7. Summary and Conclusion

The incidence rate of preterm delivery in Northeast India is alarming. Since as of date no scientific documentation on the underlying molecular etiology associated with preterm delivery and related complications as well as differences in pregnancy outcome in these cases is available from Northeast India, and given the lacunae in literature w.r.t to available global and national data on the ‘factors’ associated with the pathogenesis of preterm delivery; the study was therefore designed to elucidate the role of critical genetic, immunological and pathological risk factors associated with the susceptibility to preterm delivery and related complications including negative pregnancy outcome during pregnancy. The following are the findings of the present study:

**Association between Birth Weight and Negative pregnancy outcome:**

- The birth weight of a baby was significantly lower in all the preterm delivery case groups compared to the term delivery cases (p<0.001). The baby birth weight was also significantly differed amongst the preterm delivery sub-cohorts, the lowest being in extremely preterm delivery cases (0.925 ± 0.469) and the highest in moderately preterm delivery cases (1.94 ± 0.483).
- The lower birth weight was associated with negative pregnancy outcome (fetal death/IUD) in preterm delivery cases (p=0.071), significantly in extremely (p=0.042) and very preterm delivery cases (p=0.026).
Among multiple risk factors for preterm delivery one important factor is the genetic factor which is associated with the preterm delivery. Genetic risk factors w.r.t folate pathway and progesterone pathway, associated with the preterm delivery was studied and the data revealed the following:

- **Folate Pathway:**
  - The distribution of the variant *MTHFR* genotype was significantly higher in preterm delivery cases (29.18%) compared to term delivery cases (12.37%) (p<0.001). The results didn't show any deviation from Hardy–Weinberg equilibrium.
  - The presence of the variant *MTHFR* allele was significantly found to increase the risk of preterm delivery by more than two folds {OR=2.872, p< 0.001}.
  - The presence of *MTHFR* variant genotype was significantly associated with each sub-cohorts of preterm delivery viz., extremely (p<0.001), very (p=0.001) and moderately (p=0.002) preterm delivery cases; and resulted in significant increased risk of extremely {OR=5.903, p<0.001}, very {OR=3.420, p=0.002} and moderately preterm delivery {OR=2.449, p=0.002} compared to term delivery cases.
  - When all the term and preterm cases were considered, analysis showed that negative pregnancy outcome (IUD/fetal death) significantly correlated with *MTHFR* variant genotype (p < 0.001) and it also increased the risk of negative pregnancy by more than three folds significantly {OR= 3.421, p=0.001}. Also within the preterm delivery groups presence of *MTHFR* polymorphism (p=0.032) was found to be associated with the increased risk of negative pregnancy outcome by more than two folds significantly {OR= 2.263, p = 0.039}. 
Analysis in sub-cohort of preterm delivery separately showed that in the extremely preterm delivery cohort \textit{MTHFR} polymorphism significantly resulted in increased risk of negative outcome by eight folds (OR=8.000, p=0.043). But it non-significantly resulted in increased risk in very (OR= 1.257, p= 1.000) and moderately (OR = 1.092, p = 1.000) preterm groups.

Presence of \textit{MTHFR} variant genotype was significantly associated with low birth weight in all the pregnancy cases (p=0.001). Further wilcoxon based analysis in term and all the preterm sub-cohorts showed that \textit{MTHFR} variant genotype was correlated with low baby birth weight in extremely (p=0.006) preterm delivery cases and term delivery cases (p<0.001).

The distribution of \textit{TYMS 6 bp del} polymorphism variant was comparable between the preterm delivery (91.38\%) and term delivery cases (90.72\%) and was non significantly found to increase the risk of preterm pregnancy (OR=1.023 (0.520-2.012, p=1.000]).

The distribution of variant \textit{TYMS} genotype was found to increase the risk in case of moderately preterm group (1.780, p=0.354) compare to term delivery cases but decrease the risk of preterm delivery in extremely (OR=0.273, p=0.022) and very preterm cases (0.447, p=0.102) compared to term delivery cases.

