

The Role of the Intestinal Microbiome in Type 1 Diabetes Pathogenesis

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Abstract The gastrointestinal system represents one of the largest interfaces between the human internal microenvironment and the external world. This system harbors trillions of commensal bacteria that reside in symbiosis with the host. Intestinal bacteria play a crucial role in maintaining systemic and intestinal immune and metabolic homeostasis because of their effect on nutrient absorption and immune development and function. Recently, altered gut bacterial composition (dysbiosis) was hypothesized to be involved in mechanisms through which islet autoimmunity is triggered. Evidence from animal models indicates that alterations in the gut bacterial composition precede disease onset, thus implicating a causal role for the gut microbiome in islet destruction. However, it remains unclear whether dysbiosis is directly linked to the mechanisms of human type 1 diabetes (T1D). In this review, we discuss data implicating the gut microbiota in disease progression with an emphasis on our recent studies performed in humans and in rodent models of T1D.

Keywords Type 1 diabetes · Microbiome · Inflammation · Kilham rat virus · 16S rRNA

Introduction

Type 1 diabetes (T1D) is a proinflammatory autoimmune disorder with a yet unidentified etiology. The disease leads to the

specific loss of insulin-producing beta cells and subsequently causes dependency on daily insulin therapy for life [1]. In some cases, hyperglycemia may be preceded by a long prodromal autoimmune process that may last for many years and can be identified only by the presence of autoantibodies against β -cell autoantigens, such as GAD, islet antigen 2 (IA-2), insulin, and zinc transporter 8 (ZnT8A) [2].

More than 50 genes have been implicated in T1D mechanisms [3]. Of all the genetic factors linked with T1D, the HLA locus was found to have the strongest association with disease development, particularly *DRB1*04-DQA1*03:01-B1*03:02(DR4-DQ8)* and *DRB1*03:01-DQA1*05:01-B1*02:01 (DR3-DQ2)*, which are expressed in the majority of T1D patients who are diagnosed before 18 years of age [4]. Among other disease-susceptibility genes are insulin, cytotoxic T lymphocyte antigen (CTLA)-4, the interleukin (IL)-2 receptor, the tyrosine phosphatase PTPN22, and the intracellular viral RNA sensor MDA5 (Melanoma Differentiation-Associated protein 5) [5].

Environmental factors and changes in lifestyle over the last few decades have been hypothesized as major drivers of T1D [6•]. This possibility is supported by the rising incidence of T1D worldwide in industrialized nations, particularly in young children; this increase is occurring at a disease rate that cannot be explained based solely on genetic alterations [7]. Indeed, the annual increase in T1D incidence in European nations, such as Finland, Germany, and Norway, ranges between 2.4 and 3.3 % [7]. Strikingly, a more than 5-fold increase in the rate of new T1D cases in Finnish children younger than 15 years of age was recorded in 2006 compared to that observed in the 1950s (65 versus 12 cases per 100,000, respectively) [6•]. It was postulated that if the disease incidence continues to increase at the current pace, the global T1D incidence could double over the next decade [8]. The hypothesis that the environment is involved in diabetes progression is

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further supported by the observation that migration from countries with low incidence to countries with a high diabetes rate may increase the risk for diabetes [5]. Indeed, Pakistani children born to parents who migrated from Pakistan to the UK have a similar disease rate as the local population, which is strikingly 10-fold higher than the incidence in Pakistan [9]. Lastly, the differences in diabetes incidence between monozygotic twins further implicate the environment's role in triggering the disease [10].

Viruses and T1D

Viruses have been implicated in the mechanism triggering human T1D, but a cause-and-effect relationship between microbial infections and disease progression has not yet been established [3, 5, 11]. Previous reports have demonstrated that viruses and virus-specific antibodies can be detected more frequently in individuals with recent diabetes onset compared to healthy control subjects [12–14]. Case reports and anecdotal data have implicated viruses, such as mumps, cytomegalovirus, rubella, Epstein-Barr virus, rotavirus, and varicella zoster virus, in human diabetes [reviewed in ref. 15]. Significant attention has been paid to the role of enterovirus, and Coxsackie B virus (CVB) in particular, in triggering beta cell destruction [16, 17]. Coxsackie B virus protein-1 was detected in islets from more than 50 % of newly diagnosed subjects compared to only a few healthy subjects [18]. Furthermore, islets from patients with T1D co-expressed the enterovirus-capsid protein and proinflammatory cytokines and chemokines and were infiltrated with T cells expressing CXCR3, the receptor for CXCL-10 [19, 20].

