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**Changes in hemoglobin F levels in pregnant women unaffected by clinical fetomaternal hemorrhage**

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**Short title: Hemoglobin F in pregnant women**

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**ABSTRACT**

Complete automation of high-performance liquid chromatography (HPLC) for determination of hemoglobin F (%Hb F) and hemoglobin A1c (%Hb A1c) levels has made this procedure available in many clinical laboratories. However, the physiological changes in %Hb F during pregnancy and the effects of physiological and supraphysiological levels of %Hb A1c on measurement of %Hb F have not been studied extensively. Simultaneous determination of %Hb F and %Hb A1c was conducted in 490 blood samples obtained before ( $n = 21$ ), during 1<sup>st</sup> ( $n = 150$ ), 2<sup>nd</sup> ( $n = 116$ ), and 3<sup>rd</sup> ( $n = 192$ ) trimesters of pregnancy, and postpartum ( $n = 11$ ) from 357 women, including 60 women with hyperglycemia but unaffected by clinical fetomaternal hemorrhage, by HPLC. Mean (SD) Hb F levels were 0.71% (0.25%) before pregnancy. The value of 0.82% (0.47%) during 1<sup>st</sup> trimester decreased significantly to 0.66% (0.35%) during 2<sup>nd</sup> trimester and to 0.58% (0.38%) during 3<sup>rd</sup> trimester. The level was 0.62% (0.31%) approximately one year after delivery. Thus, %Hb F was highest during 1<sup>st</sup> trimester of pregnancy. The effects of varied %Hb A1c levels on %Hb F measurements were clinically negligible. Our data may be used as reference intervals of %Hb F determined with HPLC during pregnancy.

**Key words:** diabetes, fetomaternal hemorrhage, hemoglobin A1c, hemoglobin F, pregnancy

**Abbreviations:** HPLC, high performance liquid chromatography; Hb, hemoglobin

## 1. INTRODUCTION

Fetal blood enters the maternal circulation even during the first trimester [1, 2]. A diagnosis of clinical fetomaternal hemorrhage (FMH) is made when fetal blood loss reaches a significant level leading to fetal anemia and clinical symptoms, such as an abnormal fetal heart rate patterns and/or fetal hydrops [3]. Although clinical FMH is rare, affected fetuses may survive with cerebral palsy, which accounts for approximately 2.0% of infants with cerebral palsy caused by antenatal and/or intrapartum hypoxic conditions [4].

Fetal red cells differ from adult red cells in the variants of hemoglobin that they contain. Hemoglobin F (Hb F) is predominantly produced in fetal life, and its synthesis is normally reduced to very low levels of less than 0.6% of total hemoglobin (Hb) in adults [5]. The Hb F is restricted to a subpopulation of red cells termed “F cells”; these cells are present at levels of 0.3% – 4.4% in 85% of the normal adult population [5].

To estimate the amounts of fetal blood entering the maternal circulation, F cells are measured as the proportion of total red cells using the Kleihauer–Betke acid elution method or flow cytometry, while Hb F is measured as a proportion of the total Hb in red cell lysates by high-performance liquid chromatography (HPLC) [6]. Complete automation of the HPLC method has made this procedure available to many clinical laboratories. In this method, Hb F can be separated from Hb A and other variant Hbs, including HbA1c, which is another Hb that is frequently determined in obstetric practice. As a high Hb F level interferes with Hb A1c measurements in some commercially available assay systems [7], Hb F levels may also be affected by Hb A1c. However, the physiological changes in %Hb F during pregnancy as determined by HPLC have not been studied extensively.

We conducted this retrospective and partly longitudinal study to determine baseline values of %Hb F in the presence of varied %HbA1c levels according to stage of pregnancy.

## 2. MATERIAL AND METHODS

This partly longitudinal, and partly cross-sectional study was conducted with the approval of the Institutional Review Board of Hokkaido University Hospital. All 357 pregnant participants gave birth between January 2011 and July 2012 at our institution and were free from clinical fetomaternal hemorrhage (FMH) (Table 1). Of the 357 women, 12 (3.4%) were known to be complicated with diabetes mellitus and 48 (13.4%) were diagnosed with gestational diabetes mellitus in the current pregnancy (Table 1). A total of 490 blood

samples obtained pre-pregnancy, during pregnancy, and post-pregnancy from the 357 women were used for simultaneous determinations of both %Hb F and %Hb A1c.

