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Reference ranges of AMH and interaction with placental biomarkers in early pregnancy: the Generation R Study, a population-based prospective cohort study

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Keywords: Ovarian reserve, placental biomarker, nomogram, early pregnancy, human Choriogonadotrophin (hCG), soluble FMS-Like Tyrosine kinase-1 (sFLT), Placental Growth Factor (PLGF).

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Abstract

Objective: The primary objective of this study is to establish maternal reference values of AMH in a fertile multi-ethnic urban pregnant population and to evaluate the effect of gestational age. The secondary objective of this study is exploring the association between AMH and placental biomarkers.

Design: This study was embedded in the Generation R Study, an ongoing population-based prospective cohort study from early pregnancy onwards.

Setting: City of Rotterdam, the Netherlands, out of hospital setting.

Patients: In 5806 women serum AMH levels were determined in early pregnancy (median 13.5 weeks; 95% range 10.5-17.2).

Intervention(s): None.

Main outcome measures: Maternal AMH levels in early pregnancy and its association with placental biomarkers, including human Chorionic Gonadotrophin (hCG), soluble FMS-Like Tyrosine kinase-1 (sFLT), and Placental Growth Factor (PLGF).

Results: A nomogram of AMH in early pregnancy was developed. Serum AMH levels showed a decline with advancing gestational age. Higher AMH levels were associated with a higher level of the placental biomarkers hCG and sFLT in early pregnancy. This last association was predominantly mediated by hCG. AMH levels were negatively associated with PLGF levels.

Conclusion: In this large study we show that AMH levels in early pregnancy decrease with advancing gestational age. The association between AMH and the placental biomarkers hCG, sFLT and PLGF suggests a better placental development with a lower vascular resistance in mothers with higher AMH levels. Hence AMH might be useful in predicting adverse pregnancy outcome due to impaired placental development.

Keywords: Ovarian reserve, placental biomarker, nomogram, early pregnancy, human Choriogonadotrophin (hCG), soluble FMS-Like Tyrosine kinase-1 (sFLT), Placental Growth Factor (PLGF).

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein from the transforming growth factor-beta family and is produced by granulosa cells of antral and preantral follicles (1, 2). AMH plays an important role in ovarian function and folliculogenesis and is believed to be the best biomarker of so called ovarian reserve (1, 3). AMH has basically three different functions in the human ovary. First, it inhibits the recruitment of follicles from the primordial follicle pool. Second, it inhibits follicle stimulating hormone (FSH)-induced aromatase activity thereby increasing intra-ovarian androgen concentrations with a consequent decrease in estrogen levels. Finally, AMH decreases the individual sensitivity to FSH of large antral follicles, thereby inhibiting the selection of the dominant follicle (1).

The ovary-specific expression pattern in granulosa cells of growing non-selected follicles makes AMH an ideal indirect marker for the size of the ovarian follicle pool (2). Serum AMH is believed to be the best marker of the ovarian reserve (1, 4). It is widely used as a predictor of ovarian response in controlled ovarian stimulation and to predict age-at-menopause as well as primary ovarian insufficiency (5-7). Besides the role of AMH as a predictor of menopause and response to ovarian stimulation its role is more and more being explored in different fields, including the association with pregnancy complications and outcomes (8-10).

Studies suggest that AMH levels can fluctuate substantially during the menstrual cycle (11-14). A few studies have described AMH levels during pregnancy, with conflicting results (15-18). Some studies conclude that AMH levels remain stable throughout pregnancy (16-18), whilst others reported a more dynamic role for AMH with a decrease with advancing gestational age (15, 18-20).

Early pregnancy is characterized by a complex interplay between placental biomarkers and steroid hormones. Placental biomarkers such as soluble fms-like tyrosine kinase-1 (sFLT), human chorionic gonadotrophin (hCG) and placental growth factor (PLGF) are known to be important representatives of placental (dys)function (21). These biomarkers have been associated with pregnancy complications such as pre-eclampsia, SGA and preterm birth (22-24). Some studies also suggest that decreased preconception AMH levels might be correlated with adverse pregnancy outcome (8, 25, 26). The interplay between AMH and placental function, reflected by these biomarkers, could therefore be of interest (22).

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Considering the controversy about AMH concentrations during pregnancy and the possible correlation between AMH and adverse pregnancy outcome, we studied AMH levels in a large prospective cohort of almost 6000 pregnant women. We aimed to establish reference intervals for serum AMH and to evaluate the effect of gestational age on AMH serum levels. We also studied the association between serum AMH and sFLT, hCG and PLGF.

Materials and methods

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands (27). Pregnant women with an expected delivery date from April 2002 to January 2006 were enrolled in the study. In total, 8976 women were enrolled during pregnancy, of whom AMH measurements in early pregnancy (<18 weeks) were available in 6183 subjects. We excluded women who participated more than once in the Generation R study (n=377). Thus, the population for analysis included 5806 women (Figure 1). Written informed consent was obtained from all participants. The general design, all research aims and the specific measurements in the Generation R study have been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31).

