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Human Anelloviruses: Prevalence and Clinical Significance During Pregnancy

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Although the bacterial microbiota of various compartments (e.g. vagina, amniotic fluid, and placenta) have been studied in pregnancy, there has been far less emphasis on normal and pathological viral communities. Cumulative evidence shows the presence of a number of apathogenic viruses in various tissues of healthy people, including pregnant individuals. What role, if any, these viruses play in human physiology is unknown. Anelloviruses (family *Anelloviridae)* are circular, single-stranded DNA viruses commonly detected with high prevalence in vertebrate hosts, including primates. Humans are nearly always colonized with at least 1 of 3 *anellovirus* subtypes, namely *Alphatorquevirus* (torque teno virus, TTV), *Betatorquevirus* (torque teno midi virus, TTMDV), and *Gammatorquevirus* (torque teno mini virus, TTMV). In healthy pregnant people, the prototype anellovirus, TTV, has been found in maternal and (variably) fetal blood, amniotic fluid, cervical and vaginal secretions, breast milk, and saliva. Nonetheless, the relevance of human anelloviruses in pregnancy and labor is unclear. There is evidence suggesting a link between anellovirus colonization and preterm birth. In this review, we discuss what is known about this family of commensal viruses in health and disease, and specifically the roles they might play during pregnancy and in the timing of delivery.

Keywords: human virome, *Anelloviridae*, anelloviruses, commensal virus, pregnancy, preterm birth

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INTRODUCTION

The human body serves as a host to a highly diverse community of microorganisms. These microorganisms may benefit the host (creating a "mutualistic" relationship), harm the host (forming a "pathogenic" relationship), or have no apparent effect (a "commensal" relationship). From time to time, mutualistic or commensal microorganisms may assume a pathogenic character (for example, in the case of vaginal yeast infections). The genomes that constitute the human microbiome include bacteria, archaeans, other eukaryotes, and viruses (1). These microbial communities are highly dynamic and vary based on the individual's age and health status, the biology of the anatomical site, diet, and hygiene (2).

While research on the human microbiome has focused mainly on bacterial populations, much less is known about viral communities residing at different sites in and on the human body and their roles in health. Advances in sequencing have uncovered myriad novel viruses in humans, many of which cause no apparent illness (3). Most humans are colonized in almost every tissue type by members of *Anelloviridae*, a family of diverse, non-enveloped, circular, single-stranded

DNA eukaryotic viruses (4, 5). Thus far, anelloviruses have not been linked definitively to any disease states (6), although there is some evidence suggesting a link to human disease (7). This review discusses this novel class of human viruses, including their prevalence, genome diversity, transmission routes, and potential association with human health and disease. We focus on pregnancy, including a possible role in the timing of delivery. Anelloviruses have been detected in maternal and—to a lesser and highly variable extent, depending on the study—fetal tissues (8–10). We discuss the potential mechanisms by which anelloviruses may interact with and modulate maternal immune responses and influence pregnancy outcomes.

DISCOVERY AND NOMENCLATURE

In 1997, while searching for a viral agent responsible for non-A to E hepatitis, Nishizawa et al. found a novel DNA virus in the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology (11). The viral clone was designated TT virus (TTV) after the patient from whom it was recovered. Subsequent studies revealed TTV as a small, non-enveloped, single-stranded, circular DNA virus (12). After the discovery of the original TTV isolate, smaller variants of TTV were identified and subsequently named torque teno mini virus (TTMV) (13), and torque teno midi virus (TTMDV) (14), derived from the Latin terms *torque* meaning "necklace" and *tenuis* meaning "thin" (15). Recent changes in nomenclature have classified the 3 anellovirus genera found in humans: *Alphatorquevirus* (TTV), *Betatorquevirus* (TTMV), and *Gammatorquevirus* (TTMDV), which together comprise the human *Anelloviridae* family (16).

ANELLOVIRUS AND HUMAN DISEASE

A clear link between anellovirus positivity and human disease has not been established (6). On the one hand, the fact that anelloviruses are rarely detected earlier than 3 months of age and are acquired later in life in healthy individuals (17–19) suggests that anellovirus acquisition over the lifespan is normal. On the other hand, recent studies have suggested that certain anellovirus subtypes are associated with various illness and diseases such as unexplained fever (20), diabetes (7), cirrhosis in liver transplant patients (21), respiratory disease (22–26), cancer (27–30), and autoimmune disorders (31–33). There is some evidence suggesting a high occurrence of anellovirus with *Epstein-Barr virus* (34) and hepatitis B or C (5). Whether this means that anelloviruses have a role in enabling pathological viral infections remains to be elucidated. Given the prevalence of TTV in organ transplant recipients, TTV load has been suggested as a candidate indicator of immune suppression (35–37).