Measurement of %Hb F and %Hb A1c in the EDTA anticoagulated blood samples was performed with a Tosoh HLC-723 G8 analyzer (Tosoh Co., Tokyo, Japan) fully automated HPLC system using reagents and conditions specifically designed to separate and quantify Hb F and Hb A1c. The intra- and interassay coefficients of variation were  $< 3.3\%$  for %Hb F and  $< 0.8\%$  for %Hb A1c.

Data are presented as means  $\pm$  standard deviation. Statistical analyses were performed using the JMP8© statistical software package (SAS, Cary, NC). Differences in the means were tested using the paired *t* test or the Tukey–Kramer HSD (honestly significant difference) test between each group. Correlations between continuous variables were analyzed using the restricted maximum likelihood method. In all analyses,  $P < 0.05$  was taken to indicate statistical significance.

### 3. RESULTS

In a longitudinal study of 21 women for whom data before pregnancy were available, %Hb F level did not show a consistent change with the establishment of pregnancy; mean %Hb F level during the 1<sup>st</sup> trimester did not differ significantly from that determined before pregnancy (Fig. 1, left panel). In a cross-sectional study, %Hb F level declined significantly during pregnancy (Fig. 1, middle panel) in the absence of a significant change in Hb A1c level (see legend for Fig. 1). In a longitudinal study of 11 women for whom data after pregnancy were available, %Hb F level tended to increase after delivery ( $P = 0.0667$ ) (Fig. 1, right panel). In an analysis of 49 women for whom data of both 1<sup>st</sup> and 3<sup>rd</sup> trimesters were available, the majority of women exhibited a decrease in %Hb F level and none exhibited an increase in %Hb F by more than 0.1% (Figs. 2, 3). Level of %Hb A1c did not change significantly in this population again (see legend for Fig. 2). Women with a higher %Hb F level during the 1<sup>st</sup> trimester were significantly more likely to exhibit a larger decrease in %Hb F during pregnancy (Fig. 3). The %Hb F levels ranged from 0.1% to 2.9% with a median value of 0.7% during the 1<sup>st</sup> trimester, while values ranged from 0.1% to 2.8% with a median %Hb F value of 0.5% during the 3<sup>rd</sup> trimester (Fig. 4).

**[Hb F levels did not differ between primiparous and multiparous women \(data not shown\).](#)**

A weak but statistically significant negative correlation between %Hb F and %Hb A1c levels was observed (Fig. 5). However, mean %Hb F levels did not differ significantly between 475 samples with %Hb A1c level < 6.5% (range, 0.2% – 3.2%) and 15 samples with %Hb A1c level  $\geq$  6.5% (range, 0.3% – 1.4%) ( $0.68\% \pm 0.41\%$  vs.  $0.55\% \pm 0.27\%$ , respectively,  $P = 0.2250$ ).

#### 4. DISCUSSION

The results of the present study indicated that %Hb F level was highest during 1<sup>st</sup> trimester of pregnancy and declined gradually toward 3<sup>rd</sup> trimester. Although a weak, but statistically significant negative correlation was observed between measurements of %Hb F and %Hb A1c determined using the Tosoh HLC-723 G8 analyzer, the effect of %Hb A1c level < 9.9% on measurement of %Hb F was considered negligible in clinical practice.

In this study, %Hb F level was significantly elevated during 1<sup>st</sup> trimester compared with those during 2<sup>nd</sup> and 3<sup>rd</sup> trimesters, consistent with the results of previous studies in which the proportion of red cells containing Hb F peaked during pregnancy between 2 and 7 months of gestation [8, 9]. Although elevation of %Hb F level occurs as a result of either FMH or an increase in the number of adult F cells [6], the increase in red cells containing Hb F during the earlier stages of pregnancy has been suggested to be due to an increased number of adult F cells [8, 9]. However, fetal red cells entering the maternal circulation may contribute to some extent to the elevation of %Hb F level in the 1<sup>st</sup> trimester.

