emphasizes the need to validate each method used to analyze for microbial components. Correlation of fungal presence with WDB was inherently unreliable if only one method of analysis was used (see similar results in Jayaprakash, above). For example, aflatoxin, commonly reported by commercial labs assessing urine for mycotoxins using only one method, was not found in any of the homes reported in this study.

The study findings suggest that using multiple measurement methods may provide an improved understanding of fungal exposures in indoor environments and that

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secondary metabolites may be considered as an additional exposure. The methods used were viable culture, internal transcribed spacer (ITS) sequencing and LC-MS, with sampling done in 120 rooms in WDB.

The authors were able to identify 32 metabolites (out of 509 tested for) from 28 samples, but 7 of the 32 metabolites were found in only one sample. The number of metabolites identified per sample ranged from 9 to 18, with an average of 15. Of the 32 metabolites, cyclo (L-Pro-L-Tyr), usnic acid, asperglaucide, emodin, and averufin were detected in all samples. Lotaustralin, skyrin, brevianamide, F, 3-nitropropionic acid, integracin B, neoechinulin A, citreorosein, integracin A, linamarin, alternariol monomethyl-ether, and rugulosuvine were found in more than 50% of samples (40). None of these metabolites are reported in commercial assays.

(1→3)-B-D-glucan (a fungal cell wall component) and endotoxin (a component of the outer membrane of Gram-negative bacteria) were detected from all 27 samples analyzed.

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Except for *Aspergillus, Epicoccum,* and *Pithomyces* that showed moderate to weak agreement, the agreements for other fungal genera detected using the 2 methods were poor[40].

Sterigmatocystin, a precursor of aflatoxin biosynthesis, can be produced by more than 30 species of filamentous fungi including *Aspergillus flavus, Aspergillus nidulans, Aspergillus parasiticus,* and *A. versicolor*, but no sterigmatocystin or aflatoxin B was found.

Given that standard reporting from building inspection does not report any metabolites tested for by Sulyok, these findings indicated that the limitations of each method may result in a potentially inaccurate assessment of occupants' fungal exposure[41].

However, continued improvement in HPLC testing methods has advanced knowledge regarding confirmation of the presence of a wide diversity of fungi in WDB[41]. "Using enzymatic pre-treatment, solid-phase extraction and UHPLC separation, the sensitivity of the method was significantly higher (10-160x lower LODs) than in a previously described method used for comparison purpose, and stable isotopes provided compensation for challenging matric effects."

Biomarkers were detected in all samples. The mycoestrogen, zearalenone was the most frequently detected contaminant (82%) but also ochratoxin A (76%), aflatoxin M1 (73%), and fumonisins B1 (71%) were quantified[41].

Despite laboratory advances, assessment of the presence of specific causation cannot be made by simple identification of indicator species of fungi[42]. Although in the "mold inspector" industry, "toxicity" is often inferred from the presence of toxigenic fungi, i.e., *Stachybotrys chartarum,* there is no evidence that the detection and quantification of airborne fungi are representative of actual mycotoxin concentrations.

The trend away from culture-based diagnostics to DNA technology and other molecular methods is not a new subject in IAQ. In 2011, Pitkaranta and co-authors published an important paper[43]. The observed flora differed markedly from that recovered by cultivation, the major differences being the near absence of several "typical" indoor mold genera such as *Penicillium* and *Aspergillus*. The potential harmfulness of microbial contamination is suggested to be

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dependent on the species content and concurrently occurring species combinations, as well as on the material and growth conditions in which the microbial proliferation occurred.

The main benefits of using DNA as an identification tool instead of cultivation-based methods are the speed, accuracy and analytical sensitivity of detection and the possibility to detect and identify dead or dormant organisms. "Molecular methods most often used in fungal studies include conventional or quantitative PCR (qPCR) specific for fungal species or groups, universal fungal PCR combined with denaturing gradient gel electrophoresis (DGGE), or temperature gradient gel electrophoresis, terminal or conventional restriction fragment length polymorphism analysis. Universal fungal PCR combined with cloning and restriction fragment length polymorphism analysis and/or DNA sequencing of cloned fragments has been used as well as probing methods[43]."

In these studies, the theoretical diversity in the studied environment was estimated to comprise at least 100 and, in some studies, even more than 500 fungal phylotypes. "Full-length ITS sequences were obtained from 1,578 clones. Of these, 1,339 originated from fungi, 223 originated from plants, and 11 originated from algae, and 30 were chimeric."

"Of the total 394 sequence types, 234 were of basidiomycetous affinity and 155 of ascomycetous affinity. The present results demonstrate that more diverse microbial flora is observed from the same material by PCR and clone library sequencing compared to traditional cultivation[43]."

Little is published on the special case of high-rise buildings. Vesper, writing with NIOSH[44], suggests that ERMI could have a role. "The ERMI metric may be useful in the quantification of water-damage and mold growth in multi-level buildings." Most previous studies of mold contamination in multilevel buildings have utilized short air-samples. Park noted that air sampling for microbial agents has pitfalls and used settled-dust sampling for the study[44].

