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**University College Cork, Ireland**  
Coláiste na hOllscoile Corcaigh

A prospective study of placental growth factor in twin pregnancy and development of a dichorionic twin pregnancy specific reference range

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**Running Title:** Placental growth factor in twin pregnancy

## **Abstract**

*Objective:* The aim of this study was twofold; to develop a dichorionic twin pregnancy specific reference range for placental growth factor, and to compare gestational specific placental growth factor levels in twin pregnancies later complicated by preeclampsia, hypertensive disorder of pregnancy or fetal growth restriction to controls

*Design:* Prospective observational study

*Setting:* Single large tertiary maternity unit in Ireland

*Population or Sample:* Women with a twin pregnancy

*Methods:* Consenting pregnant women, across a variety of gestations, had a single blood sample taken at one time point only during their pregnancy. The plasma was initially biobanked and PIGF was measured later in batches using the point of care Triage® PIGF test

*Main Outcome Measures:* Development of preeclampsia, hypertensive disorder of pregnancy or fetal growth restriction

*Results:* PIGF levels in uncomplicated dichorionic twin pregnancies were significantly lower in the women who later developed preeclampsia than in the controls at all gestational intervals. In those that later developed any hypertensive disorder of pregnancy median PIGF was lower only in those recruited before 24 weeks' gestation

1 while in infants with a customised birthweight below the 3<sup>rd</sup> centile, PIGF was lower  
2 only in those sampled after 24 weeks' gestation.

3  
4 *Conclusions:* PIGF levels in twin pregnancy differ significantly between those women  
5 with a pregnancy that will later be complicated by preeclampsia and those that will not.  
6 This difference is present many weeks before clinical signs or symptoms of disease  
7 are present. Using cross sectional values from uncomplicated twin pregnancies, we  
8 have developed a dichorionic twin pregnancy specific reference range for PIGF.

9  
10 *Funding:* The Irish Centre for Fetal and Neonatal Translational Research (INFANT),  
11 Cork, Ireland provided financial support for this study. The funder had no role in the  
12 study design; collection, analysis or interpretation of data; or in the writing of the report;  
13 or in the decision to submit the article for publication

14  
15 *Keywords:*

- 16 • Hypertensive Disorders of Pregnancy
- 17 • Placental Growth Factor
- 18 • Preeclampsia
- 19 • Reference Range
- 20 • Twin Pregnancy

21  
22 **Tweetable Abstract:** PIGF levels in twin pregnancy differ significantly between  
23 women that will later develop preeclampsia and those that will not.

## **Introduction**

Preeclampsia is a common complication of pregnancy characterised by new onset hypertension and either proteinuria or other maternal organ dysfunction after 20 weeks' gestation (1). Along with other hypertensive disorders of pregnancy (HDP), it is a major contributor to maternal and neonatal morbidity and mortality (2, 3). Potentially serious maternal morbidity may arise in the form of seizures, cerebral haemorrhage, renal failure, liver rupture and disseminated intravascular coagulation (4). The only definitive treatment for preeclampsia is removal of the placenta, often resulting in iatrogenic pre-term delivery and subsequent fetal morbidity (5). Women with a twin pregnancy are at a two to three fold increased risk of developing preeclampsia, possibly due to a combination of larger placental mass and use of assisted reproductive therapy (ART), especially use of non-autologous gametes (6-8). Rates of twin pregnancy have risen over the last number of decades globally (9-12).

Although the exact aetiology of preeclampsia is not fully understood, a growing body of evidence suggests that an imbalance of angiogenic factors of placental origin play a crucial role in its development (13-17). Placental growth factor (PlGF) is an angiogenic protein and a member of the vascular endothelial growth factor family (18). Studies in singleton pregnancies have shown lowered levels of PlGF and increased levels of its soluble receptor sFlt-1 in maternal plasma, weeks prior to the clinical onset of preeclampsia (19, 20). The UK National Institute for Clinical Excellence (NICE) advocates PlGF testing, combined with routine clinical care, to help rule out pre-term preeclampsia in singleton pregnancies (21). A number of international randomised control trials (RCTs) are currently on-going, investigating the clinical impact of the integration of PlGF into clinical care pathways (22). The international INSPIRE trial evaluated the use of sFlt-1/PlGF ratio in women presenting with suspected

1 preeclampsia and showed that use of the ratio, in conjunction with standard clinical  
2 practice, significantly improved clinical precision without changing admission rates  
3 (23). The UK PARROT study, demonstrated a reduction in time taken to diagnosis  
4 preeclampsia and reduced maternal morbidity when PIGF is integrated into clinical  
5 care algorithms (24).

