



Early pregnancy reference intervals

29 serum analytes from 4 to 12 weeks' gestation in naturally conceived and uncomplicated pregnancies resulting in live births

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Abstract

Background: Pregnancy introduces major physiological changes that also alter biochemical analytes. Maternal and perinatal health can be optimized by early intervention and therefore, pregnancy-specific reference intervals (RIs) for the local population are warranted. While the second and third trimester-specific changes are well described, the first trimester is less well characterized. We therefore wanted to facilitate early detection of abnormalities by generating first trimester reference values for 29 common analytes.

Methods: In a prospective early pregnancy (PEP) cohort (2016–2017), 203 pregnant women were recruited from 4 to 8 weeks' gestation. Consecutive blood samples were drawn every 2 weeks until an ongoing second trimester pregnancy ($n=164$) or a miscarriage ($n=39$) occurred. After exclusion of women with complicated pregnancies or deliveries ($n=42$), 122 women were included. The serum samples collected at <6, 6–8, 8–10, 10–12 and >12 weeks' gestation were analyzed for 29 common analytes. Subsequently the RIs were calculated according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommendations (2.5–97.5th percentiles) and compared with the conventional RIs for non-pregnant women.

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Results: Human chorionic gonadotropin (hCG), progesterone (P4), estradiol (E2), pregnancy-associated plasma protein A (PAPP-A), cancer antigen 125 (CA125), thyroid stimulating hormone (TSH), creatinine (CREA) and albumin (ALB) showed an early pregnancy-dependent change compared with conventional limits. For ALB the change was seen at 5.5 weeks' gestation.

Conclusions: We report gestational age-specific RIs available from the early part of the first trimester applicable to everyday clinical care of pregnant women. Well-known alterations of RIs seen in later trimesters are also observed in the first.

Keywords: first trimester; pregnancy; reference interval; reference range; reference value.

Introduction

The first trimester has emerged as an increasingly accessible timespan for developing diagnostic algorithms concerning the prevalent diseases of pregnancy, e.g. preeclampsia, small-for-gestational age infants and gestational diabetes mellitus [1–6]. Declared millennium developmental goals (MDG 4 and 5) [7] by the WHO, and the International Federation of Gynecologists and Obstetricians (FIGO) has identified the first trimester as a window of opportunity for optimizing both maternal and fetal health, especially concerning the prediction and prevention of chronic diseases later in life.

Normal pregnancy and the associated surge of reproductive hormones has a major impact on maternal physiology as it increases cardiac output, expands the vascular compartments and increases stress on the liver and kidneys. This marked influence on female physiology also leads to alterations of most commonly used biochemical measurands compared with the conventional non-pregnant reference intervals (RIs) [8–10]. Therefore, the clinical care of pregnant women would benefit from reliable RIs that take demographic and societal factors such as ethnicity, dietary intake and subclinical diseases into account [11]. Modern automated equipment has improved the intra- and interlaboratory assay variations. However,

inter-instrument bias remains a challenging issue in any laboratory and uniform translation across different platforms has yet to be attained. It therefore follows that laboratories offering biochemical analyses of pregnant women ideally should provide gestational age-specified RIs for all relevant analytes across all three trimesters.

Unfortunately, most RIs for pregnant women are available from the late first or early second trimester and onwards [8–10, 12–14] or are extrapolated from a control group of participants in a trial examining a first trimester complication [10]. Accordingly, such values are not ideal for identifying early pregnancy abnormalities and often lack the statistical requirements recommended for establishing RIs by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [15].

In response to the increased focus on first-trimester diagnostics, we therefore established gestational age-specific serum-based RIs of 29 commonly used clinical analytes in an early pregnancy cohort of women that had a live birth following an uncomplicated and naturally conceived pregnancy.

Materials and methods

Study population

From June 2016 to March 2017, a prospective early pregnancy cohort (the PEP cohort) after natural conception was recruited via online add campaigns and subsequently followed at North Zealand Hospital, Hillerød or Rigshospitalet, Copenhagen, Denmark. Following a positive urinary pregnancy test, 218 women expressed interest in participating and 212 attended the first visit (four lost interest and two were not pregnant). As seen in the Figure 1, 210 were included in the cohort (two had surpassed the inclusion criterion of no more than 55 days' gestation). Participants were at least 18 years of age, able to understand and sign a written consent and had an ultrasonically confirmed pregnancy with one fetus. Women with a history of recurrent pregnancy loss (≥ 3 consecutive losses), all types of fertility treatments, known or initially diagnosed abnormalities of the uterus or tubes or an ongoing drug abuse were excluded. All participants were followed consecutively with serial blood samples and transvaginal ultrasonography every second week until they either had a miscarriage ($n=37$) or they completed the first trimester ($n=166$). Also shown in Figure 1, 203 women were included for analyses after exclusion of seven participants due to lost interest ($n=3$) and multiple gestation ($n=3$). One woman was excluded from the cohort and