Fungal assessment:

The findings of Finnish investigators [37] were paralleled by Sylvain and Adams in 2019[45]. Common indoor molds as determined by qPCR did not show a trend in relation to WDB. They found higher molecular diversity and ERMI scores in dust collected from WDB than their matched references. In contrast, elevated total concentrations of fungal biomass, total cell counts of common indoor molds or culturable fungi were not seen. The authors found water damage associated with elevated fungal diversity[45]. There were significant differences in fungal biomass in units with visible mold and no visible mold. This study demonstrates that high-throughput sequencing of fungi indoors can be a useful strategy for distinguishing distinct microbial exposures in WDB with visible and non-visible mold growth and may provide a microbial means for identifying WDB[45].

Significant differences in fungal abundance were found in settled dust from units with visible mold, units with no visible mold, and the outdoors. Outdoor samples had the greatest abundance, followed by units with no visible mold, and then units with visible mold. Settled dust samples detect a wider array of fungal classes than surface samples and a greater abundance of taxa within shared classes. By sequencing settled dust from units with visible mold, units with no visible mold, and outdoor air, the authors studied the

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impact of water damage on fungal biomass, richness, and community composition indoors. With these data, they found distinct fungal communities associated with moldy housing.

In the study of a poorly maintained building in the same region, evidence that the presence of excess water in units, judged by visible mold, allowed for the proliferation of a few indoor taxa and the development of distinct fungal communities.

Measurement of bacterial toxins:

Actinobacteria, filamentous fungi-like bacterial genera producing spores, are recognized as a potential indicator of wet conditions. Actinobacteria have been known as an etiologic agent of hypersensitivity pneumonitis (HP), such as farmer's lung disease. These Gram-positive bacteria have also been associated with other health effects among occupants of WDB[46].

Recognition that *Streptomyce*s were important genera in WDB is longstanding[47]. Although differences in morphology, pigmentation, fatty acids, biological activity, and pH tolerance indicated that strains did not necessarily match with three single phenotypes, they all appeared to belong to two or three branches of Streptomyces spp. most common in environmental isolates. Actinobacteria were detected in 74% of dust samples, with thermophilic Actinobacteria being the most predominant (81%) among the three types. Non-tuberculous mycobacteria were cultured from 23 dust samples (19%) with a maximum level of 190,000 CFU/g[48].

In 2002, Rintala[49] recognized the benefit of PCR to show the diversity of *Streptomyces* seen in WDB. Streptomycetes are known indicators for moisture and mold damage in buildings and potential health risks, but their diversity in indoor environments is still unknown. They can grow and produce spores on the surface of the building materials using the nutrients provided by the material itself, organic dust on the material, or other micro-organisms such as fungi that are colonizing the material. The majority of the sequences clustered in two groups in the phylogenetic analysis. However, the results indicate the presence of several different types of Streptomycete 16S rDNA sequences in buildings, suggesting a higher diversity.

In 2001 Roponen showed that *Streptomyces* grown on different building materials evoked different inflammatory responses in a cell culture line of macrophages[50]. "These results indicate that the ability of S. annulatus to induce inflammatory responses and cytotoxicity in macrophages is dependent on the growth conditions provided by different building materials."

In an earlier study from 2011, Taubel, Sulyok, and co-authors[51] dramatically exposed the need for ongoing measurement of Actinobacteria. This paper was the first to demonstrate the ubiquitous presence of bacterial toxins, primarily Actinobacteria, with mycotoxins, with a clear statement that linkages of human health effects need to be made to toxicologic data on inhalation exposure to assess the risk related to indoor exposure to microbial toxins. The authors identified bacterial compounds monactin, nonactin, staurosporin, and valinomycin, and found them exclusively in building materials from moist structures. These bacterial metabolites are highly bioactive compounds produced by *Streptomyces*, a group of microbes that is considered a moisture damage indicator in indoor environments. Toxic bacterial metabolites need to be considered as being part of complex and

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diverse microbial exposures in "moldy" buildings.

Bacterial toxins co-occur with mycotoxins in WDB. These compounds are measurable also in settled airborne dust, indicating that inhalation exposure takes place. In attempts to characterize exposures to microbial metabolites, mycotoxins and bacterial metabolites have to be targeted by the analytical methods applied.

In this study, 33 different microbial metabolites were detected, with all of the 69 samples positive for at least one of the analytes. The fungal compounds emodin, enniatin B, beauvericin, and the bacterial toxin chloramphenicol were overall most prevalent and were detected in 62%, 57%, 38%, and 38% of all samples, respectively[51]. The detection of 28 different fungal metabolites confirms earlier reports showing the presence of multiple mycotoxins in moisture-damaged indoor environments. The multi-analyte method showed bacterial metabolites, most of them produced by *Streptomyces* species, with five such compounds, monactin, nonactin, staurosporin, and valinomycin, being exclusively detected in building material, but not house dust. Valinomycin, monactin, and nonactin are known ionophores that disrupt transmembrane ion gradients. Monactin and nonactin are members of the macrotetrolide antibiotics family and have been shown to modulate cytokine production and T-cell proliferation[51].

