Reference Interval of Soluble FMS-like Tyrosine Kinase-1 in Non-Pregnant and Pregnant Females: A Novel Biomarker for Pre-eclampsia

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ABSTRACT
Objective: To determine the reference interval of soluble FMS-like tyrosine kinase-1 (sFlt-1) in healthy, non-pregnant and pregnant females.

Study Design: Observational study.

Place and Duration of the Study: Department of Chemical Pathology, Chughtai Institute of Pathology, Lahore, from January to May 2023.

Methodology: Blood samples were collected from 120 disease-free non-pregnant females of reproductive age group and 120 disease-free pregnant females with singleton fetuses from 15 to 28 weeks of gestational age. Healthy reference individuals were selected by correlating history with medical disorders like diabetes mellitus, hypertension, autoimmune diseases, inherited disorders, and by excluding any other drug history. All findings were recorded on health screening questionnaire. Levels of sFlt-1 were measured by a fully automated immunoassay analyser Cobas e601. Kolmogorov-Smirnov test was applied. The value of p <0.05 was considered significant. The 2.5⁰ and 97.5⁰ percentiles were computed at 90% CI by using the formula 0.025x (n+1) and 0.975x (n+1) which corresponded to rank number 1 and 7, respectively. The reference interval was calculated by the Rank-based method.

Results: Reference interval of sFlt-1 in non-pregnant and pregnant females were determined on the basis of 2.5⁰ and 97.5⁰ percentiles which were 57.7 to 118.5 pg/mL and 563.5 to 3288.0 pg/mL, respectively.

Conclusion: The present study determined reference interval of sFlt-1 in healthy, non-pregnant and pregnant females in Lahore.

Key Words: Reference interval, Soluble FMS-like tyrosine kinase-1, Pre-eclampsia, Rank-based method.


INTRODUCTION
Pre-eclampsia is one of the serious adverse outcomes of pregnancy which consists of new onset of proteinuria and hypertension in the second half of pregnancy that is associated with increased risk of fetal, neonatal, and maternal complications.¹ Worldwide pre-eclampsia complicates 2 to 8% of pregnancies and contributes to 10 to 15% maternal death.² Pre-eclampsia results from imbalance between anti-angiogenic and pro-angiogenic factors in pregnancy. In normal pregnancy levels of the anti-angiogenic factor, i.e. soluble FMS-like tyrosine kinase-1 (sFlt-1) remain stable during the early and mid-stages of gestation and increase steadily until term.³⁴

The levels of placental growth factor (PGF) rise during the first two trimesters and decline as the pregnancy progresses to term but in females who develop pre-eclampsia or are at risk of developing pre-eclampsia. PGF levels are found to be lower and sFlt-1 levels are found to be higher than the normal levels.⁵ In pre-eclampsia, elevated serum levels of the sFlt-1 are found as a result of increase in transcriptional activity of sFlt-1 in trophoblastic cells due to hypoxia. A significant rise in sFlt-1 levels has been observed in maternal serum before the onset of clinical symptoms of pre-eclampsia. Thus, antiangiogenic sFlt-1 has been proposed to be an efficient screening biomarker for pre-eclampsia.⁵⁶

Moreover, sFlt-1 being an endogenous inhibitor of vascular endothelial growth factor can act as a marker of pathological angiogenesis. High levels of sFlt-1 can be found in non-pregnant females with cardiovascular diseases and Diabetes mellitus.⁷

Clinical Laboratory Standard Institute (CLSI) and the International Federation for Clinical Chemistry (IFCC) define the reference interval as the set of values obtained by quantitative measurement of a parameter in a selected group of reference

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individuals which is based on the well-defined criteria.\textsuperscript{8,9} Nowadays, despite the remarkable technology advancement, the contributory percentage of laboratory test to clinical decision is still higher than 80%.\textsuperscript{10} Therefore, clinical laboratories must carefully establish their own reference intervals for each analyte according to the standard protocols in consideration of the biological variations such as gender, age, genetics, ethnicity, pregnancy, non-pregnancy, healthy, and unhealthy status to achieve precise clinical assessment.\textsuperscript{11}

As per the authors’ knowledge, reference interval for sFlt-1 has not been established in the Pakistani population. Thus, the rationale in the current study was to determine the reference interval of sFlt-1 in a local population in order to segregate low-risk and high-risk females for the development of pre-eclampsia and to facilitate healthcare workers in early diagnosis and management of pre-eclampsia. The objective of this study was to determine the reference interval of sFlt-1 in healthy, non-pregnant and pregnant females.