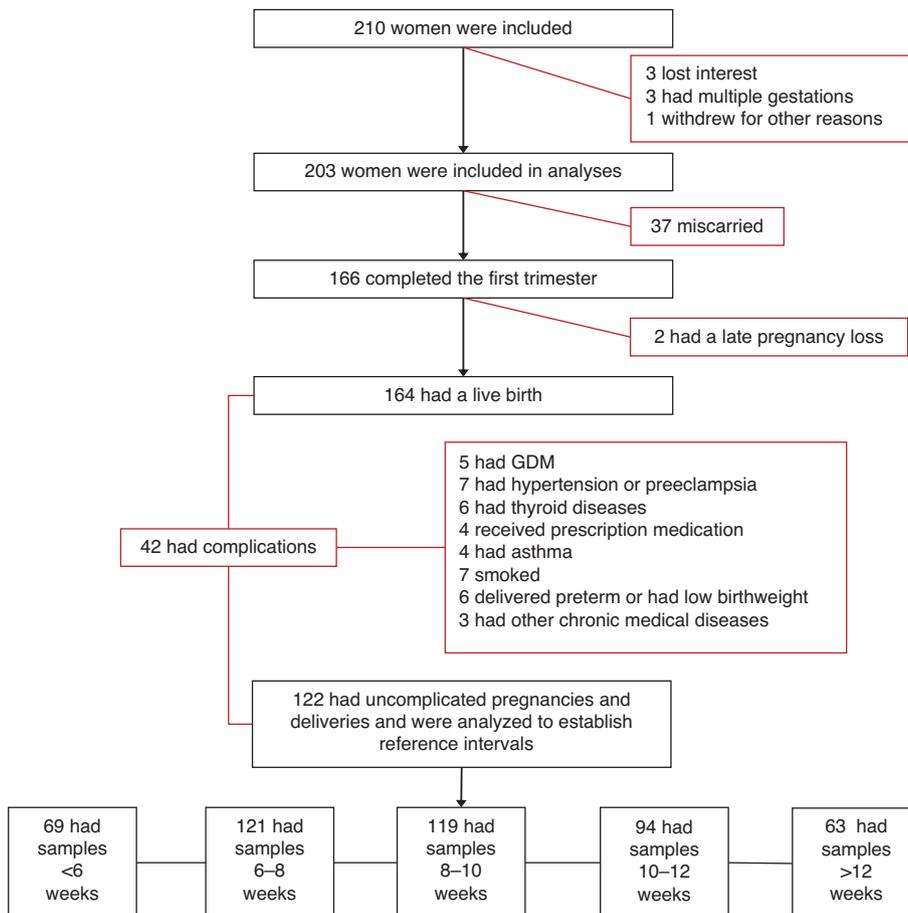


Figure 1: Flow chart of the selection of participants.

further blood sampling because her pregnancy developed ultrasonically resembling an early pregnancy loss. However, continued clinical follow-up later proved a healthy pregnancy.

A pregnancy follow-up of all participants in 2018 recorded 164 live births (two had late pregnancy loss). Within this group, RIs were based on 466 blood samples from 122 women, who fulfilled the criteria of an uncomplicated pregnancy and delivery. As described in Figure 1, exclusion criteria for this study comprised gestational diabetes mellitus, hypertension or preeclampsia, thyroid disease, prescription medication, asthma, other chronic medical diseases, smoking, preterm or low birthweight infants and 5 min Apgar scores (<7).

Gestational age estimation

All participants had a transvaginal ultrasonography (GE Voluson i BT14, GE Healthcare, Solingen, Germany) at each visit. Gestational age was determined when a crown-rump-length (CRL) could be measured [16]. Due to early inclusion, some participants did not have a CRL-measurable fetus from their initial scan, thus their gestational

age at visit 1 was calculated and registered after visit 2. Therefore, a visit 2 with CRL showing 50 days' gestation meant that a previous visit 1, 14 days earlier, without CRL estimation, was set at 36 days' gestation.

Blood samples

Each participant had an 18 mL peripheral blood sample drawn from the veins of the antecubital fossa using the Vacutainer serum separator tubes (BD Diagnostics, Franklin Lakes, NJ, USA). Fasting was not required as it was considered intolerable due to frequent nausea in the first trimester. All samples were done from 8 a.m. to 7 p.m. To allow clotting, the blood was stored at room temperature for 15 min before 10 min of 3500 g centrifuge at 5 °C (Hettich Rotina 380 R, Andreas Hettich GmbH, Tuttlingen, Germany). The separated components were finally aliquoted to polypropylene RNase-, DNase-free microcentrifuge tubes and stored at -80 °C. The samples were thawed and analyzed 8 months after the last inclusion to allow for appropriate follow up to be completed.