Evidence of an epidemic outbreak of T1D also points to the possibility that viruses could be a key component in disease pathogenesis [21]. A remarkable increase in diabetes incidence was observed in young children in Philadelphia in the first 6 months of 1993 [21]. Notably, a measles epidemic occurred in Philadelphia approximately 2 years prior to the T1D outbreak, establishing the hypothesis that the increased T1D rate might have been linked to the measles outbreak [21].

How viral infections lead to T1D is unknown. Viral infections may lead to diabetes via a number of mechanisms, including molecular mimicry, bystander activation of T cells, beta cell damage resulting in autoantigen release and activation of autoreactive T cells, and the induction of stress pathways in beta cells [5].

The Intestinal Microbiota

The intestine harbors $\sim 10^{14}$ microorganisms of more than 500 different species [reviewed in ref. 22]. The bacterial abundance is increased in the lower portions of the gastrointestinal

tract, ranging from 100 to 1000 microorganisms per ml in the acidic microenvironment of the stomach to 10^{12} per ml in the colon. These microbes use complex polysaccharides and other mucosal compounds in addition to undigested plant fiber as energy sources leading to the production of vital metabolites, such as vitamin K, biotin, and short-chain fatty acids (SCFAs). The intestine contains 500–1000 bacterial species that belong to several major phyla [22]. The number of these bacterial groups varies between individuals, but Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the most abundant phyla [22].

The gut bacterial composition can be altered as a result of environmental perturbations [23]. Antibiotic use is of the main factors that can modulate the gut microbiome, and it can lead to substantial long-term effects on the gut microbiota [6••]. Antibiotics also reduce the resistance to colonization, allowing foreign microbes to outgrow commensal bacteria, thus causing permanent changes in the structure of the microbiota [24].

The gut microbiota is required for the development of a normal immune system. Mice maintained in a germ-free environment from birth have altered intestinal morphology and function and aberrant lymphoid tissue organization and innate and adaptive immunity [25]. Emerging evidence supports the hypothesis that dysbiosis may be linked to the development of immune disorders [26]. For example, an increase in the abundance of the bacterial family *Enterobacteriaceae* and gram-negative anaerobes, such as *Bacteroides*, and a decrease in *Bifidobacterium* have been linked with inflammatory bowel disease in humans [27–29]. The intestinal microbiome has also been implicated in rheumatoid arthritis in humans [30] and in mouse models of autoimmune arthritis [31].

The hygiene hypothesis postulates that reduced exposure to microbes as a result of antibiotic use in early life results in dysregulated immunity; this hypothesis is a potential explanation for the dramatic increase in the frequency of immune-mediated diseases and T1D [32, 33]. Indeed, previous studies have demonstrated that antibiotic treatments can induce profound long-term alterations on gut bacterial composition [6••]. Antibiotic-induced alterations in the gut microbiome have been implicated in the global rise in diabetes incidence; however, there are currently no solid data linking antibiotic use in children with disease progression [34, 35].

The Immune System and the Intestinal Microbiome

Pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) expressed by immune and non-immune cells, sense pathogen-associated molecular patterns (PAMPs) expressed by bacteria, viruses, and fungi [36–38]. The activation of TLRs mediated by signaling molecules, such as MyD88 or TIR domain-containing adapter protein inducing $\text{IFN}\beta$ (TRIF) [36–38], leads to a cascade of cellular signals

involving mitogen-activated protein kinase (MAPK) activation and the expression of NF- κ B and interferon-regulatory factor 3 (IRF3), culminating in the expression of proinflammatory cytokines [36–38].