**METHODOLOGY**

This was the observational, cross-sectional study, conducted at the Department of Chemical Pathology, Chughtai Institute of Pathology, Lahore, Pakistan. It was carried out from January to May 2023 after receiving an approval from the institutional review boards of Chughtai Institute of Pathology and Sheikh Zayed Medical Centre, Lahore. The sample size was calculated in accordance with Clinical and Laboratory Standards Institute guidelines.\textsuperscript{8-13} Reference individuals were selected by purposive, non-probability sampling technique from 120 disease-free non-pregnant and 120 disease-free, singleton pregnant females with gestational age of 15 to 28 weeks. Healthy, non-pregnant, and pregnant females were included by correlating history with medical disorders like Diabetes mellitus, hypertension, autoimmune diseases, inherited disorders, and by excluding any other drug history. All findings were recorded on health screening questionnaire administered before the sample collection. Informed consent was obtained from females of reproductive age group (18-45 years) who fulfilled the inclusion criteria. From each participant, two ml of blood was drawn to obtain at least 1 ml of serum. Samples centrifugation were performed at 4500 rpm for 4 minutes to obtain clear serum. According to the exclusion criteria, females with age of less than 18 years and of menopausal age group and with any acute or chronic illness were excluded. Pregnant females with twin and triplet pregnancy and diagnosed cases of pre-eclampsia and eclampsia were excluded. Moreover hemolytic, lipemic, and icteric samples were also excluded. Levels for sFlt-1 were measured by a fully automated immunoassay analyzer (Elecys system Cobas e601) based on the electrochemiluminescence methodology. The measuring range of sFlt-1 was set as 10-85000 pg/mL\textsuperscript{14} as no reference values were mentioned in reagent kit insert. The test sFlt-1 was performed as per the manufacturer’s recommendations. Before performing the test, patient labelled samples which were frozen at -80°C were thawed and kept in the sample rack of analyzer which incubates, mix and make calculation of the test value. Controls were run before the sample analysis. Data were analyzed by using SPSS 21. Kolmogorov-Smirnov test was applied. The p-value <0.05 was considered statistically significant. The 2.5\textsuperscript{th} and 97.5\textsuperscript{th} percentiles at 90% confidence interval were calculated using the formula 0.025 x(n+1) at rank number 1 and 0.975 x(n+1) at rank number 7.

**RESULTS**

Out of the total 240 samples, 120 blood samples were taken from disease-free non-pregnant females and 120 blood samples were from disease-free pregnant females of reproductive age group from 18 to 45 years (Table I). Histogram showed parametric distribution in non-pregnant females (p >0.05) as can be seen in Figure 1, while non-parametric distribution (p<0.05) in pregnant females can be seen in Figure 2.

**Table I: Age distribution of healthy, non-pregnant and pregnant females (n=120).**

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of healthy non-pregnant females</th>
<th>Number of healthy pregnant females</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-23</td>
<td>90 (75%)</td>
<td>38 (31.7%)</td>
</tr>
<tr>
<td>24-30</td>
<td>14 (11.7%)</td>
<td>51 (42.5%)</td>
</tr>
<tr>
<td>31-35</td>
<td>7 (5.8%)</td>
<td>19 (15.8%)</td>
</tr>
<tr>
<td>36-40</td>
<td>7 (5.8%)</td>
<td>7 (5.8%)</td>
</tr>
<tr>
<td>41-45</td>
<td>2 (1.7%)</td>
<td>5 (4.2%)</td>
</tr>
</tbody>
</table>

**Figure 1:** Histogram of sFlt-1 levels (pg/mL) in healthy, non-pregnant females.

**Figure 2:** Histogram of sFlt-1 levels (pg/mL) in healthy, pregnant females.
Table II: Non-parametric determination of sFlt-1 reference interval in non-pregnant females (n=120).

<table>
<thead>
<tr>
<th>Calculation of rank numbers according to percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower 0.025×(120+1) = 3.025</td>
</tr>
<tr>
<td>Upper 0.975×(120+1) = 117.975</td>
</tr>
</tbody>
</table>

**Defining the original values corresponding to these rank numbers**

- Lower reference limit: 2.5\textsuperscript{th} percentile 57.7
- Upper reference limit: 97.5\textsuperscript{th} percentile 118.5

**Rank numbers and values of the 0.90 confidence limits of lower reference limit**

- Rank No1 and 7
  - Confidence limit 56.45 and 60.90

**Rank numbers and values of the 0.90 confidence limits of upper reference limit**

- Rank number 120+1-7=114
  - Rank number 120+1-1=120
  - Confidence limit 109.9 and 123.45

**Summary**

- sFlt-1 Lower reference limit (pg/mL) 57.7 (56.45 to 60.90)
- sFlt-1 Upper reference limit (pg/mL) 118.5 (109.9 to 123.45)

Table III: Non-parametric determination of sFlt-1 reference interval in pregnant females (n=120).

