Association of gestational diabetes mellitus and abnormal vaginal flora with adverse pregnancy outcomes

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Abstract
Gestational diabetes mellitus (GDM) is associated with adverse perinatal outcomes. This study aimed to examine the association between GDM and abnormal vaginal flora, and the association between abnormal vaginal flora and adverse pregnancy outcomes. This was a prospective study of pregnant women who visited Xuanwu Hospital of the Capital Medical University (Beijing, China) between February and October 2015. All women were screened for GDM according to the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) recommendations. Vaginal secretions were sampled at 28 to 30 and 37 to 40 weeks. Microorganisms were examined. The women were 28.3 ± 2.6 years and their body mass index was 22.8 ± 1.4 kg/m². GDM was associated with higher frequencies of vulvo vaginal candidiasis (22.6% vs 9.7%, \( P < 0.01 \)), premature rupture of membranes (PROM) (22.6% vs 11.5%, \( P = 0.004 \)), premature delivery (16.1% vs 5.5%, \( P = 0.02 \)), choioamnionitis/puerperal infection (19.4% vs 4.5%, \( P < 0.001 \)), macrosomia (9.7% vs 4.0%, \( P = 0.04 \)), neonatal hypoglycemia (5.4% vs 1.0%, \( P = 0.02 \)), and neonatal referral (15.1% vs 6.5%, \( P = 0.08 \)). Among healthy women, abnormal flora was associated with PROM (19.4% vs 7.5%, \( P = 0.02 \)) and choioamnionitis/puerperal infection (11.9% vs 3.8%, \( P < 0.001 \)). Among women with GDM, abnormal flora was associated with PROM (32.1% vs 10.0%, \( P < 0.001 \)), premature delivery (17.7% vs 6.3%, \( P = 0.04 \)), and choioamnionitis/puerperal infection (32.8% vs 2.5%, \( P < 0.001 \)). The vaginal infection rate was higher in patients with GDM compared with healthy pregnant women. GDM and abnormal vaginal flora were both associated with adverse pregnancy outcomes. The vaginal Lactobacillus species were different between the 2 groups, which could contribute to the adverse outcomes.

Abbreviations: BV = bacterial vaginosis, GDM = gestational diabetes mellitus, IADPSG = the International Association of the Diabetes and Pregnancy Study Groups, OGTT = oral glucose tolerance test, VVC = vulvovaginal candidiasis.

Keywords: bacterial vaginosis, gestational diabetes mellitus, lactobacillus, peri natal outcomes, vaginal microbiology, vulvovaginal candidiasis

1. Introduction
Gestational diabetes mellitus (GDM) is a type of diabetes diagnosed during the 2nd or 3rd trimester of pregnancy and that is clearly not overt diabetes.\[1\] Women with diabetes during the 1st trimester are classified as having preexisting pregestational diabetes.\[1\] Recently, the International Association of Diabetes and Pregnancy Study Groups (IADPSG) issued new criteria for GDM screening and diagnosis.\[2,3\] According to the IADPSG recommendations, fasting plasma glucose should be measured at the first prenatal visit and women with fasting blood glucose (FBG) >7.0 mmol/L will be diagnosed with overt DM and those with FBG >5.1 mmol/L will be diagnosed with GDM. For women with FBG <5.1 mmol/L, a 75-g 2-hour oral glucose tolerance test (OGTT) should be performed at 24 to 28 weeks: those with FBG >7.0 mmol/L will be diagnosed with overt DM; those with FBG >5.1 mmol/L, 1-hour glucose >10 mmol/L, and 2-hour glucose >8.5 mmol/L will be diagnosed with GDM; and all others will be considered insulin sensitive.\[2,3\] According to the IADPSG criteria, Zhu et al\[4\] conducted a retrospective study of 17,186 pregnant women from 13 hospitals in China between 2010 and 2011, and the incidence of GDM was found to be 17.5%. In the Arab Emirates, Agarwal et al\[2\] showed that the use of the IADPSG criteria increased the prevalence of GDM nearly 3-fold (from 12.9% using the conventional criteria, to 37.7% when using IADPSG), but the IADPSG criteria significantly simplified the diagnosis of GDM by circumventing a large number of OGTTs. In Caucasians populations, the overall prevalence of GDM was found to be 17.8% (range, 9.3%–25.5%).\[1,6\]