Focusing in 2018 on respiratory symptoms and Actinobacteria exposure, additional research from Finland, including Taubel[52], showed the highest group of Actinobacteria counts was associated with more symptoms and worse perceived health. The discussion regarding the role of exposure to Actinobacteria continues[53]. Dampness can also support the growth of dust mites and Actinobacteria, which can lead to confounders in health effects due to fungal exposure.

Growth is not only determined by moisture, but also by the pH of the environment. In 2005, documentation of preference for Streptomyces for alkaline pH was confirmed[54]. *Streptomycetes* prefer environmental pH ranging from neutral to alkaline, although they commonly occur at remarkably variable pH and nutritional conditions. Therefore, the dependence of 10 *Streptomyces spp*. on pH tolerance in nutrients was determined.

On media with starch and casein; glucose, tryptone and yeast extract; tryptone and soy peptone; and glycerol-arginine and yeast extract; strains grew over a broad pH range between 4.0-5.5 and 10.0-11.5. On glycerol-arginine and medium with Na-propionate, NH (4) NO (3), and yeast extract, Streptomycetes grew optimally at pH 7.0 and above[54].

Measurement of endotoxins

The search for data to understand the contribution of endotoxins to the chemical stew is hampered by the lack of use of immunological measures of individual reactivity[55]. It is possible that concentrations of cells obtained by QPCR and concentrations of cell wall components are not equivalent and may each be too broad a category to understand the bacterial composition and identify the sources of the microbial mixture in a home.

Endotoxin reservoirs are not well defined[56]. Floor dust samples with moderate reproducibility may be the best choice for sampling of endotoxin in large field studies. The only significant difference found

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between the high ERMI and low ERMI groups of homes was the concentration of dust-endotoxins, which was greater in the high ERMI homes. Although the dust samples from high ERMI homes contained significantly greater concentrations of endotoxins, the authors did not find a significant difference in the concentration of GNR in the dust samples between high and low ERMI homes. These results may indicate the over-production of endotoxins by the same number of GNR.

Previous investigations have shown an increased risk of adverse respiratory health outcomes associated with increased concentrations of (1→3)-B-D-glucan or endotoxin, but a protective effect has also been demonstrated for endotoxin and extracellular fungal polysaccharides. In a 7-year follow-up study, no correlation was found for dust endotoxin values measured in Year 1 and Year 7 (38). Concentrations of dust endotoxin and (1→3)-B-D-glucan were found to be significantly higher in year 7 than in year 1, whereas ERMI did not differ between these two time points. Endotoxin is a measure of GNR exposure, so its concentration in the dust is not necessarily associated with mold exposure. This finding is consistent with the results of a national study of US homes which demonstrated that about 50% of the time neither the home occupants suspected, nor the mold inspector detected the mold problem in high ERMI homes. Concentrations of endotoxin, (1→3)-B-D-glucan, and fungal spores showed inconsistent trends. These concentrations were higher in the high ERMI category compared to low ERMI[38].

Secondary microbial metabolites:

Kirjavainen, writing with Taubel and Michael Sulyok in 2016[57], also shows the absence of reliable biomarkers for adverse human health effects, and that secondary metabolites are always found in *all buildings, not just those with water damage*. This paper describes the most comprehensive characterization of microbial toxins and other secondary microbial metabolites in the indoor environment. It demonstrates the ubiquitous presence of secondary microbial metabolites in homes even *in the absence of moisture damage* or mold.

Living room floor dust was analyzed by LC-MS/MS for 333 secondary metabolites from 93 homes of year-old children. Moisture damage was present in 15 living rooms. The median number of different metabolites per house was 17 (range 8-29) and the median sum load was 65 (4 to 865) ng/m2. Overall, 42 different metabolites were detected[57].

The presence of microbial secondary metabolites was assessed in a more representative sample of Finnish homes, including both homes with and without moisture damage and mold. Six metabolites, namely brevianamide F, moniliformin, emodin, enniatins B and B1, and 3-nitropropionic acid were detected in all houses. Enniatins A and A1 were found in all but one house. The total load of metabolites in living room floor dust (ng/m2) tended to be increased if there was moisture damage in the living room or mold odor anywhere indoors during the site visit. Total metabolite loads tended to be higher in houses where the occupant reported moisture or mold damage, but not in homes where the damages had been repaired.

