Serum relaxin levels are reduced in pregnant women with a history of recurrent miscarriage, and correlate with maternal uterine artery Doppler indices in first trimester.

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Running Title: Serum relaxin during pregnancy following recurrent miscarriage.

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Condensation

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ABSTRACT

Objectives: Defective implantation is a mechanism for recurrent pregnancy loss (RPL). We sought to determine whether the serum expression of human relaxin 2 (RLX) is impaired in women with a history of RPL.

Study Design: Employing a prospective case-controlled design we studied 20 pregnant women with a history of RPL and 20 age-matched women with no history of RPL (NRPL). We measured serum relaxin 2 levels by ELISA at 6-8, 10-12, 20, 34 wks gestation and in cord blood, and maternal uterine artery Doppler resistance index (RI) at ≥ 10 wks gestation.

Results: Relaxin rose to a peak at 12 wks, and gradually declined towards term. At all gestations, women with a history of RPL had lower RLX levels than women without. At 10-12 wks uterine artery, uterine artery RI correlated with serum RLX for both RPL and NRPL and the presence of a notched waveform in the NRPL group was associated with higher RLX levels than the absence of a notch (mean 2.1 vs. 1.3ng/ml, $P < 0.05$ respectively), and also at 20 wks (2.1 vs. 0.95 ng/ml, $P < 0.05$ respectively), but did not differ in the RPL group. Umbilical venous RLX was 4-fold higher in the RPL group than the NRPL group.

Conclusion. Women with a history of RPL demonstrate attenuated levels of serum RLX across all pregnancy trimesters. How dysregulated RLX metabolism may contribute to adverse pregnancy outcome in RPL requires further investigation.

Key words: relaxin, pregnancy, recurrent miscarriage, Doppler, implantation.

INTRODUCTION.

Recurrent pregnancy loss (RPL) is a distressing clinical problem which affects 1% of all women. Although no cause is found in the majority of cases, it has been associated with the antiphospholipid syndromes (1), immunological “intolerance” from altered function of endometrial natural killer (NK) cells (2), the polycystic ovary syndrome (3) and insulin resistance (4). A common mechanism for fetal wastage in RPL is defective implantation. (2)

Even when pregnancy following RPL is ultimately successful, placental function may still be compromised, with higher rates of adverse pregnancy outcomes being reported. (5) Abnormal placentation may be associated with high resistance index (RI) in the uterine artery (UA) Doppler waveform, a finding noted to have high predictive value for conditions associated with placental dysfunction such as pre-eclampsia, fetal growth restriction and placental abruption. (6)

Several serum markers are altered in women with RPL but do not accurately predict repeat miscarriage. These markers include inhibin A (7), glycodelin (8) and microparticles (9). When RPL is associated with the antiphospholipid (APS) and polycystic ovary (PCOS) syndromes, markers of insulin resistance (4) are impaired. Leptin expression levels are low in women who subsequently miscarry (10) but do not discriminate between such pregnancies and others.

Relaxin (RLX) plays a key role in reproduction, being involved in implantation, embryogenesis, placental development and prelabour uterine preparation. (11, 12) Relaxin genes are over-expressed in preterm rupture of fetal membranes (13) and change with the remodelling of the extracellular matrix prior to parturition. (14) However RLX expression during pregnancy to women with a history of RPL has not been studied.

We compared RLX levels in serum and cord blood of pregnant women with a significant history of RPL to those women with no history of RPL, and determined whether serum RLX correlates with UA Doppler indices or pregnancy outcome.

MATERIALS AND METHODS

Subjects. Twenty women with a history of RPL (defined as the loss of three or more consecutive pregnancies before 24 wks) were consecutively recruited from the pregnancy RPL clinic. This data set comprised women with idiopathic RPL, the APS, or a history of PCOS. We included only women whose RPL was not attributable to uterine tumours, uterine malformations or proven cervical weakness. A control group of 20 age-matched women with no history of RPL or an identified association with RPL was also recruited from the booking antenatal clinics. We excluded women who smoked cigarettes from the study. Sample sizes were determined on the basis of a previous study that measured serum RLX levels and demonstrated significant differences between a group of women with abnormal pregnancies and another with normal early pregnancies. (15) We estimated that 20 subjects in each study arm would detect a 10% difference in RLX levels between groups with 80% power at the 95% confidence level. Written informed consent was obtained from each study participant. Ethical permission was obtained from the South Sheffield Research Ethics committee.