Hormonal assays

As previously described, maternal serum samples were obtained in early pregnancy (median 13.4 weeks; 90% range 10.5-17.2) from pregnant women with an expected delivery date from April 2002 to January 2006. The venous samples were taken by research nurses and stored at room temperature before being transported to the regional laboratory for processing and storage for future studies (STAR-MDC). Processing was planned to finish within a maximum of three hours after venous puncture. The samples were centrifuged and thereafter stored at −80°C (28).

AMH measurements were preformed using the AnshLabs pico AMH ELISA (AnshLabs, Webster, Tx, USA). All measurements were performed according to standard procedures between January 2018 and February 2020. The samples were thawed and measured on the same day. Loss of signal for AMH due to prolonged storage at -80° was deemed negligible given our experience with in-house used quality control materials (29). During the study, kit controls as well as pooled serum controls were used to assure accuracy. Coefficients of variation were 2.9% at 0.3 ng/mL and 6.2% at 0.1 ng/mL, respectively,

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for kit controls. For pooled serum controls, coefficients of variation were 5.4% at 0.8 ng/mL and 7.1% at 0.2 ng/mL, respectively.

Information was available for the following early pregnancy biomarkers: hCG, PLGF and sFLT. hCG was analyzed in serum using a solid-phase two-site chemiluminescent immunometric assay, calibrated against WHO 3rd IS 75/537, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA) (30). Measurements were performed in 2009. These biomarkers are stable for many years when storage at -80°(31-33). The interassay coefficient of variation was 8.0, 6.3 and 5.1 % at the concentration of 9.7, 53.1 and 821.5 IU/L, respectively (34). PLGF and sFLT were analyzed in plasma, using an immune-electrochemiluminescence assay on the Architect System. The between-run coefficients of variation for PLGF were 4.7% at 24 pg/mL and 3.8% at 113 pg/mL. The coefficients for sFLT were 2.8% at 5.5 ng/mL and 2.3% at 34.0 ng/mL (35, 36).

Covariates

Gestational age was established using data from the first ultrasound examination (37). Information on possible determinants (sociodemographic factors, life style habits as smoking and obstetrical history) was obtained from questionnaires. Sociodemographic factors included information on age, educational level and ethnicity. Ethnic background was derived from the country of birth of the woman herself and her parents. For this study we divided the women in two groups 'Caucasian' and 'other ethnicities' (38). Educational level was assessed by the highest completed education and classified into three categories: 1) primary education; 2) secondary education; and 3) university or college (39). Body mass index (BMI; in kg/m^2) was calculated from length and weight measured at enrolment. Obstetrical history included information on parity and fertility treatment.

Statistical analyses

Non-parametric specific reference ranges (RR) were determined by the 2.5^{th}-97.5^{th} percentiles for each year of maternal age. The model-based AMH reference ranges for maternal age were created using

Generalized Additive Models for Location, Size and Shape (GAMLSS). These specific statistical tools enable flexible, (semi) parametric, reference range calculations while accounting for skewness and kurtosis of the data during the modelling process. We used 2 cubic splines for maternal age, 3 cubic splines for sigma variation and a Box Cox t family distribution (after sensitivity analyses using Akaike Information Criterion and worm plots) in order to achieve the best fit (40). Subsequently, age specific Z-scores and 2.5th, 50th and 97.5th values were derived from the model. We applied the same technique for model-based AMH reference ranges for gestational age. We used two cubic splines for gestational age, no cubic splines for sigma variation and also a BCT distribution in order to achieve the best fit. Next, associations between AMH and several early pregnancy biomarkers (hCG, PLGF, and sFLT) were analyzed using multivariate linear regression analyses. Since levels of these biomarkers significantly changed during gestation, we constructed hCG, PLGF and sFLT, gestational-age adjusted standardized Multiple of the Median (MoM) scores, which we used in these analyses. MoM scores >3.0 were excluded from these analyses. The models were adjusted for maternal age, smoking, BMI, education level, maternal ethnicity (Caucasian and 'other ethnicities'), parity and fetal sex. Multivariable linear regression analyses were performed utilizing three restricted cubic splines for hCG, PLGF and sFLT, maternal age and BMI. Mediation analyses were additionally performed for hCG, PLGF and sFLT. Standardized direct and unstandardized indirect effects were computed for each of 5000 bootstrapped samples, and the 95% confidence interval was computed by determining the indirect effects at the 2.5th and 97.5th percentiles. All statistical analyses were done with SPSS version 28.01.0 (142) for Windows or R statistical software, version 3.6.1.

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Results

AMH levels during early pregnancy

The final study population consisted of 5608 pregnant women (Figure 1) of whom AMH measurements in early pregnancy (<18 weeks) were available.

Descriptive characteristics of the study population are shown in Table 1. The included women (n=5806) had a median gestational age of 13.4 weeks (range 10.5-17.2) and had a mean (SD) age of 29.6 (±5.1) years. Of the included women, 60.4% were Caucasian and the median BMI at intake was 23,6 kg/m2 (90% range 19.2 – 33.4). Of all women 36.6% had overweight or were obese. Most women (60.2%) were pregnant of their first child and a minority (1.3%) achieved pregnancy through assisted reproductive technologies (ART) (Table 1).