The authors conclude that (i) secondary microbial metabolites are ubiquitously present, albeit in relatively low concentrations, in the primary living space of residential houses even in absence of any signs of moisture damage or mold. We note that immunological reactivity is not

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concentration-dependent. The (ii) total load and number of different metabolites are moderately increased by moisture damage. Individual metabolites associated with moisture damage or moisture damage with mold included physcion, skyrin, meleagrin, ochratoxin A, enniatin A and alamethicin. Ochratoxin A and enniatin A have been detected in moisture-damaged homes but previously without comparison to undamaged homes. Finally, (iii) no macrocyclic trichothecene metabolites of *Stachybotrys chartarum*, such as satratoxins and verrocarol, were found[57].

In the study, tellingly, none of the individual secondary metabolites were detected enough in association with moisture damage or mold to promote them as potential biomarkers of moisture damage or mold, especially since microbial secondary metabolites are ubiquitously present in the floor dust of all houses with or without moisture damage and mold.

Synergistic effects of microbes:

Taubel[36], writes, "When attempting to assess the health relevance of chronic, low-level exposure to microbial metabolites in indoor environments, their variety and co-occurrence, as well as the possibility of synergistic effects of different metabolites and other microbial compounds, need to be considered." Health effects identified as well as toxicological data on inhalation exposure, will help to assess the risk related to indoor exposure to microbial toxins. Research from Finland in 2015[58] once again shows us that trying to link exposure to mycotoxins to a given building is in error, as microbial metabolites are everywhere.

The molecular basis of inflammatory responses to exposure to Actinobacteria and fungal elements was documented in 2017[59]. While the expression of proinflammatory cytokines TNF and IL-1B following exposure to single toxins was modest, low-dose co-exposure with structural components increased the responses of emodin B and valinomycin synergistically, both at the mRNA and protein level, as measured by TR-qPCR and ELISA, respectively. Co-exposure of toxins and B-glucan resulted in consistent synergistically increased expression of several inflammation-related genes. Co-exposure of toxins with either B-glucan or LPS induced mitochondrial damage and autophagocytosis. The results demonstrate that microbial toxins together with bacterial and fungal structural components found in WDB can have synergistic pro-inflammatory interactions at low exposure levels[59].

In a NIOSH study[60], interaction models showed that endotoxin modified the effects of fungi on respiratory symptoms. It was found that endotoxin exposure possibly changed the effect that fungal exposure has (and vice versa) on respiratory health. This synergism suggests that both should be assessed in investigations that look at the effect of either fungal or endotoxin exposure.

It was found that the levels of thermophilic Actinobacteria in floor dust were positively associated with any granulomatous disease-like symptoms. The association became stronger and remained significant (P-values <0.05) in models additionally adjusted for endotoxin or fungi, or similar in models adjusted for mycobacteria, total bacteria, or (1→3)-B-D-glucan.

Assessing effects of remediation:

Efforts to show salutary benefits of remediation have had a checkered past. In 2008, Huttenen and colleagues assessed the effect of remediation on the safety of two WDB[61]. The results showed that the

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renovation decreased the immunotoxicological activity of the particles collected from a damaged building, noting that no transcriptomic assessment was made. No difference was detected in the corresponding samples collected from the reference building. Interestingly, only slight differences were seen in the concentration of fungi. This finding indicates that the effects of remediation on the indoor air quality may not be readily measurable either with microbial or toxicological parameters. Use of transcriptomics would have been useful.

We have consistently seen that remediation alone is not sufficient for clearance of adverse human health effects acquired following exposure to WDB[62]. Based on microbial monitoring, an improvement was detected in one, partial improvement in two, and no improvement in two cases, whereas no follow-up was conducted in two cases. Health effect studies (mainly self-reported health status) showed improvement in one case, partial improvement in two cases, and no improvement in two cases, whereas no follow-up was conducted in one case, and in one case, follow-up failed due to low response rate.

Immunoreactivity and symptoms related to exposure:

To assess possible significance to symptoms in putative cases, NIOSH has published two pertinent papers[63.64]. It was found that in a group-based analysis of various symptoms that there were significantly increased odds, but that no individual symptom associations were found as a result of exposure to endotoxins[63]. It was found too that it was not possible to quantify health risks in a dose-related manner, due to exposure to mold or dampness. As a result, no specific threshold of safe exposure could be determined[64]. Neither of these papers cited studies of treatment of CIRS. It was also shown that the relationship between endotoxin exposure in floor dust and development of symptoms was nonlinear.

Adams has published widely, using molecular methods in her multiple international studies. Succinctly, she states[45] in 2019, "Microbial concentrations reflected well the technical condition of the construction, but the reported symptoms of the occupants did not strictly follow the timely fluctuation in microbial conditions." It is the repeated experience of the authors of this paper that the basis for this variance of exposure and symptomatic change has its origins in immunoreactivity, with Actinobacteria and endotoxins leading the way most commonly to (i) immunoreactivity; and (ii) symptoms of greater intensity and duration.