Table 1: Biochemical details of serum analytes.

Analyte (abbreviation)	CV%	Traceability	NPU code
Siemens Dimension Vista 1500 (Siemens Healthcare Diagnostics Inc., Newark, DE, USA)			
Albumin (ALB)	5.8	ERM-DA470k/IFCC	NPU19673
Alkaline phosphatase (ALP)	5.6	IFCC acc. Schumann Gen 2.	NPU27783
Alanine aminotransferase (ALT)	3.8	IFCC	NPU19651
Aspartate aminotransferase (AST)	5.7	IFCC	NPU19654
Lactate dehydrogenase (LDH)	6.7	IFCC 2002	NPU19658
Bilirubin, total (BIL)	8.5	Doumas candidate reference method	NPU01370
Iron, free (IRON)	4.2	SRM 937	NPU02508
Ferritin (FTL)	4.4	NIBSC standard 80/602	NPU19763
Cholesterol (CHOL)	4.2	ID-MS	NPU01566
High-density lipoprotein (HDL)	5.4	Internal	NPU01567
Low-density lipoprotein (LDL)	4.0	Internal	NPU01568
Triglyceride (TRIG)	5.5	ID-MS	NPU04094
Blood urea nitrogen (BUN)	5.5	ID-MS	NPU01459
Creatinine (CREA)	4.7	ID-MS	NPU04998
Sodium (Na)	1.5	NIST SRM929A	NPU03429
Potassium (K)	3.0	NIST SRM928A	EPC00026
Uric acid (URIC)	4.9	ID-MS	NPU03688
Thyroid stimulating hormone (TSH)	6.1	WHO 80/558 2nd IRP	NPU03577
C-reactive protein (CRP)	10.3	Internal method traceable to CRM 470	NPU19748
Human chorionic gonadotropin, total (hCG)	5.7	IS 75/589; proc	NPU01580
Progesterone, total (P4)	14.5	ID-GC/MS-method	NPU03242
Estradiol (E2)	7.6	ID-GC/MS-method	NPU01972
Siemens Centaur XP (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA)			
Triiodothyronine, total (T3)	3.4	Derived from USP material	NPU03625
Triiodothyronine, free (fT3)	4.4	Derived from USP material	NPU03625
Thyroxine, total (T4)	4.4	Derived from USP material	NPU03578
Thyroxine, free (fT4)	4.5	Derived from USP material	NPU03579
Cancer antigen 125 (CA125)	4.6	Derived from an internal standard	NPU01448
Kryptor Compact Plus (Thermo Fisher, Nimes, France)			
Human chorionic gonadotropin, free (fhCG)	5	1.st IRP WHO 75/551	NPU01580
Pregnancy-associated plasma protein A (PAPP-A)	5	Internal reference	NPU22157

CV, coefficient of variation; NPU, nomenclature for properties and units.

Laboratory analysis

All samples were examined at the Department of Clinical Biochemistry, North Zealand Hospital. The analytes examined, equipment manufacturers and assay coefficients of variation, traceability and nomenclature for properties and units (NPU Codes) are listed in Table 1.

Albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin (BIL), free iron (IRON), ferritin (FTL), cholesterol (CHOL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TRIG), blood urea nitrogen (BUN), creatinine (CREA), sodium (Na), potassium (K), uric acid, (URIC), thyroid stimulating hormone (TSH), C-reactive protein (CRP), total human chorionic gonadotropin (hCG), total progesterone (P4) and estradiol (E2) were analyzed using Siemens Dimension Vista 1500. Free and total triiodothyronine (fT3 and T3) and thyroxine (fT4 and T4) as well as cancer antigen 125 (CA125) were measured by Siemens Centaur XP and pregnancy-associated plasma protein A (PAPP-A) and the free beta human chorionic gonadotropin (fhCG) using the Kryptor Compact Plus.

Statistical analysis

Conditional quantile regression according to the LMS method [17] was performed using R [18] and the package `gamlss` [19]. The degrees of freedom for the spline expansions and a choice between the Box-Cox *t* distribution and the Box-Cox normal distribution were selected according to the AIC criterion. A non-parametric bootstrap procedure with 25,000 resamples was used to estimate 90% overall confidence envelopes for the 2.5% and 97.5% conditional quantile functions [20]. The envelopes were compared with the corresponding 90% confidence intervals (CIs) of the conventional RI to decide whether and, if so, when the pregnancy-dependent interval became approximately significantly different. The Reference Intervals, software package and an IFCC-recommended approach [15, 21] was used to calculate RI including 90% CI for the limits every second week of the observational period. Outliers were detected according to Horn's method [22] and evaluated with the patient chart. If a value was biologically unexplainable, suggesting measurement failure, it was removed. All missing data were assumed to be missing complete at random (MCAR) [23] due to laboratory processing glitches. Irrespective, all values were reported from a complete case analysis as RIs are usually not derived from imputed data. When the concentration was equal to or below the lower detectable limit of the assay, the value of the detection limit was used as a test result. Student's *t*-test and the chi-squared test were used to test for differences in means or proportions, respectively, and *p*-values below 5% were considered statistically significant.