GDM is associated with increased risk of maternal complications such as spontaneous preterm birth, premature rupture of membrane (PROM), choioamnionitis, traumatic complications of vaginal delivery, cesarean delivery, morbidity from operative delivery, preeclampsia, and risk of developing metabolic syndrome or DM.\[7–11\] GDM is also associated with significant risk for the fetus, including stillbirth, macrosomia, shoulder dystocia, and congenital malformations.\[11–15\] Neonatal complications

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are also more frequent and include neonatal hypoglycemia, neonatal hyperbilirubinemia, hypocalcemia, erythrocytosis, and poor feeding.\textsuperscript{7,11,16}

The vaginal flora encompasses the microorganisms that colonize the vagina. The primary colonizing bacteria in healthy women are Lactobacillus and the lactic acid, and they produce protection against pathogenic species such as Candida.\textsuperscript{17} The vaginal flora is highly sensitive to disturbances such as menses, sexual intercourse, vaginal douching, and contraception.\textsuperscript{17} In addition, pregnancy is associated with an immunonpromised state that increases the susceptibility to vaginal Candida infection.\textsuperscript{18} In itself, vaginal infection can be a cause of intrauterine infections, stillbirth, premature delivery, and neurological damage to the fetus.\textsuperscript{19–21} Since GDM is associated with poor metabolic control, higher body mass index, and impaired leukocyte function,\textsuperscript{22,23} some studies have suggested that GDM is associated with disturbances in the vaginal flora and vagina infections,\textsuperscript{24–30} but this is controversial.\textsuperscript{31} Infections caused by Candida are relatively well known to be associated with GDM,\textsuperscript{25–28} but much less is known about bacterial infections.

Based on those facts, we hypothesized that abnormal vaginal flora (such as changes in dominant bacterial strains and pathogenic bacterial infection) in patients with GDM is associated with adverse perinatal outcomes. Therefore, the aim of this study was to examine the association between GDM and abnormal vaginal flora, and the association between abnormal vaginal flora and adverse maternal and neonatal outcomes. In addition, high-throughput sequencing was used to reveal the composition and dynamic changes of the vaginal flora in patients with GDM during pregnancy. The results of this study could provide some clues about the pathogenesis of GDM, associated conditions, and potential targets for prophylaxis and treatment.

2. Methods

2.1. Study design and patients

This was a prospective study of pregnant women who visited the Xuanwu Hospital of Capital Medical University (Beijing, China) between February and October 2015. They underwent an OGTT at 24 to 28 weeks of pregnancy. The study was approved by the ethics committee of the Xuanwu Hospital of Capital Medical University (Beijing, China). All patients provided written informed consent.

All women were screened and diagnosed for GDM according to the IADPSG recommendations.\textsuperscript{2,3} The patients were grouped according to with or without GDM (controls). The inclusion criteria were Han nationality; singleton pregnancy; without sex in the past week; not taking systemic or local antibiotic; and without medical history of vaginal douching or treatment.

2.2. Sampling and data collection

Vaginal secretions were collected from the upper third of the vagina with sterile vaginal swabs, avoiding contact with the orificium vaginae or the vulva to prevent contamination. In both groups, the samples were collected at 28 to 30 and 37 to 40 weeks of pregnancy. Vaginal pH was measured and the distribution of the microorganisms was observed and assessed,\textsuperscript{22} vaginal microecology with a roughly normal pattern: grade II–III vaginal flora density; grade II–III vaginal flora diversity; Lactobacillus is the dominant bacteria; 0–5 pyocytes or leukocytes per high-magnification fields; bacterial vaginosisis (BV); according to the criteria proposed by Nugent et al,\textsuperscript{33} BV was diagnosed upon a total score ≥7; vulvovaginal candidiasis (VVC): VVC was diagnosed when spores and pseudohyphae were detected under the microscope; and BV plus VVC (BVC): co-occurrence of BV and VVC. The samples were stored at −80°C for sequencing.

2.3. Sequencing and bioinformatics analysis

In the GDM group, 30 samples were collected from 15 GDM patients. In the healthy control group, 20 samples were collected from 10 healthy pregnant women. Total DNA was extracted from the vaginal secretions. Amplicon libraries of the V4-V5 hyper-variable regions of the 16S rRNA gene were generated using specific primers. Target amplicon fragments were recovered with magnetic beads to build the library. Sequencing was conducted using a MiSeq high-throughput sequencing platform (Illumina, Inc., San Diego, CA).

