REVIEW ARTICLE

Gardnerella vaginalis in perinatology: An overview of the clinicopathological correlation

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Abstract

Gardnerella vaginalis (GV) is a facultatively anaerobic gram-variable bacillus and is the major organism involved in bacterial vaginosis. GV-associated bacterial vaginosis has been associated with adverse pregnancy outcomes include preterm parturition and subclinical chorioamnionitis. Inflammatory response induced by GV presents paediatric problems as well. Studies had shown that increased levels of proinflammatory cytokines include TNF-α, IL-1β and IL-6 following fetal inflammatory response syndrome secondary to GV-induced intrauterine infection may result in the development of periventricular leukomalacia and bronchopulmonary dysplasia in the infected fetus. There is increasing evidence that GV-associated BV infection serves as a risk factor for long-term neurological complications, such as cerebral palsy and learning disability. GV is fastidious and could elude conventional detection methods such as bacterial cultures. With current more sophisticated molecular biology detection methods, its role and pathogenic effects have been shown to have a greater impact on intrauterine inflammation and fetal/neonatal infection. This review gives an overview on the characteristics of GV and its virulence properties. Its detrimental role in causing unfavourable GV-related perinatal outcomes, with emphasis on the possible mechanistic pathways is discussed. The discovery of disease mechanisms allows the building of a strong platform where further research on innovative therapies can be based on, for instance, an anti-TLR monoclonal antibody as therapeutic agent to halt inflammation-precipitate adverse perinatal outcomes.

Keywords: Gardnerella vaginalis, perinatal outcomes, bronchopulmonary dysplasia, periventricular leukomalacia, preterm labour, intrauterine growth restriction

INTRODUCTION

Gardnerella vaginalis (GV) was first isolated by Leopold in 1953 from infected genitourinary specimens¹ and was believed to be closely related to members of the genus Haemophilus. In 1955, Gardner and Dukes found the similar Gram-negative rod from women with non-specific bacterial vaginitis,² and was assigned it as Haemophilus vaginalis. It was later reclassified as Corynebacterium vaginale in 1963³ before the name Gardnerella vaginalis was coined in honour of its original discoverer, Dr Herman L. Gardner,⁴ heretofore remains as the only species of the genus Gardnerella assigned to the Bifidobacteriaceae family.⁵

Microscopically, GV appears as a small non-motile pleomorphic rod that is non-sporulating, non-flagellate and does not possess a capsule. It has an average dimension of 0.4 x 1.0 – 1.5 μm, although it may grow up to 2 – 3 μm in length in 24 hours culture on blood agar.⁶ GV is generally described as a Gram-variable microorganism as this bacterium responds unpredictably to Gram staining (Fig. 1A), influenced by the age of cultures and the physiological states of the bacteria.⁷ On blood agar, GV grows as small pinheaded (0.5 – 1.0 mm) circular, convex-grey whitish colonies (Fig. 1B) which produce a characteristic diffuse beta-haemolysis after 48 – 72 hours of incubation (Fig. 1C), which is more prominently observed on human blood agar, compared to culture on sheep blood agar.

Biochemically, GV is typically catalase- and oxidase negative, exhibits α-glucosidase but not β-glucosidase activity.⁷ The demonstration of hippurate and starch hydrolysis and
sialidase production (Fig. 1D) permits accurate identification of GV in 99% of cases. Well established, easy-to-use commercially available biochemical strip systems, specifically API Coryne and API 20 Strept can be used for rapid identification of GV. In addition, metronidazole disk susceptibility testing with zone of inhibition clearly seen (Fig. 1E), indicates GV. Ultrastructural examination of GV cell wall reported the presence of unusually thin peptidoglycan layer within its cell wall as it ages explained the tendency to give a negative or variable reaction to Gram staining. In addition, the lack of classical lipopolysaccharide (LPS) and LPS-specific components (heptose and hydroxyl fatty acids) in its cell wall extracts unequivocally positioned GV under the group of Gram-positive bacterium.

With the advancement in molecular techniques, many molecular markers e.g. 16S rRNA, 23S rRNA, gyrB (encoding β subunit of DNA gyrase) and rpoB (encoding β subunit of RNA polymerase) had emerged to allow correct identification of specific microbial taxonomic status and their phylogeny (evolutionary relationships). With this knowledge, a universal species-specific PCR primer encoding ribosomal 16S rRNA was developed for detection of GV in clinical isolates (Fig. 1F).

Despite being the de facto barcode for bacterial identification and quantification in many metagenomic studies, there are some drawbacks. The 16S rRNA may fail to accurately distinguish closely related bacterial species. Notably, the nucleotide differences among closely related bacterial species are not evenly distributed throughout the gene, but clustered within hypervariable regions, thus making PCR difficult to detect the difference. To complicate matters further, there were nine hypervariable regions (V1 to V9) carried by 16S rRNA gene target, and not a single variable region was

![FIG. 1: GV identification methods. (A) GV appears as gram variable coccobacilli on Gram stain (Gram stain x1000). (B) Small pin-headed circular grey-whitish colonies on blood agar; (C) demonstrating beta haemolysis after 48 – 72 hours of incubation. (D) A sialidase positive reaction as indicated by a change in colour from yellow to blue; (E) susceptible to metronidazole as indicated by zone of inhibition (arrow) around a 5 µg metronidazole disk. (F) Molecular approach by polymerase chain reaction (PCR) assay targeting the 16S rRNA gene. Line 1: internal control, line 2: positive control, line 3: negative control, lines 4 - 10: positive for GV.](image-url)
found to have discriminatory power for all bacteria species.\textsuperscript{15} With the incomplete nature of 16S rRNA databases at present, placement of organisms is often limited at the genus level or above, within a phylogenetic tree.\textsuperscript{16}

