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Placental Leucine Aminopeptidase/Oxytocinase Expression in Miscarriage

Taghreed H. AL Sadoon[1*], Ferah Gh. Al-Salihi[2] and Adnan I. Al-Badran[3***]**

[1]Department of Biochemistry, College of Medicine, University of Tikrit, Iraq.
[2]Department of Chemistry, College of Sciences, University of Tikrit, Iraq.
[3]Department of Biology, Cell and Biotechnology Research Unit, College of Sciences, University of Basrah, Iraq.

Authors' contributions

This work is carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Objectives: To evaluate the role of placental leucine aminopeptidase (P-LAP) in miscarriage and searching to select the gene of this enzyme in miscarriage to exclude direct or indirect effects of abortion.
Methods: Total RNA is purified from the fresh placental tissue sample according to the protocol of the QIAamp®RNA Tissues Mini Kit (Qiagen). The sample is visualized by 0.7% agrose gel containing. Total RNA is reverse-transcribed RT-PCR carried out on the previously extracted RNA samples using (QIAGEN One Step RT –PCR Kit). Aliquots (1ml) of RT reaction samples are amplified by PCR, the PCR products (10µl) per lane are resolved by using electrophoresis on 2.0% agrose gel .Later the product is detected and examined under UV transiluminator.
Results: The obtained results indicate that there is an expression of P-LAP mRNAs in placentas of normal pregnancy women (28±SE0.45) and there is a decrease in miscarriage. Furthermore, there is a difference between single miscarriage and those with recurrent miscarriage, the mean values in single miscarriage (1st and 2nd) are (16.1%±SE0.84) and (13%±SE0.24) respectively while the mean values in recurrent miscarriage (1st and 2nd) are (12%±SE0.19) and (9%±SE0.52) respectively. The expression of P-LAP is significantly lower (p=0.05) in miscarriage and highly significant decrease in recurrent than in single miscarriage women.

**Corresponding author: Email: doctor88884@gmail.com;*

Conclusion: The decrease of P-LAP mRNA levels in miscarriage placentas could be a sign that p-LAP function is an important step in pregnancy regulation.

Keywords: Placental leucine aminopeptidase (P-LAP); complementary deoxyribonucleic acid (cDNA); β-actin as the internal control; multiplex-single step-(M-SS)-RT-PCR assay; electrophoresis.

1. INTRODUCTION

The enzyme Leucine aminopeptidase (LAP) is a glycoprotein that exists as an oligomer. It is presumably a dimer, and belongs to the family of zinc metallopeptidases. Previous work suggests that the enzyme is first synthesized as a type II integral membrane protein and then it is secreted into blood. Moreover, LAP degrades several peptide hormones such as oxytocin and vasopressin which increase during pregnancy and may have a significant effect on the uterine tonus, uteroplacental blood flow and trophoblast invasion during placental development. The enzyme may regulate the serum level of the hormones and thus maintain homeostasis during pregnancy [1].

The expression of messenger RNA (mRNA) for P-LAP is predominantly noted in the cytoplasm of cytotrophoblastic cells and a little in the cytoplasm of syncytiotrophoblastic cells [2]. On the other hand, a possible site of the production of pregnancy serum oxytocinase (P-LAP) is shown to be the lysosomes of the placenta [3] . The protease enzyme plays an important role in metabolizing vasoactive and immunomodulating peptides and its possibly derived from the fetus, so it controls the exchange of peptide hormones across the placenta in order to maintain feto-placental homeostasis [4].

Oxytocinase (EC3.4.11.3) which acts on oxytocin (OT) and vasopressin (AVP) is identical to placental leucine aminopeptidase, and increases in pregnant serum [5].The release of P-LAP into the maternal circulation through the changes in permeability of lysosomal membrane is controlled by steroid hormones in view of the fact that progesterone hormone act to destabilize the membrane of lysosome while cortisol acts to stabilize it and regulate its levels in pregnancy serum [6]. In relation to the role of oxytocin-oxytocinase system in forwarding the onset of labour, the changes in P-LAP (oxytocinase) activities during late pregnancy are suggestive of the useful role for predicting the onset of labour which is involved in both preterm labour and preeclampsia via degrading fetoplacental peptides [5]. As a result, further understanding of the pathophysiology of pregnancy and pathogenesis of trophoblastic diseases is useful. So this work aims to study the role of placental leucine aminopeptidase (P-LAP) as a promising agent to treat preeclampsia and preterm delivery (miscarriage) which are important complications in pregnancy and may lead to maternal and perinatal morbidity and mortality.