Raw sequencing data were processed by removing low-quality reads to acquire clean data.\textsuperscript{34} Then, the Fast Length Adjustment of Short reads, v1.2.11 (FLASH) software was used to assemble paired-end reads and to screen under specific standards.\textsuperscript{35} Finally, clean tags of the V4-V5 hyper-variable regions were sorted out. The USEARCH (v7.0.1090) software was applied to cluster the clean tags into operational taxonomic units (OTU).\textsuperscript{36} Then the nonphylogenetic alpha diversity, phylogenetic beta diversity, and taxonomic composition analyses were performed on the OTU table using the open-source bioinformatics pipeline QIIME.\textsuperscript{17}

2.4. Follow-up

Telephone follow-up was conducted about perinatal outcomes of maternal and neonatal complications in both groups.

2.5. Statistical analysis

SPSS 13.0 (SPSS Inc, Chicago, IL) was used for statistical analysis. Continuous data were expressed as mean±standard deviations and analyzed using the Student t test. Categorical data were expressed as frequencies and analyzed using the chi-square test. Two-sided P-values <.05 were considered statistically significant.

3. Results

3.1. Characteristics of the patients

A total of 386 pregnant women were enrolled, including 186 with GDM and 200 healthy controls. The patients were 28.3±2.6 years and their body mass index (BMI) was 22.8±1.4 kg/m\textsuperscript{2} (range, 18–28 kg/m\textsuperscript{2}).

3.2. GDM is associated with abnormal vaginal flora

In the healthy control group, vaginal pH was 3.72±0.20; VVC frequency was 9.7% (18/200); BV frequency was 9.5% (19/200); and BV plus VVC (BVC) frequency was 10.4% (19/186); BVC frequency was 22.6% (42/186); BV frequency was 10.4% (19/186); BVC frequency was 6.0% (12/186); the frequency of normal bacteria
Among the 200 healthy pregnant women, 67 had an abnormal perinatal outcomes among healthy women. The frequencies of PROM, premature delivery, and chorioamnionitis (confirmed by postoperative placental pathology) or puerperal infection were higher in the GDM group compared with the control group (22.6% vs 11.5%, \( P = .004 \); 16.1% vs 5.5%, \( P = .02 \); 19.4% vs 4.5%, \( P < .001 \), respectively) (Table 2). There were no differences between the 2 groups regarding fetal growth restriction and mild asphyxia (both \( P > .05 \)).

### 3.3. GDM is associated with worst maternal outcomes

The frequencies of PROM, premature delivery, and chorioamnionitis or puerperal infection were higher in the GDM group compared with the control group (22.6% vs 11.5%, \( P = .004 \); 16.1% vs 5.5%, \( P = .02 \); 19.4% vs 4.5%, \( P < .001 \), respectively) (Table 2).

### 3.4. Association of GDM with worst neonatal outcomes

Compared with the healthy control group, the GDM group showed higher frequencies of macrosomia (9.7% vs 4.0%, \( P = .04 \)), neonatal hypoglycemia (5.4% vs 1.0%, \( P = .02 \)), and neonatal referral rate (15.1% vs 6.5%, \( P = .008 \)) (Table 3). There were no differences between the 2 groups regarding fetal growth restriction and mild asphyxia (both \( P > .05 \)).

### 3.5. Abnormal vaginal flora is associated with adverse perinatal outcomes among healthy women

Among the 200 healthy pregnant women, 67 had an abnormal vaginal flora. Compared with the women with a normal flora, those with an abnormal flora showed higher frequencies of PROM (19.4% vs 7.5%, \( P = .02 \)) and chorioamnionitis or puerperal infection (11.9% vs 0.8%, \( P < .001 \)), while there was no difference regarding premature delivery (\( P = .19 \)) (Table 4).