6 Few studies to date have evaluated the levels of circulating angiogenic factors during  
7 twin pregnancy. In those that have been described, huge variations exist in; the  
8 primary outcome (i.e preeclampsia, fetal growth restriction or other adverse clinical  
9 outcome); the definition/classification of the primary outcome; the gestational age at  
10 time of sampling; and the immunoassay used for quantification. (25-33). The aim of  
11 this study was twofold; to develop a dichorionic twin pregnancy specific reference  
12 range for PIGF and secondly to compare gestational specific PIGF levels in twin  
13 pregnancies complicated by preeclampsia, any hypertensive disorder of pregnancy  
14 (HDP) or fetal growth restriction to controls.

## 16 **Materials & Methods**

### 17 **Setting and Design**

18 This study was conducted in a single maternity hospital in Ireland with over 8000  
19 deliveries per annum. The study was a prospective cross-sectional cohort study of  
20 PIGF in twin pregnancy. From the start of July 2015 to the end of December 2017,  
21 women attending the hospital's dedicated twin pregnancy clinic were approached to  
22 participate in the study. Any woman with an uncomplicated twin pregnancy from 12+0-  
23 36+6 weeks' gestation inclusive, without signs/symptoms or a diagnosis of  
24 preeclampsia was eligible for inclusion. Those with complications such as a known

1 congenital anomaly in either baby, severe early onset growth restriction or twin-to-twin  
2 transfusion syndrome (TTTS) were excluded from recruitment. Following informed  
3 patient consent, a 3ml ethylenediaminetetraacetic acid (EDTA) blood sample was  
4 taken, centrifuged, divided into aliquots and the plasma biobanked at -80C within 3  
5 hours of sampling. All sampling, processing and biobanking was carried out within the  
6 same building according to previously published Standard Operating Procedures  
7 (SOPs) (34). Women had venepuncture performed at one random gestational time  
8 point only. Clinically relevant outcome data such as the diagnosis of any HDP (chronic  
9 hypertension, gestational hypertension, preeclampsia or superimposed preeclampsia)  
10 and infant birthweights were taken from medical notes following delivery. Anonymised  
11 clinical and demographic data pertaining to the participant and their offspring were  
12 recorded in the study database. For our study, the NICE definitions of hypertensive  
13 disorders of pregnancy were utilised (35). Fetal growth restriction was calculated based  
14 on actual birthweight, gestation at birth, fetal gender and maternal ethnicity, parity and  
15 BMI using the Gestation Related Optimal Weight (GROW) centile calculator (36).  
16 Patients were not involved in the development of this research study and a core  
17 outcome set was not used.

#### 18 Placental Growth Factor Immunoassay

19 Biobanked plasma samples were analysed in batches for circulating levels of PIGF  
20 using a point of care immunoassay; the Triage® PIGF test (Quidel Inc., San Diego).  
21 This test is not routinely available in the hospital for clinical use. It was purchased by  
22 our research centre for the purpose of this study. The test manufacturers had no part  
23 in the study design, conduct, analysis or manuscript development. The immunoassay  
24 was performed as per manufacturer's instructions, in a single freeze thaw cycle to  
25 minimise protein denaturation. The results are displayed on the meter screen in



approximately 15 minutes and have a measurable range from 12-3000 pg/ml. The Triage® has a reported measurable range from 12-3000 pg/ml. The manufacturers report total precision on plasma controls at concentrations of 85.2 and 1300 pg/mL as 12.8% and 13.2% respectively. For the purposes of this study, any result obtained <12 pg/ml was allocated the value of 10 pg/ml.

## Statistics

SPSS Version 23 and Stata 15 were used to analyse the data.

Part 1: Descriptive statistics were employed to examine the baseline maternal demographics, clinical outcomes and the PIGF distribution in the cohort. When developing a reference range for PIGF, all cases where a stillbirth was diagnosed in either of the twins as well as cases where any form of HDP later developed were removed. Secondly in order to facilitate development of a reference range for PIGF in an uncomplicated dichorionic twin pregnancy all monochorionic twin pregnancies were removed. Lastly, cases that developed fetal growth restriction resulting in both twins having a customised birthweight of less than the 3<sup>rd</sup> centile were then removed. The remaining women were divided according to gestational age at recruitment and PIGF ranges calculated for each gestational week were calculated. A heterogeneous regression of log(PIGF) on gestational age was used to develop a reference range for PIGF in uncomplicated dichorionic twin pregnancies.. The mean of log(PIGF) was modelled using fractional polynomials and the variance was modelled as proportional to some power of gestational age (37). The residuals were assumed to follow a Normal distribution at each gestational age. Centiles of log(PIGF) were exponentiated to yield centiles

Part 2: To examine the effect of hypertensive disorders and placental dysfunction on PIGF, the entire cohort including abnormal cases, was divided into 2 groups based on the woman's gestational age at time of her enrolment to the study and hence sampling of maternal plasma PIGF; <24 weeks' gestation and  $\geq 24$  weeks' gestation. This gestational cut-off was employed as pregnancy related hypertensive complications are unusual prior to this timepoint and also it equated well with the median of the cohort.