In 2015, Murtoniemi and colleagues published an instructive paper that foreshadowed our work with immunoreactivity[65]. In WDB, the environmental conditions, as well as the dominant microbial species, will vary, leading to a diversity of microbes and continual changes in the different microbial populations. Among the tested microbes and their combinations, the spores of S. californicus proved to be the most potent inducer of cytotoxicity and inflammatory responses. Spore-induced cytotoxicity and production of inflammatory markers increased during the period from 5 to 10 weeks, suggesting that the immunotoxic potency of spores increases with time.

A basic question for causation of illness comes from the route of exposure. Are we assuming that elements found in reservoirs of settled dust are the source of immunoreactivity? Or should we be looking at airborne samples reflecting personal

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exposure[66]? Personal exposures to viable fungi and bacteria were compared with the concentrations being assessed by stationary samples in-home and workplace microenvironments. The samples with higher fungal concentrations also had a higher diversity of fungi than samples with lower concentrations. The total number of fungal genera recovered was 39 for personal, 34 for home, and 23 for work samples. These results also indicate that the presence of a certain fungus in a microenvironment does not necessarily mean similar findings in personal exposure samples.

In another special case paper from 2017, NIOSH noted the adverse impact of exposure to a building with excess asthma and a cluster of sarcoid cases[48]. In multivariate regressions, thermophilic Actinobacteria (median = 529 CFU/m2) in dust were associated with asthma and granulomatous disease-like symptoms. Findings show that Actinobacteria were the dominant microbial flora in the dust, implying that these bacteria might be partly responsible for various health effects in these occupants. They reported that bacteria (*S. californicus*) and *Pseudomonas fluorescens* (GNR) were stronger inducers of inflammation than fungi for both types of cell lines.

Fungi or (1→3)-B-D-glucan in dust or heat pump traps were not associated with any health outcomes. Thermophilic Actinobacteria and non-tuberculous mycobacteria may have played a role in the occupants' respiratory outcomes in this water-damaged building. This indicates that occupants in damp buildings are simultaneously exposed to multiple microbial agents from both bacteria and fungi. This mixed exposure is likely to result in interactive effects among various microbial agents and produces more complicated health outcomes. Some genera of bacteria may be more prevalent than others in indoor environments[48]. We must have NGS data to decide.

In a series of papers, Adams has published widely on the intersection of public health, microbiology, and building science, primarily by using molecular methods. In a current paper (published 9/2020[67]), she summarizes what is known and poses remaining research questions in a seminal paper that unfortunately does not include human proteomic and transcriptomic data. This absence does not permit the additional insight that comes from defining when exposure, particularly to Actinobacteria, leads to metabolic complications based on immunoreactivity.

Urinary mycotoxins

A common concern of food scientists is safety of food despite the ubiquitous contamination of food with mycotoxins and their metabolites. In Europe, more than the USA, attempts have been made to establish guidelines for food safety by measuring mycotoxins in urine, and then deriving exposure limits. However, this approach of not assessing reactivity cannot be used for estimation of effect of exposure in a defined patient. As this paper has suggested, correlation of exposure to microbial compounds, and especially mycotoxins, is nearly impossible in the absence of prospective exposures that fulfill a case definition, using objective parameters of illness. Confounding the definition of adverse effects of ingested mycotoxins are differences by type, source, co-exposures and species of host. Studies of toxicity in pigs[68,69,70] confirm the greater sensitivity of swine to ingested mycotoxins, as shown by inflammatory markers in the gut, but not when the pig diet has been supplemented with glutamate in measurable quantities.

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Concerns for the safety of food would be reduced if glutamate, in protein-deficient diets, were confirmed to prevent toxicity in humans.

Conversely, studies stratifying gastrointestinal illness by dietary glutamate in patients with ongoing mycotoxin illness could be done but in the absence of widespread human disease, in patients consuming known unsafe doses of mycotoxins, data could not be interpreted. With widespread-contamination of food and the widespread presence of urinary mycotoxins in healthy people, no widespread-illness causation has been shown. What one must conclude is that the null hypothesis, one that states that there is no significant dose/response relationship for illness causation by mycotoxins, is confirmed.

In another paper from Sulyok and colleagues[71], published in 2018, a newer approach to dietary mycotoxins was presented. Using enzymatic pre-treatment, solid phase extraction and UHPLC separation, the sensitivity of the method was significantly higher (10-160x lower LODs) than in a previously described method used for comparison purpose, and stable isotopes provided compensation for challenging matric effects.

Biomarkers were detected in all samples. The mycoestrogen, zearalenone, was the most frequently detected contaminant (82%) but also ochratoxin A (76%), aflatoxin M1 (73%) and fumonisins B1 (71%) were quantified in a large share of urines. The extreme sensitivity permitted use of this model for assessment of chronic, low dose effects of mycotoxin exposure. We can only consider how much stronger this model would be were transcriptomic assays added.