PREVALENCE OF ANELLOVIRUS BY AGE AND GENDER

Anelloviruses are reported at a high prevalence in the general population across the globe (38). TTV, the prototypical anellovirus, is multitropic, i.e., found in nearly every body site, fluid, and tissue tested, as summarized in **Table 1**.

A plethora of evidence suggests that anelloviruses are detected by PCR in all age groups. A study analyzed fecal specimens collected longitudinally from day of life 1–4 (month 0) and at 3, 6, 12, 18, and 24 months of age from 4 healthy twin pairs (18). Anelloviruses were rarely detected earlier than 3 months of age. Thereafter the prevalence increased significantly, peaking at 6–12 months of age, and began to decline at 18 and 24 months of age. Among 8 infants enrolled in the study, 1 infant harbored no less than 47 anellovirus species at 12 months of age. In some infants, the same anelloviruses could be detected from fecal samples collected up to 12 months apart, suggesting persistence and expansion of anellovirus richness in the gut of infants. Another study of 20 twin pairs (0–30 months of age) showed the abundance of anellovirus species increased until 15–18 months of age, after which time abundance diminished (66). A prospective single-center study of 98 clinically healthy breastfeeding infants (1–12 months of age) demonstrated a significant increase in whole blood anellovirus load during the first year of life, reaching a plateau after 6 months of age (17).

A study investigated the epidemiology of anellovirus in blood samples derived from healthy children (1–14 years) and healthy blood donors (18–59 years) (67). Among 208 children, 141 were

TABLE 1 | Tissues tested for anellovirus.

	Citations
Tissues with detectable anellovirus	
Whole blood, plasma, or serum*	(3, 39–48)
Peripheral blood mononuclear cells (PBMCs)	(43, 49–51)
Exosomes-enriched vesicles from plasma	(52)
Bone marrow, lymphoid tissue, thyroid gland, muscle, pancreas, spleen, kidney, lung	(48)
Bronchoalveolar lavage	(53)
Nasal or throat swabs	(24, 53)
Saliva*	(10, 40–42, 54)
Liver	(43, 55)
Bile	(56)
Feces	(41, 42, 46, 49, 56–59)
Urine	(60, 61)
Skin, hair follicle	(62)
Tears	(42)
Semen	(42, 54)
Amniotic fluid*	(9)
Cervix*	(63, 64)
Vaginal secretions*	(65)
Umbilical cord blood*	(9, 10)
Breast milk*	(9, 45, 47)
Tissues without detectable anellovirus	
RBCs	(50, 51)
Platelets	(50, 51)
Sweat	(42)

Tissues tested in pregnant or post-partum people.

TTV-positive. TTV prevalence was highest in 1–2-year-olds, lower for 8-year-olds, and higher again in 14-year-old children. Among 196 healthy blood donors representing the normal population, 103 were TTV-positive; there was no difference in the TTV DNA prevalence with age. However, other studies (68–70) with larger sample sizes have consistently demonstrated positive correlations between anellovirus prevalence and age in healthy populations. Phylogenetic analyses did not find associations between anellovirus genotypes and particular age groups (67) or geographic locations (68, 71). One study (68) noted viral loads were highest in blood donors more than 50 years old, but a longitudinal analysis of plasma TTV loads after 2 years showed minimal changes in TTV viremia (70). The findings suggest that although anelloviruses are acquired over the lifetime, healthy aging causes only minimal increases in TTV viremia.

Anellovirus prevalence and viral load may be gender-specific. One study found TTV prevalence was significantly higher in males than in females (70). A separate study found that young women (20–30 years) had lower plasma loads of anellovirus than men in the same age group (19).

Substantial evidence suggests that anellovirus load is governed by the immune system (72). Although the mechanisms by which the immune system reacts to anellovirus colonization are unknown, studies have shown that people receiving a solid organ transplant (73–77), and those with cancer (47), HIV infection (78), and sepsis (79) have higher plasma anellovirus loads than healthy donors. Other studies have shown an inverse correlation between levels of TTV and CD4+ lymphocytes in HIV-positive patients (80) and pediatric lung transplantation patients (81). The latter study findings revealed that patients with low anellovirus genome copies are at risk of transplant rejection or death. There is also evidence of increased anellovirus DNA concentrations after antiviral therapy (6). Thus, it appears that anellovirus load is inversely correlated to and may serve as a marker of general immune status.