Due to predominance of \textit{TYMS 6 bp ins/del} genotype, distribution of homozygous 6 bp del/del condition was studied compared to presence of either 6 bp ins/ins or 6 bp ins/del and it was found that 6 bp del/del genotype was associated with increased risk of preterm delivery compared to term delivery [OR=2.801, p=0.002] also risk was increased in extremely [OR=3.094, p=0.079], very [OR=2.707, p=0.063] and moderately preterm delivery [OR=2.647, p=0.007] compared to term delivery; thereby
underlying the protective role of the 6 bp ins in preterm delivery compared to 6 bp del genotype.

- The presence of TYMS 6 bp del variant genotype was found to be significantly associated with negative pregnancy outcome in preterm delivery cases (p<0.001) compared to term delivery cases.
- When analysis was performed in all the preterm sub-cohorts separately the presence of TYMS 6 bp del variant genotype was associated with the reduce risk of negative pregnancy outcome in extremely {OR=0.750, p=1.000}, very {OR=0.395, p=0.353} and moderately {OR=0.104, p=0.047} preterm delivery cases.
- The presence of heterozygous 6 bp ins/del and homozygous 6 bp del/del genotype was associated with lower baby birth weight compared to 6 bp ins/ins allele in both term and preterm delivery groups.
- Within the preterm delivery groups the presence of TYMS 6 bp del variant genotype was significantly associated with low birth weight in very (p=0.024) and moderately (p=0.045) preterm delivery cases.
- MTHFR and TYMS combined variant genotype was significantly (p=0.042) associated with the low baby weight.
- The ELISA based data for serum levels of homocysteine was significantly higher in preterm delivery cases (16.169±4.368 umol/L) compared to the term delivery cases (5.7± 0.899 µmol/L) (p<0.001). Further the results of IHC also demonstrated the upregulation in protein level expression of homocysteine in preterm placenta compared to term placenta.
- When all the term and preterm cases were considered, the higher homocysteine levels were significantly associated with fetal death in preterm delivery cases (p=0.013).
Further analysis within the preterm groups also showed the association of higher homocysteine levels with the negative pregnancy outcome (p= 0.090).

- When all the term and preterm delivery pregnancy cases were considered it was found that the presence MTHFR polymorphism was associated with higher homocysteine levels (p= 0.065) in preterm delivery cases showing higher levels of homocysteine (µmol/l) in variant type (14.34±2.73) compared to wild type (12.67±4.26).

- On considering all enrolled pregnancy cases, the presence of TYMS 6 bp del/del genotype was significantly associated with higher homocysteine levels (p=0.008) 6B). Within the preterm delivery group also TYMS 6 bp del/del genotype polymorphism was associated with higher homocysteine levels compared to ins/ins (p=0.005) and ins/del (p=0.062) genotypes.

- The expression of FR-α was downregulated in preterm delivery cases (0.322±0.263 folds) compared to term delivery cases at mRNA level in the placenta tissue. Further the data for FR-α expression in blood was also found to be downregulated in preterm delivery cases (0.898±0.551 folds) compared to term.

- In between the sub cohorts of preterm delivery cases the expression of FR-α was found to be downregulated in very preterm delivery compared to extremely (fold=0.364, p=0.263) and moderately (fold= 0.236 p=0.284) preterm delivery cases and in extremely preterm compared to moderately preterm delivery cases (fold = 0.648, p= 0.564).

- The protein level expression of FR-α was found to be downregulated in the placenta of preterm delivery cases compared to term delivery cases.
When all the term and preterm cases were considered, analysis showed that negative pregnancy outcome (IUD/fetal death) significantly correlated with the downregulation in FR-\(\alpha\) expression (\(p=0.010\)).

The expression of FR-\(\alpha\) and the low birth weight of a baby was directly correlated with each other significantly \(\{\text{Pearson correlation} = 0.428, p = 0.001; \text{Spearman’s rho} = 0.311, p = 0.0.020\}\).