As mentioned, the search for mycotoxin exposure in food is advanced in Europe. The BIOMYCO study is reported here[72]. The direct measurement of biomarkers of exposure in biological fluids has been proposed as a suitable alternative to perform an accurate mycotoxin exposure assessment at individual levels. For this reason, the BIOMYCO study was designed to assess mycotoxin exposure in Belgian adults and children using urinary biomarkers of exposure.

These urine samples were analyzed for the presence of 33 potential biomarkers with focus on aflatoxins, citrinin (CIT), fumonisins, trichothecenes, ochratoxin A (OTA), zearalenone and their metabolites using two validated LC-MS/MS methods. Deoxynivalenol-15-glucuronide was the main urinary DON biomarker and was found in all urine samples in the ng/ml range. The study showed a clear exposure of a substantial portion of the Belgian population to ochratoxin, citrinin and DON. No human health data was reported.

In a recent study from Sweden[73], pigs were used to assess differences in grain contamination from different areas in Sweden. Urinary concentrations of four mycotoxins, (deoxynivalenol (DON), zearalenone (ZEA), fumonisins B1 (FB1), and ochratoxin A (OTA)), and four key metabolites, (diepoxy-deoxynivalenol (DOM-1), aflatoxin M1 (AFM1, biomarker of AFB1), a-zearalenol (a-ZOL), and B-zearalenol (B-ZOL) were identified and measured by UPLC-MS/MS.

Urinary mycotoxin biomarker concentrations were used to estimate mycotoxin intake and the level of mycotoxins in feeds consumed by the monitored pigs. The introduction of an enzymatic digestion step in the sample preparation of urine was used in several

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analytical methods to hydrolyze conjugated mycotoxins, phase II metabolites and conjugated phase I metabolites of mycotoxins into free analytes. Mean urinary excretion rate of each mycotoxin in 24 h post dose in piglets (36.8% for total ZEA, 27.9% for total DON, 2.6% for FB1, 2.6% for OTA and 2.5% for AFB1, excreted as AFM1[58]. No health assessment was done.

In a paper[74] from 2019 in China, human mycotoxin exposure was studied using simultaneous blood and urine mycotoxins. Despite finding carriage of potentially carcinogenic aflatoxin (AFB1) and confirming multiple mycotoxins appearing in urine, no health assessment was done.

A study from Sweden in 2015[75], looked at 4-day diet diaries and measuring urinary mycotoxins. Over 69% of the 252 participants had more than one type of mycotoxin found.

Transcriptomic studies, while in their infancy, have been done[76] in lab rats. Apoptosis genes were deregulated in vitro but less affected in vivo; activation of several MAPKs was observed. Many genes related to oxidative stress or involved in cell-to-cell interaction pathways or cytoskeleton structure appeared to be deregulated either in vitro or in vivo. Genes related to OTA transport (OATs) and metabolism (CYPs) appeared downregulated in vivo. The correlation with human exposures cannot be determined.

A reasonable concern regarding the use of urinary mycotoxins clinically stems from massive under-reporting of toxin congeners, epitopes, and degradation daughters. One example comes Ghent, Belgium in 2018[77]. In an animal study, the urinary biomarker analysis revealed that DON and DON-3 glucoside were rapidly absorbed, distributed, metabolized, and excreted. Sixty-four % of the administered DON and 58% of DON-3 glucoside was recovered in the urine collected within 24h. The analysis of urinary glucuronides is crucial for the study of trichothecene biomarkers because approximately 90% of DON excreted via urine is conjugated.

However, previous urinary biomarker-analysis of DON represents some uncertainties and limitations: (i) biomonitoring data are dependent on the sample collection, (ii) there is a lack of information on the absorption and exertion rate of DON; and (iii) the contribution of DON modified forms like DON-3-glucoside or acetyl-deoxynivalenol (ADON) remains unclear.DON-15-glucuronide is the major urinary biomarker after DON administration, with a constant ratio around 4/1 (DON-15-glucuronide/DON-3-glucuronide). DON-3-glucoside has a similar excretion profile with DON-15-glucuronide being the most abundant metabolite.

Besides the excretion in urine and feces, a small amount can be recovered in the bile, as observed in a study on sheep after oral exposure. The biliary excretion does not play any important role in the elimination of DON from sheep, however, the low recovery observed in sheep could be due to the subsequent conversion of DON to unidentified metabolites in the liver. The authors do not discuss enterohepatic recirculation of these toxins. No animal health studies were reported.

Given the apparent difficulty in reporting adverse health effects associated with urine mycotoxin studies, the use of blood has potentially greater sources of aberrant results. This problem might be solved as suggested in a 2017 paper from Germany[78]. Detection of aflatoxins (AFB1, AFB2, AFG1, AFG2,

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AFM1), trichothecenes (deoxynivalenol, DON; DON-3-glucuronic acid, DON-3-GlcA; T-2; HT-2; and HT-2-4-GlcA), fumonisins B1 (FB1), ochratoxins (OTA and its thermal degradation product 2'R-OTA; OT a; 10-hydroxychratoxin A, 10-OH-OTA), citrin (CIT and its urinary metabolite dihydrocitrinone, DH-CIT), zearalenone and zearalenone (ZEN, ZAN), altenuene (ALT), alternariols (AOH; alternariol monomethyl ether, AME), enniatins (EnA, EnA1, EnB, EnB1) and beauvericin (Bea) was validated for two matrices, serum (DSS) and whole blood (DBS).