AMH in early pregnancy versus maternal age

Population-based, maternal age specific reference ranges for AMH in pregnancy are shown in Table 2. AMH reference ranges (μg/L) were calculated according to a population-based approach in the whole study population per maternal age category (years [yrs]). In addition, model based reference centile curves are depicted in Figure 2. Serum AMH levels seemed to remain rather constant until the age of 25 years. After 25 years of age we observed a steady decline.

AMH in early pregnancy versus gestational age

Throughout gestation we observed a decline in serum AMH between 8 and 12 weeks of gestation (Table 3 and Figure 3). AMH reference ranges (μg/L) were calculated according to a population-based approach in the whole study population per gestational age category (weeks gestation [wks]

Association of serum AMH levels with markers of placental function

Next, we analyzed the association of serum AMH levels with markers of placental function. Table 4 shows the decline in AMH serum levels during early pregnancy coinciding with an increase in hCG, PLGF and sFLT.

Over the full spectrum, there was a significant positive association between AMH levels and hCG ($P < 0.0001$) as well as sFLT ($P < 0.05$). Higher AMH levels were associated with higher hCG and sFLT levels. On the contrary, AMH was negatively associated with PLGF levels ($P < 0.01$). (Figure 4).

We used mediation analyses to examine the mediation impact of hCG on the relationship between placental biomarkers (sFLT and PLGF) and AMH. We identified that the relationship between sFLT and AMH was fully mediated by hCG (Figure S1). The relationship between PLGF and AMH was not mediated by hCG (Figure S2).

Discussion

Main Findings

In this large cohort study, a nomogram of AMH serum concentrations during early pregnancy was developed, demonstrating that serum AMH levels decrease already early in the first trimester of pregnancy. Finally, this decrease in AMH levels seems to be associated with a significant decrease in PLGF and an increase in hCG and sFLT levels.

Strengths and limitations

A major strength of this prospective cohort study is the large sample size and the long term follow up. To the best of our knowledge this is the largest study in the field addressing AMH serum concentrations in early pregnancy. In this study AMH was measured in the population at different gestational age (62,1% gestational age <14 weeks). We demonstrated a suppression of AMH levels already early in pregnancy. A potential limitation of this study is the spread of gestational age at enrollment. Knowing that gestational age lead to different suppression of AMH levels. An important limitation of this study is the absence of an AMH measurement before pregnancy. Another limitation of this study is the fact

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that we not have blood samples available from all the initial participants, therefore we could not determine AMH levels of all participants of Generation R. This can lead to selection bias. We looked at the differences between both groups (included women n=5608 and excluded women n=2793). There are small differences. The most remarkable difference is that the women with a known AMH level have e more favorable BMI profile and probably reflecting the more healthier group.

Interpretation

Our results confirm the results of other studies, showing that AMH levels decline during early pregnancy. Most studies reported a decline of AMH levels from the late first trimester of pregnancy onwards (20, 41, 42). Others observed a decline earlier in pregnancy, between 7 and 14 weeks of gestation (43). The analyzed AMH levels in those studies were not adjusted for maternal age, smoking, BMI, education level, maternal ethnicity, parity or fetal sex. The decrease in AMH levels during pregnancy is probably due to the suppression of the hypothalamic pituitary gonadal axis which leads to a change in follicle dynamics and a decrease in AMH levels (44). Indeed Durlinger et al. showed that suppression of the hypothalamic pituitary ovarian axis using a GnRH antagonist in mice leads to different follicle class distribution and a different AMH expression. Due to low FSH levels, the growth of the small follicles is slower and the granulosa cell mass seems to be less resulting in lower AMH levels (44). Moreover, combined oral contraceptive pill use suppresses the hypothalamic pituitary-ovarian axis through an increase in negative feedback and thereby inhibits FSH and LH release from the pituitary preventing dominant follicle selection causes similar changes in AMH output (45, 46). During pregnancy the gonadotropin dependent stages of folliculogenesis are also inhibited. Indeed, the ovary seems to be suppressed in pregnancy mimicking the prepubertal quiescent state (47). Hence, AMH serum concentrations decrease from early pregnancy onwards due severely depressed FSH as well as LH levels caused by high serum levels of estrogen and progestogens originating from the corpus luteum and later on from the placenta. Indeed, Koniger et al. found an AMH decline during pregnancy followed by a rapid increase of AMH to near pre-pregnancy levels within a few days after delivery (20).

Different other underlying mechanisms of AMH suppression in pregnancy have been explored including the influence of fetal sex and maternal BMI. Stojsin-Carter et al. found a trend that fetal-sex was linked with differences in maternal AMH levels in cattle. That might be driven by a decrease in maternal AMH production coupled with sex-dependent fetal AMH production (48). In a large study performed in a healthy general female population, AMH was negatively related to BMI, the relationship was age dependent. AMH levels decreased and BMI increased with age. The correlation between AMH and BMI was secondary to the stronger relationship of the two variables with age (49). Part of the observed reduction in AMH levels during pregnancy could also be explained by the pregnancy-associated hemodilution and increased plasma-protein binding (50).