Ethics

All participants received oral and written information about the study prior to participation and signed an informed consent according to the standards of the Helsinki declaration. The study was approved by the local Regional Ethical Committee (H15018059) as well as the Danish Data Protection Agency (NOH-2015-042) and

was published at clinicaltrials.org prior to first patient enrollment (NCT02761772).

Results

All participants delivered a healthy singleton neonate during 2017. Table 2 shows the study population demography compared with the 2017 nationwide Danish birth data [24]. Participants were younger (mean \pm SD; 29 ± 4 vs. 30 ± 5 years, $p=0.01$) due to 62% being <30 years of age (46% nationwide, $p<0.001$). Participants were less likely to be nulliparous (27% vs. 39% nationwide, $p=0.02$) and birthweights were marginally higher than the nationwide mean (3592 ± 457 vs. 3474 ± 609 g, $p=0.005$). All participants were Caucasian.

Table 3 shows RIs (2.5–97.5th percentiles) and the 90% CI for these limits per 2 weeks' gestation of each analyte. As also shown in Figure 2: ALB, hCG, P4, E2, PAPP-A, CA125, TSH and CREA, already exhibited a notable change during the earliest part of the first trimester. ALB values reduced almost linearly from a starting range (36–48 g/L) equal to the conventional RI (37–48 g/L) and finished with the median below the 2.5 percentile of the conventional (>12 weeks' RI 31–42 g/L). The pregnancy-dependent markers of hCG, fhCG and PAPP-A developed expectedly showing surges around 6–9 weeks' gestation. hCG plateaus about 9 weeks' gestation before decreasing in the remaining period. The P4 and E2 97.5th percentile surpassed the luteal phase-based conventional equivalent throughout the observation and were both significantly increased after 4.5 weeks' gestation. Additionally, the E2 2.5th percentile exceeded the conventional 97.5th at about 8 weeks' and became significantly higher after 11 weeks' gestation. CA125 levels developed a peak at 6 weeks' gestation (<6 weeks RI 7–170 U/mL) with the 97.5th percentile significantly higher already after 4.5 weeks. Levels decreased thereafter and at 11 weeks' gestation the 97.5th percentile were within conventional limits. TSH described a U-shaped curve with a minimum at 8–10 weeks' gestation (0.10–4.1 mU/L) resembling the conventional range (0.30–4.0 mU/L). About half of CRP values (279/463, 60%) were at the lowest limit of assay detection, although a tendency of higher values with gestation was seen. Early CREA were marginally higher than values by 9 weeks' gestation (<6 weeks: 42–68 vs. 8–10 weeks: 39–66 μ mol/L) with an almost steady curve thereon. Compared with conventional limits, the 97.5th percentile was reduced throughout the observation and the 2.5th became significant after 4.5 weeks' gestation.

Table 2: Cohort demographics at the time of inclusion compared with the 2017 nationwide Danish birth data.

	Study population		Danish 2017 births		p-Value
n	122		60,871		
Age, years, mean, SD ^a	29.3	(4.1)	30.3	(5.0)	0.01
Age groups, n (%) ^b					
< 30 years	76	(62.3)	27,694	(45.5)	<0.001
30–35 years	33	(27.0)	23,547	(38.7)	
> 35 years	13	(10.7)	9630	(15.8)	
BMI, kg/m ² , mean, SD ^a	24.1	(4.4)	24.8	(8.2)	0.07
BMI groups, n (%) ^b					
Normal weight	76	(62.3)	35,189	(57.8)	0.08
Underweight	3	(2.5)	2579	(4.2)	
Pre-obese	34	(27.9)	12,683	(20.8)	
Obese	9	(7.4)	8009	(13.2)	
Missing	NA		2411	(4.0)	
Former pregnancies, n (%) ^b					
0	33	(27.0)	23,637	(38.8)	0.02
1	42	(34.4)	19,395	(31.9)	
2+	47	(38.5)	17,839	(29.3)	
Parity, n (%) ^b					
0	51	(41.8)	29,490	(48.4)	0.1
1	53	(43.4)	20,913	(34.4)	
2+	18	(14.8)	9532	(15.7)	
Gestational age at delivery, mean (SD)	280	(7.70)	NA		
Method of delivery, n (%) ^b					
Acute CS	9	(7.4)	6520	(10.7)	0.4
Planned CS	14	(11.5)	5548	(9.1)	
Vacuum extraction ^c	3	(2.5)	NA		
Vaginal	96	(78.7)	48,803	(80.2)	
Infant sex, n (%) ^b					
Boy	63	(51.6)	31,166	(51.2)	0.9
Girl	59	(48.4)	29,705	(48.8)	
Infant weight, g, mean, SD ^a	3592	(457)	3474	(609)	0.005
Apgar <7 at 5 min, n (%)	0	(100.0)	NA		

^aT-test, ^bchi-squared test, ^ccensored from test. SD, standard deviation; n (%), column % per level; NA, not applicable.