"Besides positive findings of OTA and 2-R-OTA, all samples were positive for EnB.
Those mycotoxins and metabolites are frequently determined in human fluids such as urine, human breast milk, and blood, or serum and plasma, respectively. Their reliable analysis in a multi-mycotoxin approach for physiological samples is a challenging task but allows to determine the individual mycotoxin exposure of humans and animals."

Human blood and serum samples were provided by healthy volunteers giving written consent as participants of biomonitoring studies. As some mycotoxins or metabolites are mainly detectable in urine (e.g., DON-3_GlcA) 10, 11) and others such as OTA, 2'R-OTA, Enb mainly in the blood, the analysis of both matrices is recommended to evaluate the human and animal exposure to mycotoxins. Unfortunately, we see no human health studies reported. Detection of aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1), trichothecenes (deoxynivalenol, DON; DON-3-glucuronic acid, DON-3-GlcA; T-2; HT-2; and HT-2-4-GlcA), fumonisins B1 (FB1), ochratoxins (OTA and its thermal degradation product 2'R-OTA; OT a; 10-hydroxychratoxin A, 10-OH-OTA), citrin (CIT and its urinary metabolite dihydrocitrinone, DH-CIT), zearalenone and zearalenone (ZEN, ZAN), altenuene (ALT), alternariols (AOH; alternariol monomethyl ether, AME), enniatins (EnA, EnA1, EnB, EnB1) and beauvericin (Bea) was validated for two matrices, serum (DSS), and whole blood (DBS). Besides positive findings of OTA and 2-R-OTA, all samples were positive for EnB.

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The Actinobacteria/immunoreactivity study; transcriptomics leads the way.

METHODS:

To approach a data-driven definition of immunoreactivity to *Actinobacteria* in known cases of CIRS, a retrospective review of a transcriptomic ("GENIE") data subset was conducted using a waiver provided by CGIRB. We stratified the stage of treatment of CIRS (0-5); presence of molecular hypometabolism (MHM); fungal rosters of HERTSMI-2 and ERMI; endotoxins; Actinobacteria, CD14/TLR4; MAPK of 6 subtypes; TGF beta, TGF beta Receptor 1, 2, 3; HLA DR by PCR haplotype; VCS results; and symptoms assessed by cluster analysis.

The data set included 6 controls (Stage 0); 96 in Stage 1, treatment-naive; 91 in Stage 2, after use of a published protocol; Stage 3, after the protocol and on nasal instillation of

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VIP; Stage 4, off VIP; and Stage 5, relapse (within 2 days). These data were extracted from test results requested from treating physicians as part of the background for performance of a GENIE test. Not all physicians reported all human health metrics; not all patients had all environmental assays.

Environmental sampling with Swiffer cloths for settled dust was forwarded to a commercial laboratory, Envirobiomics, San Antonio, Texas. Fungal testing was done using MSQPCR, with 10 being the threshold of abnormal for HERTSMI-2. Endotoxin testing was performed using the kinetic chromogenic method to ensure high-quality endotoxin analysis, with 99 being the threshold of abnormal. The Actinobacteria Analytical Report of species abundance from EnviroBiomics was used for Actinobacteria values, ranging from 0-40, with 15 being the threshold of abnormal.

CD14 and TLR4 were abnormal if the z score was $\geq$ 1.37, as was MAPK, TGF beta-1 and TGFBR 1, 2 3. HLA DR was recorded using a previously published haplotype roster (15). VCS and symptom cluster scoring were taken from previously published rosters (21).

RESULTS:

Controls had no positive findings.

Stage 1 had 96 patients, 27 positive Actinobacteria exposed were noted with 22 with positive MAPK, with a mean number of subtypes of 1.5 (out of 6), similar to a prior unpublished report (Lessons from GENIE, volume 3, 2020) value mean of 1.6. The combination of + exposure, + MAPK and + TGFBR was found in 19. One other had + TGFBR but was negative for Actinobacteria exposure. Two had positive exposure and + TGFBR only. Four had exposure only two had exposure and +MAPK.

All patients had baseline VCS and cluster analysis done, with 94% positive VCS compared to literature results of 92% positive. Cluster analysis was 92%, as seen also in published literature. All had susceptible HLA with relative risk haplotypes > 2.0. HLA haplotype 4-3-53 was found to have incidence in stage 1 of 36% (literature reported value of 3.0). For the "triple positive," 4-3-53 was 26%.