The rapid increase in AMH levels post-partum suggests a physiological cross-talk between the corpus luteum and later on via the placenta (through sex steroid feedback) on the one hand and the ovary (through reduced secondary cyclic recruitment of follicles) on the other hand resulting in suppressed AMH serum levels during pregnancy (20). Moreover, since the menstrual cycle is not restored immediately after delivery and during the puerperium it also suggests that placental factors might play a role in suppressing AMH levels during pregnancy. Placental biomarkers cross-talk with other organs, such as the thyroid, pituitary and the ovary. hCG causes the so called “luteal rescue” and a subsequent increase in estrogen and progesterone production in the corpus luteum until the luteo-placental shift takes place (51). Hence it is the indirect driver of the suppression of the GnRH pulse generator during pregnancy and contributes in that way to the decrease in pituitary gonadotrophins causing a decrease in AMH.

Fetal growth is dependent on adequate development of the placenta (52). Korevaar et al. showed that a higher hCG MoM was associated with a higher placental weight (53). Other important placental biomarkers, associated with placental (dys)fynction, are sFLT and PLGF. Impaired angiogenesis and vasculogenesis in early pregnancy compromises placental and embryonic development (52). sFLT is an anti-angiogenic factor that binds to free circulating vascular endothelial growth factor and PLGF, thereby inhibiting blood vessel growth (22). PLGF is the most abundantly regulated angiogenic factor in first trimester decidua (54). In other studies it has been demonstrated that higher sFLT levels in early

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pregnancy are associated with lower placental vascular resistance leading to higher placental weight as well as birth weight (22). The positive association, we observed, between AMH, hCG and sFLT was fully mediated by hCG.

The negative association between AMH, hCG and PLGF was not mediated by hCG. Upregulation of PLGF leads to the activation of an inflammatory state, with a subsequent release of different cytokines. These cytokines can modulate the cells of the immune system and could therefore interfere with adequate vascular development of the placenta (55). PLGF supports the early events of implantation and placental development. Upregulation of PLGF leads to the activation of an inflammatory state, with a subsequent release of different cytokines. These cytokines can modulate the cells of the immune system and could therefore interfere with adequate vascular development of the placenta (55).

Taken together the significant association between AMH and the placental biomarkers sFLT mediated by hCG suggests that higher AMH levels are coinciding with a lower vascular resistance in the early placental bed. Similarly, higher AMH levels are associated with lower PLGF levels and this might prevent the release of cytokines that interfere with proper placental development. Hence AMH might be useful in predicting adverse pregnancy outcome due to impaired placental development. The Generation R study population is a relatively healthy group. The incidence of preeclampsia was 2,2%, and IUGR 1,9%. Overall is the incidence of preeclampsia 2-8% (56). We found no significant correlation between AMH and preeclampsia (OR 0,96) or IUGR (OR 1,04). Moreover, studies that assess AMH levels pre-pregnancy, during gestation, and postpartum may help to better understand the mechanism of how the ovary might influence the placenta and vice versa and how this interaction impacts on follicular recruitment during pregnancy and after delivery. Prospective pregnancy studies that evaluate maternal and pregnancy outcomes in addition to other biomarkers in pregnancy are important to better understand how AMH is related to maternal and fetal outcomes of pregnancy.

Conclusion

AMH levels in pregnancy decrease with advancing gestational age. Higher AMH levels are associated with a better placental development and a lower vascular resistance in the early placental bed. The

underlying mechanism may be due to the cross-talk with placental biomarkers. AMH is significantly associated with placental biomarkers as hCG and PLGF. The significant association between AMH and sFLT was mediated by hCG. Those biomarkers are correlated with placental development. Therefore, they are potential candidates for predicting adverse pregnancy outcomes.

Declaration of interest

J.S.E.L. has received fees or grant support in the most recent 5-year period from the following organizations (in alphabetical order): Ansh Labs, Dutch Heart Foundation, Dutch Medical Research Counsel (ZonMW), Ferring, Roche Diagnostics and Titus Healthcare.

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Author contribution statement

As part of the Generation R study research project, this study was planned and designed by JL, ES, SS, YL and RD. SB and BL preformed all the laboratory tests. SV RD, AA and SS preformed the statistical analysis. RD, SS, YL, TK and JL interpreted the data and wrote the manuscript. All authors contributed substantially to revisions of the manuscript and approved the final version.

Details of Ethical approval

The study was approved by the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam, the Netherlands (MEC 198.782/2001/31). Written informed consent was obtained from all participants.

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Figure legends

Figure 1. Population for current study – AMH and pregnancy

Figure 2. Maternal age specific reference ranges for AMH

Maternal age (years) specific reference ranges for AMH levels (µg/L). AMH reference values were calculated according to a (semi) parametric 2.5th-97.5th percentiles by GAMLSS modeling in a population-based approach. Colored lines depict the maternal age specific centiles for AMH levels.

Figure 3 Gestational age specific reference ranges for AMH

Gestational age (weeks) specific reference ranges for AMH levels (µg/L). AMH reference values were calculated according to a (semi) parametric 2.5th-97.5th percentiles by GAMLSS modeling in a population-based approach. Colored lines depict the maternal age specific centiles for AMH levels.

Figure 4. Associations of AMH with hCG, sFLT and PLGF

Graphs show the significant associations between maternal levels of hCG, sFLT and levels of AMH with 95% confidence interval. Analyses were performed on gestational age specific Z-scores for AMH and gestational age specific Multiple of the Median (MoM) scores for hCG and sFLT. MoM scores >3.0 were excluded from the analyses. Analyses were additionally adjusted for maternal age, educational level, ethnicity, parity, BMI, smoking and fetal sex. Analyses were performed using linear regression analyses utilizing two restricted cubic splines for hCG, $P< 0.001$. Analyses were performed using linear regression for sFLT, $P\ 0.02$.