In a study of pregnant women in the 1<sup>st</sup> or 2<sup>nd</sup> trimester using a flow cytometry immunophenotyping technique [10], fetal red cells defined as those showing high levels of Hb F expression accounted for 0.01% – 0.015% of the total number of circulating red cells in 5 of 21 control women not undergoing invasive tests and 0.015% – 0.15% in 53% of 153 women after invasive tests, such as chorionic villous sampling and amniocentesis [10]. Fetal red cells during the 1<sup>st</sup> trimester exclusively contain Hb F [11], while adult F cells contain both Hb F and Hb A, with a variable proportion of Hb F ranging up to 25% of the total Hb in adult F cells [12]. The correlation between %Hb F level and %F cells among non-pregnant adult subjects suggests that an increase in number of adult F cells by 1.0% results in an increase in %Hb F by 0.1% [13] implying that Hb F accounts for an approximately 10% of the total Hb in adult F cells. Thus, as Hb F content per cell may be 10-fold higher in fetal red cells than in adult F cells, an increase in the number of fetal red cells by 0.01% in adults, as seen in normal women in 1<sup>st</sup> and 2<sup>nd</sup> trimesters [10], may correspond to an increase in %Hb F of 0.1% in pregnant women. Thus, physiological FMH may have contributed partly to the elevation of 1<sup>st</sup> trimester Hb F levels.

Some investigators may think that Hb F level is higher in multiparous women than in primiparous women. Fetal red cells in the maternal circulation are shown to be detectable up to 119 days in a postpartum follow-up study [14]. As time interval after the previous childbirth until the current pregnancies may have been longer than 119 days in the majority women, effect of parity on the Hb F level was not seen in this study.

Generally, an increase in the number of adult F cells occurs as a result of an increased demand for red cells [5]. As pregnancy is accompanied by a marked increase in the circulating blood volume by 40% [15], acute erythropoiesis that may occur in 1<sup>st</sup> trimester may also have contributed to the elevation of 1<sup>st</sup> trimester Hb F levels seen in the present study. However, a significant increase in %Hb F level during 1<sup>st</sup> trimester compared with that before pregnancy was not confirmed in this study, perhaps partly due to the small size of the study population.

The incidence of FMH > 75 mL (> 150 mL whole blood) is approximately 1 in 3000 deliveries as determined by the acid elution test [16]. Determination of %Hb F by HPLC is suggested to be of limited clinical value as a diagnostic tool for clinical FMH [6]. HPLC separates and quantifies the types of Hbs in hemolyzed blood samples, but does not differentiate between fetal red cell Hb F and adult F cell Hb F. Chambers et al. [6] reported that %Hb F level rarely exceeds a cut-off level of 5.0%, set for antenatal screening of thalassemia [17], even in cases with clinical FMH. However, the prevalence of thalassemia is very low among Japanese women and clinical problems related to thalassemia are hardly encountered in obstetric practice in Japan. As shown in Fig. 4, the frequency of pregnant women with %Hb F levels > 3.0% was < 1.0% among Japanese women in the present study. In addition, none of the women exhibited elevation of %Hb F by more than 0.1% during pregnancy. These observations may be helpful in suggesting clinical FMH in women exhibiting %Hb F > 3.0% or %Hb F elevation > 0.1% during pregnancy. Although the reason why %Hb F decreases in the later stages of pregnancy remains to be determined, it may be due to deceleration of erythropoiesis during the latter half of pregnancy.

In conclusion, this retrospective study of 357 pregnant women unaffected by clinical fetomaternal hemorrhage determining %Hb F and %Hb A1c levels indicated that %Hb F was highest during the 1<sup>st</sup> trimester and declined toward the 3<sup>rd</sup> trimester. Our data may serve as reference intervals for %Hb F during pregnancy determined by HPLC.

## **ACKNOWLEDGEMENT**

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## **DISCLOSURE**

The authors have no conflicts of interest.