## **Results**

In total, 275 women with a twin pregnancy were recruited. There were no withdrawals or losses to follow up. Three women (1% of the cohort) had a stillbirth occur in one of the twins while in 4.7% (n=12) of women, an anomaly of one or both twins was diagnosed. Given with twin pregnancy there is differing placental volumes present dependent on chorionicity, circulating levels of PIGF may also vary in line with chorionicity. We found that PIGF was lower in monochorionic twin pregnancy (data not shown) but given the high incidence of complications as well as the small numbers present (n=40) in this subgroup, further analysis was not possible. We limited our analysis to dichorionic cases only for development of the reference range.

### **Part 1:**

#### ***Reference Range Demographics***

Removal of those with an abnormal pregnancy outcome (preeclampsia or HDP in the mother, stillbirth of either twin or where both twins had a customised birthweight of <3<sup>rd</sup> centile at delivery), or a monochorionic pregnancy left 173 women with an uneventful

dichorionic twin pregnancy for inclusion in the reference range analysis (Supplemental Material Figure 1). Median maternal age was 34 years, booking BMI was  $<30\text{Kg/m}^2$  for the majority (81.5%;  $n=141$ ) and most were Caucasian (93.6%;  $n=162$ ). Over half of the group were multiparous (56.1%;  $n=97$ ), just over a third (35.1.1%;  $n=60$ ) had conceived the twin pregnancy with use of ART. All women with pre-existing renal disease or essential hypertension developed superimposed preeclampsia in their pregnancies and hence were not included in the reference range cohort (Supplemental Material Table 1). Comparison of participant characteristics between each gestational group showed no significant difference in enrolment characteristics (Supplemental Material Table 2).

#### *Reference Range Development*

The 3<sup>rd</sup>-97<sup>th</sup> centiles of PIGF as a function of gestational age concentrations were calculated (Table 1). With progressing gestational age the median PIGF was seen to rise, simultaneously to the development and maturation of the placentae, peaking at 25 weeks gestation, and then steadily decreased towards term. The lowest acceptable PIGF value for each gestational week is presented (Figure 1). Removal of cases where women developed any form of HDP or where both twins had a customised birthweight  $<3^{\text{rd}}$  centile did not alter the reference range significantly. These data provide a valid reference range for PIGF in a normal dichorionic twin pregnancy (Figure 2).

#### Part 2:

##### *Comparison of Gestational PIGF*

The second aim of this study was to compare gestational PIGF in twin pregnancies complicated by preeclampsia, HDP or customised birthweight of both twins  $<3^{\text{rd}}$  centile, to controls. To this end, the entire cohort ( $n=275$ ) was divided into 2 groups

1 based on the woman's gestational age at time of her enrolment to the study and hence  
2 gestational age at time of sampling of maternal plasma PIGF; <24 weeks' gestation  
3 and  $\geq 24$  weeks' gestation. Just under half the cohort (43.6%; n=120) were recruited  
4 at <24 weeks' gestation with the remainder (56.4%, n=155) recruited at  $\geq 24$   
5 gestational weeks. The groups were then stratified by presence of preeclampsia, HDP  
6 or customised birthweight <3<sup>rd</sup> centile for both infants.

### 7 8 *Demographics of Entire Cohort*

9 The maternal age of the study group ranged from 20 to 50 years, with 134 women  
10 (48.7%) aged >35 years at booking. The majority of the cohort had a Body Mass Index  
11 (BMI) of <30Kg/m<sup>2</sup> at booking (78.9%; n=217) and were of Caucasian ethnicity  
12 (93.8%; n=258). Just under half the cohort were nulliparous (46.9%; n=129). The  
13 majority of the group were dichorionic twin pregnancies (81.5%; n=224) and  
14 approximately two thirds (65.5%; n=180) of the population studied had conceived the  
15 twin pregnancy spontaneously. Where assisted reproductive therapy (ART) was  
16 utilised, almost a fifth (17.1%; n=47) had conceived through the assistance of In Vitro  
17 Fertilisation (IVF) and a large proportion of these using a donor oocyte (12%; n=33).  
18 There was a small number of women with pre-existing renal disease or hypertension  
19 (1.8%; n=5). The two gestational groups were well matched, with no differences seen  
20 in BMI <30, ethnicity, parity or chorionicity. However, there were significantly more  
21 women with ART assisted pregnancies (40.2%; n=48 v 27.8%; n=42, p=0.04), oocyte  
22 donation (18.5%; n=22 v 7.3%; n=11, p=0.009) and those with a maternal age >35  
23 years (57.5%; n=69 v 41.9%; n=65, p=0.01) sampled in the <24 weeks' gestational  
24 group compared to the  $\geq 24$  weeks group (Supplemental Material Table 3).