ANELLOVIRUS GENOME

Despite their nucleotide sequence diversity, anelloviruses share virion structure and genomic organization (13). Electron microscopy of the prototype anellovirus, TTV, isolated from serum specimens (82) and a TTV-infected HEK293 cell line (83) demonstrate TTV as an unenveloped icosahedral virus with a diameter between 30 and 50 nm. As indicated by their names, the human anelloviruses differ in genome size: 3.9 kb for TTV, 3.2 kb for TTMDV, and 2.8–2.9 kb for TTMV. The TTV genome consists of an untranslated region (UTR) of ∼1.2 kb and a potential coding region of ∼2.6 kb. The non-coding UTR of the TTV genome contains a GC-rich segment (>60% GC) flanked by a TATA box upstream of the coding region and a poly-A sequence downstream (84), and multiple stem-loop structures that facilitate virus replication (71). The coding region consists of 3–5 overlapping open reading frames (ORF1-5) which encode at least 6 proteins with structural (85), host immune suppression (86, 87), cell cycle regulation, and apoptosis-inducing properties, respectively (88). ORF1 also contains hypervariable regions where mutations occur more frequently than in other regions. These hypervariable regions help the virus evade the immune system (89).

Genetic Heterogeneity

In addition to size, the 3 *Anelloviridae* genera can be grouped according to their degree of genetic similarity in the ORF1 region. TTV, TTMV, and TTMDV have at least 105, 68, and 34 species, respectively. Phylogenetic analysis of TTV isolates recovered from disparate locations have identified 7 major clusters, with genomic sequence differences of up to 35% (90). It has been hypothesized that in a given individual, genetic variability within a viral group is high, and that coinfection by distinct viral strains in blood and other tissues is common (91, 92). A study investigated possible relationships between the number of genogroups carried and the total TTV load present in 239 TTV-positive subjects (93). Individuals with high viral loads tended to possess more TTV genogroups than those with low viral loads. TTV genogroups 1 and 3 were the most prevalent, followed by genogroups 4 and 5, while genogroup 2 was rather infrequent.

DETECTION AND QUANTITATION OF ANELLOVIRUSES

To date, polymerase chain reaction (PCR) is the most prominent method used to detect anellovirus. Because of the extensive heterogeneity among the genomes of anelloviruses, detection of the entire spectrum of the anellovirus variants is impossible using a single set of primers. For genotyping, primer pairs designed either in the ORF1 region or the sequences spanning 5' or 3' UTRs are widely used (93, 94). Taking advantage of these regions, nested and semi-nested PCR assays are developed in which the genomic DNA of all anelloviruses is amplified by first-round PCR with universal primers, and then species-specific DNA are amplified by using a second set of primers (7). In a number of studies, sequences spanning N22-ORF1 regions are utilized for the detection of anellovirus DNA (11, 92). However, this strategy allows detection of only some genotypes of TTV, a genus with more than 30 genotypes (95). For example, N22 primers can efficiently amplify genotypes in group 1, but amplifies certain genotypes in group 2 less efficiently (96) and fails to amplify many genotypes in groups 2, 3, and 4 at all. Over time, studies have increasingly focused on utilizing degenerate primers and highly conserved regions located just downstream of the TATA box to potentially detect all known genetic forms of anelloviruses (97–99). The results are validated across multiple iterations followed by phylogenetic analysis (94, 100, 101). In recent studies 5'UTR primer sets are often used, but these primers differ in their abilities to detect TTV and related genotypes by PCR (95). Therefore, differences in primer selection could explain some of the considerable variation in estimates of anellovirus prevalence between studies. Even within a single healthy cohort, TTV detection ranged from 53% (251/471) to 90% (90/100) depending on which primers were used (68). In addition, measuring prevalence of detectable TTV is highly dependent on the type of specimen analyzed—for example, TTV titer is higher in whole

blood than in plasma (70). Therefore, TTV negativity in a sample could be a laboratory artifact due to sub-optimal sensitivity of the detection methods. A study validated the commonly used PCR primer sequences to detect TTV and TTV-like virus in different populations (102). Primer alignment and PCR product characterization consistently indicated that a minimum of five primer sets (NG, TT, TLMV-S, TLMV-L, and a genotype 21-specific set of primers) are required to detect all known genotypes of TTV and TTV-like viruses in healthy individuals.