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## FIGURE LEGENDS

### **Fig. 1: Changes in the %Hb F levels before, during, and after pregnancy**

The %Hb F and %Hb A1c levels were determined simultaneously in 490 blood samples from 357 women (data for %Hb A1c are not shown in this figure). Each line without vertical bar indicates change in %Hb F level for an individual case. Of the 150 women examined during the 1<sup>st</sup> trimester, 21 were examined before pregnancy (at  $20.4 \pm 21.6$  weeks prior to the establishment of pregnancy), giving mean (SD) %Hb F levels, 0.71% (0.25%) before pregnancy and 0.75% (0.30%) during the 1<sup>st</sup> trimester (at  $10.5 \pm 1.7$  weeks of gestation) for these 21 women (left panel). The mean %Hb (SD) A1c levels at the corresponding times were 5.77% (1.02%) and 5.52% (0.63%), respectively. Totals of 150, 116, and 192 women were examined during the 1<sup>st</sup> (at  $10.8 \pm 1.4$  weeks of gestation), 2<sup>nd</sup> (at  $21.1 \pm 4.0$  weeks of gestation), and 3<sup>rd</sup> trimesters (at  $32.3 \pm 2.9$  weeks of gestation), giving mean (SD) %Hb F levels of 0.82% (0.47%), 0.66% (0.35%), and 0.58% (0.38%), respectively (middle panel) and mean (SD) %Hb A1c levels of 5.37% (0.42%), 5.20% (0.47%), and 5.44% (0.42%), respectively. Of the 192 women examined during the 3<sup>rd</sup> trimester, 11 were examined after pregnancy ( $338 \pm 77$  days after delivery), giving mean (SD) %Hb F levels of 0.52% (0.20%) during the 3<sup>rd</sup> trimester ( $33.5 \pm 2.8$  weeks of gestation) and 0.62% (0.31%) after pregnancy (right panel) for these 11 women. The mean (SD) %Hb A1c levels at the corresponding times were 5.64% (0.29%) and 5.72% (0.64%), respectively.

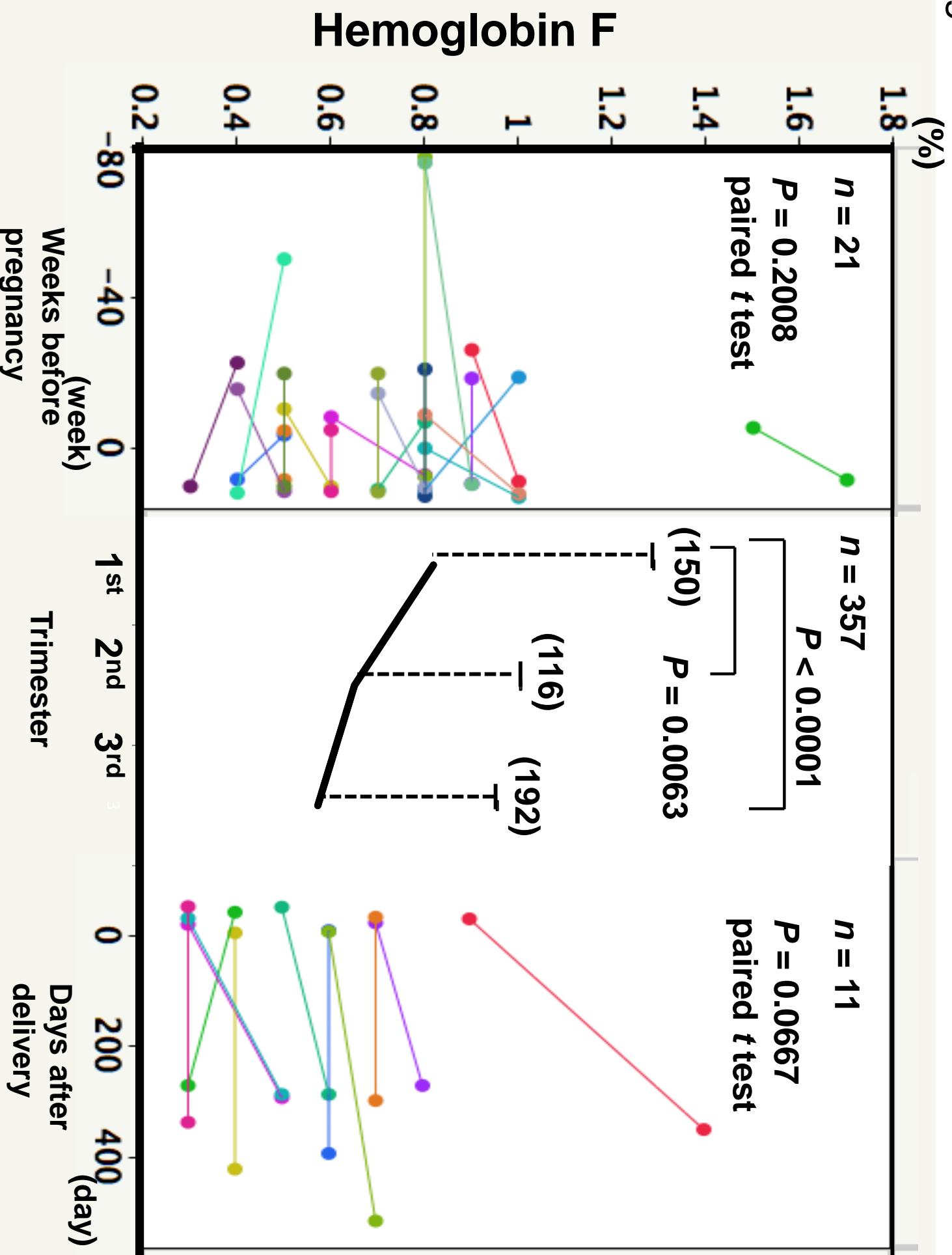
### **Fig. 2: Longitudinal changes in %Hb F levels during pregnancy in 49 women**