As shown in Supplementary Figure 1, other markers were either aligned with the conventional RI or rather were unchanged during the entire first trimester.

Discussion

This study successfully established detailed RIs for 29 common analytes in the first trimester of women who had a naturally conceived and uncomplicated pregnancy with the delivery of a healthy singleton neonate. Primarily, the study provided novel and detailed data from 4 to 8 weeks' gestation. This allowed for an assessment of when these analytes deviated from the RIs of non-pregnant women and hence could become clinically important. Furthermore, by determining the RIs for 29 biomarkers of endocrine, renal, liver and inflammatory

function the study provided an overview of the many-faceted early reprogramming of body physiology induced by pregnancy.

Endocrine

The production of hCG has been detected in plasma as early as 6–14 days after fertilization [25]. In our assay, hCG and fhCG were primarily increased after placenta-tion (week 6), as shown in Figure 2. Hereafter, the production increased markedly until week 9. PAPP-A is a protease that cleaves IGFBP4 causing the release of IGF1 and IGF2. PAPP-A, like hCG, are synthesized by the syncytiotrophoblast and secreted into the maternal circulation in increasing concentrations until term [26]. Low levels of PAPP-A are therefore associated with restricted fetal growth as IGF markedly influence fetal intrauterine

Table 3: Reference intervals per 2 weeks' gestation.

Analyte (abbreviation), conventional range, unit, outliers	Weeks' gestation	n	Reference interval		90% CI for the reference interval percentiles			
			2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile
Human chorionic gonadotropin (hCG) total, U/L	<6	69	226	32,660	0	364	21,755	48,387
	6–8	120	10,622	161,144	5422	15,571	124,479	200,000
	8–10	117	45,180	200,000	36,685	68,721	200,000	207,915
	10–12	93	36,709	171,645	34,758	38,585	143,290	194,872
	>12	63	22,314	143,568	14,363	26,780	118,943	181,980
Human chorionic gonadotropin (fhCG) free, U/L	<6	70	0.1	27.1	0.0	0.2	26.0	32.8
	6–8	121	7.9	155.3	3.8	11.2	127.3	206.9
	8–10	118	20.5	223.8	10.8	31.0	120.6	257.1
	10–12	93	21.0	211.6	18.9	25.7	201.0	265.3
	>12	63	11.4	118.4	7.8	13.5	86.5	155.0
Pregnancy-associated plasma protein A (PAPP-A), U/L	<6	70	0.00	0.24	0.00	0.01	0.00	0.46
	6–8	121	0.02	0.46	0.01	0.02	0.36	3.48
	8–10	118	0.13	1.87	0.12	0.19	0.00	2.19
	10–12	93	0.46	5.79	0.33	0.54	0.34	6.60
	>12	63	0.90	15	0.45	0.96	13	19
Estradiol (E2) 0.25–1.2 nmol/L (luteal phase)	<6	69	0.4	3.0	0.3	0.5	2.4	3.6
	6–8	120	0.8	5.8	0.7	0.9	5.1	6.3
	8–10	119	1.0	8.6	0.4	1.3	7.1	10
	10–12	90	1.5	10	0.9	2.0	9.6	12
	>12	54	2.2	10	1.5	2.4	9.7	11
Progesterone (P4), total 20–100 nmol/L (luteal phase)	<6	70	21	138	13	26	123	152
	6–8	120	27	147	20	30	118	154
	8–10	118	28	156	23	31	144	185
	10–12	92	38	177	28	48	173	206
	>12	59	53	172	48	54	161	187
Cancer antigen 125 (CA125) <35 U/mL	<6	70	7	170	4	7	131	254
	6–8	120	8	98	4	14	82	119
	8–10	118	8	69	5	11	32	76
	10–12	94	6	50	4	8	32	59
	>12	63	6	47	4	7	38	62
Albumin (ALB) 37–48 g/L	<6	70	36	48	34	36	47	50
	6–8	119	36	46	35	37	44	46
	8–10	118	34	45	33	35	43	46
	10–12	94	32	43	31	33	43	44
	>12	63	31	42	29	31	42	43
Alkaline phosphatase (ALP) 37–106 U/L	<6	69	33	108	25	43	106	125
	6–8	118	33	96	29	39	79	111
	8–10	118	32	117	29	37	108	148
	10–12	94	32	101	29	33	90	124
	>12	63	28	127	23	28	117	164
Alanine transaminase (ALT) 8–46 U/L	<6	69	10	49	9	10	30	63
	6–8	117	8	43	7	10	31	53
	8–10	118	7	53	6	8	40	70
	10–12	94	7	38	6	7	28	50
	>12	63	6	28	5	7	27	31
Aspartate transaminase (AST) 13–37 U/L	<6	70	9	33	8	10	31	42
	6–8	118	9	24	9	10	20	27
	8–10	118	8	26	7	9	24	30
	10–12	94	8	26	7	10	21	31
	>12	63	9	19	8	9	19	21
Lactate dehydrogenase (LDH) 103–204 U/L	<6	69	109	205	103	118	136	235
	6–8	118	109	175	104	114	152	187
	8–10	118	105	172	100	136	164	176
	10–12	94	98	184	83	102	175	202
	>12	63	83	186	57	110	185	208