In addition to the PCR method, antibody-based detection of TTV has also been developed and used for the diagnosis of TTV colonization (103).

SITES OF ANELLOVIRUS REPLICATION

Despite decades of research, the main site of anellovirus replication remains unknown. Studies have indicated the association of TTV with peripheral blood mononuclear cells (PBMCs) and distinct distribution of TTV subtypes between plasma and PBMCs (104, 105). Research has also shown that TTV is abundant in granulocytes compared with other peripheral blood cell types in healthy individuals (51). Given the reported evidence of elevated TTV titers with immunosuppression and transplant-related complications (6, 106), a study investigated TTV levels in plasma samples and potential sites of TTV replication in individual blood cell types derived from pediatric allogeneic hematopoietic stem cell transplant (HSCT) recipients (107). Among 43 HSCT patients enrolled in the study, 34 had detectable TTV in plasma before transplantation, and all patients tested positive for TTV by day+50 post-transplant. TTV copies reached peak titer around day+100, and then gradually declined to pre-transplantation levels over a period of about 2 years. TTV DNA was not present in NK cells, B- and T-cells. On the other hand, granulocytes isolated from peripheral blood or bone marrow were invariably positive in post-transplant samples of all patients. Until day+30 post-transplantation, TTV tested either near or below the detection limit in granulocytes, but dramatically increased between days +30 and +100 days post-transplantation in peripheral blood and bone marrow granulocytes. At the same time, TTV DNA was absent in granulocytes derived from healthy immunocompetent controls throughout the study period. Together, these findings suggest granulocytes as potential TTV replication sites, particularly in immunosuppressed individuals.

Evidence comparing viral titers between different tissues within a single patient suggests anellovirus replication can occur in bone marrow (108), liver (109, 110), lungs (111), lymphoid tissue (112), oropharyngeal and/or salivary glands (40). These findings suggest that viral replication takes place in multiple tissues at distinct levels in infected individuals (48).

Attempts to replicate anellovirus *in vitro* have been unsuccessful thus far. Human cell lines, including Chang liver (109), HEK293TT (113), lymphoma and T-cell leukemia (83), and the Raji cell line (109), have demonstrated TTV infection in initial passages, but the virus did not propagate to later passages (83, 113, 114).

IMMUNOBIOLOGY OF HUMAN ANELLOVIRUSES

Toll-like receptors (TLRs) are members of a family of cell-surface proteins responsible for recognition of a diverse spectrum of pathogens and generation of an innate immune response. TLR9 recognizes intracellular unmethylated heterodimers of guanosine and cytosine (CpGs), which are abundant in the genomes of DNA viruses. Depending on the number of nucleotides flanking CpGs, this may stimulate the production of either pro- or anti-inflammatory cytokines (115). It has been reported that the genome as well as the replicative intermediates of anellovirus are unusually rich in CpG sequences (116). The DNA of 1 genogroup of anellovirus (ViPiSAL strain) was found to provoke robust activation of TLR9 and the production of proinflammatory cytokines in *ex vivo* mouse spleen cells (117). Nevertheless, the genomes of other anellovirus strains failed to promote inflammatory responses. These findings may indicate that the effects of anelloviruses on the host's inflammatory status vary depending on genogroups.

Due to the lack of an efficient culture system to support TTV replication, the transcription profile of TTV has been largely gained from human cell lines (COS1, HEK293, and L428) transfected with TTV plasmids (87, 118). Three spliced mRNAs of TTV that produce at least 6 proteins by alternative translation initiation have been reported (85). At present, the functional role of ORF2 protein is well-characterized. Overexpression of TTV ORF2 encoded protein has been shown to suppress NF-κB activation elicited by TNFα in various human cancer cell lines, including HeLa and HepG2, and in the mouse macrophage line RAW 264.7 (86). Further analyses revealed that TTV ORF2 protein has the ability to suppress NF-κB activity *in vitro* in a dose-dependent manner, affecting translocation of NF-κB p65 and p50 subunits to the cell nucleus, thus inhibiting the transcription of downstream genes such as interleukin (IL)-6, IL-8, and cyclooxygenase-2. Together these findings indicate that TTV ORF2 protein may be involved in negative regulation of host cell inflammatory responses.

Evidence suggests that TTV encodes microRNAs (miRNA) that cooperate with viral proteins to regulate the expression of viral genes involved in replication, pathogenesis, inflammation, and immune evasion (119). The functional relevance of proteins translated from other TTV ORFs and TTV-encoded miRNAs warrant further study.