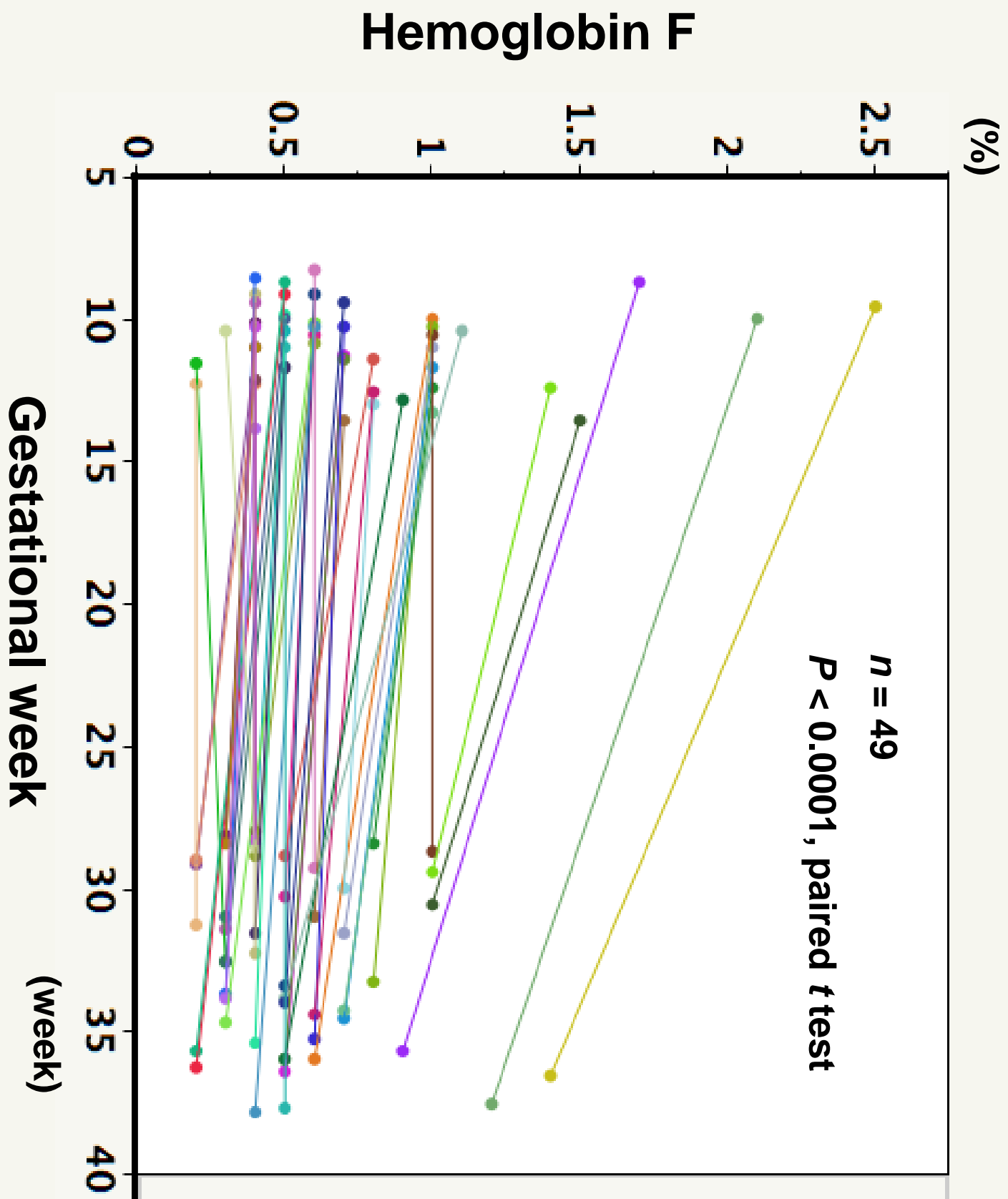
Of the 357 women, 49 women were examined during both 1<sup>st</sup> and 2<sup>nd</sup> trimesters. Each line indicates change in %Hb F level for an individual case. In the 49 women, mean (SD) %Hb F level of 0.74% (0.46%) in the 1<sup>st</sup> trimester declined significantly to 0.52% (0.27%) in the 3<sup>rd</sup> trimester, while mean (SD) %Hb A1c level did not change significantly over the period from 5.61% (0.61%) to 5.59% (0.47%).