## *Clinical Outcomes*

Overall, the incidence of a subsequent diagnosis of HDP was 15.3% (n=42) and of these 11.3% (n=31) developed preeclampsia (Supplemental Material Table 4). Of the 532 infants with maternal BMI information available, 11.8% (n=65) had a customised birthweight <3rd centile with both twins <3rd customised birthweight in twelve cases. Gestation at delivery ranged from 23 to 38 weeks' gestation, with two thirds of the cohort delivered via Caesarean section (66.5%, n=183). Pre-term delivery at <35 weeks occurred in almost a fifth of the cohort (17.8%, n=49) and in over half of cases was iatrogenic (59.2%, n=20). Preterm delivery at <32 weeks was less common (6.9%, n=19), and again half of cases were iatrogenic (47.4%, n=9). There were no significant differences between the two gestational groups in terms of incidence of HDP or preeclampsia, nor were there any differences in pre-term delivery or mode of delivery.

## *Comparison of PIGF*

The median PIGF was 230.5 pg/mL when sampling occurred at <24 weeks and 276 pg/mL when sampling was ≥24 weeks. The cohort was then stratified by subsequent diagnosis of preeclampsia, HDP or customised birthweight <3<sup>rd</sup> centile in both twins. PIGF levels were 0.6 times higher in the controls than in the women who later developed preeclampsia at <24 weeks gestation (247 pg/ml vs 153 pg/ml) and 2.0 times higher at >24 weeks gestation (304 pg/ml vs 99.8 pg/ml.) (Table 2). In those that subsequently developed any form of HDP, PIGF was 0.7 times higher in the >24 weeks group (250 pg/ml vs 150 pg/ml) (Table 3). In those that subsequently had either twin born at a birthweight <3<sup>rd</sup> customised centile, PIGF was 0.8 times higher in the group recruited <24 weeks (170 pg/ml vs 304pg/ml) (Table 4).

## **Discussion:**

### **Main Findings**

This study demonstrates that maternal plasma PIGF in twin pregnancy follows the same gestational pattern as described in singletons (38, 39); a steady rise corresponding with development of the placenta, peaking slightly earlier at approximately 25 weeks' gestation, and then declining thereafter. It also shows that maternal plasma PIGF is significantly lower in twin pregnancies that will later develop preeclampsia but not other HDP, independent of gestational age at time of sampling of PIGF, compared to controls. PIGF was also noted to be lower in those babies with a customised birthweight <3rd centile when maternal sampling occurred after 24 weeks gestation.

### **Interpretation**

To our knowledge, this is the largest prospective study of PIGF in twin pregnancy from a single site. This allows us to describe the twin pregnancy specific distribution of gestational PIGF, as well as develop a dichorionic specific reference range for PIGF in twin pregnancy, which has not been previously described. This is also the only study to date examining PIGF in twin pregnancies specifically using the Triage® PIGF test. The Triage® PIGF test is currently the only point of care test on the market for measuring PIGF, is CE marked and has been endorsed by NICE for use in further research (21).

Previous studies of angiogenic factors in twin pregnancy have had limited numbers of participants, varied gestations at quantification, varied outcome measures and often involve pooled results from a number of sites or countries across a variety of time periods (40-42). Often these studies require shipment of specimens to laboratories in

1 other countries, which may affect the quality of samples. In contrast, all of the  
2 laboratory analysis in our study was performed on site, by a single researcher, in a  
3 single freeze thaw cycle, to minimise the chance of protein denaturation.

4 A Spanish study in 2011 examined first trimester levels of circulating angiogenic  
5 factors in 61 women with a twin pregnancy (40). Using a R&D systems immunoassay,  
6 they reported higher serum concentrations of both PIGF and sFlt-1 in twins compared  
7 to matched singletons. They also reported maternal serum sFlt-1 levels were higher  
8 in twin pregnancies conceived through ART compared to spontaneous twin  
9 conceptions, supporting the well-accepted concept that ART pregnancies are at  
10 increased risk of preeclampsia development.

11 A study from Boston in 2012 (41) described 79 women with a twin pregnancy  
12 presenting with suspected preeclampsia in the third trimester. Serum PIGF and sFlt-1  
13 from the women was quantified using the Roche Elecsys immunoassay Ratio test.  
14 The outcome measure utilised was the diagnosis of an adverse clinical event in the  
15 subsequent fortnight, of which 52 women met the criteria. The authors reported  
16 median PIGF was significantly reduced, while median sFlt-1 was elevated in those that  
17 did develop an adverse event indicating that these angiogenic factors have potential  
18 utility as prognostic indicators in twin pregnancies with suspected preeclampsia.

19 A German group in 2014 published on a small cohort of 49 women with a twin  
20 pregnancy, 18 of which developed preeclampsia. Maternal serum PIGF and sFlt-1 was  
21 quantified again using the Roche Elecsys immunoassay Ratio test. The researchers  
22 reported PIGF levels were decreased and sFlt-1 levels increased in the preeclampsia  
23 cases at time of presentation with preeclampsia symptoms compared to the twin

controls, indicating the potential for integration of angiogenic factors into clinical care pathways for investigation of suspected preeclampsia in twin pregnancy (42).