2. MATERIALS AND METHOD

The purpose of this study is to confirm the presence of LAP in human placentas which will be helpful to elucidate the physiological and clinical roles of P-LAP during miscarriage. So we evaluated 40 miscarrige cases; 10 (25)% 1st trimester single miscarriages ,7 (17.5)% 2nd trimester single miscarriages ,12 (30)% 1st trimester recurrent miscarriages and 11(27.5)% in 2nd trimester recurrent miscarriages**.**

2.1 Total RNA Purification from Placental

Total RNA is purified from fresh placental tissue samples according to the protocol of the QIAamp®RNA Tissues Mini Kit (Qiagen).Then the sample is visualized by 0.7% agarose gel which contains ethidium bromide. The concentration of the RNA in µg/ml is calculated by spectrophotometer using the guide that 1ml of a solution have an absorbance at 260nm which is equivalent to 40µg of single strand RNA. Total RNA in the previously extracted RNA samples is reverse-transcribed using (QIAGEN One Step RT-PCR Kit) in a total volume of 50µl. According to the manufacturer's instructions. Ten µl of a template RNA is added to the individual PCR tubes. The PCR is optimized for the amplification of the cDNA coding sequence under the following conditions:94°C for 1min, 60°C for 1min and 72°C for 1min; for the P-LAP using the following primers: PLAPsense: (5'-GGGCACAGATCAGGCTTCCCACT-3'); P-LAP anti-sense:(5'-GATCTCAGCTTGTTTTTCTTGGCTTG-3'). RT-PCR for β-actin as the internal control using sense(5'-AACCGCGAGAAGATGACCCAG-3') and anti-sense (5'-CTCCTGCTTGCTGATCCACAT-3'; have been performed as well under the same PCR conditions. 30 PCR cycles were established in preliminary experiments within the exponential phase of the amplification. The PCR products (10µl) per lane are resolved by electrophoresis in 2.0% agarose gels. Then the product is detected and examined under the UV transilluminator.

3. RESULT

The total RNA is isolated from placenta tissues and its concentration has been determined to be 38.5µg/ml .The quality and purity of the extracted RNA were checked by using agarose gel electrophoresis ,which shows the bands of 28SrRNA and 18SrRNA as shown in Fig.1. The agarose gel electrophoresis clearly illustrate that RNA does not undergo any degradation during extraction [7,8].

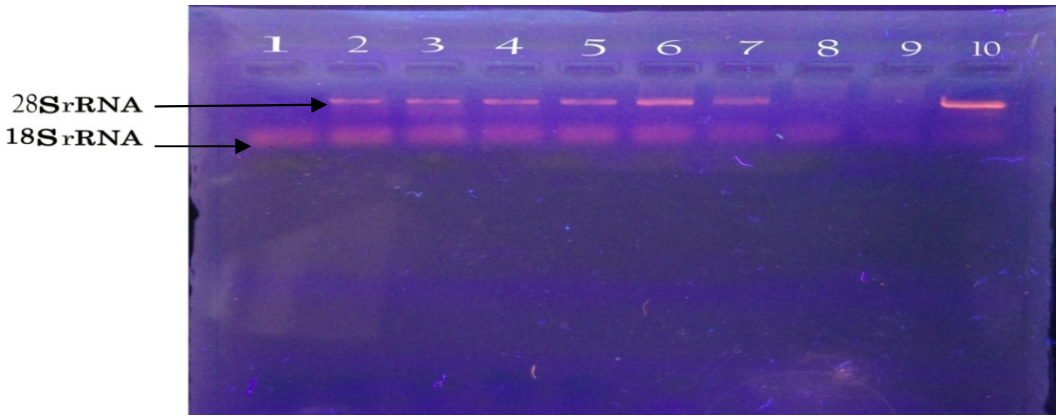

Fig. 1. Agarose Gel Electrophoresis of placental tissue, in which total RNA Bands Show the Discrete 28S &18S ribosomal RNAs. All Lanes represent positive Samples (presence of RNA), Lane 1,8,9 abnormal m-RNA from miscarriage

Amplified product 621 bp is detected by using RT-PCR for β-actin. The size of Lanes (SM_1,$SM_2$and SM_3) are 100-bp transcript which corresponds to that of cDNA of P-LAP in

single miscarriage (1st trimester) and absences of the band at SM_4 as shown in Fig. 2. Electrophoresis is carried in 2% agarose gel at (4V/cm) for 60 minutes.