Table 3 (continued)

Analyte (abbreviation), conventional range, unit, outliers	Weeks' gestation	n	Reference interval		90% CI for the reference interval percentiles			
			2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile
Bilirubin (BIL) total 5–24 µmol/L	<6	70	4	29	4	4	29	39
	6–8	118	4	20	3	4	11	24
	8–10	118	3	20	3	4	12	24
	10–12	94	3	21	2	3	19	25
	>12	63	3	16	3	3	12	21
Cholesterol (CHOL) total 3.4–6.9 mmol/L	<6	70	2.6	6.6	2.2	2.8	5.9	7.7
	6–8	117	2.8	5.5	2.7	3.4	5.4	5.8
	8–10	118	2.9	5.9	2.6	3.3	3.9	6.2
	10–12	94	3.2	6.4	3.0	3.4	4.9	6.9
	>12	63	3.0	8.0	2.4	3.1	7.7	9.9
Low-density lipoprotein (LDL) 1.4–4.7 mmol/L	<6	70	1.1	4.9	0.8	1.5	4.3	5.7
	6–8	117	1.0	3.9	0.5	1.5	3.8	4.4
	8–10	118	1.1	4.2	0.9	1.5	2.6	4.7
	10–12	94	1.5	4.3	1.3	1.7	2.6	4.5
	>12	63	1.1	6.1	0.5	1.7	6.0	8.1
High-density lipoprotein (HDL) 1.0–2.6 mmol/L	<6	70	1.1	2.3	1.0	1.1	2.1	2.5
	6–8	118	1.0	2.5	0.9	1.2	1.9	2.8
	8–10	118	1.1	2.6	1.0	1.2	1.6	2.8
	10–12	94	1.1	3.0	0.9	1.2	2.4	3.3
	>12	63	1.2	3.2	1.0	1.3	2.6	3.8
Triglyceride (TRIG) 0.47–2.6 mmol/L	<6	70	0.42	2.3	0.40	0.44	1.5	3.0
	6–8	118	0.43	2.3	0.39	0.50	1.8	2.7
	8–10	118	0.46	3.1	0.33	0.59	3.0	3.8
	10–12	94	0.56	2.4	0.49	0.60	2.4	2.7
	>12	63	0.70	2.9	0.64	0.70	2.5	3.5
Sodium (Na) 137–145 mmol/L	<6	70	132	143	130	135	140	150
	6–8	120	134	141	133	135	140	141
	8–10	117	133	141	132	134	140	141
	10–12	94	134	141	134	135	140	141
	>12	63	135	142	133	134	139	146
Potassium (K) 3.6–4.6 mmol/L	<6	69	3.3	4.4	3.1	3.3	4.1	4.6
	6–8	120	3.6	4.3	3.4	3.6	4.2	4.5
	8–10	117	3.5	4.4	3.4	3.6	4.3	4.5
	10–12	94	3.5	4.4	3.5	3.6	4.3	4.5
	>12	63	3.4	4.3	3.3	3.4	4.2	4.4
Creatinine (CREA) 51–84 µmol/L	<6	70	42	68	37	43	67	71
	6–8	118	43	67	42	48	61	69
	8–10	118	39	66	37	43	60	70
	10–12	94	40	64	40	41	62	67
	>12	63	39	64	36	40	59	68
Urea nitrogen (BUN) 2.7–6.4 mmol/L	<6	70	2.1	6.3	1.8	2.4	5.7	6.6
	6–8	118	2.2	5.7	2.0	2.2	5.3	6.0
	8–10	118	2.1	5.6	1.9	2.5	5.3	6.1
	10–12	94	2.0	5.5	1.8	2.0	4.2	5.6
	>12	63	1.9	5.2	1.6	2.1	4.9	5.6
Uric acid (URIC) 0.15–0.35 mmol/L	<6	70	0.12	0.30	0.10	0.12	0.27	0.34
	6–8	118	0.10	0.27	0.08	0.10	0.24	0.29
	8–10	118	0.10	0.26	0.09	0.12	0.19	0.28
	10–12	94	0.09	0.25	0.08	0.10	0.22	0.27
	>12	63	0.10	0.26	0.09	0.10	0.24	0.28
Thyroxine (T4) total 60–140 nmol/L	<6	70	67	118	62	70	118	124
	6–8	120	71	142	66	77	126	166
	8–10	118	79	151	71	86	150	159
	10–12	94	77	164	61	80	144	174
	>12	63	80	167	66	89	164	182