Clearly, potential exists for use of PIGF and sFlt-1 as biomarkers for prediction of preeclampsia in twin pregnancies. However, before these biomarkers are introduced into clinical use for twins, it is important that relevant cut-offs are developed and validated specifically for this group and specific to each PIGF platform. Differences in PIGF results may arise between commercially available platforms owing to measurement of different PIGF isoforms by each assay gene (43). The Triage immunoassay predominantly measures PIGF Isoform-1, the R&D System assay detects PIGF-2 and PIGF-3 isoforms in addition to PIGF-1 while the Roche is a ratio of PIGF to sFLT-1 (44, 45).

Several large prospective observational studies have published on clinically relevant cut-offs for use in singletons. The PROGNOSIS study, using the Roche Elecsys immunoassay Ratio test in 550 women with suspected pre-eclampsia, reported a sFlt-1:PIGF ratio of  $\leq 38$  as having a negative predictive value for preeclampsia in singletons in the subsequent 7 days of 99.3% (46). The PELICAN study, using the Triage® PIGF test in 625 women with suspected pre-eclampsia, reported a PIGF of  $>100$  pg/ml as having a 98% negative predictive value for preeclampsia in the subsequent 14 days in singletons presenting at  $< 35$  weeks' gestation (47).

A 2018 Dutch study compared PIGF and sFlt-1 levels in normotensive and pre-eclamptic singleton and twin pregnancies using the Roche Elecsys immunoassay Ratio test (48). Numbers were small, with only 22 twin pregnancies included. Again, differences in serum sFlt-1 and PIGF levels were noted in the normotensive twins compared to the matched singletons and in the pre-eclamptic twin cases compared to



the twin controls. Importantly, they demonstrated that the previously defined sFlt-1/PIGF ratio cut-off of  $\leq 38$  for predicting short-term absence of preeclampsia in singleton pregnancies is not applicable to twin pregnancies. Importantly this demonstrates that established reference ranges for PIGF/sFlt-1 in singletons are not transferrable to twin or higher order multiple pregnancies. This highlights the need for quality prospective observational studies of women with twin pregnancy presenting with suspected preeclampsia, in order to develop and validate clinically useful cut-offs for PIGF/sFlt-1 in twins.

It appears there may be a role for PIGF in identification of pathologically related growth restriction in twin pregnancy, given the lower PIGF levels we observed in the maternal plasma of those women that later went on to deliver babies <3rd CBW centile. Studies in singleton pregnancies have demonstrated the utility of PIGF in discriminating pathological growth restriction from constitutional smallness and in the prediction of adverse perinatal outcomes (49, 50).

### **Strengths and Limitations.**

We recognise there are limitations to our study specifically the use of a customised birthweight centile not specific to twin pregnancy and the exclusion of cases where only both twins were <3<sup>rd</sup> customised centile. This choice was pragmatic given our numbers however we recognise that reduced placental volume in either twin may affect the circulating maternal PIGF levels. Normal twin growth patterns are the subject of much debate with differing opinion as to which is the most appropriate growth curve to use in clinical practice (51, 52). Concerns exist that twin specific growth charts, adjusted to reflect the smallness of twins compared to singletons, may not identify growth restricted twins with underlying placental pathology, thereby resulting in

increased perinatal morbidity (53). There is no consensus as to whether fetal growth charts should be customised by factors such as ethnicity, height, weight and parity or not and there is also no agreement regarding which is the most appropriate growth calculator to use (54-59).

A second limitation of the study was single sampling of participants. Serial sampling of maternal PIGF may have provided a much more robust, informative account of PIGF distribution. However repeated phlebotomy, solely for the purposes of a research study, may have deterred many women from taken part and given that participation was truly altruistic, a single timepoint only approach was adopted. Our population is largely homogenous; white Caucasian and non-obese which potentially limits extrapolation to minority ethnic groups however this is representative of the twin pregnancy population that attends our unit. Although a large number of women with a twin pregnancy were enrolled, we do not have sufficient power at present to develop a monochorionic twin pregnancy specific reference range, although it would be possible to expand on the study and add to our numbers in the future to achieve this. An additional limitation of our study is the use of only one automated commercial platform for quantification of PIGF alone and not s-FLT1, rather than on multiple commercially available platforms such as the DELFIA Xpress PIGF 1-2-3 test, Brahms Kryptor and the Roche Elecsys ratio test, as advocated by NICE (21). Comparative studies performed in singleton pregnancies have shown similar performance of all three platforms in ability to rule out preeclampsia (60). As sufficient plasma remains biobanked in our site, this is an area for potential future research subject to funding and ethical approval.