Graphs show the significant associations between maternal levels of PLGF levels of AMH with 95% confidence interval. Analyses were performed on gestational age specific Z-scores for AMH and gestational age specific Multiple of the Median (MoM) scores for PLGF. MoM scores >3.0 were excluded from the analyses. Analyses were additionally adjusted for maternal age, educational level, ethnicity, parity, BMI, smoking and fetal sex. Analyses were performed using linear regression for PLGF, $P=0.01$.

Figure S1 Relationship between sFLT and AMH as mediated by hCG

The total effect (c) is significant. The direct effect (c') of sFLT on AMH was not significant and fully mediated by hCG. * P value< 0.05.

Figure S2 Relationship between PLGF and AMH as mediated by hCG

The total effect (c) is significant. The direct effect (c') of PLGF on AMH is significant and therefore not mediated by hCG. * P value< 0.05

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Table 1. Baseline characteristics (n = 5806)

Outcome	Women
Gestational age at blood sampling, median (90% range), (weeks)	13.4 (10.5; 17.2)
<8.00	0.5
8.01-10.00	2.6
10.01-12.00	17.3
12.01-14.00	41.7
>14.00	37.9
Age mother at enrollment, mean (SD), (years)	29.6 (5.1)
<25	20.0
25-30	28.6
30-35	37.9
>35	13.4
Ethnicity, %	
Caucasian	60.4
Non-Caucasian	39.6
Education level, %	
Primary education	10.3
Secondary education	46.1
University or college	43.6
BMI at intake, median (90% range), (kg/m2)	23.6 (19.2-33.4)
<25kgm2	63.4
25-30kg/m2	24.7
>30kg/m2	11.9
Smoking	
Never smoked	71.5
Smoked until pregnancy	9.6
Continued smoking in pregnancy	18.9
Pregnant %	
Spontaneously	98.7
ART	1.3
Parity	
Nulliparous	60.2
Para-1	27.4
Para-2 or more	12.4

Abbreviations: BMI, body mass index. Values are valid percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (90% range) for continuous variables with a skewed distribution.

Table 2. Maternal age specific reference ranges for AMH

Age (years)	n	Median	2.5th	97.5th
<16	4	2.952	0.723	8.215
16	7	2.920	0.710	8.162
17	30	2.888	0.696	8.109
18	58	2.856	0.683	8.055
19	107	2.825	0.669	8.000
20	127	2.792	0.655	7.947
21	167	2.757	0.640	7.896
22	191	2.720	0.622	7.848
23	224	2.680	0.602	7.803
24	244	2.635	0.579	7.760
25	280	2.586	0.553	7.719
26	291	2.531	0.523	7.682
27	291	2.470	0.490	7.648
28	382	2.403	0.455	7.608
29	416	2.329	0.419	7.553
30	487	2.246	0.383	7.467
31	504	2.153	0.348	7.340
32	464	2.051	0.312	7.174
33	407	1.942	0.278	6.979
34	341	1.826	0.243	6.760
35	259	1.705	0.210	6.522
36	167	1.581	0.178	6.263
37	121	1.455	0.149	5.983
38	94	1.327	0.122	5.685
39	59	1.202	0.098	5.373
40	30	1.079	0.077	5.047
41	20	0.960	0.059	4.706
42	17	0.845	0.045	4.346
43	7	0.732	0.033	3.957
>43	3	0.621	0.024	3.529

AMH reference ranges (μg/L) were calculated according to a population-based approach in the whole study population per maternal age category (years [yrs]).

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Table 3. Gestational age specific reference ranges for AMH

Gestational Age (wks)	n	Median	2.5th	97.5th
5	3	3.809	0.643	11.809
6	4	3.592	0.593	11.236
7	10	3.373	0.544	10.657
8	34	3.152	0.497	10.038
9	79	2.930	0.451	9.418
10	167	2.712	0.408	8.798
11	453	2.505	0.367	8.202
12	1258	2.325	0.333	7.684
13	1173	2.186	0.305	7.298
14	900	2.094	0.285	7.054
15	670	2.037	0.270	6.925
16	502	1.989	0.256	6.829
17	388	1.946	0.244	6.744
18	159	1.898	0.232	6.419

AMH reference ranges (μg/L) were calculated according to a population-based approach in the whole study population per gestational age category (weeks gestation [wks]).