Study design: Investigations were carried out on participants at 6-8, 10-12, 20, and 34 wks gestation. At each visit 10mls of blood was obtained. A further blood sample was obtained from the umbilical cord vein immediately after delivery of the baby. Samples were transported to the laboratory immediately and centrifuged to obtain serum which was aliquoted and stored at -80°C for subsequent batch analysis. At ≥ 10 wks, fetal biometry and UA Doppler indices were measured by transabdominal ultrasound. (16)

Laboratory analysis: Serum RLX was measured using the Quantikine Human Relaxin-2 Immunoassay kit (Catalog Number DRL200, R&D systems, Minneapolis,

USA). This assay is based on the quantitative sandwich enzyme technique and employs a monoclonal antibody specific for Human Relaxin-2 pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Relaxin-2 present is bound by the immobilized antibody. Following addition of an enzyme-linked polyclonal antibody specific for Relaxin-2 a substrate solution is added to to enable colour to develop in proportion to the amount of Relaxin-2 bound in the initial step. The assay measures the concentration range 2–250 pg/mL. The inter-assay coefficient of variation (CV) for this kit is 10.2% and 5.5% for 40 and 220 pg/mL relaxin respectively, and the intra-assay CV is 4.7% and 2.3% for 34 and 206 pg/mL, respectively. All samples were blinded for the analyst and measured simultaneously. Samples above 250 pg/mL were diluted and re-analysed. Quantitation of serum RLX has been previously described. (15, 17)

Doppler studies: Maternal UA Doppler velocimetry was evaluated using a pulsed wave technique at each visit ≥10 wks by transabdominal ultrasonography. The RI has been shown to be the most repeatable and least variable of the UA Doppler indices. (16). The average RI of the left and right UA Doppler waveform was used for tests of association. The presence of a dicrotic notch in either or both uterine arteries was also used compare RLX levels in both study groups. All clinical details, including the pregnancy outcome for both groups, were recorded.

Outcome measures: The primary outcome measure was serum RLX and UA RI indices. Clinical outcomes that were tested for association with serum RLX and uterine RI were spontaneous miscarriage, premature delivery before 34 wks gestation, small for gestational age (birth weight less than the 10th customized centile for gestation, (18)), placental abruption and stillbirth.

Data analysis: Serum RLX and UA Doppler indices were compared by one-way ANOVA, the student t-test or the Mann-Whitney U test with appropriate post hoc correction (the Bonferroni test) for repeated measures and multiple comparisons. Binary logistic regression analyses and the area under the Receiver Operator Characteristic (ROC) Curves were used to test association between RLX and UA RI and clinical outcome variables. Where data regarding cord blood RLX were missing a listwise deletion approach was adopted for analysis.

RESULTS:

General clinical and laboratory details. Table 1 summarises the clinical details of all participants and the outcome of their pregnancies. Of the 20 participants with a history of RPL, 12(60%) had idiopathic RPL, 5(25%) had the APS, and 3(15%) had the PCOS associated with suspected luteal phase deficiency. All those with the APS were treated with low molecular weight heparin (LMWH) and low-dose aspirin throughout pregnancy. There was no significant difference in the rates of preterm birth, prelabour rupture of fetal membranes and miscarriage between study groups and between aetiological association subtypes in women with RPL.

Serum relaxin levels did not differ with maternal gravidity or parity. At all gestational time points, except at 6-8 wks, RLX was significantly lower in the RPL compared to the NRPL group (Table 1). Amongst women with RPL serum RLX levels did not differ by cause of miscarriage or by treatment with aspirin or LMWH (data not shown). In NRPL but not RPL, RLX at 6-8 wks markedly correlated with levels at 12 and 20 wks gestation.