Chaperonin-60 (variously known as GroEL in \textit{E. coli}, MopA and Hsp60), a 60 kDa heat shock protein found in eukaryotes and virtually all bacteria, plays a key role in post-translational cellular protein folding and assembly.\textsuperscript{17} The chaperonin-60 universal target (\textit{cpn60} UT) gene sequence (549 – 567 bp) can serve as a tool for GV detection, identification and quantification. This 549 – 567 bp segment of the \textit{cpn60} coding gene is can be amplified by universal, polymerase chain reaction (PCR) primers. Unlike 16S rRNA with dispersed variable regions between highly conserved sequences, \textit{cpn60} UT coding region is fairly uniform throughout. Furthermore, its relatively small size allows high throughput next generation sequencing approaches. With these properties, \textit{cpn60} UT target gene is highly discriminatory and provides more accurate phylogenetically informative data on bacterial species that allows accurate species-level identification.\textsuperscript{18}

\section*{1. Aetiopathogenesis of \textit{Gardnerella vaginalis}}

In 1955, Gardner and Dukes were the first who identified GV as the aetiologic agent in bacterial vaginosis (BV), in which GV (formerly known as \textit{Haemophilus vaginalis}) was isolated in 92% of women presented with primary diagnoses of “bacterial vaginitis”.\textsuperscript{2} In addition, some reported a high rate of recurrence in those successfully treated BV-infected women with sexual re-exposure by their male sex partners harbouring GV in their urethra.\textsuperscript{19} It is well known that not all individuals harbouring the organism eventually develop an infection but remain asymptomatic. Host susceptibility or the virulence of the subspecies may have a role in the pathogenesis of GV.

Bacterial vaginosis (BV) is one of the leading causes of vaginal disorders among women of childbearing age globally, with the highest prevalence seen in Africa. The prevalence of BV reported ranges from 6\% to 68.3\%, depending on geographical region and ethnicity.\textsuperscript{20} The prevalence and epidemiology of BV worldwide is summarised in Fig. 2. In Malaysia, the prevalence of BV in pregnant women in a single tertiary referral centre was 10.1\% (unpublished

\begin{figure}[h]
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\caption{Global burden of bacterial vaginosis (2013). Data extracted from a systematic review report.\textsuperscript{20}}
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data). BV is often described as a disturbed equilibrium in the vaginal ecosystem with a shift in the normal vaginal microflora, replaced by mixed anaerobes. The common BV-associated microbes are *Mycoplasma hominis*, *Atopobium vaginae*, *Peptostreptococcus* sp., *Prevotella* sp., *Mobiluncus* sp. and *Bacteroides*, with GV being the most frequently isolated.

Clinically, the women may have classical symptoms of grey, profuse homogenous malodourous vaginal discharge, though up to 80% of the infected individuals are asymptomatic. Clinical criteria described by Amsel et al. is employed for diagnosing BV, based on the fulfillment of three out of the four stated composite criteria. These include 1) vaginal pH of greater than 4.5; 2) increase in characteristic thin, homogenous vaginal discharge; 3) release of fishy amine odour when vaginal secretions are mixed with 10% potassium hydroxide (KOH) (positive Whiff test) and 4) the presence of clue cells in wet smears.

Besides the unpleasant symptoms, BV is associated with adverse obstetric and gynaecologic sequelae. Infected women are at risk of pelvic inflammatory disease and the development of postoperative infections. It could potentiate the acquisition and transmission of sexually transmitted bacteria such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis*. There are also reports linking BV to human immunodeficiency virus (HIV) infection. The increase in vaginal pH may reduce defense against HIV and allow its adherence and survival. Proinflammatory cytokines particularly IL-8 induced by BV, recruits immune cells, at the same time stimulates HIV-1 replication in both macrophages and T lymphocytes that may increase host susceptibility to HIV infection.

Noteworthy, GV in rare circumstances has been linked to various extragenital infections, such as osteoarticular infection, non-gonococcal urethritis in men, retinal vasculitis, septicaemia with pyelonephritis and infective endocarditis. Asymptomatic GV carriers in oral and anal cavities had also been previously described. Reported clinical conditions that are associated with GV/BV are summarised in Table 1.

### 1.1 Genetic Diversity of *Gardnerella vaginalis*

In order to establish the cause-and-effect relationship between GV and BV, numerous studies were conducted to identify particular

<table>
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<tr>
<th>TABLE 1: Reported pathologies and clinical conditions associated with BV/GV</th>
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<td><strong>BV/GV-related Diseases / Clinical Conditions</strong></td>
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<tr>
<td>Bacterial vaginosis</td>
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<td>Pelvic inflammatory diseases</td>
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<td>Cervicitis, salpingitis, endometritis</td>
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<td>Postpartum endometritis</td>
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<td>Subclinical chorioamnionitis</td>
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<td>Preterm labour</td>
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<td>Premature preterm rupture of membrane</td>
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<td>Intrauterine infection</td>
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<td>Fetal inflammatory response syndrome</td>
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<td>Intrauterine growth restriction</td>
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<td>Bronchopulmonary dysplasia</td>
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<td>Periventricular leukomalacia</td>
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<td>Non-gonococcal urethritis</td>
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<td>Increase susceptibility to HIV infection</td>
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<td>Septicaemia with infective endocarditis</td>
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<td>Osteoarticular infection</td>
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<td>Vertebral osteomyelitis with discitis</td>
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<td>Spinal epidural abscess</td>
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<td>Pyelonephritis</td>
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<td>Post-operative bacteraemia</td>
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<td>Asymptomatic carrier in oral and anal cavities</td>
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virulent subtypes of GV over the past 40 decades. Eight biotypes of GV were identified by Piot et al. based on bacterial enzymatic reactions for lipase, β-galactosidase and hippurate hydrolysis, however failed to link the occurrence of BV to any specific GV biotype. Genotypic and molecular methods such as classical ribotyping with Southern blot detection, PCR ribotyping and restriction analysis of 16S-23S rRNA intergenic spacer sequences and amplified ribosomal DNA restriction analysis (ARDRA) were employed to recognise different GV genotypes responsible for BV, but found limited success.

Research interest on GV had ceased until the advancement of molecular genetics a decade later when the whole genomes of GV strains were sequenced, assembled and annotated in Human Microbiome Project supported by the United States National Institutes of Health (NIH). To date, there were over 40 research articles on
Vaginolysin proposed that during the formation of biofilm, GV served as an early coloniser displacing the existing vaginal lactobacilli, and acted as a scaffold which enhanced the subsequent adherence and growth of successive species in BV, particularly Prevotella bivia and Fusobacterium nucleatum, in a symbiotic manner. Gene encoding type I and II pili that mediates cytadhesion as well as type II glycosyltransferases and biofilm-associated protein (BAP) have been demonstrated in BV-associated GV strains. These are crucial for the biosynthesis of EPS for biofilm production.