Table 4. AMH and placental biomarkers according to gestational age

Weeks of gestation	**4.0-6.0**	**6.1-8.0**	**8.1-10.0**	**10.1-12.0**	**12.1-14.0**	**14.1-16.0**	**16.1-18.0**	
n	**4**	**29**	**163**	**1088**	**2452**	**1350**	**777**	***P values***
AMH (**ug/L)**	2.87 (1.56-3.10)	3.51 (0.48-8.70)	2.89 (0.70-7.93)	2.27 (0.53-6.64)	2.11 (0.42-5.88)	1.96 (0.37-5.73)	1.93 (0.42-5.65)	<0.005
hCG (IU/L)	3659 (455-8077)	60887 (22716-137849)	75533 (33133-129909)	58234 (25731-106628)	49844 (23379-94075)	33525 (14324-72545)	23410 (8154-52436)	<0.005
PLGF (pg/ml)	12.30 (8.80-13.30)	14.00 (8.10-500.30)	19.85 (12.20-33.24)	28.30 (15.14-58.00)	37.20 (19.16-87.90)	67.80 (30.10-163.40)	113.50 (49.10-252.69)	<0.005
sFLT (ng/ml)	0.21 (0.12-0.36)	3.99 (1.13-14.26)	5.18 (2.45-12.08)	5.08 (2.38-11.27)	5.07 (2.29-11.84)	5.25 (2.14-12.78)	5.17 (2.05-13.05)	<0.005

Abbreviations: hCG, human chorionic gonadotropin; PLGF, placental growth factor; sFLT, soluble fms-like tyrosine kinase-1.Values are medians (90% range) for continuous variables with a skewed distribution. Presented values are not imputed. Differences between different groups of gestation were tested through one-way ANOVA.

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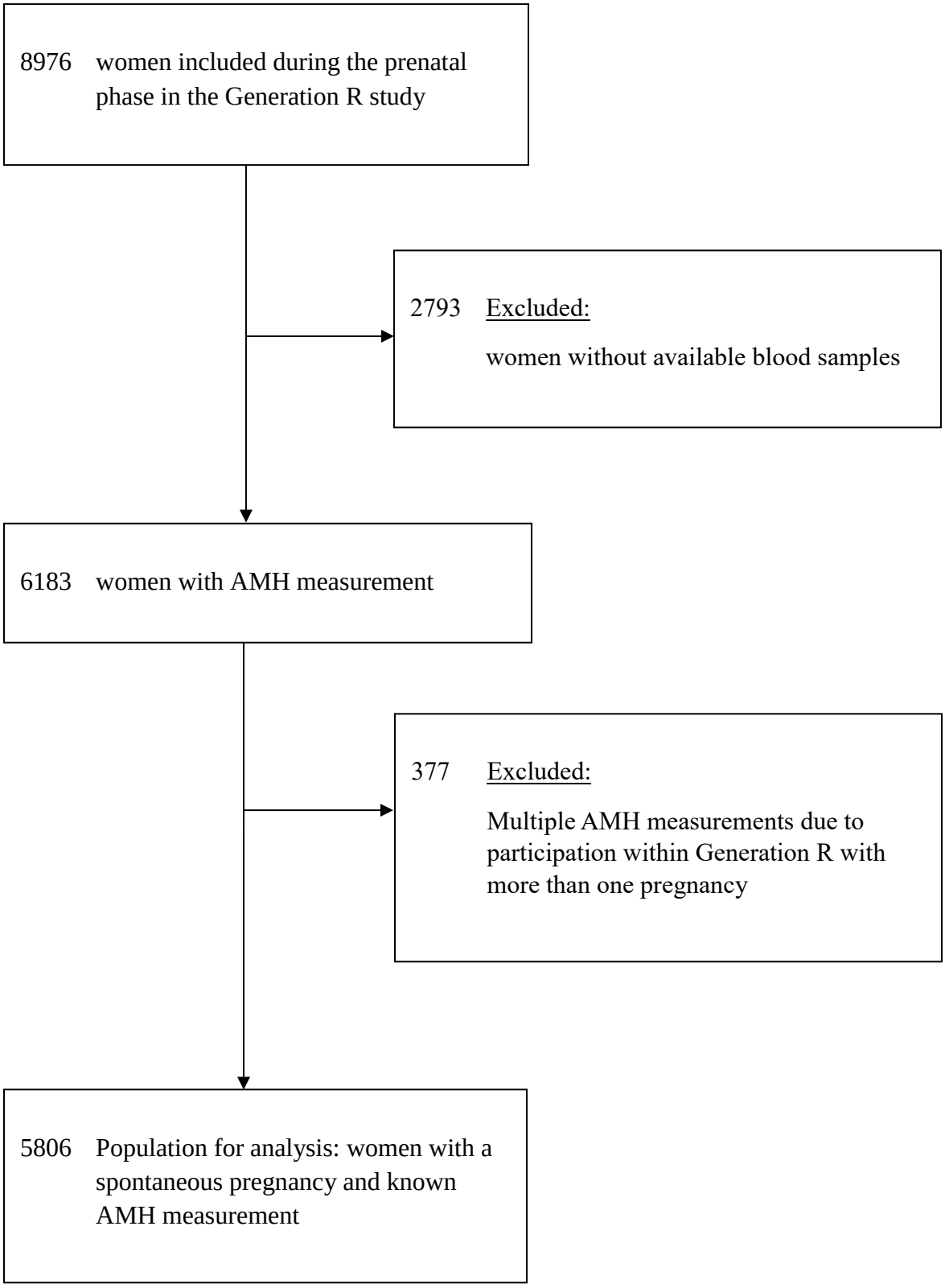