### 3.6. Abnormal vaginal flora is associated with adverse perinatal outcomes among patients with GDM

Among the 186 pregnant women with GDM, 106 carried an abnormal vaginal flora. Compared with the patients with GDM and a normal flora, those with an abnormal flora showed higher frequencies of PROM (32.1% vs 10.0%, \( P < .001 \)), premature delivery (17.7% vs 6.3%, \( P = .04 \)), and chorioamnionitis or puerperal infection (32.8% vs 2.5%, \( P < .001 \)) (Table 5).

### 3.7. Changes in the composition of the vaginal flora during pregnancy

Among the 30 specimens from the GDM group and the 20 specimens from the control group and after primer removal, 1,909,133 clean tags were obtained from all samples (average of 38,182±454 tags/sample and average length of 378±1 base pairs). Clean tags were clustered into OTUs based on 97% similarity: 169 OTUs arose from the 30 samples. By blasting with databases, the taxonomy of OTUs was determined at the levels of phylum (Fig. 1), genus (Fig. 2), and species (Fig. 3).

In the GDM group, the most abundant phylum was *Firmicutes* and the second was *Proteobacteria* (Fig. 1). In the control group, the most abundant phylum was *Bacteroidetes* and the second was *Firmicutes* (Fig. 2). The composition of the vaginal flora was significantly different between the 2 groups (\( P < .001 \)).

### Table 1

Comparisons of the vaginal flora.

| Group     | Normal, % | VVC, % | BV, % | BVC, % | Suppression of normal flora, % | Changes in dominant bacterial strains, % |
|-----------|-----------|--------|-------|--------|-------------------------------|-----------------------------------------|
| GDM       | 80 (43.0) | 42 (22.6) | 19 (10.4) | 12 (6.0) | 5 (2.7) | 28 (15.1) |
| Normal    | 133 (66.5) | 18 (0.7) | 19 (0.5) | 10 (2.5) | 3 (1.5) | 17 (8.5) |
| \( P       | <.001     | <.001   | .865   | .662   | .490   | .056    |

BV = bacterial vaginosis, BVC = bacterial vaginosis plus vulvovaginal candidiasis, GDM = gestational diabetes mellitus, VVC = vulvovaginal candidiasis.

### Table 2

Comparisons of the maternal outcomes in the perinatal stage.

| Group     | PROM, % | Premature delivery, % | Chorioamnionitis or puerperal infection, % |
|-----------|---------|-----------------------|------------------------------------------|
| GDM       | 42 (22.6) | 23 (16.1) | 36 (19.4) |
| Normal    | 23 (11.5) | 11 (5.5)  | 9 (4.5)  |
| \( P       | .004     | <.001     | <.001    |

GDM = gestational diabetes mellitus, PROM = premature rupture of membranes.

### Table 3

Comparisons of the neonatal outcomes in the perinatal stage.

| Group     | Macrosomia, % | FGR, % | Mild asphyxia, % | Hypoglycemia, % | Neonatal referral rate, % |
|-----------|---------------|--------|------------------|-----------------|--------------------------|
| GDM       | 18 (0.7)      | 3 (1.6) | 3 (1.6)          | 10 (5.4)        | 28 (15.1)                |
| Normal    | 8 (4.0)       | 1 (0.5) | 1 (0.5)          | 2 (1.0)         | 13 (6.5)                 |
| \( P       | .040         | .306   | .306             | .017            | .008                     |

FGR = fetal growth restriction, GDM = gestational diabetes mellitus.

*Neonatal hypoglycemia detected within 72 h after birth. |

†Given that the neonatal ward was not set in the trial-launching hospital, the newborns would be immediately transferred to other hospitals upon the incidence of lung edema, pneumonia, severe jaundice, or neonatal malformation.

### Table 4

Association between the vaginal flora and perinatal outcomes in healthy pregnant women.

| Group     | PROM, % | Premature delivery, % | Amnionitis or puerperal infection, % |
|-----------|---------|-----------------------|-----------------------------------|
| Abnormal vaginal flora | 13 (19.4) | 6 (9.0) | 9 (11.9) |
| Normal vaginal flora | 10 (7.5) | 5 (3.8) | 1 (0.8) |
| \( P       | .018     | .186     | <.001    |

PROM = premature rupture of membranes.