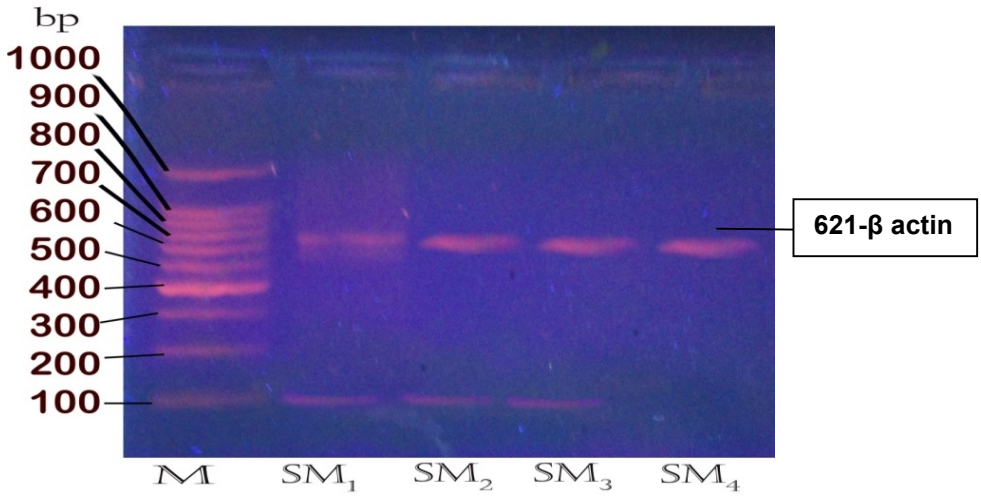

Fig. 2. Expression of Leucine aminopeptidase (LAP) $_c$DNA in placentas of 1st trimester ((SM) represents single miscarriages, (bp) represents Base pairs)

In the present study, the same primer nucleotide fragment which was previously used by Ikoma et al. [9] has been used to amplify the P-LAP cDNA and it is detected in placentas of normal Pregnancy NP with a size of transcript corresponding to that cDNA (175bp).

Expression of P-LAP-mRNA is significantly higher in the placentas of women with NP compared with miscarriage. These results are in agreement with those reported by Zhang et al. [10] who suggest that the P-LAP gene play an important role in trophoblast invasion during placental development. Amplified products of P-LAP patients show a missing band derived from SM4. The RT-PCR reaction is repeated for those patients using Monoplex-Two steps-RT-PCR for amplifying the internal control and fusion gene separately (data not show). In this case, the band that refers to normal β-actin is seen in all those samples indicating that there is no failure in P-LAP expression and there is no probability of primer dimmer formation between primers' mixture used in the M-SSRT-PCR reaction.

Amplified product 621 bp was detected as well by using RT-PCR for β-actin and lanes (SM_5,SM_6,SM_7 and SM_8) with size of 100-bp transcript corresponds to that of cDNA of LAP in 2nd trimester single miscarriage. Electrophoresis is carried out in 2% agarose gel at (4V/cm) for 60 minutes. The results of RT-PCR for P-LAP in patient`s samples of 2nd trimester single miscarriage revealed that they are positive when detected in different intervals from starting gestation to labor as shown in Fig. 3.

The assay was considered satisfactory for interpretation when the internal control (β-actin) present was positive control. Expression of P-LAP was significantly lower($p<0.05$) in 2nd trimester SM than that in 1st trimester These results are in agreement with those reported by Zhang et al. [10].