Table 3 (continued)

Analyte (abbreviation), conventional range, unit, outliers	Weeks' gestation	n	Reference interval		90% CI for the reference interval percentiles			
			2.5 percentile	97.5 percentile	2.5 percentile	5 percentile	95 percentile	97.5 percentile
Thyroxine (fT4) free 12–21 pmol/L	<6	67	11	17	11	12	17	18
	6–8	112	11	19	11	12	18	20
	8–10	114	11	18	10	12	15	18
	10–12	90	11	18	11	11	18	20
	>12	63	11	17	10	11	17	18
Triiodothyronine (T3) 1.1–2.5 nmol/L	<6	70	1.3	2.6	1.2	1.3	2.4	3.2
	6–8	120	1.3	2.4	1.3	1.4	2.4	2.5
	8–10	118	1.5	2.9	1.4	1.6	2.7	3.3
	10–12	94	1.5	2.9	1.4	1.6	2.7	3.2
	>12	63	1.5	3.0	1.3	1.5	3.0	3.3
Triiodothyronine (fT3), free 3.9–6.8 pmol/L	<6	67	3.6	6.2	3.2	3.8	6.2	6.6
	6–8	112	3.7	6.0	3.3	3.9	5.7	6.4
	8–10	113	3.5	5.6	3.2	3.7	5.6	5.9
	10–12	90	3.8	5.4	3.7	4.1	4.9	5.5
	>12	63	3.7	5.2	3.4	3.7	5.2	5.4
Thyroid stimulating hormone (TSH) 0.30–4.00 mU/L	<6	70	0.68	5.29	0.34	0.86	4.72	6.42
	6–8	117	0.35	4.05	0.30	0.40	1.82	4.55
	8–10	117	0.10	4.14	0.03	0.14	3.56	5.00
	10–12	94	0.08	5.76	0.00	0.12	4.28	8.17
	>12	63	0.37	7.41	0.22	0.48	3.89	11.5
Iron (IRON), free 9–34 µmol/L 1 outlier removed	<6	69	5	26	3	7	25	29
	6–8	118	7	30	4	9	22	32
	8–10	118	4	33	3	6	32	38
	10–12	94	5	34	3	6	28	40
	>12	63	4	29	1	4	27	32
Ferritin (FTL) 15–200 µg/L	<6	70	13	141	7	21	124	154
	6–8	120	17	146	6	20	124	171
	8–10	118	14	149	9	15	103	172
	10–12	94	12	161	6	14	153	179
	>12	63	9	109	1	9	76	138
C-reactive protein (CRP) <3 mg/L	<6	70	2.9	19	2.9	2.9	12	22
	6–8	118	2.9	18	2.9	2.9	8.5	30
	8–10	118	2.9	22	2.9	2.9	14	44
	10–12	94	2.9	17	2.9	2.9	13	34
	>12	63	2.9	31	2.9	2.9	16	32

growth restriction. However, our data display great variability in what was needed to maintain the pregnancy, demonstrated by one participant who had all three proteins consistently below the 2.5th percentile limit throughout the first trimester. Despite this, the pregnancy was uneventful and ended up in the delivery of a healthy and normal-weight neonate. This illustrates the difference between RI and establishing clinically applicable decision limits, for example, “lowest values associated with live birth”. Accordingly, diagnostics based on our reported limits should be cautiously done and serial measurements are warranted [27].

As expected, total T4 and T3 increased while the levels of the free hormones were unchanged. Increased total concentrations are caused by the well-known increase

of thyroglobulin binding protein (TBG) beginning at the time of placentation [28–30]. The TSH concentration was inversely linked to hCG attributed to their structural similarities; they share the same alpha subunit causing cross reactivity at the TSH receptor. Therefore, hCG, when in abundance, acts in a thyrotrophic manner in the pituitary and produces a negative feedback [31].