Intestinal cells, such as Paneth and goblet cells, sense bacterial number and location via TLR pathways. TLR activation in these cells leads to the expression of the antimicrobial lectin regenerating islet-derived protein 3 γ (REG3 γ) [39, 40]. Adaptive immune mechanisms are also involved in defending the host from invading pathogens via immune cells present in the gut-associated lymphoid tissue (GALT), such as Peyer's patches, Th17 cells [40], and immunoglobulin A [41]. Moreover, the microbiome itself serves as a barrier against potential pathogens [42]. The microbiome further promotes the maintenance of the intestinal barrier by promoting epithelial cell turnover and mucin production and by competing with pathogens for available nutrients and space [43].

The intestine is the largest organ of the immune system in the body and is inhabited with an enormous amount of microbes living in symbiosis with the host [44]. Recent studies have shed light on the interplay between the immune system and the intestinal microbiota that begin to interact with one another immediately after birth [44, 45]. A key element that allows a peaceful host-commensal bacteria mutualism is the separation of microbes from the host internal environment by barriers such as epithelial and mucosal layers and innate and adaptive immune mechanisms sensing and eliminating harmful pathogens [43]. Disrupting these mechanisms can lead to inflammation and autoimmunity [46].

A major function of the host intestinal immune system is to keep inflammatory responses in check to allow the survival of beneficial bacteria and simultaneously preserve the ability to fight potential pathogens [44]. Thus, interactions between commensal bacteria and host cells in the intestine are tightly regulated [42]. A key innate immune mechanism allowing the symbiosis between gut bacteria and the host immune system involves TLRs and NOD-like receptor (NLR) signaling pathways [42]. For example, the capsular polysaccharide A (PSA) of *Bacteroides fragilis* induces Treg cells that secrete the anti-inflammatory cytokine IL-10, thus preventing inflammation in the gut [47, 48]. Likewise, commensal bacteria, such as *Bacteroides thetaiotaomicron*, inhibit proinflammatory cytokine expression in the intestine by modulating nuclear factor- κ B (NF- κ B) [49]. Intestinal dendritic cells (DCs) contribute to the microbiome-host coexistence by sampling a small number of live commensal bacteria from the intestinal lumen to the mesenteric lymph nodes to induce a protective IgA response [50].

Rat Models of T1D and Virus-Induced Islet Destruction

The LEW1.WR1 and BioBreeding diabetes-resistant (BBDR) rat models of virus-induced T1D have a normal immune

phenotype and do not develop T1D when housed in specific pathogen-free facilities [51–53]. However, infection with the parvovirus Kilham rat virus (KRV) leads to beta cell inflammation and destruction in 50 and 25 % of infected BBDR and LEW1.WR1 rats, respectively; this phenomenon is detectable 14–28 days following virus inoculation [51–53]. Another rat model of T1D is the diabetes-prone BioBreeding (BBDP) rat, which is severely lymphopenic. Unlike the BBDR and LEW1.WR1 rats, BBDR rats develop diabetes spontaneously [54, 55]. The disease in the rat is characterized by the specific loss of islet beta cells, glycosuria, ketonuria, and polyuria in a strain-specific manner [52, 53]. Susceptibility to virus-induced disease is dependent on the presence of class I A^u and class II B/D^u [52, 53]. We recently hypothesized that the innate immune system plays a key role in the mechanism triggering T1D in the BBDR and LEW1.WR1 rats [51, 56–58]. Indeed, activation of the innate immune system with TLR agonists, such as the viral mimic polyinosinic:polycytidylic acid (poly I:C) or CpG DNA, followed by infection with KRV substantially exacerbates T1D [59, 60]. Moreover, infection with KRV leads to a robust proinflammatory response associated with the up-regulation of a vast array of proinflammatory cytokines and chemokines in the spleen, pancreatic lymph nodes, and Peyer's patches via mechanisms that involve TLR9 pathways [51, 58, 59]. Finally, modulation of virus-induced innate immunity with steroids [59], IL-1 receptor antagonists [57], and histone deacetylase inhibitors can prevent disease progression.