Stepwise logistic regression analysis (factors included in model: maternal age and body mass index, serum relaxin) demonstrated that serum RLX in RPL was lower

than in NRPL group at 10-12wks gestation (regression coefficient -1.1, SE 0.5, OR 0.33, 95% CI 0.12, 0.95, P < 0.05), at 20 wks gestation (regression coefficient -2.0, SE 0.9, OR 0.14, 95% CI 0.03, 0.75, P < 0.05), and at 34 wks gestation (regression coefficient -1.3, SE 0.7, OR 0.26, 95% CI 0.07, 1.06, P = 0.05). The association between low RLX and a history of RPL is depicted in Figure 1 as Receiver Operator Characteristic (ROC) curves: at 10-12 wks gestation RLX ≤ 1.7ng/ml was best associated with a history of RPL [sensitivity 95%, specificity 42%, LR+ 1.64 95% CI 1.0 – 2.8, LR- 0.12, 95% C1 0.02 – 0.8, P < 0.05)] whilst at 20 wks gestation RLX ≤ 0.7ng/ml was best associated with a history of RPL [sensitivity 79%, specificity 61%, LR+ 2.0 95% CI 1.3 – 3.1, LR- 0.3 95% C1 0.3 – 1.0, P < 0.01)]. Although mean RLX was lower in RPL than NRPL at 6-8 and at 34 wks, the levels were not significantly associated with a history of RPL.

Correlation of serum RLX levels with uterine artery resistance index. This is summarised in Table 2. At 10-12 wks gestation there was moderate positive correlation (correlation coefficient between 0.4 and 0.6) between the average UA RI and serum RLX for both RPL and NRPL (Figure 2). The incidence of uni- or bilateral notching did not differ between RPL and NRPL (Table 1). However at 10-12 wks mean ± SE serum RLX was higher in the NRPL group with UP notching (2.1±0.4 ng/ml) compared to those without UP notching (1.3±0.2 ng/ml, P < 0.05), but did not differ by notching in those with a history of RPL (1.1±0.2 vs. 0.9±0.2 ng/ml). Similarly at 20 wks gestation serum RLX was higher in the NRPL group with UP notching (2.1±0.7 ng/ml) compared to those without UP notching (0.95 ± 0.1 ng/ml, P < 0.05) but did not differ in those with a history of RPL (0.6 ± 0.1 vs. 0.4 ± 0.21 ng/ml). In neither group did RLX differ with UA notching at 34 wks gestation. In both groups UA pulsatility index did not correlate with serum RLX at any gestation.

Serum RLX levels and pregnancy outcomes.

Serum RLX at 34 wks gestation was not significantly different in women who had prelabour rupture of membranes (mean ± SE 0.8 ±0.1) compared to women who went into labour with intact membranes (0.9 ±0.2 ng/ml), and did not correlate with fetal birth weight or gestational age at delivery for either group.

Cord blood relaxin levels. Cord blood was obtained in 15 (75%) RPL and 13 (60%) NRPL participants: RLX levels were 4-fold higher in the RPL group than the NRPL group but this did not attain statistical significance (Table 1) but did not correlate with antenatal RLX levels, Doppler indices, fetal birth weight, or gestational age at delivery. In RPL, women with prelabour rupture of membranes showed a trend towards higher cord RLX levels than women without prelabour amniorrhexis (0.16 ± 0.11 vs. 0.04 ± 0.02 ng/ml, P = 0.15 respectively) whilst in the NRPL group cord RLX did not appear to differ by timing of fetal membrane rupture (0.26 ±0.01 vs. 0.20 ±0.00, P = 0.48 respectively).

COMMENTS.

This is the first report that serum relaxin levels are decreased throughout pregnancy in women with a history of RPL compared to those without such history. Additionally we noted correlation between 1st and 2nd trimester serum RLX levels in the women without a history of RPL but not in the group with a history of RPL, suggesting that dysregulation of relaxin metabolism may be important in the pathogenesis of recurrent early pregnancy failure. The positive correlation of serum relaxin with UA RI at 10-12 wks is consistent with a putative role for this hormone in the early regulation of uteroplacental haemodynamics. (19)

Our gestational changes in serum RLX levels confirm the only previous report: in normal pregnant women serum RLX concentrations increased during the first 14 wks of pregnancy, decreasing thereafter until about 24wks when it then remained constant, with no changes evident immediately before or during spontaneous delivery. (15) One study reported lower RLX levels during miscarriage and ectopic pregnancies compared to viable early pregnancies (15) whilst another reported unchanged levels. (17) We could not confirm or refute these observations as only one woman in our RPL group miscarried. Any temporal relationship between changes in serum RLX levels and clinical pregnancy failure remains to be determined.