## **Conclusion**

We have shown that PIGF levels in twin pregnancy differ between those pregnancies that later will be complicated by preeclampsia and those that will not. This difference is present many weeks before clinical signs or symptoms of disease are present. We provide a valid overall reference range for PIGF in a normal twin pregnancy and specifically in a normal dichorionic twin pregnancy. With further research, PIGF has potential as an adjunct to clinical care as a predictor of evolving preeclampsia and/or adverse clinical outcomes in twin pregnancy.

## **Financial Disclosure**

All authors report no financial disclosures

## **Conflict of Interest**

All authors report no conflicts of interest

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## **Contribution to Authorship**

All authors contributed to the overall study design and specific methodologies. KOD and LK conceived and designed the study with DHR. DHR conducted the data collection with assistance from CN and EOM. DHR conducted the analysis with assistance from SM and AF. DHR drafted the manuscript with assistance from SM and

KOD. All authors have critically read, contributed with inputs and revisions and approved the final manuscript.

#### **Details of Ethics Approval**

Ethical approval for the study was granted from the Cork Research Ethics committee (ECM 3 (PPP) 19/05/15).

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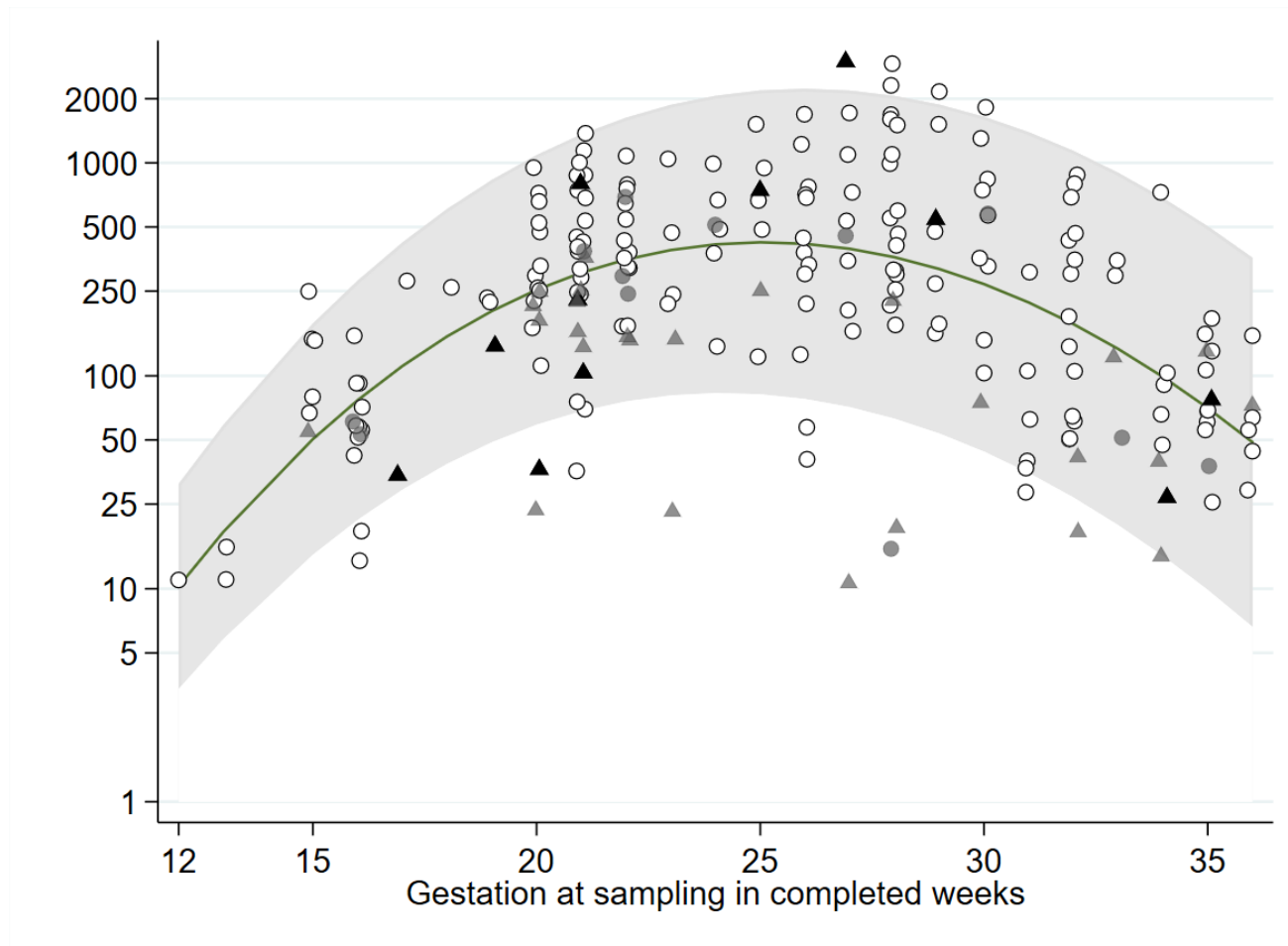
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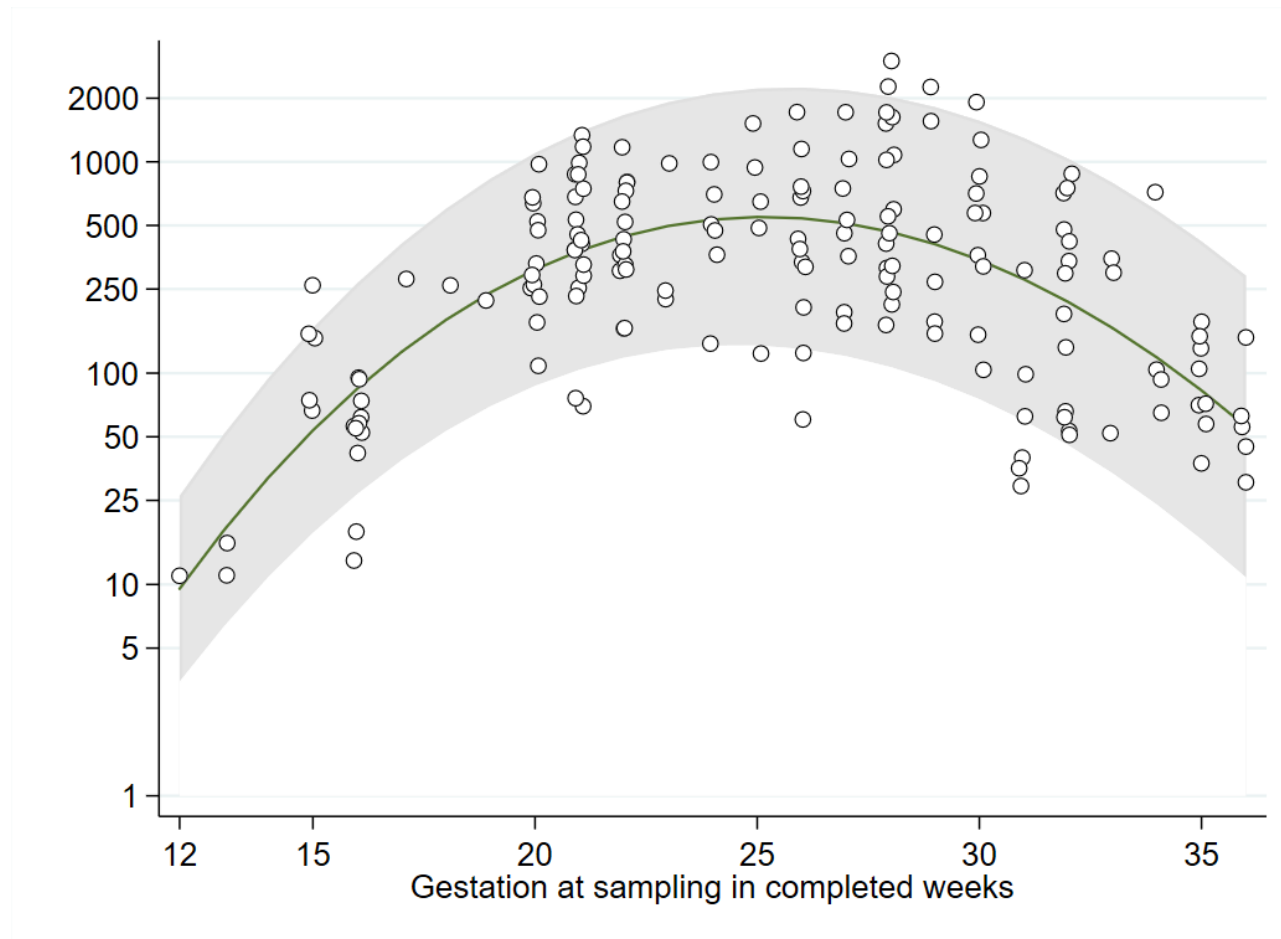