### Table 5

Association between the vaginal flora and perinatal outcomes in pregnant women with GDM.

| Group     | PROM, % | Premature delivery, % | Amnionitis or puerperal infection, % |
|-----------|---------|-----------------------|-----------------------------------|
| Abnormal vaginal flora | 34 (32.1) | 18 (17.7) | 34 (32.8) |
| Normal vaginal flora | 8 (10.0) | 5 (6.3) | 2 (2.5) |
| \( P       | <.001   | .041     | <.001    |

GDM = gestational diabetes mellitus, PROM = premature rupture of membranes.
the most abundant phylum was also *Firmicutes* and the second was *Proteobacteria* (Fig. 1).

*Lactobacillus* was detected in most samples from both groups (Fig. 2). *Lactobacillus* was not detected in 1 patient with GDM during gestation, while *Atopobium* was the primary genus in this woman. In general, at the genus level, *Lactobacillus* remained the dominant genus during pregnancy. Over 96% of bacteria in vaginal flora were *Lactobacillus, Corynebacterium, Achromobacter, Strepacoccus, Ato...*. In the GDM group, 64 genera were detected in vaginal secretions at 28 to 30 weeks of pregnancy while 36 genera were detected at 37 to 40 weeks of pregnancy (Fig. 2). In the GDM group, 64 genera were detected in vaginal secretions at 28 to 30 weeks of pregnancy while 36 genera were detected at 37 to 40 weeks of pregnancy (Fig. 2). The GDM group harbored 26 genera that were absent in the control group, and the control group had 2 unique genera.

Taxonomic analysis at the species level was conducted (Fig. 3). In the control group, the most abundant bacteria in vaginal flora was *Lactobacillus crispatus*, accounting for 28.5%; the second ones were *Lactobacillus inersclone* and *Lactobacillus acidophilus*, respectively, accounting for 20.9% and 13.7%. In the GDM group, the sequential order of *Lactobacillus* abundance in vaginal flora was: *L acidophilus*, 28.3%; *L crispatus*, 15.1%; and *L...* Other *Lactobacillus* species were also detected in vaginal secretions from both groups, but with lower abundance. The ratio of *L jensenii* was higher in the GDM group. In the GDM group, the composition of the vaginal flora also
included *Lactobacillus listeri, Lactobacillus amylovorus,* and *Lactobacillus fructivorans,* which were absent in the vaginal secretions from healthy pregnant women. On the contrary, *Lactobacillus salivarius* was specific to healthy pregnant women.

4. Discussion

GDM is associated with adverse perinatal outcomes and with disturbances in the vaginal flora.\textsuperscript{25–30} The association between candidiasis and adverse pregnancy outcomes is well known,\textsuperscript{25–28} but less is known about the association between bacterial species and pregnancy outcomes.\textsuperscript{31} Therefore, the present study aimed to examine the association between GDM and abnormal vaginal flora, and the association between abnormal vaginal flora and adverse pregnancy outcomes. The results strongly suggest that the vaginal infection rate was higher in GDM compared with healthy pregnant women. GDM and abnormal vaginal flora were both associated with adverse pregnancy outcomes. Vaginal *Lactobacillus* species were different between the 2 groups, which could contribute to the adverse outcomes.

The vaginal microecological environment is complex, it is affected by a variety of factors, and it is subject to dynamic changes.\textsuperscript{177} In the present study, there was no significant difference regarding the frequency of BV between GDM patients and healthy pregnant women, but a significant difference was found regarding VVC. Pregnancy and DM are 2 independent causative factors for VVC.\textsuperscript{291} VVC is a type of vaginitis caused by the overgrowth of the conditional pathogenic microorganism *Candida,* and it is one of the most common types of vaginitis.\textsuperscript{177,20,22,23,38} *Candida* proliferates when the homeostasis of the vaginal microenvironment is disturbed, including changes in mucosal acidity and hormone levels. According to a previous study, the lifetime occurrence of at least 1 VVC event in healthy women of childbearing age is 75%, with recurrences in 50% of them.\textsuperscript{139} During pregnancy, elevated hormone levels and glycogen accumulation in the vagina lead to a rise in VVC frequency, about 2 folds compared with nonpregnant women.\textsuperscript{23,24,29–31,40} It is generally believed that pregnant women with GDM are predisposed to *Candida* colonization of the vagina.\textsuperscript{23,28} Elevated glycemia in the vaginal tissue increases fungus adhesion and growth, predisposing the vaginal epithelial cells to binding to *Candida albicans* cells.\textsuperscript{23,24} In addition, glycemia of 10 to 11 mmol/L could impair host defense mechanism, and hyperglycemia decreases neutrophil nonpurposeful migration and weakens their chemotactic and phagocytic powers, thereby elevating diabetic patient’s sensitivity to VVC.\textsuperscript{23,24}