ROUTES OF TRANSMISSION

Numerous studies have suggested horizontal and vertical TTV transmission routes. Horizontal transmission includes parenteral, fecal-oral, and sexual. Vertical transmission involves the possible passage of virus from mother to fetus during pregnancy and breast feeding.

Parenteral Route

Since bone marrow cells and activated PBMCs are recognized as potential sites of TTV replication (120, 121), blood and blood products could be among possible routes of TTV transmission.

Therefore, people with blood-related diseases such as hemophilia and thalassemia (122–124), blood donors (5), patients having multiple blood transfusions (124–126), and patients who have undergone organ transplantation (73, 127–131) are more likely to have TTV colonization.

Fecal-Oral Route

To examine patterns of anellovirus shedding into the circulation and the GI tract after new infection, 2 naïve chimpanzees were injected intravenously with bacteria-free (filtered) fecal supernatant or serum from human newborns with documented acute TTV infection (132). Serum and fecal specimens obtained weekly from experimentally infected chimpanzees were tested for TTV DNA by nested PCR. In the chimpanzee that received TTV-positive human serum, TTV DNA was detected in serum starting 5 weeks post-inoculation (PI) and remained positive until 15 weeks PI. In the chimpanzee that received fecal supernatant, TTV DNA was detected in serum samples 7–12 weeks PI and peaked at 14–16 weeks PI and continued to be positive for longer than 30 weeks. TTV DNA was detected in fecal specimens from the chimpanzee inoculated with TTV-positive human fecal supernatant after 16 weeks PI (coincident with high-titer TTV DNA in the serum). However, fecal specimens obtained at 24 weeks PI (when serum titers were low) were negative for TTV DNA.

Sexual Contact

Detection of TTV DNA in semen (54), and vaginal fluid (64, 133), suggests possible TTV transmission during sexual intercourse.

Transplacental Route

The published information on transplacental TTV transmission is inconsistent. In a prospective cohort study, paired maternal and cord bloods were examined for the presence of TTV DNA (69). Among 105 participants enrolled in the study, 37 mothers were TTV DNA-positive, and 7 cord blood samples from the 37 TTV-positive mothers were also TTV-positive. All cords from TTV-negative mothers were TTV-negative. In another study (134) TTV DNA was present in the blood of 57 of 138 mothers. Among the 57 TTV-infected mothers, 19 cord sera were positive for TTV DNA. A follow-up of 3 randomly selected infants with TTV sequences in their cord blood showed positivity persisting for 8 weeks after birth. The finding of TTV in the cord blood of between 1/5 and 1/3 of colonized mothers is consistent with transplacental passage of virus, however other routes are possible, as is contamination of the cord specimens by maternal blood.

A separate study analyzed plasma samples from 54 mothers and their newborns for TTV DNA (135). Though TTV-DNA was detected in 49 of 54 mothers, only 4 (8%) infants tested positive.

By contrast, another study analyzed TTV DNA in maternal and fetal cord blood collected postpartum from 100 mother-child pairs (44). TTV DNA was detected in 84% of maternal samples, while cord blood was devoid of TTV.

The sum of these findings call into question whether transplacental transmission of TTV occurs in human pregnancy.

Breast Feeding

Several studies provide evidence of anellovirus transmission by breast feeding (9, 134, 136). In a cohort study, blood was sampled from 300 normal pregnant people (60 of whom were TTV-positive). Twenty infants born to TTV-positive women in the cohort who delivered vaginally ($n = 10$) or by C/S ($n = 10$) were sampled at both 5 days and 3 months after birth. Half the infants in each group were also tested at 6 months after birth. Additionally, breast milk was collected from 30 TTV-positive nursing women (137). All infants from TTV-positive mothers were TTV-negative at both 5 days and 3 months after birth, regardless of delivery method, arguing against TTV transmission either transplacentally or during the birth process. By 6 months after birth, 4 of the 10 infants born to TTV-positive parents were TTV-positive. TTV DNA was detected in the breast milk of 7 of 30 TTV-positive patients.

An earlier study in Germany looked for TTV in 46 women who collectively birthed 47 children. Of this cohort, 22 maternal serum samples tested positive for TTV. Notably, TTV DNA was detected in 22 of 23 serum samples of 1-week-old infants who were born to TTV-positive parents. Twenty four TTV-negative individuals gave birth to 24 TTV-negative children who remained negative throughout the study period of 28 months. TTV DNA was detected in 77% of breast milk samples from TTV-positive patients and in none from TTV-negative individuals (45).