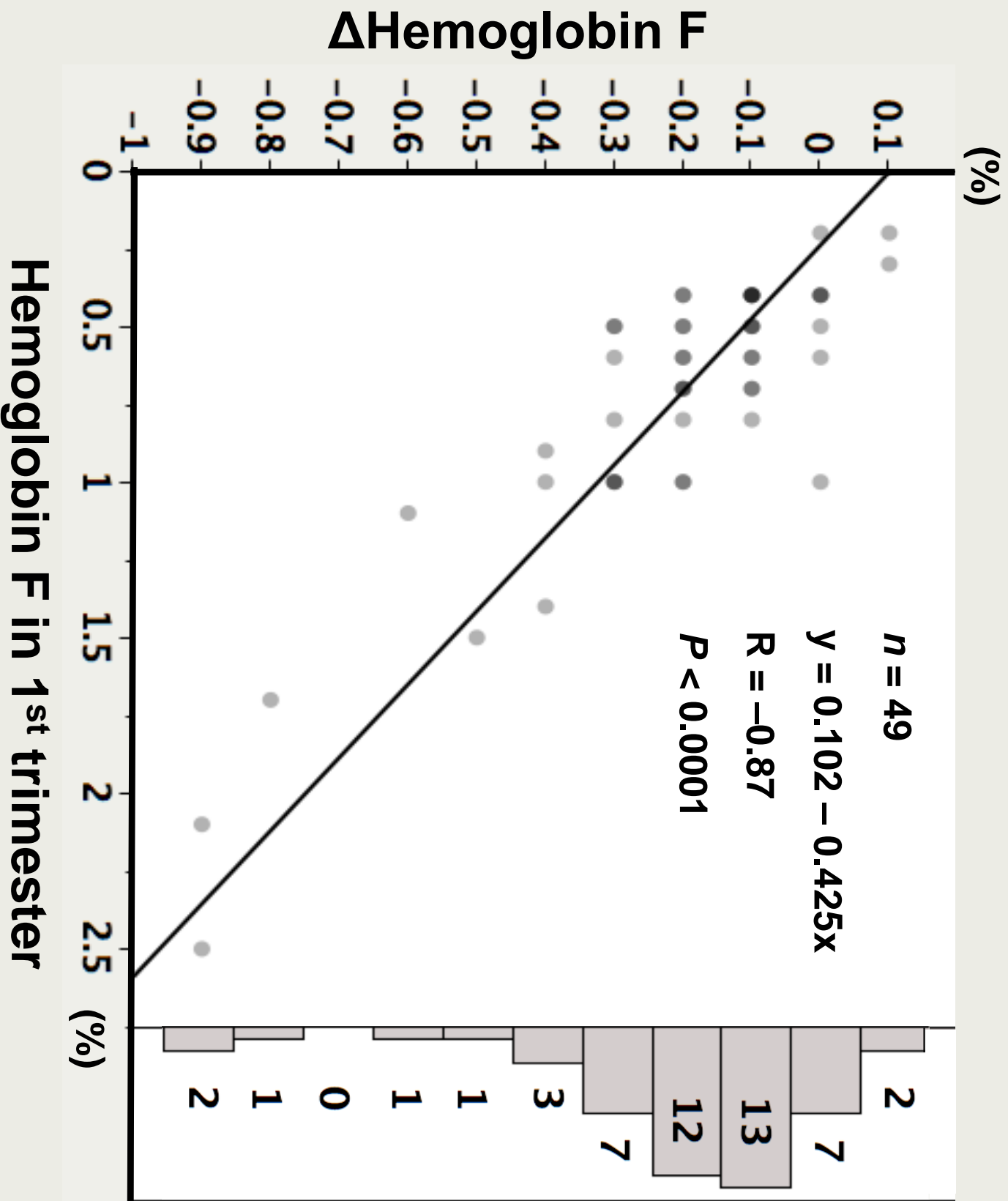
### **Fig. 3: Correlation between %Hb F levels during the 1<sup>st</sup> trimester and net decreases in %Hb F levels during pregnancy**