**Progesterone Pathway:**

- Study of \(PR\) mutation analysis showed that the distribution of \(PR\) mutation was higher in preterm delivery cases (5.26\%) compared to term delivery cases (2.06\%) (\(p=0.088\)).
- The presence of \(PR\) mutation increased the risk of preterm delivery cases by two times non significantly \(\{\text{OR}=2.652, p=0.115\}\).
- The presence of \(PR\) mutation was not associated significantly with the occurrence of extremely (\(p=0.058\)), very (\(p=0.329\)) or moderately preterm delivery (\(p=0.152\)). But it was found that the \(PR\) mutation increased the risk of extremely \(\{\text{OR}=4.750, p=0.116\}\), very \(\{\text{OR}=2.317, p=0.299\}\) and moderately preterm delivery \(\{\text{OR}=2.427, p=0.215\}\).
- When all the term and preterm cases were considered, data showed that negative pregnancy outcome (IUD/fetal death) significantly correlated with the presence of \(PR\) mutation (\(p=0.005\)) and it increased the risk of negative pregnancy non significantly by 1.7 folds \(\{\text{OR} = 1.707, p = 0.367\}\). But within the preterm delivery groups presence of \(PR\) mutation was not found to be associated with negative pregnancy outcome (\(p=0.866\)).
• Analysis in sub-cohort of preterm delivery separately showed that in the extremely (p=0.456) and very (p=0.401) preterm delivery cohort PR mutation did not resulted in increased risk of negative pregnancy outcome. Meanwhile PR mutation was found to be significantly associated with negative pregnancy outcome in the moderately preterm group (p= 0.033) and it also resulted in increased risk of negative pregnancy outcome in this group \{OR= 5.689, p= 0.090\}.

• Presence of PR mutation was significantly associated with low birth weight in all the pregnancy cases (p=0.035). Further wilcoxon based analysis in term and all the preterm sub-cohorts showed that PR mutation was correlated with low baby birth weight in very (p<0.001) and moderately (p<0.001) preterm delivery cases.

• The expression based study at mRNA level in placenta showed that the expression of PR was downregulated in preterm delivery cases compared to term delivery by 0.568 ± 0.45folds.

• In between the sub-cohorts of preterm delivery cases the expression of PR was found to be downregulated in extremely preterm delivery cases compared to both moderately \{0.554folds, p=0.042\} and very preterm delivery cases \{0.418fold, p=0.317\}; and in moderately preterm cases compared to very preterm cases \{0.819folds, p=0.035\}.

• The protein level expression of PR was found to be downregulated in the placenta of preterm delivery cases compared to term delivery cases.

• When all the term and preterm cases were considered, analysis showed that negative pregnancy outcome significantly correlated with the downregulation in the expression of PR (p=0.052).

• The expression of PIBF at mRNA level was downregulated in preterm placental tissue compared to term by 0.345±0.29 folds.
• In between the sub cohorts of preterm delivery cases the expression of PIBF was found to be downregulated in extremely preterm delivery {0.702 fold, p=0.505} and moderately preterm delivery {0.422 folds, p= 0.018} compared to very preterm cases; as well as in moderately preterm delivery groups {0.710 folds, p=0.497} compared to extremely preterm cases.

• The PIBF mRNA expression at blood level was also found to be downregulated in preterm cases (0.217± 0.12 folds) compared to term.

• The down-regulation in PIBF mRNA expression was significantly associated with negative pregnancy outcome in preterm cases (p=0.024).

• The positive correlation between PR and PIBF placental expression (Pearson correlation = 0.301, p = 0.225 and spearman’s rho = 0.655, p = 0.003) was observed when all the preterm cases were considered indicating that the expression of PIBF is dependent on PR .The positive correlation was also observed in all the sub-cohort of preterm delivery cases.

• Considering the association of deleterious immune response of the mother toward the fetus in the occurrence of preterm delivery we sought to study the role of differential immuno-modulatory and Th1/Th2 profile in predisposition to preterm delivery and outcome. The data obtained revealed the following:

• Whole blood based analysis of NK cells profile showed that the NK-cells activation was predominant in preterm delivery (24.97±9.77%) compared to term delivery (17.93±6.398%) (p=0.448).
Whole blood based analysis of NKT like cells profile showed that the activation of NKT like cells was predominant in preterm delivery (32.40±19.62) compared to term delivery (22.54±4.43%).