<table>
<thead>
<tr>
<th>Calculation of rank numbers according to percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower 0.025×(120+1) = 3.025</td>
</tr>
<tr>
<td>Upper 0.975×(120+1) = 117.975</td>
</tr>
</tbody>
</table>

**Defining the original values corresponding to these rank numbers**

- Lower reference Limit: 2.5 percentile 563.50
- Upper reference Limit: 97.5 percentile 3288.0

**Rank numbers and values of the 0.90 confidence limits of lower reference limit**

- Rank No.1 and 7
  - Confidence limit 423.2 and 793.0

**Rank numbers and values of the 0.90 confidence limits of upper reference limit**

- Rank number 120+1-7=114
  - Rank number 120+1-1=120
  - Confidence limit 3153.0 and 3431.0

**Summary**

- sFlt-1 Lower reference limit (pg/mL) 563.50 (423.2 to 793.0)
- sFlt-1 Upper reference limit (pg/mL) 3288.0 (3153.0 to 3431.0)

Table IV: Comparison of reference values of sFlt-1 in healthy, non-pregnant and pregnant females.

<table>
<thead>
<tr>
<th>Percentile</th>
<th>sFlt-1 values in non-pregnant, healthy females</th>
<th>sFlt-1 values in pregnant, healthy females (15 to 28 weeks of gestation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textsuperscript{th} percentile</td>
<td>60.0 pg/mL</td>
<td>712.0 pg/mL</td>
</tr>
<tr>
<td>50\textsuperscript{th} percentile</td>
<td>84.4 pg/mL</td>
<td>1578.5 pg/mL</td>
</tr>
<tr>
<td>95\textsuperscript{th} percentile</td>
<td>111.8 pg/mL</td>
<td>3201.4 pg/mL</td>
</tr>
</tbody>
</table>

For the current study, the authors opted for non-parametric method (Rank-based method)\textsuperscript{12,13} for both non-pregnant and pregnant females as Rank-based method is IFCC and CLIA recommended method for determination of the reference values.\textsuperscript{12,13} Data values were arranged in the ascending order and rank number was allotted. Reference intervals of sFlt-1 in non-pregnant and pregnant females were determined on the basis of 2.5\textsuperscript{th} and 97.5\textsuperscript{th} percentiles, and was found to be 57.7 to 118.5 pg/mL (Table II) and 563.5 to 3288.0 pg/mL (Table III), respectively, while the cut-off values for pre-eclampsia in pregnant females from 15 to 28 weeks of gestation at 5\textsuperscript{th}, 50\textsuperscript{th} and 95\textsuperscript{th} percentile were 712, 1578.5, and 3201.4 pg/mL, respectively (Table IV).

Table V: Comparison of reported reference values of sFlt-1 in different studies.

<table>
<thead>
<tr>
<th>Percentile</th>
<th>Verlohren et al.\textsuperscript{17} (2014)</th>
<th>Mitlid-Mork et al.\textsuperscript{18} (2020)</th>
<th>Current study (2023)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textsuperscript{th} percentile</td>
<td>15-20weeks 708 pg/mL</td>
<td>37-40weeks 1612 pg/mL</td>
<td>15-28weeks 712 pg/mL</td>
</tr>
<tr>
<td>50\textsuperscript{th} percentile</td>
<td>24-28weeks 1355 pg/mL</td>
<td>37-40weeks 3470 pg/mL</td>
<td>15-28weeks 1578.5 pg/mL</td>
</tr>
<tr>
<td>95\textsuperscript{th} percentile</td>
<td>24-28weeks 3205 pg/mL</td>
<td>37-40weeks 8267 pg/mL</td>
<td>15-28weeks 3201.4 pg/mL</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Reference intervals/values are the most common decision making tool used to interpret the pathology reports. Different screening models use high levels of sFlt-1 as a biomarker to predict the risk of early onset pre-eclampsia in pregnant females and associate increased levels of sFlt-1 with cardiovascular diseases in non-pregnant females\textsuperscript{15} but there is limited data available for establishment of reference interval of sFlt-1 and its cut-off values in pregnant and non-pregnant females, especially in the local population. The current study aimed to determined the reference...
interval of sFlt-1 in healthy, non-pregnant and pregnant females as a predictive biomarker for pre-eclampsia in the Pakistani population.