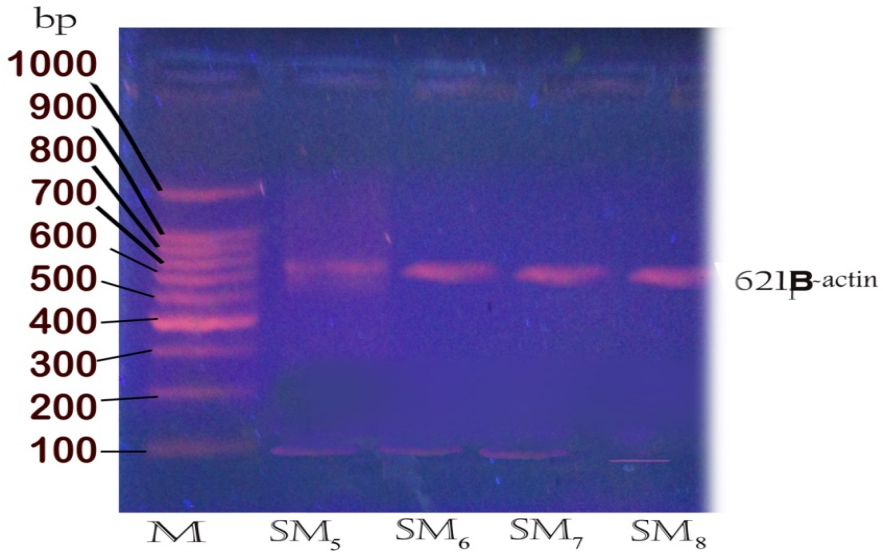

Fig. 3. Expression of Leucine aminopeptidase (LAP) $_c$DNA in placentas of 2nd trimester ((SM) represents single miscarriages, (bp) represents Base pairs)

Amplified product 621 bp was also detected by using RT-PCR for β-actin and Lanes (RM_1,R M_2,RM_3 ,RM_4): The size of 100-bp transcript corresponded to that of cDNA of P-LAP in 1st trimester recurrent miscarriage as shown in Figs. 4 and 5. Electrophoresis was carried in 2% agarose gel at (4V/cm) for 60 minutes.

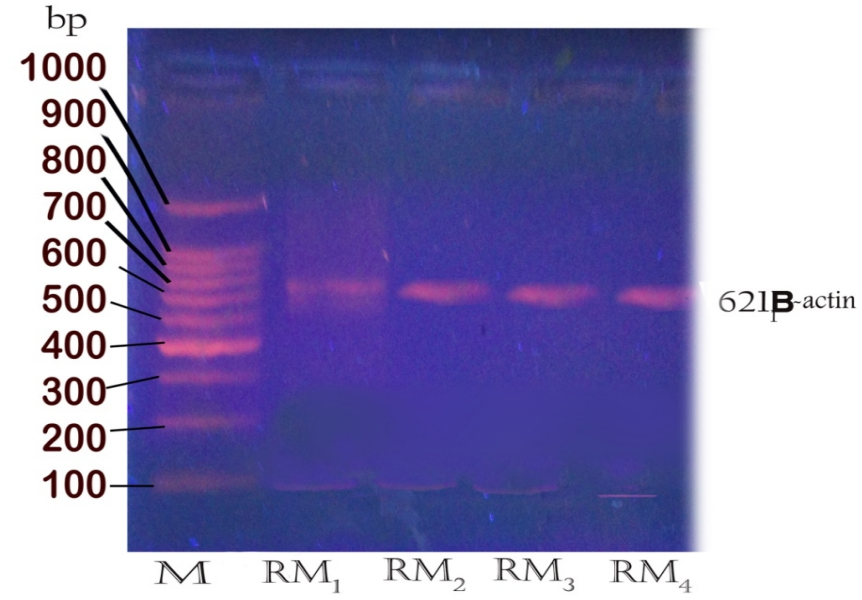

Fig. 4. Expression of Leucine aminopeptidase (LAP) $_c$DNA in placentas of 1st trimester recurrent miscarriages (RM). Base pairs (bp)