The Role of Gut Bacteria in Virus-Induced T1D

Recent evidence has suggested that the intestinal microbiome is involved in the pathogenesis of T1D in rat models. BBDR rats that developed T1D had reduced levels of *Bacteroides* compared to rats that remained diabetes-free and rats with antibiotic therapy-ameliorated T1D [61]. In addition, the transfer of *Lactobacillus johnsonii* N6.2 from the BBDR intestine to BBDR rats delayed disease development in a bacteria-specific manner [62]. Our own studies indicated that infection with KRV resulted in alterations in the gut microbiome reflected by increased abundances of the *Bifidobacterium* and *Clostridium* genera on day 5 following viral infection, prior to insulinitis or hyperglycemia [51]. Furthermore, oral therapy with the broad-spectrum antibiotic sulfatrim (sulfamethoxazole plus trimethoprim) reversed virus-induced alterations in the gut microbiome and protected rats from insulinitis and islet destruction [51]. The effect of sulfatrim on disease progression was specific because treatment with ampicillin, metronidazole, or neomycin sulfate did not alter the course of islet destruction. It remains unclear whether KRV-induced alterations in the gut microbiome are directly linked to diabetes progression. The observation that

KRV alters the abundance of *Bifidobacterium* and *Clostridium* in the intestine prior to hyperglycemia may imply that infection with KRV results in conditions that favor the development of islet autoimmunity. This possibility requires testing in future studies.

The mechanism by which manipulation of the intestinal microbiota prevents islet destruction could be associated with interference with virus-induced adaptive and innate immunity [ref. 51 and Fig. 1]. Indeed, sulfatrim reduced the number of B lymphocytes in the pancreatic lymph nodes and Peyer's patches and downregulated KRV-reactive T cells in the spleen [51]. Moreover, sulfatrim induced a reduction in the transcript levels of KRV-induced proinflammatory cytokines and chemokines in the pancreatic lymph nodes and Peyer's patches [51]. The notion that the gut microbiome is involved in innate and adaptive immune responses outside the gut is perhaps not surprising because gut bacteria are involved in numerous physiological responses that can affect various organs and cells, such as nutrient adsorption, vitamins and hormone production, and the prevention of colonization by pathogens [63]. In any case, the evidence that manipulating the gut microbiome results in altered innate and adaptive immunity outside of the gastrointestinal tract and interferes with autoimmunity is consistent with previous observations from other immune disorders, such as allergies, asthma, diabetes, obesity, and cancer [63].

The Role of TLRs and the Gut Microbiome and Diabetes

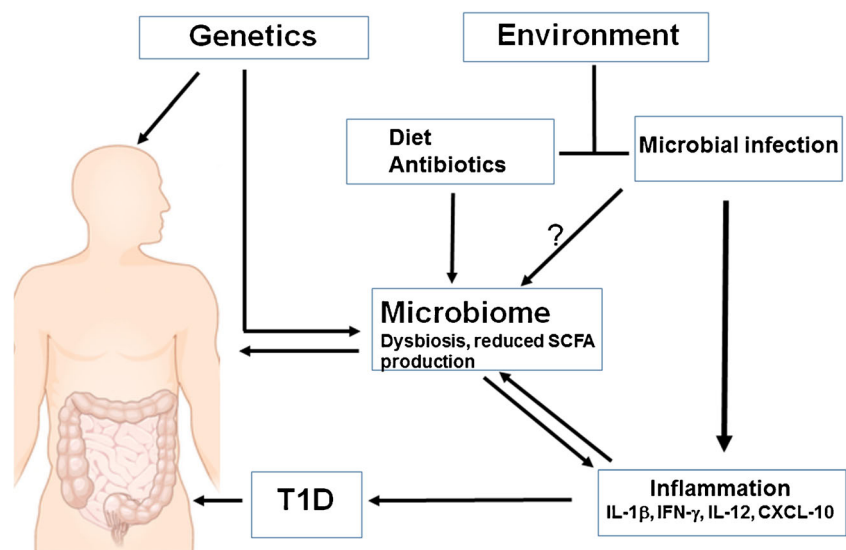
As discussed earlier, TLRs play a key role in innate immune recognition of pathogens and commensal bacteria and as such are a critical component in mechanisms that maintain gut homeostasis and control of the intestinal microbiome [64, 65].

Mice lacking the expression of TLR5, which binds bacterial flagellin, had alterations in their gut bacterial composition [66]; however, differences in the gut bacterial composition between wild-type and TLR-deficient mouse lines could be a result of divergence of the gut microbiota after long-term housing in isolation from one another [67].