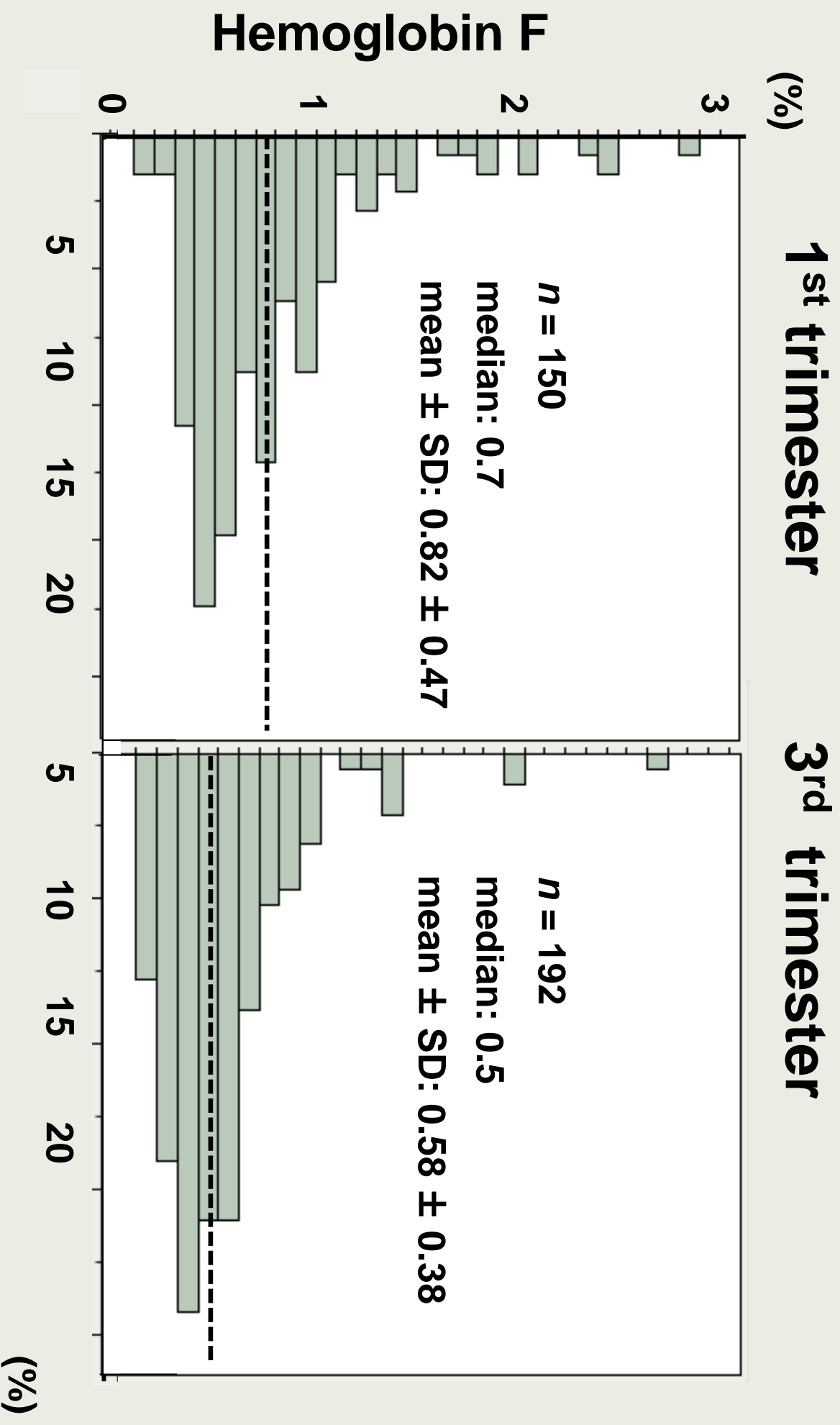
### **Fig. 4: Distribution of %Hb F levels determined during 1<sup>st</sup> and 3<sup>rd</sup> trimester**