Reduced serum RLX2 in women with a history of RPL is likely to mirror lower production in reproductive tract tissue, the primary site of relaxin production and action. Increasing blood flow to endometrial or placental tissue requires neo-vascularisation promoted by the key angiogenic growth factors vascular endothelial growth factor (VEGF) and placental growth factor. (20) Relaxin upregulates VEGF and increases blood flow to the endometrium and other reproductive tract tissue (21).

Reduced functional activity of RLX from the corpus luteum (22) is therefore likely to contribute to defective endometrial implantation and recurrent pregnancy failure. We hypothesise that a critical level of defective RLX activity is required before a pregnancy fails. Consequently the pregnancies to our cohort of women with a history of RPL may have had favourable outcomes because the reduced expression of RLX did not attain the critical levels required to result in miscarriage or other adverse outcomes.

Serum relaxin levels peak by the end of the first trimester, coinciding with the switch of RLX synthesis from the systemic corpus luteum to the paracrine placenta and fetal membranes. The order of expression of RLX gene transcripts is: corpus luteum of pregnancy > corpus luteum of the cycle > placenta and prostate > decidua parietalis (23), somewhat consistent with our temporal observations through pregnancy. The reported wide variations in expression levels of RLX genes from tissue to tissue and within each tissue within the term placenta (23) may explain the lack of correlation at 34 wks between serum RLX levels and UA Doppler indices on the one hand and serum RLX and pregnancy outcome on the other. Furthermore, RLX has paracrine functions within tissues close to its site of production. Systemic RLX, derived mainly from the corpus luteum (24), may therefore not parallel local functional levels in the placenta, decidua or fetal membranes. (25) We did not measure serum RLX immediately prior to labour because RLX levels do not appear to change significantly at term and prior to parturition. (15)

We noted a 4-fold increase in cord blood RLX levels in the women with a history RPL compared to those without. However our data only approached statistical significance possibly because our sample size was small. It may be hypothesized that deranged maternal RLX metabolism associated with RPL leads to a compensatory increase in fetal relaxin expression through as yet unidentified regulatory feedback mechanisms

similar to the fetal hyperinsulinaemia/hypoglycaemic response to maternal hyperglycaemia. Interestingly relaxin belongs to the insulin family of peptide hormones.

We observed modestly increased cord blood relaxin levels in the subset of the RPL group who had prelabour membrane rupture. A similar increase has been described in preterm decidual tissue and placental syncytiotrophoblast from women with preterm premature rupture of membranes compared to those with intact membranes. (13) Our study of systemic rather than local tissue RLX levels may have precluded identification of any relationship between RLX expression and timing of fetal membrane rupture.

We studied the expression levels of human RLX2 rather than RLX1 because only the former isoform appears to have a functional role in reproductive tract tissue. (26). It is unlikely that the serum RLX that we measured originated from non-gestational tissues as gestational relaxin is only expressed in placental syncytiotrophoblast, decidua, and corpus luteum. (27) In the nonpregnant state, relaxin was only demonstrable in the corpus luteum and secretory phase endometrium and not in myometrium, cervix, vagina, and Fallopian tubes.

We did not exclude women whose previous miscarriages were due to embryonic chromosomal aberrations because this information was not always available. Such chromosomal aberrations in past pregnancy losses may have confounded the results. Only one patient in our study group subsequently miscarried, suggesting an extremely high live birth rate or 95%. The reason for the highly successful birth outcome in our cohort is unclear but may be attributable in part to our care philosophy that spans from conception to birth in one unified service provided by

specialist nurses, midwives, gynaecologists with an interest in RM and fetal medicine specialists.