Serum level of proinflammatory cytokine TNF-α was found to be higher in preterm delivery cases (19.76±2.03 pg/ml) compared to term delivery cases (16.4±3.99 pg/ml) (p=0.195) and non-pregnant control cases (17.13±2.34 pg/ml) (p=0.115).

Serum levels of proinflammatory cytokine, IL-12 was higher in preterm delivery cases (3.61±1.62 pg/ml) compared to term delivery cases (2.71±0.53 pg/ml) (p=0.213) and non pregnant control cases (3.01±0.76) (p= 0.436 ).

The differential serum level expression of IFNγ was also higher in in preterm delivery cases (24.14±2.20 pg/ml) compared to term delivery (20.12±1.93 pg/ml) (p=0.020) and non pregnant control cases (23.52±3.76 pg/ml) (p= 0.796).

Serum level expression of anti-inflammatory cytokine IL-10 was downregulated in preterm delivery cases (12.26±2.38pg/ml) compared to term delivery cases (21.63±4.65pg/ml) (p=0.020) and non-pregnant control cases (20.65±5.17 pg/ml) (p=0.019).

The TNF-α by IL-10 ratio was found to be higher in preterm groups (1.67±0.38 folds) compared to term delivery (0.76±0.054 folds) (p=0.020) and non-pregnant controls (0.94±0.46 folds) (p=0.071)

The IL-12 by IL-10 ratio was found to be higher in preterm groups (0.28±0.06 folds) compared to term delivery (0.14±0.02 folds) (p=0.206) and non-pregnant controls (0.16±0.09 folds) (p=0.319).
• The IFN\(\gamma\) by IL-10 ratio was found to be higher in preterm groups (2.00±0.28 folds) compared to term delivery (0.88±0.12 folds) (p=0.046) and non pregnant control (1.36±0.79 folds) (p= 0.317).

• In preterm delivery cases it was observed that the higher TNF-\(\alpha\) levels (p= 0.517), IL-12 levels (p=0.784), IFN\(\gamma\) levels (p=0.038) and lower IL-10 levels (p=0.364) was associated with the negative pregnancy outcome.

• On studying the association of elevated Th1/Th2 cytokine with negative pregnancy outcome in preterm delivery cases it was found that the higher TNF-\(\alpha\) by IL-10 ratio (p=0.302); IL-12 by IL-10 ratio (p=0.518) and IFN\(\gamma\) by IL-10 ratio (p=0.513) was associated with negative pregnancy outcome.

• The mRNA based expression in preterm placental tissue showed increased fold change in proinflammatory cytokines (TNF-\(\alpha\)= 1.96±1.51 fold, IL-12= 1.16 ± 0.712 fold) and decreased fold change in anti-inflammatory cytokine (IL-10= 0.612 ± 0.398 fold) compared to term placenta.

• Based on IHC the protein level expression of TNF-\(\alpha\) was found to be significantly upregulated in preterm placental tissue (p=0.044) compared to term placental tissue.

• When all the term and preterm cases were considered the data analysis showed that the higher expression of TNF-\(\alpha\) (p=0.109) and IL-12 (p=0.582) and the lower expression of IL-10 (p=0.395) at mRNA level in placental tissue was associated with the negative pregnancy outcome in preterm delivery.

• Differential mRNA expression base study showed sharp upregulation of NF-\(\kappa\)\(\beta\)p65 expression in the placenta of preterm delivery cases with a fold change of 53.21±10.82, compared to term delivery cases.
• The mRNA level expression of NF-κβp65 was upregulated in extremely preterm delivery compared to very (p= 0.317) and moderately preterm delivery (p= 0.064), and also in very preterm cases compared to moderately preterm delivery groups (p=0.203).
• The differential expression of NF-κβp65 in placental tissue at protein level showed higher expression of total NF-κβp65 in preterm delivery cases (0.35±0.14 μg/ml) compare to term delivery cases (0.285±0.19 μg/ml) (p=0.439).
• Expression for total NF-κβp65 protein was also analysed between three stratified cohort of preterm delivery cases and the data showed that there was upregulation in the expression of NF-κβp65 in extremely preterm delivery (0.42±0.035 μg/ml, p=0.121) and very preterm delivery (0.41±0.16 μg/ml, p=0.439) compared to moderately preterm delivery group.
• The increased expression of NF-κβp65 was found to be significantly associated with the negative pregnancy outcome in preterm delivery cases. (p = 0.037).