### **Fig. 5: Association between levels of %Hb F and %Hb A1c in 490 blood samples from the 357 women**









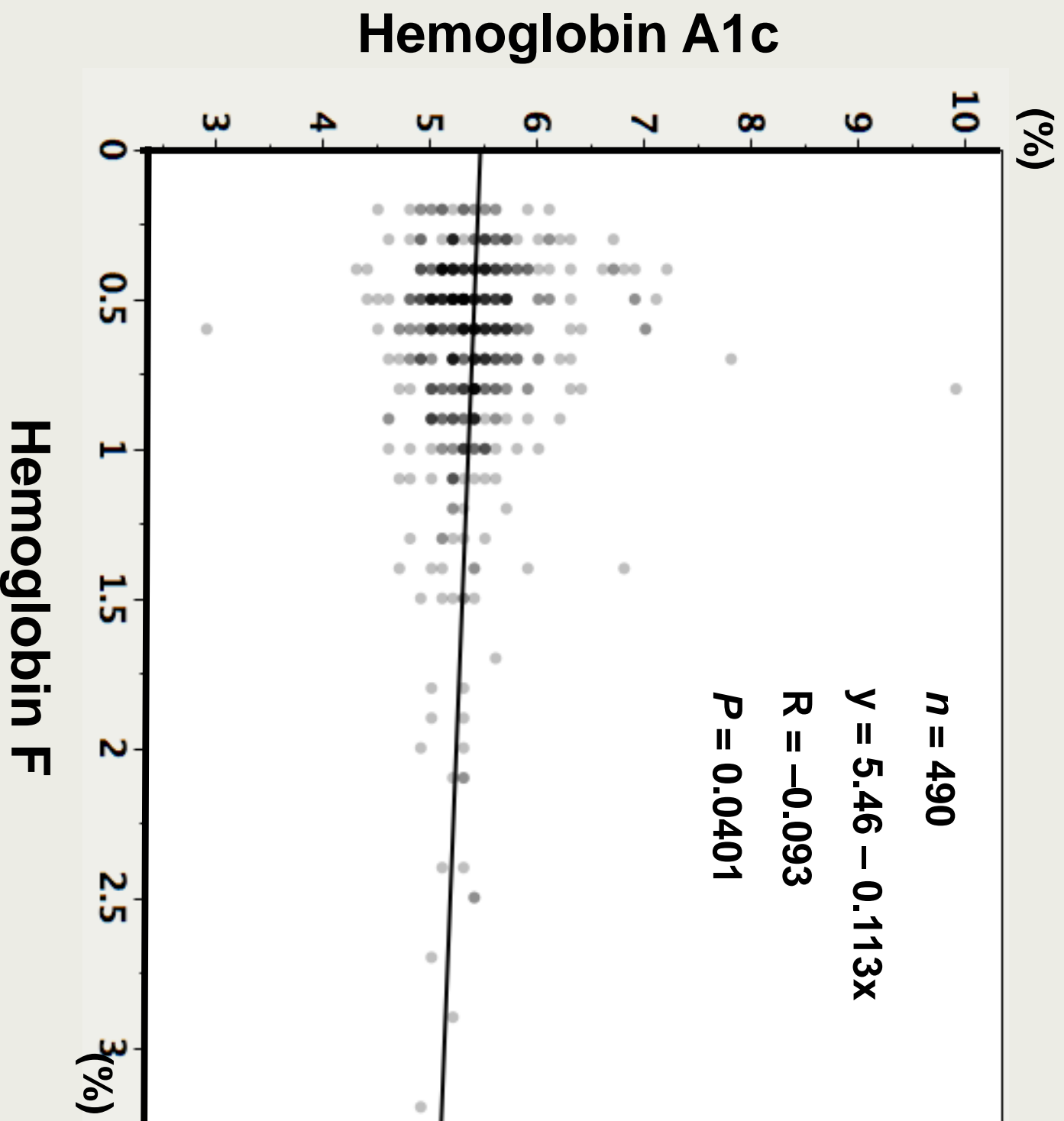




Table 1. Clinical background of the 357 subjects

Age (years)	32.4 <del>4</del> ± ± 5.2
Nulliparity	211 (59.1%)
Pre-pregnancy BMI (kg/m <sup>2</sup> )	22.0 ± ± 4.3
Diabetes mellitus	12 (3.4%)
Gestational diabetes	48 (13.4%)
Twin	22 (6.2%)
Triplet	3 (0.8%)
Delivery (week)	37.6 ± ± 3.2
Birth weight (g)	2701 ± ± 692

~~Diabetes mellitus did not include gestational diabetes or overt diabetes in pregnancy.~~

BMI, body mass index

書式変更: フォントの色 : 青