In conclusion, we have demonstrated that serum relaxin levels are reduced across all gestations of viable pregnancies to women with a history of RPL. Taken together with studies that show reduced relaxin levels in abnormal and non-viable pregnancies, our data suggest that dysregulation of RLX functional expression may play a role in habitual early pregnancy failure possibly by suppressing endometrial and placental angiogenesis. Further studies are required to explain these observations.

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TABLES/FIGURE CAPTION LIST.

Table I. Subject characteristics and pregnancy outcome.

Table 2. Correlation matrix, serum relaxin levels vs. uterine artery Doppler resistance index in women with a history of RPL and a control group of women with no history of RPL (NRPL).

Figure 1. ROC curves depicting association of serum relaxin levels at various gestations with a history of RPL (RLX human relaxin 2, AUC Area under the Curve, * $P < 0.05$, ** $P < 0.01$)

Figure 2. Scatter diagram showing correlation of average uterine artery resistance index with serum RLX levels at 10-12wks (open circles: women with history of RPL, triangles: women with no history of RPL).

Table 1 Subject characteristics and pregnancy outcome.

	History of recurrent pregnancy loss	No history of recurrent pregnancy loss	P value
n	20	20	
Median (range) age (yrs)	32.5 (20 – 43)	31.0 (24–41)	0.8
Body mass index	27.3 (5.5)	25.7 (5.8)	0.3
Mean (SE) gestational age at delivery (weeks)	38.4 (1.0)	38.3 (1.1)	0.95
Delivery before 37 weeks, n (%)	3 (15)	3 (15)	NS
Mean (SE) birth weight (g)	3040 (206)	3364 (216)	0.15
Pre-labour spontaneous rupture of fetal membranes, n (%)	5 (25)	6 (30)	NS
Infant birth weight <2500g, n (%)	7 (35)	3 (15)	0.15
Small for gestational age (birth weight <10th centile)	5 (25%)	0	0.048
Still births	0	0	
Miscarriage, n (%)	1(5)	0	
Mean (SE) serum RLX levels in ng/ml)			
6-8wks	1.06 (0.14)	1.49 (0.21)	0.09
10-12 wks	1.07 (0.11)	1.62 (0.21)	0.03
20 wks	0.59 (0.09)	1.14 (0.18)	0.01
34 wks	0.58 (0.09)	1.07 (0.21)	0.03
Cord serum	0.08 (0.04)	0.02 (0.00)	0.21
Mean (SE) uterine artery Doppler resistance index			
10-12 wks	0.66 (0.03)	0.61 (0.04)	0.47
20 wks	0.64 (0.10)	0.55 (0.03)	0.76
34 wks	0.54 (0.02)	0.48 (0.02)	0.12
Uterine artery Doppler (diastolic notch, n %)			
10-12 wks	8 (40)	8 (40)	1
20 wks	6 (30)	4 (20)	0.43
34 wks	3 (15)	1 (5)	0.55

Table 2. Correlation matrix, serum relaxin levels vs. uterine artery Doppler resistance index in women with a history of RPL and a control group of women with no history of RPL (NRPL). Data shown are coefficients of correlation.

	RLX at 6 -8wks		RLX at10-12wks		RLX at 20wks		RLX34wks		Cord blood RLX		UA RI,10-12wks		UA RI, 20wks	
	RPL	NRPL	RPL	NRPL	RPL	NRPL	RPL	NRPL	RPL	NRPL	RPL	NRPL	RPL	NRPL
1. RLX at 6 -8wks														
2. RLX at10-12wks	0.4	0.7(**)												
3. RLX at 20wks	0.2	0.9(**)	0.7(**)	0.7(**)										
4. RLX34wks	0.3	0.8(**)	0.9(**)	0.8(**)	0.7(**)	0.9(**)								
5. Cord blood RLX	-0.1	0.3	-0.1	0.6(*)	0.1	0.3	-0.1	0.3						
6. Uterine artery RI,10-12wks	0.3	0.3	0.5(*)	0.5(*)	0.5(*)	0.4	0.2	0.5(*)	-0.01	0.5				
7. Uterine artery RI, 20wks	0.6(*)	0.3	0.3	-0.1	0.2	0.1	0.3	-0.0	-0.1	-0.2	-0.3	-0.3		
8. Uterine artery RI, 34wks	0.2	-0.1	0.1	-0.4	0.0	-0.2	0.2	-0.2	-0.6 (*)	-0.3	0.2	-0.2	0.1	0.1

Correlation coefficient is significant: * at the 0.05 level,** at the 0.01 level. RI resistance index, RPL recurrent pregnancy loss.