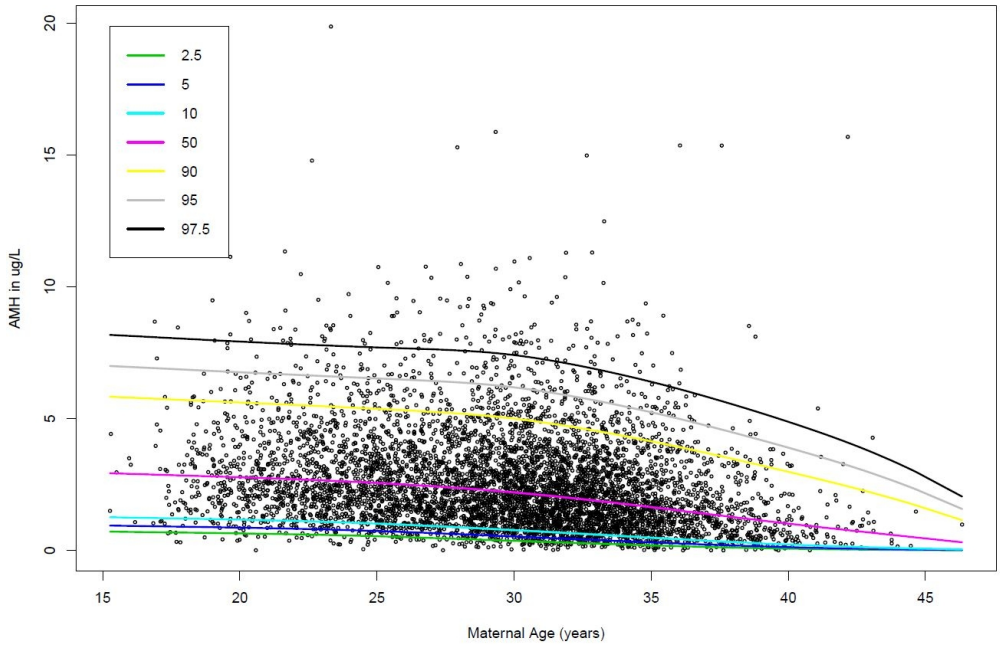

Maternal age specific reference ranges for AMH

Maternal age (years) specific reference ranges for AMH levels (µg/L). AMH reference values were calculated according to a (semi) parametric 2.5th-97.5th percentiles by GAMLSS modeling in a population-based approach. Colored lines depict the maternal age specific centiles for AMH levels.

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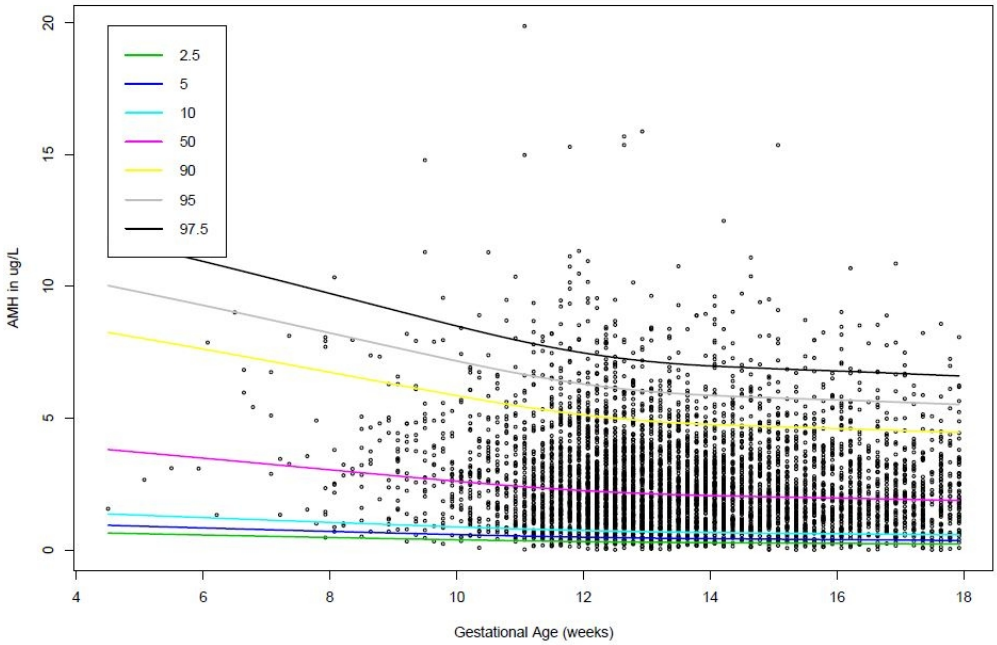

Gestational age specific reference ranges for AMH

Gestational age (weeks) specific reference ranges for AMH levels (µg/L). AMH reference values were calculated according to a (semi) parametric 2.5th-97.5th percentiles by GAMLSS modeling in a population-based approach. Colored lines depict the maternal age specific centiles for AMH levels.