A prospective single-center study in Russia analyzed whole blood TTV load in 98 clinically healthy breastfeeding infants of 1–12 months of age to determine TTV dynamics during the first year of life (17). The findings revealed a significant increase in TTV copy number for the first 60 days, before plateauing after 6 months, with viral loads correlating with age.

In sum, these findings suggest that newborns can acquire TTV through breast milk, but acquisition from either parents or others via alternative routes was not ruled out. There is some evidence that among infants who are breast-fed, the prevalence of TTV positivity increases with prolonged lactation (136).

Horizontal Transmission Could Be the Major Route of Anellovirus Colonization in Infants

A study determined whether the predominant route of transmission of TTV in children is horizontal, vertical, or both, by testing infants born to TTV-positive mothers (138). Serum samples were obtained from 12 mothers on the day of delivery or within 1 month after delivery. Among 12 mothers, TTV DNA was detected in 10 (83%) cases. Serum samples were obtained from infants at 0.5–3 month intervals from 1 to 12 months of age. All infants, aside from 1 born by C/S, were delivered vaginally. The prevalence of TTV in infants born to TTV-positive and TTV-negative mothers were 9/10 (90%) and 0/2 (0%) respectively. Serum TTV DNA was not detected in any infant at 1 month of age but was detected for the first time at 1.5–8 months of age, and thereafter TTV positivity persisted throughout the follow-up period. Detection of TTV in 9/10 infants born to TTV-positive mothers and 0/2 infants born to TTV-negative mothers suggests that

TTV transmission from mothers to their infants postpartum is possible.

To confirm the transmission route, a homology search was performed in 7 randomly selected TTV-positive mother-infant pairs. Although only a few clones tested for each case were sequenced, the degree of homology varied considerably in most matched mother-infant pairs. One of the 7 mother-infant pairs showed a high degree of similarity for all TTV clones (98.7–100%), 2 pairs had 88–99% homology, and the remaining 4 showed 83.6–89% nucleotide identity. While these findings indicate that colonization with maternal TTV can occur, most acquired TTV is not identical to maternal strains.

These findings suggest a predominance of horizontal, rather than vertical transmission of TTV to infants, whether from their mothers or from other sources.

HUMAN ANELLOVIRUS COLONIZATION AND PREGNANCY-RELATED COMPLICATIONS

Although the bacterial microbiota of various compartments (e.g., vagina, amniotic fluid, and placenta) have been studied in pregnancy (139–143), there has been far less emphasis on the normal or pathological viral community (144, 145). Given the prevalence of anelloviruses in various tissues and body sites of healthy asymptomatic pregnant individuals, several studies have attempted to understand what impact, if any, TTV colonization has on pregnancy, labor, and birth.

Anellovirus Colonization May Have a Role in Determining the Timing of Parturition

Evidence suggests that overt maternal viral infection with influenza (146), hepatitis (147, 148), HIV (149), and herpes (150) can lead to preterm labor and delivery. Although the mechanisms underlying these associations are not clear, it has been suggested that maternal viral infection may predispose toward an exaggerated pro-inflammatory response to a secondary inflammatory stimulus (such as bacterial infection), leading to labor through a "double-hit" mechanism (151, 152). With this premise, we examined the association of virus colonization with a preterm "initiating event of labor" [either spontaneous labor with intact membranes or premature rupture of membranes in the absence of labor (PROM)] using a prospective case-control study (153). We hypothesized that patients experiencing a preterm initiating event of labor (< 37 weeks, "cases") would be more likely to harbor viruses than patients who enter labor at term ("controls"). An initial unbiased screen for viruses performed with next-generation sequencing in serum pooled from 8 cases identified 7 unique viral sequences, all TTVs. Subsequently, 72 patient samples were analyzed individually by nested and semi-nested PCR to identify other anellovirus subtypes. Among patients experiencing spontaneous labor, TTV and TTMV were significantly more prevalent in cases than controls, while TTMDV was not different between the 2 groups. Cases were more likely to harbor at least 1 member of the anellovirus family (91% vs. 68%). In the subgroup of subjects experiencing spontaneous labor with intact membranes, the incidence of TTV was significantly higher in preterm patients (23 of 24 cases) than in controls (8 of 13), whereas there was no difference in TTMDV and TTMV. There were no significant differences in viral subtypes in serum from patients with PROM.