Cytotoxic Activities (Vaginolysin and Sialidase)
Cytotoxic activities had been demonstrated in GV, which may warrant separate new species designation.

1.2 Virulent Properties of Gardnerella vaginalis

Biofilm Formation
In trying to establish the plausible role of GV in BV, numerous research on its virulent properties have been carried out. Generally, biofilm formation is crucial as the first step in establishing an infection, with GV playing a predominant role. This includes the initial adherence of GV to host cells which induces the formation of biofilm. This is cytologically identified as clue cells (squamous epithelial cells heavily coated with bacteria in situ) (Fig. 3A). Biofilm is defined as assemblage of microorganisms that are tightly held together, encased within an extracellular polymeric matrix containing exopolysaccharide (EPS).

Ultrastructural examination by Swidsinski et al. revealed that biofilm formation associated with GV was highly organised, composed of confluent layers of GV adhered tightly to the vaginal surface epithelium. Many other anaerobes especially Atopobium, Prevotella and Mobiluncus were also detected within GV-related biofilms. However, they were found to be variably intermixed with no perceivable structural organisation. Biofilm formation is necessary to allow the survival of GV and other anaerobes in the hostile acidic vaginal environment. Furthermore, this biofilm-related infection has been reported to be highly associated with antibiotic resistance and prone to disease recurrence.

Macho et al. proposed that during the process of biofilm formation, GV served as an early coloniser displacing the existing vaginal lactobacilli, and acted as a scaffold which enhanced the subsequent adherence and growth of successive species in BV, particularly Prevotella bivia and Fusobacterium nucleatum, in a symbiotic manner. Gene encoding type I and II pili that mediates cytadhesion as well as type II glycosyltransferases and biofilm-associated protein (BAP) have been demonstrated in BV-associated GV strains. These are crucial for the biosynthesis of EPS for biofilm production.

Genomic analysis of GV published, providing a plethora of genetic information on this enigmatic organism. Significant genomic and metabolic divergence were observed among the GV strains isolated from symptomatic and asymptomatic patients, in which these symptomatic BV-associated GV strains possessed greater virulence potential by encoding mucin-degradable protein and biofilm-associated protein (BAP) gene.

More recent phylogenetic profile of cpn60 UT sequences analysis on GV strains reliably defines four distinct GV subgroups (A – D) focusing on the sialidase activity that plays a key role in BV pathogenesis. Nonetheless, the clinical significance of sialidase activity in these subgroups is yet to be established. There is increasing molecular evidence to suggest the existence of GV subspecies with variable virulence determinant and clinical relevance, which may warrant separate new species designation.

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Recently, a putative GV sialidase A gene was detected in vaginal specimen in most of the GV-positive cases. However, the mere presence of sialidase A gene does not predict the actual sialidase activity, as it may not be translated into protein. Further studies should look into the expression pathways of sialidase enzyme at molecular level. Interestingly, sialidase activity detection kit e.g. BVBlue® (Gryphus Diagnostics, L.L.C) (Fig. 1D), a chromogenic diagnostic kit could be used as simple point-of-care (POC) quick test for the diagnosis of BV. High level of sialidase activity (≥ 7.8 U) present in the vaginal fluid hydrolyses the chromogenic substract to sialic acid and IBX-4050, which exhibits intense blue colour upon addition of BVBlue® developer solution. Importantly to note, this virulent determinant is strain-dependent, typically identified in GV strains isolated from symptomatic BV cases.

2. Significance of Gardnerella vaginalis Infection in Perinatal Pathology

2.1 Gardnerella vaginalis and Intrauterine Infection

In healthy pregnancy, the amniotic cavity is believed to be a sterile environment. Infectious agents can be transmitted to the offspring through several routes from the mother. Four acquisition pathways of intrauterine infection have been proposed, with ascending microbial invasion from the lower genital tract being the main pathway of intrauterine infection. Evidence of ascending trafficking of carbon particles deposited in the posterior vaginal fornix into the abdominal cavity in nonpregnant women was demonstrated by Egli and Newton in 1961, supporting the existence of vaginal-uterine cavity migration route. Other routes include transplacental passage, haematogenous spread in systemic maternal infection and uncommonly, accidental introduction of pathogens during invasive procedures such as amniocentesis and percutaneous umbilical cord blood sampling.

Similar to other microbial pathogens, there are four-stage processes involved in ascending route of GV-associated intrauterine infection as speculated by Romero et al. Stage I requires an overgrowth of vaginal microbial flora, frequently manifested as BV. The microorganisms reside in the decidua of the supravisceral region after gaining access to the intrauterine cavity (Stage II). Intra-amniotic infection may follow (Stage III) if the infection progresses to invade the amniotic cavity (amnionitis) or the fetal vessels (chorionic vasculitis). Stage IV involves fetal invasion following several ports of entries, include i) direct aspiration of infected amniotic fluid by fetus leading to congenital pneumonia, ii) direct invasion of microorganisms from infected fluid results in localised infections (conjunctivitis, otitis and omphalitis) and/or iii) seedling of microorganisms into the fetal circulation from any of these infected sites causes bacteraemia and sepsis.

2.2 Maternal Response to GV-associated Bacterial Vaginosis

GV-associated intrauterine infection denotes a disease with a relative scarcity of polymorphonuclear leukocytes response (Fig. 3 B & C) contrasting to conventional acute chorioamnionitis (Fig. 3D) and chorialnic vasculitis. Despite the mere absence of clinical signs of acute inflammation, many believed that there is a significant proinflammatory impact at mucosal level in GV-infected individuals which may lead to adverse pregnancy outcomes.