The first observation pointing towards the role of TLR signaling and the gut microbiota in T1D occurred in the NOD mouse model in which gut bacteria were shown to play a protective role in disease development [68]. NOD mice with disrupted MyD88 signaling developed T1D when housed in specific pathogen-free conditions, but this effect was not observed in mice lacking TLR2, TLR3, and TLR4 [68]. The protective effect observed in these mice was hypothesized to be linked with altered responses to gut bacteria because MyD88-deficient NOD mice housed in germ-free conditions or treated with antibiotics had increased disease incidence [57].

The role of TLR signaling and the gut microbiome in T1D development was further addressed by us in RIP-B7.1 mice lacking the expression of critical TLR signaling molecules. RIP-B7.1 mice express the B7.1 costimulatory molecule under the control of the rat insulin promoter [69], and administering these mice Poly (I:C), which is a ligand of TLR3 and RIG-like helicases, results in insulinitis and diabetes via mechanisms linked with type I interferon pathways [70] and the up-regulation of antigen presenting cells (APCs) and autoreactive T cells [71]. Data obtained from RIP-B7.1 mice with disrupted TLR pathways support the hypothesis that interactions between the innate immune system and the gut microbiota are involved in diabetes development. RIP-B7.1 mice with and without disrupted MyD88, TLR3, or TLR9 pathways housed under normal conditions remained diabetes-free [72]. In sharp contrast, wild-type RIP-B7.1 mice with intact TLR signaling or without TLR9 expression who were administered Poly

Fig. 1 The potential role of the intestinal microbiome in T1D progression. Environmental and genetic factors play a key role in triggering type 1 diabetes. Virus infection leads to early inflammation in the periphery or intestine, which in turn leads to alterations in the gut microbiota towards a proinflammatory microbiome. Environmental risk factors, such as a high-fat diet and antibiotics, can also contribute to the development of dysbiosis. A shift in the abundance of the gut microbiome could result in altered functionality, such as a reduction in the production of anti-inflammatory SCFAs



(I:C) and oral sulfatrim developed diabetes, whereas RIP-B7.1 mice deficient in MyD88 and TLR3 treated under the same experimental conditions were protected from disease development [72].

Diabetes-susceptible TLR9-deficient mice had different intestinal microbiomes than those of diabetes-resistant TLR3- and MyD88-deficient RIP-B7.1 mice following treatment with sulfatrim plus Poly (I:C) [72]. This was reflected by an increase in the bacterial diversity in TLR9-deficient RIP-B7.1 mice compared to TLR3- and MyD88-deficient mice. Furthermore, the overall intestinal microbiome of TLR9-deficient mice either untreated or administered with sulfatrim plus Poly (I:C) was different than that of diabetes-resistant mice. Lastly, sulfatrim plus Poly (I:C) modulated the relative abundances of individual bacteria, including the Actinobacteria phylum and the *Bifidobacterium*, *Lactobacillus*, and *Clostridium* genera in TLR9-deficient versus disease-resistant mice. Together, these data support the possibility that the mechanism of diabetes in RIP-B7.1 mice is linked with altered gut bacterial composition. How Poly (I:C) alters commensal bacteria in the gut and how shifts in bacterial abundance lead to disease onset will require further investigation.

The Role of the Intestinal Microbiota in Human T1D

It is hypothesized that the dietary intake in developed nations, which has shifted to a high-fat, high-carbohydrate, low-fiber diet (“Western diet”), may have resulted in functional changes in the intestinal microbiota [73–76]. Such diet-induced alterations to gut bacterial communities are postulated to play a key role in the rising incidence of proinflammatory immune disorders in the developed world, including obesity, type 2 diabetes, and inflammatory bowel disease [77]. SCFAs, such as butyric acid and acetic acid, produced by the fermentation of indigestible dietary plant fiber by the intestinal microbiota [reviewed in refs. 78, 79] can enter the circulation (27) and regulate innate and adaptive immunity.