These observations led us to hypothesize that anelloviruses may have a role in determining parturition timing. A potential mechanism for such a phenomenon is through modulation of the inflammatory and immune landscape (154), lowering the threshold for a labor response to stimuli, such as subclinical bacterial infection or non-infectious stimuli, that on their own would be insufficient to induce parturition. It is also possible that, due to the predilection of anelloviruses for leukocytes and the changes in leukocyte populations induced by labor, premature onset of the parturition process is a cause, rather than a consequence, of increased anellovirus recovery in these subjects. Given that the findings are qualitative and were made in a small group of subjects, confirmatory studies are needed.

Anellovirus May Associate With Other Maternal Microbiomes to Precipitate Preterm Birth

A nested case-control study analyzed the vaginal eukaryotic DNA virome and its associations with the bacterial vaginal community and preterm birth (155). Viral communities were analyzed according to diversity, dynamics over time, and association with bacterial community in vaginal swabs collected longitudinally from 60 subjects across pregnancy. Overall, 6 families of human DNA viruses were detected in vaginal samples from pregnant patients, including *Papillomaviridae, Polyomaviridae, Herpesviridae, Poxviridae, Adenoviridae,* and *Anelloviridae*. Anelloviruses were the most common viruses, detected in more than 40% of the patients. Viral richness diminished through the trimesters of pregnancy in subjects who had term delivery. Changes in vaginal virome diversity were similar to changes in the vaginal bacterial microbiome over pregnancy. The 24 pregnant subjects who delivered preterm showed higher viral richness compared to term birth patients. Although higher viral richness was significantly associated with both spontaneous and indicated preterm birth subtypes, no single virus or viral community was associated with preterm birth. Nonetheless, individuals who had both high bacterial diversity [as is seen in bacterial vaginosis, itself associated with preterm birth (156)] and high viral diversity early in pregnancy had the highest risk for preterm birth.

Evidence links the composition of the vaginal microbiome with immune status and variations in cervical length in pregnant people (157, 158). Specifically, when *Lactobacillus crispatus* is dominant, the vaginal level of D-lactic acid isomer is high, matrix metalloproteinase (MMP)-8 is low, and vaginal inflammation tends to be absent. Conversely, when *Lactobacillus iners* or bacteria other than lactobacilli are dominant, D-lactic acid levels are low, and MMP-8 levels are high, which is associated with a more pro-inflammatory vaginal environment and overall shorter cervical lengths (159). A recent cohort study of 121 pregnant subjects investigated TTV presence in vaginal secretions, and

how its occurrence and/or titer varies with the dominant bacteria in the vaginal microbiome (65). Vaginal secretions collected from pregnant individuals in their first trimester (≤12 weeks), third trimester (28–38 weeks), and 28–45 days postpartum were analyzed for TTV DNA by quantitative PCR. Approximately 40% of pregnant individuals who delivered a healthy baby at term had TTV detected in their vaginal secretions during at least 1 of these time points. In subjects who were tested at all time points ($n = 33$), those who were TTV-positive in the first trimester were equally likely to became negative or remain positive throughout the other sampling time points. These findings suggest that vaginal TTV colonization is most often associated with healthy gestation and normal outcomes. However, the correlation between vaginal TTV and features of bacterial vaginosis provides a mechanism by which anellovirus colonization may lead to preterm delivery. In the first trimester, *L. crispatus* was dominant in 66.7% of pregnant individuals who were negative for TTV, as opposed to 25% of those who were TTV-positive, and D-lactic acid levels were diminished in TTV-positive patients. Similarly, in the third trimester, *L. crispatus* was dominant in 50% of pregnant individuals who were TTV-negative and only 6% of those who were TTV-positive. In summary, vaginal TTV colonization appears to correlate with features of bacterial vaginosis (diminished predominance of *L. crispatus*, higher MMP-8, and lower D-lactic acid levels).

Adverse Pregnancy Outcomes May Not Be Associated With Anellovirus Presence or Quantity

A study determined the prevalence of viruses in matched maternal-infant preterm cohorts and ascertained whether viral presence or load correlates with histologic chorioamnionitis, spontaneous preterm labor, and preeclampsia (160). Preterm labor was defined as spontaneous preterm labor or preterm premature rupture of membranes that resulted in very premature delivery <31 weeks. Histological chorioamnionitis was determined by placental pathology, and preeclampsia was based on clinical diagnosis. Whole blood or plasma collected from 56 matched mothers and premature infants was analyzed for the presence and quantity of anellovirus and 8 other viruses by qPCR. Twenty-nine of the 56 maternal samples contained viral nucleic acid, of which anellovirus was most prevalent (26 samples). However, there was no association of presence or quantity of viral load in samples from mothers with or without preeclampsia, histological chorioamnionitis, or preterm labor. Taken together, this study suggests no clear relationship between TTV load and perinatal morbidity or spontaneous preterm labor, though its small size and focus only on extreme prematurity are limitations that require validation.