Innate Immunity

Mucosal immune system notably the innate defense mechanisms provide the first line protection against invading microorganisms. Generally, the innate immune system is activated following the recognition of pathogen-associated molecular patterns (PAMPs) of variety microbial pathogens via pattern recognition receptors (PRRs) which include toll-like receptors (TLRs). Eleven TLRs have been reported in humans at RNA level. TLR-2 alone or in combination with TLR-1/TLR-6 recognise peptidoglycan cell wall components of gram-positive bacteria, while TLR-4 interacts with lipopolysaccharide (LPS) of gram-negative microbes. When triggered, sequence of events leading to influx of inflammatory cells and the release of proinflammatory cytokines including interleukin (IL)-1β, tumour necrosis factor-alpha (TNF-α), IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and CXCL6 will ensue.

Inflammatory Mediators

Numerous inflammatory markers were measured in cervicovaginal fluid of women with BV, but yielded disparate results. Proinflammatory cytokine levels, notably IL-1β and IL-8 were significantly raised in GV-associated BV,
while anti-inflammatory molecule secretory leukocyte protease inhibitor (SLPI) levels were expectedly low. IL-1β, a key mediator in innate inflammatory response, is produced and secreted by many different cell types including monocytes and macrophages. It is released in response to various inflammatory stimuli, which orchestrates the inflammatory downstream cascade and stimulates the release of IL-6, IL-8, TNF-α and matrix metalloproteinases (MMPs) by the target cells such as vaginal epithelial cells, in cases of GV-associated BV. A study of 18 pregnant rhesus monkeys was conducted to determine the role of these proinflammatory cytokines in initiating preterm labour. They were infused with IL-1β, TNF-α, IL-6 and IL-8 via intraamniotic route, and found that IL-1β and TNF-α, but not IL-6 and IL-8 stimulated intense uterine contractions, resulting in preterm parturition in all the animals.

IL-8 is a potent chemoattractant which plays a key role in neutrophil recruitment to fight off microorganisms. A wide range of IL-8 concentrations were detected in vaginal fluid of BV-positive non-pregnant women. In those with low IL-8 responders group, Cauci et al. also observed a low leukocyte count, a low anti-GV haemolysin (Gvh) IgA level and high microbial enzymatic (sialidase and prolidase) activities associated. It is conceivable that the low levels of IL-8 released failed to mount a robust local innate immune response in these individuals, which explained the faint inflammatory signs that was observed. Such impaired IL-8 response may be attributed to a direct degradation of chemokine by proteolytic enzymes associated with GV. Likewise, the impairment of mucosal adaptive antigen-specific immune response against GV-associated BV as indicated by unusually low anti-Gvh IgA levels was likely associated with the similar microbial enzymatic activities.

The immunomodulatory mechanisms of GV...
in those with high IL-8 responders group and pregnant women who have constantly elevated IL-8 concentration but lacking histological evidence of acute inflammation are poorly understood. Lee et al. proposed that the abrogation of innate immune response may be inhibited by the accumulation of polyunsaturated fatty acids that are frequently accumulated in GV-infected individuals, while some argued that the unknown bacterial products from GV may degrade TLRs directly or induce the release of anti-inflammatory cytokines such as IL-10.

2.3 Adverse Perinatal Outcomes in GV-associated Intrauterine Infection

Preterm Labour
Preterm labour is defined as birth before 37 completed weeks of gestational age or before 259 days according to the World Health Organisation (WHO). It represents one of the major clinical problems associated with substantial perinatal mortality and severe neonatal morbidity. While the causes of spontaneous preterm birth are multifactorial, infection is widely accepted as an important factor associated with preterm delivery in up to 40% of cases. Maternal vaginal colonisation by GV manifested as bacterial vaginosis was implicated in the aetiology of preterm labour. The exact mechanisms of how infection induces preterm birth are not well established. It is believed that this organism ascends from the lower genital tract into the uterus and choriodecidual space which subsequently triggers an inflammatory response in the placenta, membranes and decidua.

Menard et al. conducted a large scale molecular quantification study on vaginal bacterial loads in 813 preterm births and concluded that high vaginal concentration of GV (at least 10^7/mL) was significantly associated with preterm delivery. Their findings are supported by a meta-analysis on 20,232 patients, which revealed that GV-associated BV patients had two-fold the relative risk of preterm delivery, after controlling other confounding major risk factors. In addition, GV is one of the abnormal genital tract flora frequently isolated in the amniotic fluid of women presented with premature delivery and preterm premature rupture of membrane. Arguably, association does not necessarily imply causation and microbial invasion of the amniotic cavity is perhaps merely the sequelae of labour. The causal link of GV infection in preterm labour was later proven valid following the criteria outlined by Sir Bradford Hill.

Recently, amniotic fluid sludge, a sonographic finding of free-floating hyperechogenic particles within amniotic fluid in close proximity to the uterine cervix has been identified as an independent risk factor for impending preterm labour. Evidence determines that this material represents a biofilm formed by amniotic fluid bacteria, including Streptococcus mutans and Mycoplasma hominis. Bacteria are seen embedded within exopolymeric matrix substance, typical of a biofilm demonstrated with scanning electron micrograph. Besides being a marker to indicate intra-amniotic inflammation, biofilm is highly resistant to antibiotics. Interestingly, GV, a vaginal microbial notorious for its biofilm formation on cervical epithelium in bacterial vaginosis has not been implicated in sludge formation.

Microbial intrusion of amniotic cavity elicits a robust intrauterine inflammatory reaction. Fetal skin, umbilical cord, amnion and chorion-decidua are among the tissues responsible for the intra-amniotic inflammatory cascade. The release of proinflammatory cytokines particularly IL-1β and TNF-α in turn, can stimulate maternal prostaglandins production by decidua which initiate myometrial contractility, degradation of fetal membranes’ extracellular matrix and cervical ripening by matrix metalloproteinases leading to the onset of parturition. High levels of vaginal IL-1β and TNF-α that were detected in GV-associated BV infected pregnant women could explain the occurrence of preterm birth associated with this organism.

The onset of intrauterine infection-driven preterm parturition is believed to be adaptive in nature, i.e. to allow the survival of the fetus by exiting the hostile intrauterine environment, as well as the survival of the mother by removing the infected fetus.