Correlation analysis for expression of key markers in preterm delivery reveals the following findings:
• The expression of PR was negatively correlated with the proinflammatory cytokine levels in the placental tissue.
• The correlation analysis of PIBF with proinflammatory cytokine, anti-inflammatory cytokine and NK, NKT like cells showed a negative or inverse correlation between PIBF expression and proinflammatory cytokine as well as NK, NKT-like cells whereas it showed positive correlation with anti-inflammatory cytokine i.e. IL-10.
To study the pathological aspect associated preterm delivery we attempt to the evaluate the association and correlation of altered folate and progesterone pathways, and immune status in deciding the fate of delivery and outcome in Hepatitis E virus infected pregnancy cases. Our findings reveals the following:

- In HEV infected pregnancy cases the fetal death (p=0.009) and maternal death (p=0.012) were significantly higher in FHF compared to AVH and higher incidence of preterm delivery was observed in FHF (69.23%) compared to AVH (56%).
- The results of genotyping analysis showed the presence of only HEV genotype 1 and the viral load quantification data showed viral load was significantly higher in FHF cases compared to AVH. Increased in viral was significantly associated with fetal death in both FHF (p=0.006) and AVH (p=0.011) indicating the increased severity in pregnancy complications with the increase viral load.
- **MTHFR** genotype was significantly higher in HEV related preterm delivery cases compared to healthy term delivery case (p<0.001) and increased the risk of HEV related preterm delivery compared to healthy term cases [OR=9.167, p<0.001].
- The presence of variant **MTHFR** genotype was associated with negative pregnancy outcome in AVH [OR=1.333,p=0.810] and FHF [OR=5.00,p=0.102] cases within their respective groups.
- The presence of **TYMS 1494del6** polymorphism was found to be associated with increased risk of HEV infected preterm delivery compared to healthy term delivery cases [OR=1.636] and preterm delivery in both AVH-E [OR=2.00] and FHF-E [OR=5.00] infected preterm delivery cases compared to the term delivery cases of their respective categories.
• The presence of variant TYMS 1494del6 del/del genotype was associated with increased risk of negative pregnancy outcome in AVH [OR=1.125, p=0.456] cases within their respective groups.

• Homocysteine levels were significantly increased in AVH (p=0.023) and FHF (p<0.001) compared to healthy pregnancy cases. Also within AVH and FHF group the higher homocysteine levels was significantly associated with preterm delivery and fetal death (p<0.001).

• The expression of FR-α was downregulated in AVH (0.1077±0.0676 folds) and FHF (0.0481±0.0108 folds) preterm delivery cases compared to term delivery cases. FR-α mRNA was significantly down-regulated in FHF-E cases compared to both AVH-E (p=0.015) and preterm delivery cases, and in AVH-E cases compared to preterm delivery cases.

• Downregulation in the expression of FR-α mRNA expression was found to be associated with negative outcome in both AVH and FHF preterm delivery group (p<0.001).

• PR mutation was significantly higher in HEV related preterm delivery cases (11.76%) compared to healthy term delivery case (2.06%) (p=0.021), and resulted in increased risk of HEV related preterm delivery compared to term cases [OR=6.33, p=0.076] but no significant association was observed between the groups.