## Figure Legend



**Figure 1:** Scatter plot of gestational PIGF. Shaded area represents the reference range from the 5<sup>th</sup> to 95<sup>th</sup> percentiles (n=222).

○ uncomplicated dichorionic twin pregnancy cohort, ▲ HDP and PET present, ▲ HDP present, ● both neonates <3<sup>rd</sup> CBW



**Figure 2:** Scatter plot of gestational PIGF for the uncomplicated dichorionic twin pregnancy cohort. Shaded area represents the reference range from the 5<sup>th</sup> to 95<sup>th</sup> percentiles (n=173).

**Table 1:** Normal Reference Range percentiles of PIGF by gestational age interval quantified using the Triage® PIGF test (n=173)

Gestational age at sampling (weeks)	Percentile of PIGF (pg/ml)								
	3rd	5th	10th	25th	50 <sup>th</sup>	75th	90th	95th	97th
12	3.0	3.5	4.3	6.3	9.5	14.5	21.0	26.2	30.3
13	5.5	6.3	8.0	11.8	18.1	27.8	40.9	51.5	59.9
14	9.3	10.9	13.8	20.6	32.1	50.0	74.6	94.7	110.7
15	15.0	17.6	22.5	33.9	53.6	84.7	127.9	163.7	192.1
16	22.7	26.8	34.5	52.8	84.5	135.4	206.9	266.6	314.4
17	32.8	38.8	50.3	77.8	126.2	204.8	316.5	410.7	486.5
18	44.8	53.3	69.7	108.9	178.9	293.8	459.3	600.0	713.7
19	58.5	69.8	91.8	145.1	241.1	400.8	633.3	832.7	994.7
20	72.7	87.1	115.3	184.1	309.6	520.7	831.3	1100.0	1319.4
21	86.2	103.8	138.2	223.0	379.3	645.2	1040.7	1385.5	1668.4
22	97.9	118.3	158.5	258.2	444.0	763.7	1244.1	1666.1	2014.0
23	106.4	129.1	173.9	286.0	497.2	864.4	1422.0	1915.3	2324.1
24	110.8	134.9	182.8	303.5	533.3	936.8	1555.7	2107.4	2566.6
25	110.7	135.3	184.3	308.9	548.2	973.0	1630.8	2221.4	2715.2
26	106.2	130.2	178.3	301.6	540.7	969.5	1639.6	2245.4	2754.2
27	97.8	120.4	165.8	282.9	512.1	927.3	1582.1	2178.3	2681.0
28	86.7	107.0	148.1	255.0	466.1	852.1	1466.5	2029.5	2506.3
29	73.9	91.5	127.3	221.0	407.9	752.7	1306.5	1817.3	2251.7
30	60.6	75.4	105.3	184.4	343.4	639.6	1119.5	1565.0	1945.3
31	47.9	59.7	83.9	148.1	278.3	523.1	923.1	1296.8	1617.1
32	36.5	45.6	64.4	114.6	217.3	412.0	732.9	1034.6	1294.1
33	26.8	33.6	47.6	85.4	163.4	312.6	560.6	795.0	997.5
34	19.0	23.9	34.0	61.4	118.5	228.7	413.2	588.7	740.9
35	12.9	16.3	23.4	42.6	82.9	161.3	293.6	420.3	530.5
36	8.5	10.8	15.5	28.5	55.9	109.7	201.3	289.4	366.3

*Centiles were calculated based on a Normal distribution for  $\log(\text{PIGFR})$  where*

$$\mu = \exp(-19.8165 + 6.9434 \times \sqrt{G} - 0.01375 \times G^2)$$

$$\sigma = \exp(-1.5851 + 0.4422 \times \log(G)) \text{ and } G = \text{gestational age (weeks)}$$

**Table 2:** PIGF by gestational group at enrolment in twin pregnancies complicated by <sup>A</sup> Preeclampsia (de novo or superimposed) compared to those that were not, quantified using the Triage® PIGF test (n=275)

<b>Gestation at recruitment (weeks)</b>	<b>Median (IQR) PIGF pg/mL (n=275)</b>	<b>Median (IQR) PIGF PE<sup>A</sup> present pg/mL (n=31)</b>	<b>Median (IQR) PIGF PE not present pg/mL (n=244)</b>
<24	230.5 (79.4-437.8)	153 (54-224)	247 (81-489)
≥24	276 (71.6-577)	99.8 (24-273)	304 (73-652)

**Table 3:** PIGF by gestational group at enrolment in twin pregnancies complicated by <sup>B</sup> Hypertensive Disorder of Pregnancy compared to those that were not, quantified using the Triage® PIGF test (n=275)

<b>Gestation at recruitment (weeks)</b>	<b>Median (IQR) PIGF pg/mL (n=275)</b>	<b>Median (IQR) PIGF HDP<sup>B</sup> present pg/mL (n=42)</b>	<b>Median (IQR) PIGF HDP not present pg/mL (n=233)</b>
<24	230.5 (79.4-437.8)	150 (45-229)	250 (84-490)
≥24	276 (71.6-577)	123 (32-425)	304 (73-598)

**Table 4:** PIGF by gestational group at enrolment in offspring of twin pregnancies complicated by <sup>C</sup> birthweight <3rd customised centile compared to those that were not, quantified using the Triage® PIGF test (n=532)

<b>Gestation at recruitment (weeks)</b>	<b>Median (IQR) PIGF pg/mL (n=532)*</b>	<b>Median (IQR) PIGF pg/mL CBW &lt;3rd<sup>C</sup> (n=109)</b>	<b>Median (IQR) PIGF pg/mL CBW not &lt;3rd<sup>C</sup> (n=423)</b>
<24	230.5 (79.4-437.8)	234 (54.2-460.5)	231 (105-413)
≥24	276 (71.6-577)	170 (42.7-462)	304 (73-652)

\*BMI not available for 9 women so CBW not available for 18 offspring