Fetal Inflammatory Response Syndrome
Microbial attack of fetus can occur following intra-amniotic infection. As mentioned, bacteria can gain access to fetus via respiratory tract, gastrointestinal route or direct contact through skin, conjunctiva and ear. The recognition of microbial agents by PRRs such as TLRs that are expressed in fetal mucosa will initiate a downstream inflammatory signalling cascade leading to a localised and subsequently systemic inflammatory reaction referred to as fetal inflammatory response syndrome (FIRS).
related organisms (anaerobes, *Mycoplasma hominis* and GV) are among the lower genital tract colonisers in association with FIRS.99

FIRS, similar to systemic inflammatory response syndrome (SIRS) manifested in adult patients, is a form of serious acute-phase response occurring during fetal life that was first coined by Gomez *et al*. in 1998.90 It is characterised by systemic activation of the fetal innate immune system leading to multiple organ failure associated with sepsis. In reference to the American College of Chest Physicians and the Society of Critical Care Medicine guidelines, SIRS in adults is defined by clinical criteria which include temperature > 38°C or < 36°C, tachycardia, tachypnoea and white cell count > 12,000/uL or < 4,000/uL or left shift in myeloid maturation.91 Unlike in adults, SIRS criteria are not applicable to fetus as the vital signs (with the exception of heart rate) could not be readily accessed during intrauterine period. Hence, elevated circulating level of an inflammatory biomarker, IL-6, in fetal cord blood plasma is used to define FIRS, which is easily measured via immunoassays.92

Histological evidence of chorioamnionitis and chionic vasculitis could represent FIRS as these features were positively associated with higher fetal plasma IL-6 concentration.93 Interestingly, despite the lack of histologic chorioamnionitis and/or funisitis seen in GV-infected placenta, IL-6 level is constantly elevated.94 This may be explained by the different signalling pathways involved by IL-6 as recently proposed by Noda-Nicolau and colleagues.94 They revealed that IL-6 was pleiotropic, exhibited its distinctive bioactivities either through classical or trans-signalling mechanisms by interacting with different receptors. The authors observed that through classical signalling pathway, GV generated an anti-inflammatory effect through membrane-bound IL-6 receptor (mIL-6R), in contrast to trans-signalling pathway where proinflammatory effect was produced through soluble IL-6 receptor (sIL-6R).94 Large scale studies on immunomodulatory role of GV in perinatal infection are lacking, hence requires further exploration.

Substantial evidence indicate that fetuses with FIRS can progress towards multi organ damage including the haematopoietic system, the adrenals, lungs, heart, brain and skin and long-term neonatal handicaps such as bronchopulmonary dysplasia, periventricular leukomalacia (PVL) and cerebral palsy.92,95,96

It is noteworthy that the damage incurred by multiple organs in developing fetus is thought to be the result of an exaggerated inflammatory reaction rather than the virulence of the infecting microorganism itself. However, it remains to be determined whether novel immunomodulatory strategies in fetus affected with FIRS may help improve perinatal outcomes.

**Fetal Lung Injury**

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease typically affecting infants born prematurely, prior to 28 weeks of gestational age. BPD is inconsistently defined by its treatment, characterised by the need for prolonged respiratory support in these affected babies.97 BPD is believed to be multifactorial and represent the end result of insults related to preterm birth that interfere with normal lung development. Injurious insults include chronic intrauterine infection, sepsis, severe prematurity at 22-23 weeks of gestational age, mechanical ventilation-related lung injury, chronic hypoxia and pulmonary vascular disease.98

The insurgence and progression of BPD rests on the timing of abnormal environmental triggers which disrupt normal lung morphogenesis. There are five distinct stages in normal human lung development: embryonic, pseudoglandular, canalicular, saccular and alveolar stages.99 Current evidence suggests that BPD is likely to develop in preterm infants who are born in the late canalicular or early saccular stage of lung development.100,101 During the late canalicular stage (22-25 weeks of gestation), functional gas-exchange units are formed by further expansion of distal airspace structures along with microvascular development directed by vascular endothelial growth factor (VEGF) as early preparation for respiration at birth.102,103

In early saccular stage (26-28 weeks of gestation), extensive maturation of alveolar epithelial cells into type I and II pneumocytes with surfactant production, complex vascular network with enlargement of terminal saccules ensues.103 In a premature baby, this programmed developmental process is interrupted leading to incomplete alveolarisation and vascular maldevelopment.

The pathophysiology of BPD has been a focus of research over the past decade. The development of BPD is complex and multifactorial. At least three different pathogenic pathways had been postulated depending on the timing and severity of the injurious exposures, ranging from the
“old” structural lung damage to the “new” early pulmonary developmental arrest to the current vasculogenesis impairment predispose to the evolution of BPD. This vascular theory was supported by the increase risk for developing pulmonary hypertension in those infants who survived later in life. Nonetheless, genetic predisposition could influence the disease risk and/or outcome. Here, our discussion focuses on the possible mechanisms related to intrauterine infection-induced BPD.

Published data showed a possible link between subclinical intrauterine infection with fetal lung injury and consequential development of BPD. Ghezzi et al. observed that patients whose neonates subsequently developed BPD had elevated concentrations of amniotic fluid IL-8 compared with those who did not, in agreement with others. Similarly, higher proinflammatory cytokines IL-1β, IL-6 and IL-8 in the tracheobronchial secretions of preterm infants had been reported predictive of the development of BPD.

Intra-amniotic proinflammatory cytokines come in contact with the fetal lungs via normal swallowing in utero initiate local innate immune response by attracting influx of inflammatory cells into the lung and the release of proteases namely elastase, matrix metalloproteinase and trypsin by the inflammatory cells. The imbalance between the proteases and proteases inhibitors further aggravates pulmonary tissue damage, by facilitating inappropriate elastin breakdown.

Besides, activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signalling by IL-1β and TNF-α in the developing lung exaggerates pulmonary inflammatory response which initiates the development of BPD. As a corollary, NF-κB inhibitors may have therapeutic role in this context. The therapeutic benefit of NF-κB inhibitors however was proven otherwise given the contrasting complex physiologic functions of NF-κB in the developing lung. NF-κB mediates both pro- and anti-inflammatory actions influenced by the timing and extent of inhibitory signals. Blackwell et al. in their animal model proved the pivotal role of alveolar macrophages specific NF-κB activation in the pathogenesis of BPD. The authors revealed that these macrophages were required to generate a local inflammatory reaction in the fetal lung and disrupted airway morphogenesis, while airway branching was preserved in macrophage-depleted fetal lung explants or those pre-treated with targeted NF-κB inhibitors following exposure to LPS.