Vaginal microecological abnormality is closely associated with adverse pregnancy (such as premature birth, PROM, and puerperal infection) and neonatal outcomes. VVC could cause retrograde infection, which gives rise to intrauterine infection, chorioamnionitis, and endometritis, causing PROM and abortion, premature birth, and intrauterine fetal death.\textsuperscript{41} Gestational VVC without treatment can lead to vaginal injury during delivery, puerperal infection, and poor wound healing after perineal cut and cesarean section.\textsuperscript{140} This is consistent with the present study since we also observed associations between abnormal vaginal flora and higher frequencies of PROM and chorioamnionitis or puerperal infection in healthy women, and with higher frequencies of PROM, premature delivery, and chorioamnionitis or puerperal infection in women with GDM.

Healthy women carry a variety of normal microorganisms in the vagina, of which *Lactobacillus* is the dominant bacteria, comprising 50 species, with a separation rate of 80% to 90%.\textsuperscript{139} The present study showed that *Lactobacillus* was the dominant vaginal bacteria in both GDM and healthy pregnant women. The present study also found that the most abundant species among *Lactobacillus* was *L. crispatus* in the vagina of healthy pregnant women, followed by *L. inersclone* and *L. acidophilus,* which is consistent with previous study results.\textsuperscript{42–45} On the contrary, in the vagina of pregnant
women with GDM, the descending order was L. acidophilus, L. crispatus, and L. inersclone.

In vitro experiments by Mirmonef et al.[46] showed that the free glycolgen content in vaginal secretions could affect pH values and colonization by various Lactobacillus species. Indeed, along with rising free glycolgen levels in vaginal secretions, pH values decline and the levels of L. crispatus and L. jenseni raise, while L. inersclone remains unchanged.[46] Nevertheless, the present study revealed that the ratio of L. crispatus in the vagina of pregnant women was 15.1% versus 28.5% in the GDM group versus healthy controls. Hence, L. crispatus levels showed a decline, not an elevation, with descending vaginal pH values. This discrepancy could be attributed to a number of reasons, including differences in vitro versus in vivo and population-specific differences.

Vaginal Lactobacillus produces hydrogen peroxide to regulate microecological homeostasis, thereby preventing infections from external pathogens.[47,48] Lamont et al.[49] reported that hydrogen peroxide was generated by 100% of L. crispatus, but only by 80% of L. acidophilus. The present study showed that even though the constitutional ratio of L. acidophilus rose in the vagina of GDM pregnant women, the ratio of L. crispatus declined, which could weaken the overall production of hydrogen peroxide, thereby favoring the growth of diverse conditional pathogens.

Compared with healthy pregnant women, the constitutional ratio of vaginal L. inersclone was smaller in GDM pregnant women. Mirmonef et al.[46] revealed that the detection rate of vaginal L. inersclone was 62.5% in healthy pregnant women, but only 8.3% in patients with gestational VVC. Thereby, L. inersclone was proposed as a potential biomarker for vaginal microecological changes.[50] This decrease of L. inersclone was also observed in GDM patients in the present study.

MacIntyre et al.[51] showed that there was no significant difference in vaginal pH values or the detection rate of Lactobacillus during pregnancy. In the present study, irrespective of GDM or healthy pregnant women, the abundance and diversity of vaginal flora showed no significant difference between 28 to 30 and 37 to 40 weeks of pregnancy, and Lactobacillus was the dominant bacteria at both stages.

The present study is not without limitations. The sample size was small and from only 1 hospital. Only a few parameters were examined and inflammation parameters were not assessed.

In conclusion, the infection rate was higher in GDM compared with healthy pregnant women. GDM and abnormal vaginal flora were both associated with adverse pregnancy outcomes. Vaginal Lactobacillus species were different between the 2 groups, which could contribute to the adverse outcomes. The results of this study provide clues about the pathogenesis of GDM and associated conditions, as well as about potential targets for prophylaxis and treatment.

Author contributions

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