• The PR (p=0.005) and PIBF mRNA expression (p=0.007) was significantly down-regulated in HEV preterm cases compared to HEV term pregnancy cases. The PR (p=0.440) and PIBF mRNA expression (p=0.064) was significantly down-regulated in HEV cases with negative pregnancy outcome.
- The PR and PIBF mRNA expression correlated positively significantly (Pearson’s correlation = 0.628, p=0.021; Spearman’s rho=0.666, p=0.013) in HEV infected pregnancy cases.
- Downregulation of PR (p=0.009) and PIBF (p=0.028) was significantly associated with preterm delivery in AVH-E cases compared to AVH-E infected term delivery cases and was also associated with negative pregnancy outcome in AVH-E infected term and preterm delivery cases.
- NK cells count in blood was significantly higher in HEV related pregnancy cases (29.36±12.34%) compared to non-HEV infected term delivery cases (17.93 ± 6.398%) (p=0.037) whereas NKT like cell levels were comparative between HEV infected pregnancy cases and non HEV infected term delivery cases.
- In case of HEV infected pregnancy cases compared to non infected pregnant cases, the serum levels of Th1 type cytokines TNF-α (p<0.001) and IFNγ (p=0.034) was significantly upregulated whereas the Th2 type cytokine i.e. IL-10 (p<0.001) was significantly downregulated.
- On comparing Th1/Th2 type cytokine it was found that TNF-α/IL-10 (p=0.016) and IFNγ/IL-10 (p=0.098) ratio in HEV infected cases was higher than the non-infected term delivery cases.
- The levels of TNF-α was also found to be inversely correlated with IL-10 levels significantly (Pearson’s correlation= -0.598, p=0.046; Spearman’s rho= -0.674, p=0.031).
- Serum level TNF-α was significantly higher in FHF cases (48.77±7.82pg/ml) compared to AVH (31.09±11.73 pg/ml) pregnancy cases (p<0.001). Further the TNF-α
levels was higher in AVH preterm cases (34.54±5.66 pg/ml) compared to AVH term cases (29.84±8.38 pg/ml) (p=0.292).

- TNF-α expression at mRNA level in the placental tissue was elevated in HEV infected pregnancy cases when compared with non-infected term delivery (3.64±1.96 folds), as well as significantly upregulated in FHF cases compared to AVH cases (p=0.016).

- Proinflammatory NF-κβp65 in placental tissue at mRNA level was upregulated in HEV infected pregnancy cases (4.58±2.09 folds) compared to healthy term delivery cases.

- NF-κβp65 expression was upregulated in AVH preterm delivery cases compared to AVH term delivery cases (p=0.302), and was also associated with negative pregnancy outcome in AVH–E pregnancy cases (p=0.417).

- Placental PR mRNA expression inversely correlated with NF-κβp65 expression, and placental TNFα expression positively correlated with NF-κβp65 expression, thereby underlying the importance of downregulation of PR in preterm delivery cases.

**Conclusions:**

The present study strongly highlights the association of deregulated folate and progesterone pathway along with the deleterious immune responses in non-pathological and HEV infected pregnancy resulting in predisposition to preterm delivery and related complications, and negative pregnancy outcome. Based on the study results under different objectives it may be stated that:
Genetic alteration in folate pathway genes i.e. *MTHFR, TYMS*, resulting hyperhomocystenemia and deregulation in FR-α expression plays a critical role in preterm delivery and outcome as well low baby birth weight.

Genetic alteration in *PR* gene along with alteration in expression profile of PR and PIBF gene involved in progesterone pathway are critically associated with preterm delivery and related complications.

A significant Th1-biased immunological status with increased NK/NKT-like cells activity and increased NF-κβ expression is critically associated with preterm delivery and outcome.

The Th1 biased immunomodulation in preterm delivery is associated with upstream deregulation in PR pathway.

Alterations in folate pathway, progesterone pathway and associated Th1 biased altered immunomodulation status is found to be correlated with the HEV related pregnancy complications and outcome.

Finally to conclude, through this novel study it has been possible to identify a panel of genetic, biochemical and immunological factors viz., *MTFHR C677T* polymorphism, *TYMS1494 del6* polymorphism, homocysteine levels, FR-α expression, PR and PIBF expression, TNF-α and IL10 levels which may both suitably serve as biomarkers to be used for stratifying pregnancy cases with risk of preterm delivery irrespective of underlying pathology, as well as serve as therapeutic targets for controlling preterm delivery and associated complications; and thereby enhancing the possibilities of controlling the fetal morbidity and mortality associated with preterm delivery.