A number of recent studies implied that the increase in the incidence of T1D may be linked to alterations in the gut microbiome, particularly those associated with SCFA-producing bacterial species [ref. 66 and Fig. 1]. A metagenomics analysis performed in four cases and four controls from Finland revealed that the level of genes associated with carbohydrate metabolism, adhesions, motility, phages, sulfur metabolism, and stress responses was higher in cases [80]. The authors hypothesized that increased adhesion and flagella synthesis in autoimmune subjects may be linked with triggering islet autoimmunity. The 16S rRNA data from the same subjects suggested a higher proportion of butyrate-producing and mucin-degrading bacteria in controls compared to cases [80]. Another study performed in 18 seropositive

subjects from Finland suggested a link between a low abundance of lactate-producing and butyrate-producing bacterial groups with islet autoimmunity [81]. Moreover, a study performed in 76 Finnish children, of whom 22 converted to seropositivity and later developed T1D, suggested an increase in the abundance of *Bacteroides dorei* in cases compared to individuals who did not convert to autoimmunity prior to seroconversion [82]. Data from a study that included 11 seropositive children from Finland and Estonia, of whom 4 progressed to T1D, demonstrated a reduction in bacterial diversity after autoantibody appearance prior to disease diagnosis [83]. Lastly, a study that included 28 diabetic children, of whom 18 were from Finland and the rest were from France, Greece, Estonia, and Lithuania, suggested that non-diabetic children have a more balanced microbiota and higher abundance of butyrate-producing bacterial groups [84].

We recently performed a cross-sectional study in stool samples from subjects living in Colorado [85]. The study included 35 new onset patients (up to 6 months following diagnosis), 21 first-degree relatives (FDRs) positive for at least one islet autoantibody, 32 seronegative FDRs, and 23 unrelated healthy subjects without a family history of T1D. The 16S rRNA data suggested that there is no clear bacterial signature of predominant bacterial communities in subjects with islet autoimmunity prior to or following disease diagnosis. However, the gut microbiomes of seropositive subjects and seronegative FDRs displayed an overall similarity to one another but were distinct from those of new onset patients and unrelated healthy controls [85]. Stratification of the seropositive cohort based on the expression of multiples versus one autoantibody further pointed towards an increase in the abundance of *Bacteroides* and a reduction in *Prevotella* and the phylum Firmicutes in the multiple autoantibody group. An increase in *Bacteroides* has been linked to the “Western diet”, which is characterized by a content high in protein and fat and low in plant fiber, whereas an increase in *Prevotella* has been linked to a diet rich in plant fibers [86]. *Prevotella* was found to be highly prevalent in African children with a diet rich in grains [87], and taxa from both *Prevotella* and Firmicutes can digest plant polysaccharides [86, 88] and promote the production of SCFAs, which are known for their anti-inflammatory properties [89].

Collectively, the available human data suggest that shifts in the gut microbial composition may occur in genetically susceptible individuals prior to seroconversion or hyperglycemia. However, it is difficult to assess whether these alterations represent an “autoimmune microbiome” with a causal role in disease progression, partially because of the variability observed between the various studies. The data variability could result at least in part from differences in sample size, methods of sample collection, geographical origins and climate, cultural differences, ethnicity, and data analysis. The mechanism by which the gut microbiota may promote islet autoimmunity is currently unclear. It is thought to be associated with its ability

to modulate immunity and/or alter epithelial barrier function [35]. A recent study suggested that T cell cross-reactivity between islet antigens and microbes may be involved in the process of beta cell destruction [90]. Lastly, it was hypothesized that the translocation of intestinal bacteria to pancreatic lymph nodes and the subsequent activation of the nucleotide-binding oligomerization domain containing two pathways play a role in disease development [91].

Conclusions

It is becoming increasingly apparent that there is a link between the gut bacterial composition and various aspects of human health. Indeed, evidence from animal studies implicates the intestinal microbiome in the development of autoimmune disorders, including T1D. Studies conducted in individuals at risk for T1D have suggested that disease progression is associated with dysbiosis. Further longitudinal studies performed prior to and following seroconversion and disease diagnosis are required to establish a correlation between shifts in the intestinal microbiota and disease progression. Parallel studies performed in animal models of diabetes will be necessary to dissect the mechanisms by which intestinal microbes mediate disease and to develop microbiome-based therapies for disease prevention.

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Compliance with Ethical Standards

Conflict of Interest James C. Needell and Danny Zipris declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Our studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in our studies. The studies involving animals were in accordance with the ethical standards of the University of Colorado Denver.

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