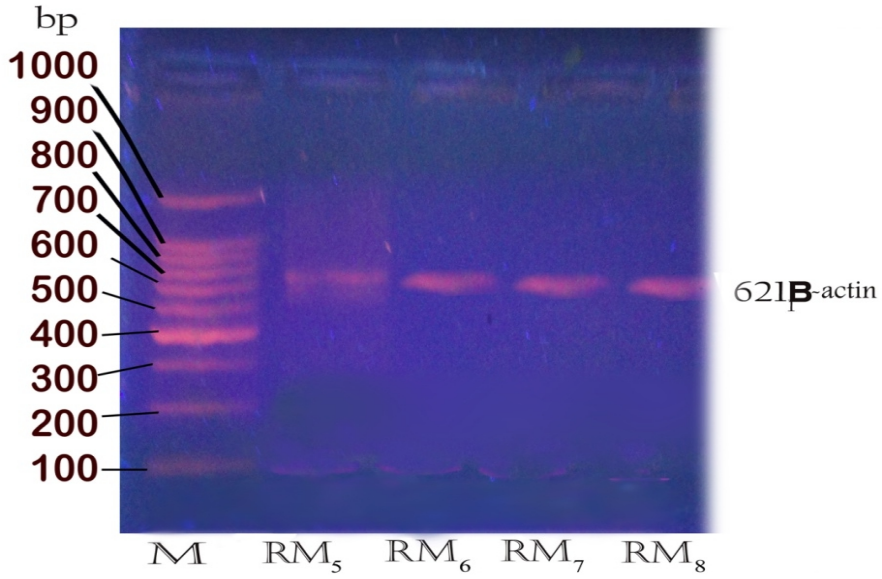

Fig. 5. Expression of Leucine aminopeptidase (LAP) $_c$DNA in placentas of 2nd trimester recurrent miscarriages (RM). Base pairs (bp)

Table 1. The ratio between transcripts of (P-LAP/ β-actine×100) of women with single and recurrent miscarriages

Factors	n (%)	Mean ±SE
Single miscarriage		
First –Trimester SM	10(25) (%)	16.1%±0.84
Second-Trimester SM	7(17.5) (%)	13.0%±0.24
Recurrent miscarriage		
First –Trimester RM	12(30) (%)	12.0%±0.19
Second-Trimester RM	11(27.5)(%)	9.0% ±0.52

Insufficient expression of (LAP) in placenta is demonstrated in women with RM , the results revealed that the expression of (P-LAP) is gradually declined in miscarriage, these results are in agreement with those reported by Qiao & Lin [11] .

In this study, the results of RT-PCR reaction are recorded as a ratio between transcripts of (P-LAP/ β-actine×100) as shown in Table 1.The mean values of ratio in single miscarriage at (1st and 2nd trimester) were (16.1%±SE0.84) and (13%±SE0.24) respectively. While in the case of recurrent miscarriage at (1st and 2nd) the ratio percent were (12%±SE0.19) and (9%±SE0.52) respectively. The expression of P-LAP is significantly lower (p=0.05) in recurrent miscarriage than in single miscarriage women. Expression of LAP in placentas of NP women (28±SE0.45) is highly significant in comparison with SM and RM women as illustrated in Fig. 6.

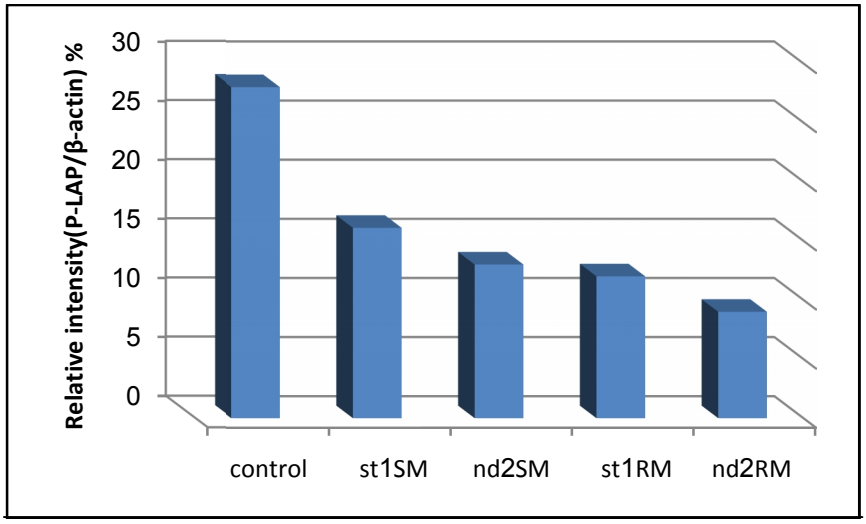

Fig. 6. Semiquantitative analysis of LAP m-RNA expression in the placenta of normal pregnancy (NP) and in Single Miscarriage(SM) and Recurrent Miscarriage (RM). The relative density of each gene product was compared between the two examined groups. *p<0.05

4. DISCUSSION

The results illustrated in Fig. 1 indicate that the two clear bands which represent ribosomal RNA (rRNA) subunits (18s and 28s) are present in the total RNA samples and they are clearly visible, which is in agreement with the results of Schroeder et al. (2006) [7], who suggest that the assessment of RNA integrity is a critical first step for obtaining meaningful gene expression data. Band Lane1,8,9 has an abnormal m-RNA from miscarriage. This finding reveals that there are various problems such as early abortion or Infertility, and inadequate placentation as a result of m-RNA testing. Other studies suggesting that m-RNA expression test is a highly sensitive, simple and rapid developed to detect the rare abnormal gene transcripts for both *In-vitro* and vivo derived embryos that can lead to abnormal differentiation of the cell compartments forming the blastocyst [8].We recommended that in cases of vitro fertilization IVF in order to success pregnancy (when appropriate) must first perform the expression of these mRNA test. Moreover pre implantation genetic diagnosis PGD is desired for choosing the embryos to be replaced after PCR results.