Figure 1

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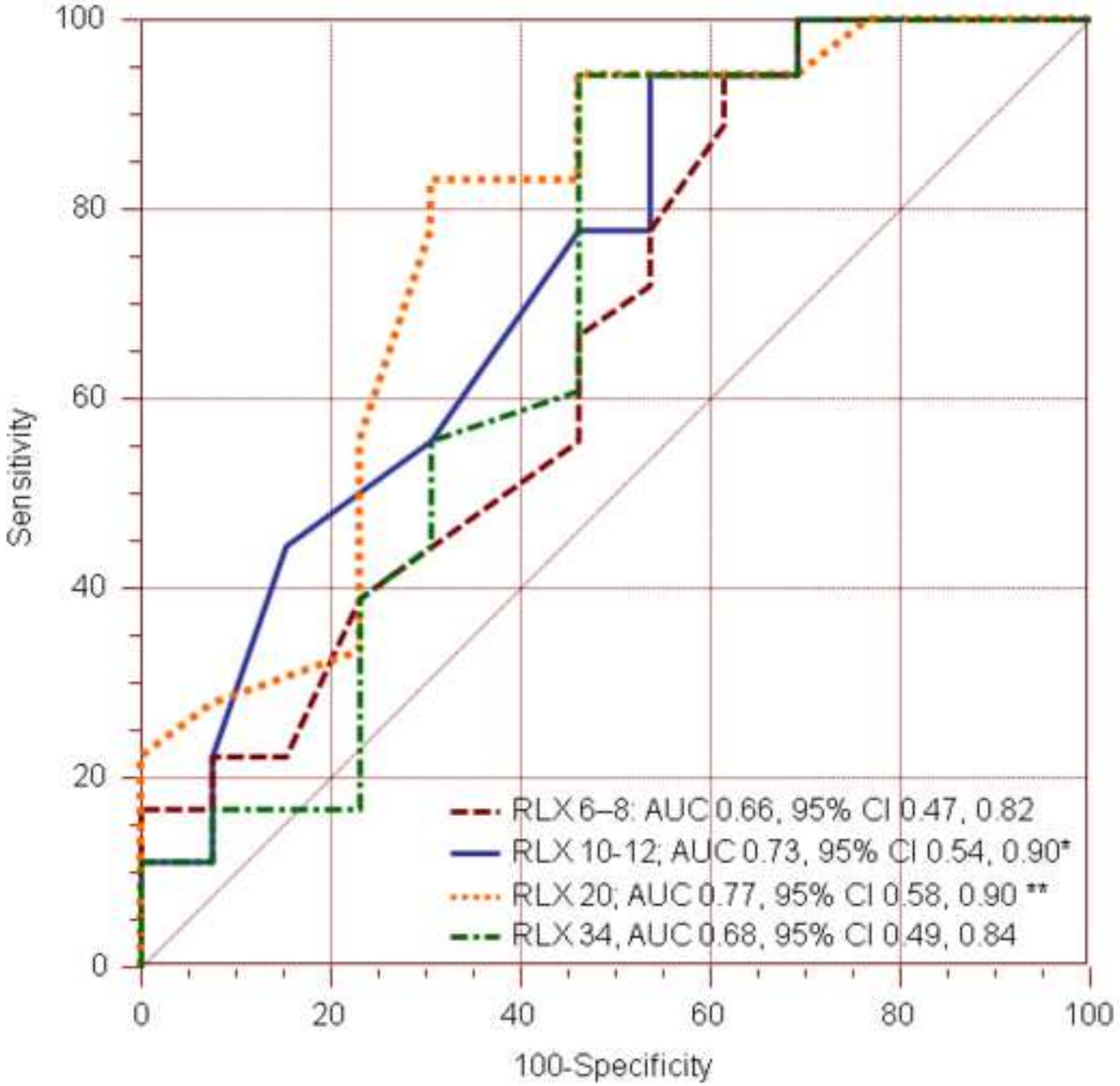