The American College of Obstetricians and Gynaecologists, the International Society for the Study of Hypertension in Pregnancy, and the National Institute for Health and Care Excellence suggested the screening of pregnant females for pre-eclampsia in the first trimester of pregnancy with certain maternal risk factors and with anti-angiogenic factor sFlt-1 which circulate in the maternal blood, and increase in pregnancies complicated by pre-eclampsia. The hypoxic placenta releases sFlt-1 in elevated concentration to promote peripheral vasoconstriction ultimately raising maternal blood pressure and increasing the flow of oxygenated maternal blood through the intervillous space.\textsuperscript{16}

A prospective, multicentre study was conducted by Verlohren et al. to define the reference interval of sFlt-1 for normal pregnancies in which 877 pregnant females (normotensive) from Europe (Germany, Austria, Spain, Switzerland, and Czech Republic) were included.\textsuperscript{17} All females had a singleton pregnancy with normal pregnancy outcome (i.e. no IUGR, no preeclampsia or HELLP syndrome). Levels of sFlt-1 were determined by Elecsys sFlt-1 assay. They reported cut-off value of sFlt-1 according to gestational age (15 to 28 weeks) at 5\textsuperscript{th} percentile 708 pg/mL, at 50\textsuperscript{th} percentile 1355 pg/mL, and at 95\textsuperscript{th} percentile 3205 pg/mL.\textsuperscript{17} These findings supported the current research findings (Table V).

A retrospective, cross-sectional study was conducted by Mittid-Mork et al. on 146 pregnant females from 37 to 40 weeks of gestation with an uncomplicated delivery outcome.\textsuperscript{18} They reported concentrations of sFlt-1 1612 at 5\textsuperscript{th} percentile, 3470 at 50\textsuperscript{th} percentile and 8267 pg/mL at 95\textsuperscript{th} percentile.\textsuperscript{18} This study’s findings also provided comparison of sFlt-1 levels between 2\textsuperscript{nd} and 3\textsuperscript{rd} trimesters. The levels of sFlt-1 increases in late third trimester (37 to 40 weeks) as compared to mid-gestational phase levels of sFlt-1 reported in the present study (Table V).

A case-control study was conducted by a Taraseviciene et al. on 144 pregnant females from 26-40 weeks of gestation in which cut-off value of sFlt-1 9581 pg/mL was reported in pre-eclampsia group and levels of sFlt-1 1731.5 pg/mL was reported in the control group (i.e. healthy, pregnant females without pre-eclampsia).\textsuperscript{19} These findings also supported our hypothesis that sFlt-1 has a role as predictive biomarker for pre-eclampsia and its levels are significantly high in pre-eclamptic patients as compared to the healthy, pregnant females.

During pregnancy, normal sFlt-1 levels facilitate the avoidance of unnecessary medical interventions and surveillance, whereas sFlt-1 levels higher than normal reference value justify the provision of vigilant maternal monitoring and administration of low-dose aspirin in the high-risk group. Regarding fetal care, high-levels of sFlt-1 justify additional interventions such as optimally and timely administration of steroids for fetal lung maturation, administration of intensive--care monitoring, and avoidance of iatrogenic preterm birth.\textsuperscript{20,21} In addition to supporting the integration of sFlt-1 testing in routine antenatal investigation, the current study’s findings supported a role for sFlt-1 testing as a contingency screening tool integrated into remote communities and primary care centres. The associated risks of early onset pre-eclampsia, pre-term delivery, and still-birth may warrant referral of high-risk females with high sFlt-1 levels to higher-level healthcare centres.\textsuperscript{22}

The main limitation of the present study was the small sample size. Such studies should be conducted on a large scale to get more accurate estimation of reference interval and cut-off value. Moreover, these reference interval studies should be done in diagnosed cases of pre-eclampsia and eclampsia to see the comparison of sFlt-1 levels between healthy and disease groups.

CONCLUSION

This study established reference interval of sFlt-1, a novel biomarker for pre-eclampsia in healthy, non-pregnant, and pregnant females for the local population. Due to limited data on reference interval of sFlt-1, the current study’s findings can contribute to compare reference interval of sFlt-1 in healthy, non-pregnant and pregnant females. The current study’s findings will help the gynaecologist, clinicians, and pathologists to interpret the results. It can further help in decreasing maternal morbidity, mortality, and fetal complications related to pre-eclampsia. Moreover, it can be cost effective for healthcare system by reducing unnecessary hospitalisation and investigations in females at low-risk of pre-eclampsia.

ETHICAL APPROVAL:

An ethical approval was received from IRB of Chughtai Institute of Pathology, Lahore (Ref. Letter No. CIP/IRB/1089) on 26\textsuperscript{th} October 2021.

PATIENTS’ CONSENT:

Patients’ consents were obtained before the blood collection and history, and patients were assured that all information would be kept confidential while publishing the data.

COMPETING INTEREST:

The authors declared no competing interest.

AUTHORS’ CONTRIBUTION:

AR: Main author, literature search, data collection, data analysis, article writing.
MDK: Critical review, overall supervision of the study.
HB: Statistical analysis, proof-reading.
ASC, OC: Overall supervision of study and final approval.
SS: Data collection.
All authors approved the final version of the manuscript to be published.
REFERENCES