In addition, impaired pulmonary angiogenesis is also a key feature in BPD. Abnormal alveolar microvessels and decreased expression in pulmonary angiogenic growth factors [vascular endothelial growth factor (VEGF) and platelet and endothelial cell adhesion molecule 1 (PECAM-1)] were demonstrated in preterm infants with severe BPD. A decrease in VEGF receptors in pulmonary microvascular endothelium was reported. Collectively, disruption of normal pulmonary morphogenesis results in lung remodelling and consequential evolution of BPD.

Morphologic changes in classic “old” BPD following severe airway injury including alternating emphysema and atelectasis, fibrosis, smooth muscle hypertrophy of airways as well as pulmonary vasculature predispose the BPD survivors to subsequent pulmonary hypertension. On the contrary, the “new” milder form of BPD characterised histologically by large, simplified alveolar spaces with generally decreased secondary septation and hence reduction in numbers of alveoli, disordered capillarisation and variable degrees of interstitial fibrosis.

In an attempt to establish the effects of GV infection on fetal lung development, we induced intrauterine infection by direct GV inoculation into uterus of pregnant rabbits. In this experimental model, we observed that the average weight of GV-infected fetal rabbit lungs was significantly lower than the non-infected control group, while the infected lungs showed a significant degree of alveolar septa thickening, suggesting impaired lung morphogenesis in these infected fetuses similar to those of human.

**Fetal Brain Injury**

Intrauterine infection resulting in FIRS is known to cause direct fetal neuronal injury, leading to various neurodevelopmental disorders. These fetal neuronal aberrations are not due to prematurity per se. When comparing preterm birth in murine models induced by inflammation and non-infectious agent respectively, Burd et al. observed that only the intrauterine inflammation model exhibited raised inflammatory cytokines and altered fetal neuronal morphology.

GV-associated bacterial vaginosis in pregnancy has been associated with adverse neurological outcomes in the offspring, which include periventricular leukomalacia and cerebral
palsy. In an attempt to establish a causative link of GV-associated intrauterine infection with fetal brain injury, Field et al. inoculated GV direct into the intrauterine cavity of pregnant rabbits. Severe neuronal necrosis predominately in the fetal cerebellum and hippocampus regions was observed in the GV-inoculated study group compared with the control group, suggesting the detrimental role of GV infection in the perinatal brain. White matter however was not analysed in those infected fetal rabbits. Their findings were in agreement with the work by McDuffie et al., in similar rabbit model, where histological evidence of apoptosis with additional white matter lesions were observed in the fetal brain in chronic GV-induced intrauterine infection.

One may argue that the brain is an immune-privileged site, which is protected from circulatory components by a selective blood-brain barrier (BBB) tightly regulated by endothelial cells to maintain brain homeostasis. Nonetheless, proinflammatory cytokines in the fetal circulation may gain access into the brain via various mechanisms such as i) breakdown or increase leakage of the BBB induced by neuroinflammatory reaction, ii) prostaglandin-dependent, cytokine-mediated pathway, iii) through carrier-mediated transporter mechanisms across the BBB or iv) direct damage to BBB by binding of endotoxins particularly LPS on TLRs present on microglia or cerebral endothelial cells.

Of note, the timing of intrauterine infection during pregnancy may determine the neurodevelopmental outcome of the fetuses since the brains are affected at different stages of development. During the late second trimester in normal human corticoneurogenesis, migration of neurons derived from the germinative neuroepithelium from the ventricular zone to the cerebral cortex takes place. Influx of inflammatory cytokines following intrauterine infection may disrupt the process of migration of post-mitotic neurons to the neocortex, resulting in aberrant cortical development and neuronal dysfunction. Immature oligodendrocyte progenitor cells (OPCs) and activated microglia that are present in abundance during this critical period (24 to 32 weeks gestation) make the developing white matter of the brain vulnerable to injurious inflammation and immunological stress. Late gestational intrauterine infection confers a higher risk for neuropsychotic disorders in the resultant offspring corresponding to schizophrenia and autism in human, demonstrated by Meyer et al. on pregnant mice. Generally, axon and astrocytes are more resistant to injurious insults; and OPCs are the predominant cell types that are susceptible within the critical window.

The mechanistic pathways of neuronal injury initiated by GV-associated intrauterine infection are still poorly defined to date, awaiting further elucidation. We hypothesised that intrauterine infections secondary to GV or other pathogens share a common pathway resulting in neuronal injury i.e. by initiating FIRS. As discussed earlier, FIRS had been implicated in triggering neuronal damage in the developing brain. Microglial activation in response to FIRS plays a crucial role in the pathophysiology of white matter damage in the immature brain. Upon crossing the BBB, circulatory proinflammatory cytokines notably TNF-α, IL-1β and IL-6 in turn may directly cause injury to oligodendrocytes and neurons, and at the same time activate adjacent astrocytes and microglia to release cytotoxic inflammatory mediators, excitotoxic metabolites e.g. glutamate and quinolinic acid and oxidative products.

Several putative immune-precipitated mechanisms of neuronal injury had been advocated. Microglial product notably TNF-α has direct cytotoxic effect on oligodendrocyte and its progenitors leading to myelination defects. At molecular level, Kadhim et al. proposed that TNF-α exerts its neurotoxicity effects by triggering apoptotic cascades directly via p75TNFR2 or p55TNFR1 receptors or indirectly through stimulating nitrosative-induced neuronal apoptotic pathway. Other potent proinflammatory cytokines IL-1β and IL-6 also have deleterious effects on the maturation of OCPs. Elevated levels of IL-1β had resulted in increased numbers of unmyelinated axons while overexpression of IL-6 contributed to severe neurological defects seen in the affected mice. In addition, oxidative and nitrosative products which generate reactive oxygen and nitrogen species can cause damage to the cell membrane and initiate cell death. Collectively, hypomyelination, dysmyelination and maturation arrest of OPCs to form myelinating oligodendrocytes will follow, contributing to the insurgence of periventricular leukomalacia (PVL) in premature infants and subsequent cerebral palsy later in life.