A MECHANISM BY WHICH ANELLOVIRUS COLONIZATION COULD INFLUENCE THE TIMING OF PARTURITION

The link between infection and preterm labor has long been recognized. In some instances, this may entail the initial presence of microorganisms (whether bacterial, viral, or fungal) which creates a favorable environment or amplifies the effect of a secondary infection. As noted above, experimental models illustrate the potential for synergy between viral and bacterial infections leading to amplification of host responses. Polyinosinic:cytidylic acid [poly(I:C)] is a TLR3 ligand and synthetic analog of double-stranded RNA, which is a replication intermediate for most viruses, including DNA viruses. Poly(I:C) induces preterm delivery when injected either into the uterus (152) or systemically (161) in mid- to late gestation and greatly amplifies the potency of bacterial products in mice when injected into the uterus (162). In a mouse model, it has been demonstrated that viral infection of the cervix during pregnancy reduces the capacity of the female reproductive tract to prevent bacterial infection of the uterus (163). Similarly, sub-clinical viral infection in pregnant mice has been shown to sensitize them to bacterial infection, leading to preterm delivery (151). These findings suggest the existence of synergism during combined viral and bacterial infection. This "2 hit" trigger and existence of synergism might be a beneficial strategy to a host, as it would blunt the maternal response to mild insults (such as subclinical infection), while providing for rapid and efficient amplification of the labor response in cases of a superimposed or more severe infection. Given the higher prevalence of circulating anellovirus in preterm than in term patients (153), and TTV's association with other bacterial communities linked to preterm birth (65, 155), we propose that anellovirus colonization during gestation might affect the onset of labor through lowering the threshold for a response to stimuli, such as subclinical bacterial infection, that on their own would be insufficient to induce parturition.

On the other hand, pregnant patients who have a normal term pregnancy and give birth to a healthy infant may harbor viral sequences or genogroups that protect against preterm labor. Functional studies have revealed that apathogenic, endogenous retroviruses (ERV), and ERV-derived proteins found in the placenta mediate cell-cell fusion, suppress maternal immunity, and protect the fetus from exogenous viruses (164). Given the evidence that TTV ORF2 protein suppresses NF-κB pathways and inhibits transcription of proinflammatory cytokine genes (86), it is possible that TTVs may act as "little helpers" in shaping the gene networks of innate and adaptive immune responses to maintain normal pregnancy.

In the majority of human body sites, microbial diversity is considered a signature of health (1). If multiple variants or genotypes of anellovirus ("anellome") found in healthy humans remain stable for a long time, they may make up the personalized and healthy part of the host microbiome (92). The gene products of anellovirus might help to maintain the composition and fitness of other (beneficial) microbial communities by preventing colonization by pathogens. At the same time, the host immune system, through immunosurveillance, may maintain a safe balance, thus protecting the body from the pathogenic effects of the virus (165). However, as noted above, microbial diversity (including anellovirus) in the pregnant vagina is associated with premature timing

of delivery. In summary, under physiological conditions, human anellovirus is unlikely to be pathogenic *per se*. Nonetheless, perturbations in host defense and microbial composition may allow anellovirus to achieve an opportunistic pathogen status.

FUTURE DIRECTIONS

At present, the quality and number of studies on the association of anelloviruses with pregnancy outcomes are limited. Large cohort studies are important to clarify the role, if any, of anellovirus colonization in the timing of labor. Investigations are warranted with a focus on determining the kinetics of anellovirus colonization over the course of pregnancy, and whether certain genogroups promote or suppress preterm birth. Studying anellovirus abundance in other conditions associated with pregnancy, such as miscarriage, preeclampsia, and gestational diabetes, will provide more detailed insight in the relationship between anellovirus colonization and clinical outcomes.

CONCLUSIONS

The impact of anelloviruses on human health remains incompletely characterized. Although the possible pathogenicity of anelloviruses is still an open question, further study of anellovirus colonization during pregnancy and in mother-infant pairs will help determine whether and how these ubiquitous viruses affect microbial infection-associated preterm labor and preterm birth.

AUTHOR CONTRIBUTIONS

CK, MS, and EH contributed to the literature review and composition of the present text. All authors reviewed and approved the final version of the manuscript.

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