Stage 2 had 91 patients, 18 Actinobacteria exposed and 15 with positive MAPK, with a mean of 1.7. Four patients had a positive TGFBR with the triple positive; 3 had +MAPK/ (-) TGFBR.
HLA: All had haplotypes, as seen in stage 1, with increased relative risk.
VCS showed a positive test in 29/47 patients, with 11/47 showing a positive cluster analysis.

Stage 3 had 35 patients, with 4 Actinobacteria exposed with all 4 showing -MAPK/-TGFBR.
VCS numbers and cluster numbers were too small for analysis, as was HLA.

Stage 4 had 10 patients, with 1 Actinobacteria exposed. None were + MAPK/+TGFBR.
VCS all negative. Cluster all negative. HLA numbers were too few.

DISCUSSION:

For the first time, we see a possible clue to immunoreactivity in response to Actinobacteria exposure with a massive and unique increase in triple positive results compared to all other groups sorted by the three variables of exposure, MAPK and TGFBR. This finding must be replicated in large studies, but preliminary results suggest that exposure to actinomycetes has an

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immunoreactive signature that permits assessment of specific causation.

VCS was not discriminatory; neither was cluster analysis. HLA susceptibility was present in all cases, with 4-3-53 overly represented in all comers at a factor of 12 times predicted. In the triple positive, 4-3-53 was nearly as over-represented as the entire cohort (9-fold increase) but one cannot explain the triple positive as related solely to a disproportionate susceptibility to that single HLA haplotype.

The critical point is that this early evidence suggests that Actinobacteria exposure activates MAPK and TGFBR as part of immmunoreactivity, with all the TGFBR-related nuclear transcription factors involved in as undetermined fashion. To our knowledge this report is the first to identify a new mechanism of immune injury in CIRS, one that could, if validated, confirm specific causation of injury from Actinobacteria exposure. The missing additional information regarding downstream effects of TGF beta-1 receptor signaling possibly activating SMAD genes which will act as nuclear transcription factors following exposure to novel Actinobacteria gene products[79,80,81].

The role of TGFBR as a metabolic gene activator has not been discussed concerning CIRS to date. Given the tight relationship of MAPK and TGFBR, this finding suggests (i) co-activation; (ii) consistent with immunoreactivity to Actinobacteria exposure; and the (iii) downstream upregulation of Akt, P1,3K, mTOR by TGFBR, as well as other genes also activated by insulin receptor activation; (iv) additional attention must be devoted to Actinobacteria responses, including anabolic gene functions.

Future prospective GENIE studies on putative specific causation are planned, adding metabolic genes and avoiding incomplete data recording.

CONCLUSIONS

We have presented published environmental studies on WDB that confirm our observations of results of environmental sampling: without the use of NGS, continued use of outdated tests will invariably lead to unreliable data. In parallel, without transcriptomic data obtained by stage of treatment, trying to confirm health data from CIRS patients leads to guessing and assumption when prospective studies are not engaged. Moreover, specific causation of illness cannot be validated without confirmation of immunoreactivity acquired in conjunction with exposure. Exposure alone is not enough to prove causation, as shown by the most accurate urine mycotoxin testing, for example With use of new molecular methods, there is a learning curve, as the jargon of gene names can be off-putting, but the inexorable march of science demands that all of us keep pace.

The future is bright, however, as new diagnostic and treatment approaches based on transcriptomics are revealed. Now that we know the majority of illness in CIRS WDB comes from Actinobacteria and endotoxins, or their interactions, with fungi and mycotoxins playing minor roles, we can begin to assemble a new list of hazardous compounds found in WDB, only this list will codify an array of bioactive and immunoreactive compounds made by Actinobacteria.

Transcriptomics and GENIE provide a means to assess immunoreactivity based on patterns of gene expression to determine molecular hypometabolism, proliferative physiology, activation of MAPK, TGFB1R, CD14, TLR4 and many other genes involved in defining

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CIRS. These data are the critical link between exposure and illness, determining treatment, with resolution providing hope for cure.

For all the excitement surrounding use of molecular tools, we are reminded that individual health factors need to be taken into consideration when determining the effectiveness of remediation, as the threshold for when remediation is completed will be determined by the current health and effectiveness of treatment for a patient living in a WDB.

More questions are yet to be answered: Do different triggers have different levels or types of immunoreactivity? Transcriptomics provides a means to begin to assess this unknown. The combination of (i) NGS for building sampling; (ii) transcriptomics and (iii) NeuroQuant allow us to begin to see specific causation effects and determine which individuals are demonstrating immunoreactivity, versus exposure but no reactivity.

Beyond those concepts are the unfathomable human costs of chronic and daily manifestations of fatigue, pain, non-restorative sleep, cognitive deficits, neuropathic pains, gastrointestinal issues, musculoskeletal compromise, post-exertional malaise, dermatological, ophthalmic, endocrine, and other chronic multisystem symptoms in tens of millions of Americans and hundreds of millions globally. Resources must be directed to elucidate this critical information and stop CIRS, a potentially preventable disease.

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