PVL, a major cause of cerebral palsy is characterised by multifocal (coagulative) necrosis of the developing white matter, particularly involving the lateral ventricles with adjacent reactive gliosis. Movement disorders
such as spastic quadriplegia, diplegia and dyskinesia, epilepsy, mental retardation and delayed language development are among the common clinical manifestations associated with PVL. A decrement in the volume of cerebral cortical grey matter secondary to neuronal degeneration and gliosis following PVL may explain the cognitive and motor impairments in long term survivors of prematurity.

**Intrauterine Growth Restriction**

In order to achieve a successful uncomplicated pregnancy, normal development and function of the placenta is critical. Forbes and Westwood (2010) have identified a number of growth factor receptors e.g. insulin-like growth factor receptor (IGF), vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) that are indispensable in regulating fetal growth and development.  

IGFs and their receptors exert a key role in feto-placental growth throughout gestation from as early as six-week gestation. In placenta, IGF1R is expressed in trophoblast, villous endothelium and mesenchymal core; while IGF2R in microvillous and plasma membranes of trophoblast. Ligand binding to the IGF1R activates two main signalling cascades, the phosphoinositide 3-kinase (PI3K) pathway or the mitogen-activated protein kinase (MAPK) pathway, which then promote cell proliferation, differentiation and protection from apoptosis. IGF2R, conversely, does not have tyrosine kinase activity. It involves in regulating fetal growth by clearing IGF-II from the circulation.

EGFR, one of the members in ERBB family of receptor tyrosine kinases, modulates many aspects of cellular biology including cell proliferation, differentiation, apoptosis and survival. Defective EGFR activity is associated with intrauterine growth retardation or delayed embryonic development. Likewise, TGF-β, a prototype member of a family of multifunctional cytokines, plays a critical role in immunomodulation, vasculogenesis and cell cycle. Larsson et al. demonstrated that mice lacking TGFβ-1R died suffering from severe defects in yolk sac vasculogenesis and haematopoiesis.

VEGF, as the name implies, induces angiogenesis, promotes cell migration and inhibits apoptosis. Study had shown that VEGF expression was raised in the placenta of mothers with hypertension. Inhibition of VEGFR-2 during pregnancy contributes to adverse outcomes, i.e. preeclampsia and intrauterine growth restriction. Wada et al. revealed that the vascular development in labyrinthine zone of placenta and various fetal organs were suppressed by testing VEGFR-2 tyrosine kinase inhibitor on pregnant mice. PDGFR-A and PDGFR-B play an essential role in promoting cell proliferation, migration and survival. Dimerisation of both receptors by ligands activates PI3K and MAPK signalling cascades, thus inducing cell growth. Hitherto, studies on the role of PDGR in regulating human fetal growth are limited. Jarvenpaa et al. reported a reduced expression of PDGFRα in intrauterine growth retardation (IUGR) placentas. Studies in mice have demonstrated that knockout of growth factor receptor genes could result in severe fetal growth restriction and in worse scenario, intrauterine death.

It has been reported that GV-associated BV is an independent risk factor for low birth weight and even IUGR. A large scale cohort study conducted on Danish pregnant women demonstrated that as high as 56% of fetuses with IUGR had been linked with BV-infected pregnant mothers. Likewise, in three pregnant rabbit models including one of ours had revealed that the fetuses that were infected with GV suffered from lower birth weight compared with the matched non-infected group. The exact biological mechanisms and sequences of infective processes which affect intrauterine growth however are far from being completely elucidated. Virtually no study on the effects of GV, a low virulence pathogen, on the growth factor receptors has been performed thus far, and hence warrants further investigations.

Besides the immune effector cells, intriguingly TLR-2 and TLR-4 were found to be highly expressed in the epithelial cells of female reproductive tissues and at maternal-fetal interface in human term placenta. Engagement of TLR-4 by LPS evokes a classical inflammatory immune response, characterised by release of proinflammatory cytokines such as TNF-α, IL-6 and IL-8 and recruitment of PMN leukocytes to the site of infection. Interestingly, instead of upregulating the production of cytokines, ligand of TLR-2 by a gram-positive bacterium expressing peptidoglycan or lipoteichoic acid triggered apoptosis of the trophoblast cells. Elevated trophoblast cell apoptosis is frequently seen in placenta complicated with IUGR and preterm birth. Further investigation is needed.
to understand whether GV-associated intrauterine infection promotes direct trophoblast cell death via initiation of apoptotic pathway by activation of caspases upon the recruitment of Fas-associated death domain or by activating TLR-2.

3. Animal Model Studies on *Gardnerella vaginalis*

An ideal animal model for medical research should possess a taxonomic equivalency, biological and genetic properties parallel to that of humans. The development of a suitable animal model for the study of vaginosis however is far from satisfactory thus far.

The earliest animal model for the study of vaginosis was in 1984 on primates (pig-tailed macaques, tamarins and chimpanzees) but with limited success. The infected animals failed to show characteristic features of BV (pathognomonic clue cells, increased succinate to lactate ratio, increased in vaginal pH) following intravaginal inoculation with GV. This may be attributed to the physiological and microbiological differences in the vaginal environment between primates and humans. Mardh et al. explored the possibility of inducing BV in grivet monkey by inoculating vaginally several strains of BV-associated pathogens (GV and presumed *Mobiluncus* sp.). They found that these monkeys that were co-infected with GV and presumed *Mobiluncus* sp. had profuse vaginal discharge, typical to that of BV after five days of inoculation, but not with GV alone. However, clue cells were not demonstrated from the vaginal secretions.