Figure 2

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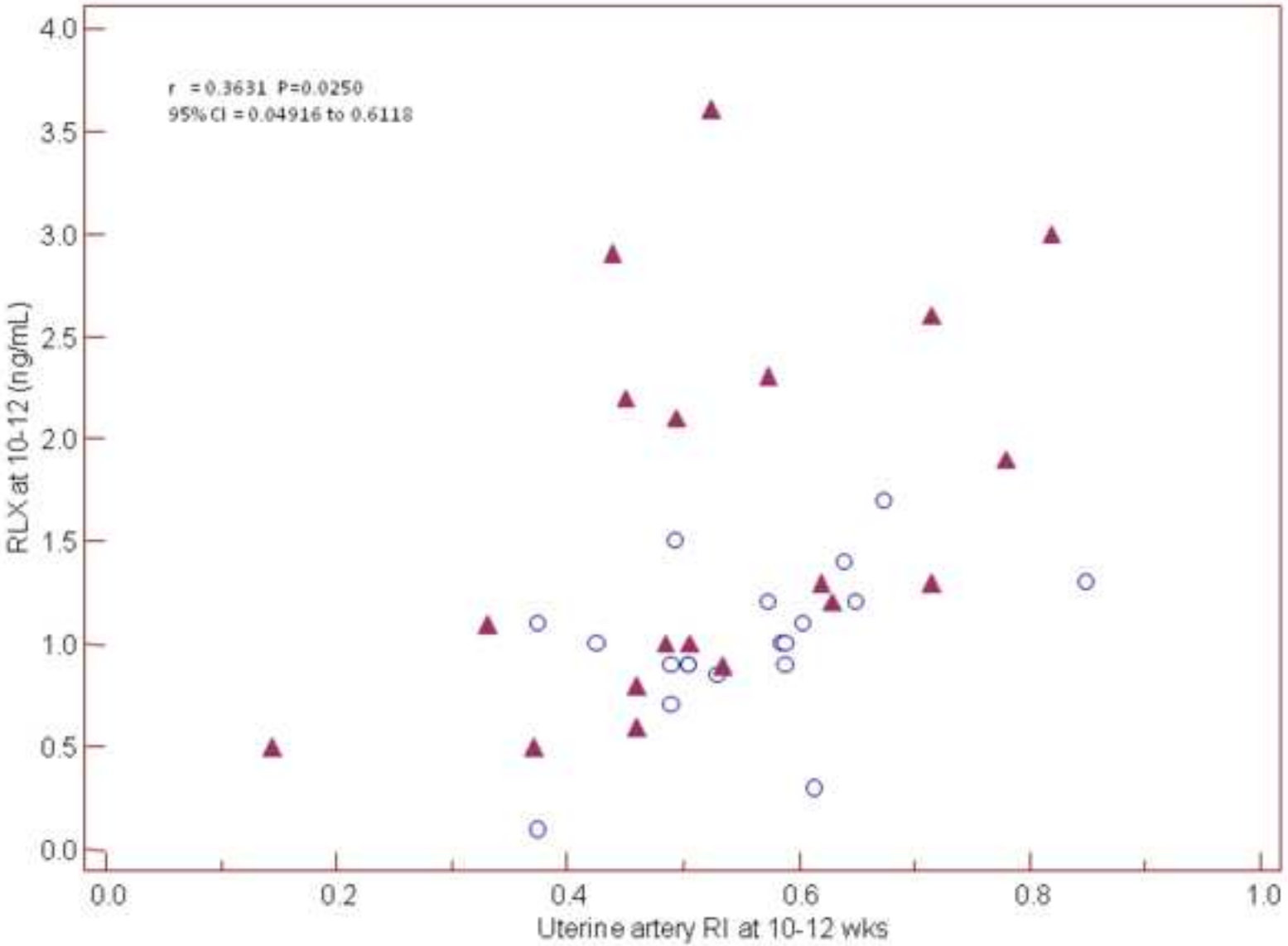

Figure 3