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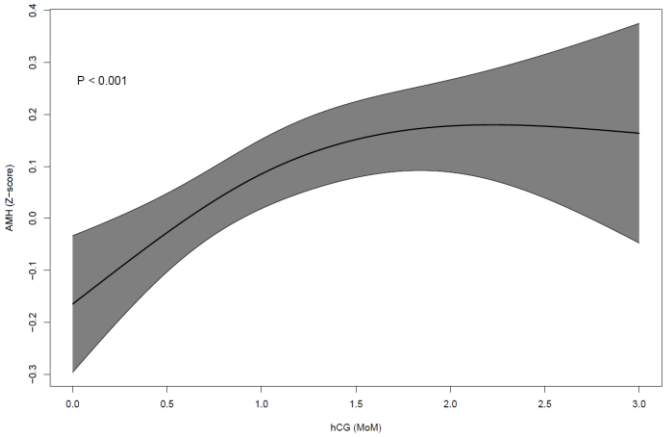

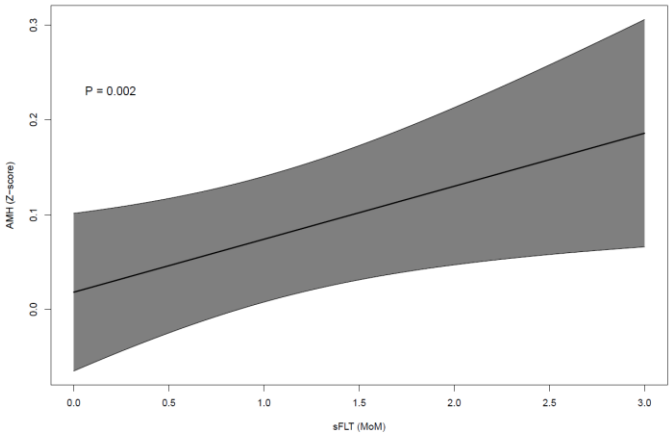

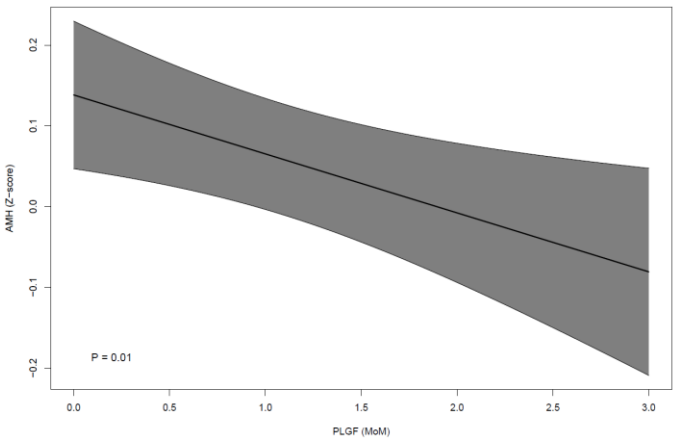

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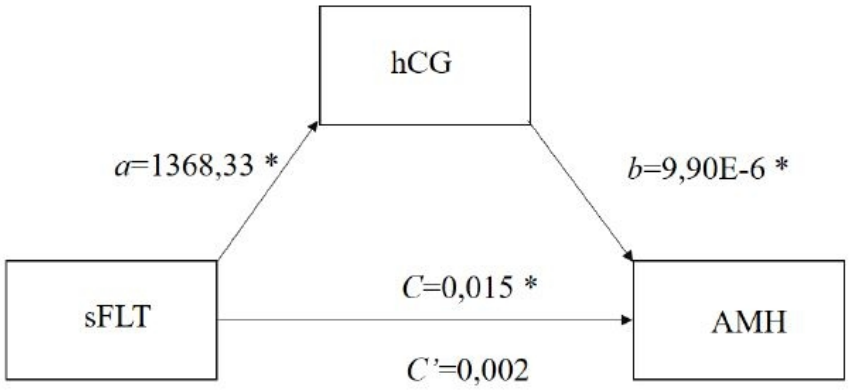

Relationship between sFLT and AMH as mediated by hCG

The total effect (c) is significant. The direct effect (c') of sFLT on AMH was not significant and fully mediated by hCG. * P value< 0.05.

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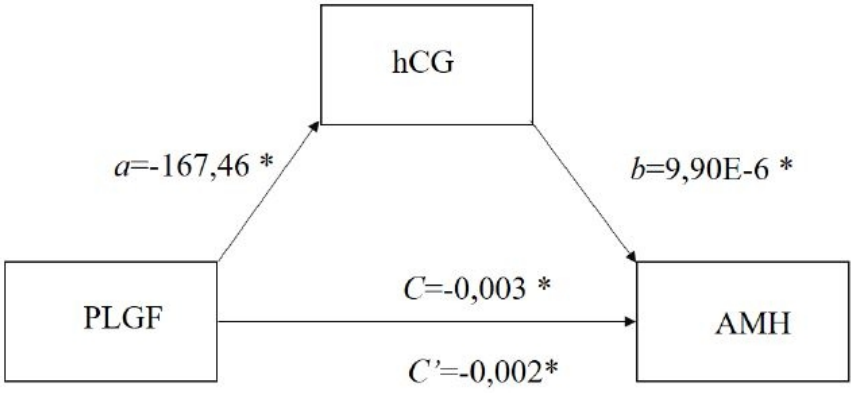

Relationship between PLGF and AMH as mediated by hCG

The total effect (c) is significant. The direct effect (c') of PLGF on AMH is significant and therefore not mediated by hCG. * P value< 0.05.

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