The rationale behind the failure in establishing a successful primate model system for vaginosis remained inexplicable until recently. Yildirim et al. reported an interesting observation that the vaginal microbiomes of nonhuman primates including chimpanzees, baboons, colobus, vervets, mangabeys and monkeys showed limited congruence with humans. All primates exhibited greater vaginal microbial diversity with low abundance of *Lactobacillus* species. Conversely, humans were predominantly colonised by *Lactobacillus* species. Studies involving smaller animal models were also explored which yielded varying degrees of success. Dukes and Gardner failed to induce systemic infection in small animal systems (mice, guinea pigs, rats and rabbits) following GV inoculation through intravenous, subcutaneous and intraperitoneal routes. Field and colleagues (1993) developed a rabbit model of GV infection in pregnancy and observed GV-induced amnionitis and deciduitis. It was associated with minimal maternal morbidity. However, the affected fetuses that survived had significantly decreased birth weight and severe brain injury. They concluded that GV acted selectively as “a fetal, but not maternal pathogen” in rabbits. Their work was later supported by McDuffie et al. who used a similar model, and successfully yielded positive cultures of GV from uterus, lungs and brains of rabbit fetuses with histological evidence of brain injury.

We have also developed a GV infected pregnant rabbit model and found significant difference in birth weight between the GV-inoculated group and saline-inoculated group. All the fetuses in GV-inoculated group had lower birth weight. Histologic examination of the fetal lung and placenta showed no significant inflammation. Interestingly, the number of placental syncytial knots was increased. This suggests that GV infection in pregnancy might induce a hypoxic state to the fetus. Further work on this model may shed light into the key mechanisms related to intrauterine growth restriction in fetal rabbits.

Most murine models were focusing on the therapeutic strategies for GV-infected rodents. Studies showed that antibiofilm strategy with DNase and intravaginal treatment with *Lactobacillus johnsonii* HY7042, a probiotic strain can inhibit GV colonisation. Gilbert et al. was the first who yielded key BV phenotypes parallels to that of human in GV-infected murine model, and also described the presence of vaginal sialidase activity, host epithelial exfoliation in the absence of histological inflammation and formation of clue cells. However, it is difficult to determine whether the murine model is translatable to human BV infection.

A robust gnotobiotic (germ-free) mice model system was used to study the antagonistic effects of probiotic *Lactobacillus* strains with GV isolated from women with or without BV. A protective effect against GV infection was observed in mice previously mono-associated with *L. johnsonii*, with significant reduction in vaginal population levels of GV. Such models offer a more realistic environment to understand further microbe-microbe interactions, avoiding the potential confounding effects from the commensal microbiota that are originally present in conventionally raised mice. As discussed in the earlier section, human CD59 facilitated the
process of vaginolysin-mediated lysis in non-susceptible cell lines. Hence, transgenic animal model expressing human CD59 might serve as a suitable model for mechanistic studies of GV with the background understanding of such interactions.

4. Future Prospects

There is incremental evidence that GV is a significant genital tract pathogen involved in BV and its association with poor reproductive health outcomes. However, whether GV acted alone or synergistically with other anaerobes to trigger the associated disease remains an enigma. We cannot also explain why GV is frequently isolated in the vagina of healthy women. More work is still needed to understand the complex aetiology of BV. With the recent advancement in molecular genomic analysis, GV can be more clearly divided into pathogenic and nonpathogenic strains with diverse virulent potentials. Functional characteristics and virulence properties, particularly biofilm formation associated with sialidase production between pathogenic and nonpathogenic strains as well as their interspecies interaction with other bacteria warrant further studies.

Specific mechanisms of immune activation during pregnancy against this microbial species remain unresolved. There is increasing evidence that trophoblast cells are actively involved in innate immunity. Through the expression of TLRs, these cells are able to recognise and respond to pathogens. The potential role of TLR-2 that is highly expressed in trophoblast cells, which recognises mainly Gram-positive bacteria may be worthwhile in further exploring the role in GV-related fetal growth interference.

While numerous studies reported a higher vaginal level of IL-1β, IL-6 and IL-8 among pregnant women infected with BV, histological examination of GV-infected placenta and fetal tissue surprisingly displayed no signs of PMN leukocytes recruitment. Immunomodulatory effects of GV had been explored, however, targeted mostly on non-pregnant women. Further study is needed to provide a better understanding of the mechanisms of immune subversion by GV at molecular level in BV-infected pregnant mothers. Moreover, it is believed that the placenta may orchestrate or have paracrine effects on the development of the fetus in the setting of GV infection of the placenta. This has not been studied and more so, the fetal growth and development impact from intrauterine GV infection requires further investigation. The occurrence of low fetal weight may be related to increase apoptosis of trophoblast cells. The question of whether GV infection induces direct trophoblast cell death through initiation of apoptotic pathway needs to be investigated. On the other hand, GV infection could have effects on growth factor receptors in trophoblast cells and influence the growth of the fetus. Further study should be directed to elucidate the complex mechanism involved in the development of intrauterine growth restriction of the fetus in antenatal GV infection.

It is noteworthy that not all antenatal GV infection will eventually lead to adverse perinatal outcomes. Genetics may play a pivotal role in this context. Early evidence suggested that individuals who carry a certain TNF-α genotype were able to mount an overt inflammatory reaction with significantly higher TNF-α plasma levels to BV-associated organisms, not limited to GV, increasing the risk of perinatal morbidity. Plasma TNF-α level may serve as a potential biomarker to help identify such high risk fetuses with less favourable outcomes in the future.

The issue concerning treatment failure in BV with high rate of recurrences remains unresolved due to lacking of a reliable animal model. Ongoing clinical trials in the treatment of BV and the current United States Food and Drug Administration (US FDA) recommendations are beyond the scope of discussion in this paper. Again, the challenge lies on which animal model system to choose that is more representative and translatable to the human population. Since there is no ideal single model that exists which represents all the complex vaginal microenvironment of human at present, this called for combinatory approaches by using both in vitro and in vivo model systems in refining the gaps in GV research. In addition, the development of humanised small animal model with transgenic hCD59 receptor, though technically burdensome, may be of potential use in this regard.

In conclusion, precise molecular mechanisms by which an infection can lead to fetomaternal complications in the absence of overt inflammatory response remain largely undefined thus far, leaving a research gap in this area (Fig. 4). A better understanding in the mechanisms of disruption of fetal development in GV infection is crucial in creating a newer therapeutic intervention in GV-infected pregnant
mothers by targeting growth receptors and related signalling pathways.

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