The results of agarose gel electrophoresis of P-LAP PCR amplified Products are represented in Figs. 2,3 .The appearance of just one band of about (621 bp) in the lanes refers to the internal control for β-actin, while the size of (100 bp) transcript corresponds to that of cDNA of LAP.

There is a missing band derived from SM4 as shownin Fig. 2, this negative result indicates that miscarriage may be due to P-LAP deficiency which induces widely continuous dropping incidences of preeclampsia and preterm labour.

It has been found out that placental expressions of P-LAP mRNAs in recurrent miscarriage are lower than in single miscarriage women, as shown in Figs. 4,5. This suggests that

P-LAP may contribute to the limited trophoblast invasion in miscarriage. The secretion of proteolytic enzymes such as, the aminopeptidase degrades the components of the extracellular matrix (ECM) then increases the invasive capacity of the trophoblast. It is reasonable to infer that P-LAP genes are involved in promoting the trophoblast invasion during normal pregnancy. These results are in agreement with those reported by Nardo et al. [12]. There is another evidence for the role of the aminopeptidase regulation of trophoblast invasion and spiral artery remodeling in early placentation as reported by Naruse et al. [13].

Maintenance of normal pregnancy depends on the dynamic balance between promoting the expression of genes and inhibiting invasion in placenta whether the activation and inhibition of the invasion genes that are controlled by independent factors or are known or not. Previous studies have identified several putative nucleotide consensus sequences such as binding sites for activator protein-2 (AP-2), selective promoter factor 1 (Sp1), nuclear factor-kB (NF-kB) and NF-interleukin-6 (NFIL6) .Patients with spontaneous preterm delivery have higher concentrations of inflammatory cytokines and lower P-LAP activities than those with normal delivery suggesting that placental leucine aminopeptidase P-LAP are cytokine-induced nuclear proteins. Several putative cytokines are associated with P-LAP gene expression Ikoma et al. (2003) [9]. P-LAP and APA represent promising agents for the treatment of preeclampsia and preterm labour by degrading bioactive hormones which are derived from the feto-placental circulation Mizutani et al. [14]. Controlled balance between promoting and suppressing invasion genes are largely responsible for pathological pregnancies.

The obtained results revealed that placental expressions of LAP mRNAs are lower in miscarriage than women with normal pregnancy infer that in normal pregnancy sufficient levels of P-LAP degrade OT and vasoactive peptide AVP to protect the mother. However, a drop in the activity of the enzyme permits elevated levels of vasoactive peptide hormones from the feto-placental circulation to leak into the maternal circulation resulting in vasoconstriction (hypertension) and uterine contraction (onset of labour and preterm labour) Kozaki et al. [15]. So serial assays of P-LAP may be useful as a rough guide for predicting the onset of labour. While at term increases in fetal development OT overwhelms P-LAP resulting in contractions of the uterus and then triggering the onset of labour [16]. In the current study a new evidence can provide regarding the role of the P-LAP gene in the pathogenesis of pregnancy which may have significant clinical implications and can be used as predicting factor to avoid any complications lead to miscarriage.

4. CONCLUSION

This study investigates that there is an association between P-LAP genotype and miscarriage as risk factors and focuses on finding such biomarkers (in placentas) that are linked with the diseases .Which will be helpful to elucidate an assays or sophisticated tests that can detect changes during miscarriage.

5. STUDY LIMITATIONS

Limitations of our study are mainly the observational not randomized character which doesn't prevent bias through unknown impacting parameter.

1. Long standing hypertension, chronic disorder and cardiovascular events should have been monitored and excluded.

2. The expression of the mRNA must be performed on fresh placental tissue in order to get successful results.
3. Screening programs may also be more effective as well as improve the clinical assessment of the pregnancy. This study shows that a substantial portion of the population can be identified as 'at risk' and appropriately referred to general practice for follow-up based on the P-LAP results.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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