Another feature of Figure 2 is the luteoplacental shift traditionally defined as the onset of placental steroidogenesis of P4 and E2. This juncture is clinically important, especially in reproductive endocrine therapy of infertile women seeking fertility treatment. Theoretically, the onset of endogenous placental sex steroid production is the earliest time when exogenous steroid administration may be withdrawn. The exact timing of this shift has been

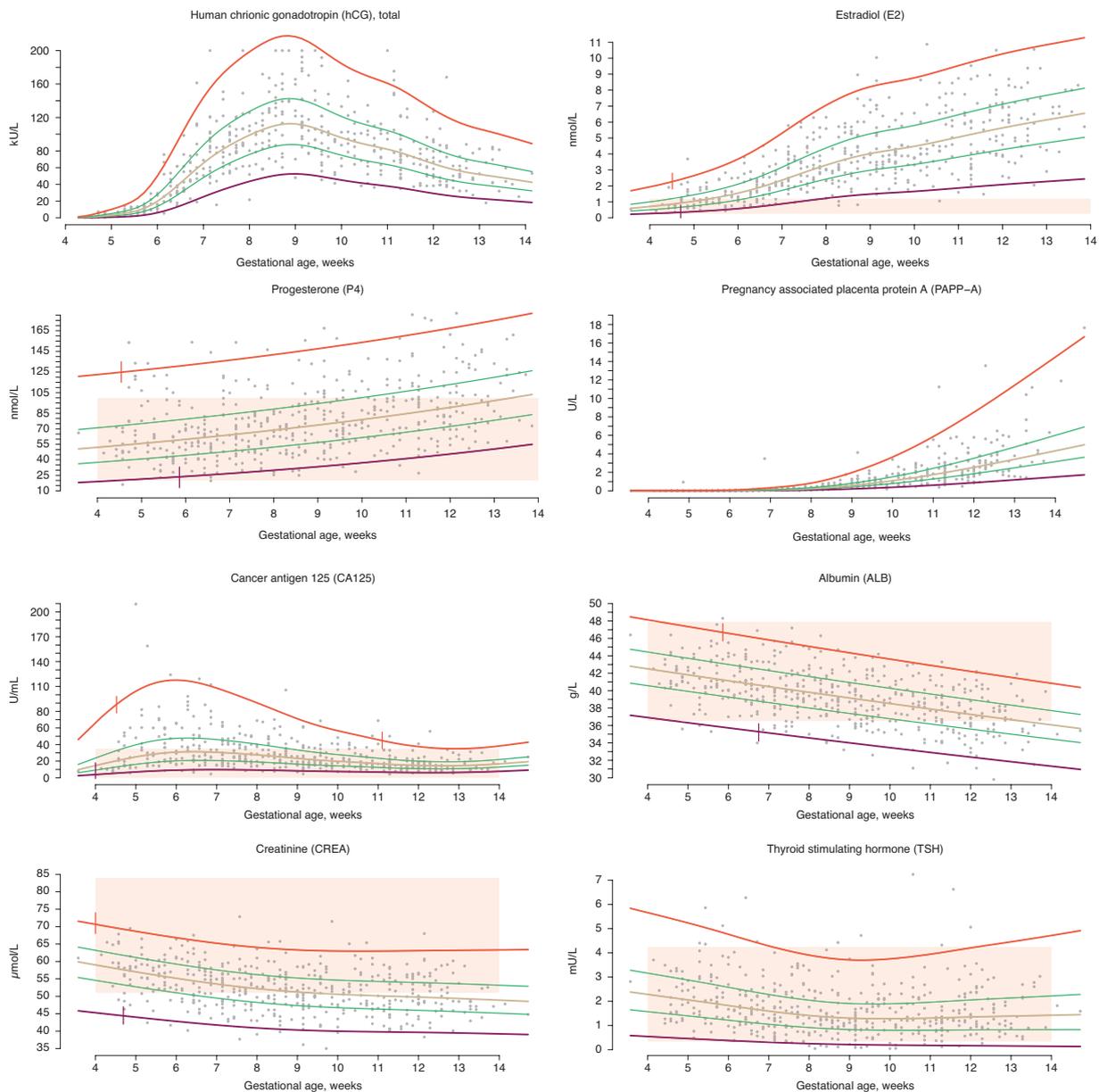


Figure 2: Scatter plot of the eight analytes with the most prominent changes early in pregnancy.

Every observed value and smoothed percentiles (red: 97.5th; maroon: 2.5th; green: 25–75th and yellow: the median) are plotted to the gestational age in weeks 4–14. Vertical lines mark the timing of the 2.5th (maroon) and the 97.5th (red) centile of the calculated reference interval becoming statistically significantly different from the conventional limits if available (orange boxes).

debated. In 1990, Devroey et al. [32] reported a significant E2 and P4 rise by 7 and 9 weeks' gestation, respectively, examining 18 pregnancies from oocyte donation in agonal women. One year later, Scott et al. [33] performed a similar study and observed similar elevations already at 6 and 7 weeks' gestation, respectively. These studies provided a near-optimal *in vivo* model of placental steroidogenesis as the endogenous contribution from the corpus luteum was unavailable. Nonetheless, as the developing conceptus is dependent on the corpus luteum, these protocols had to supply exogenous sex steroids that potentially disturb

the estimation of onset timing. Conversely, our setup displayed the continuum of E2 and P4 levels attributed by both the corpus luteum and the developing placenta in a non-supplemented pregnancy. Therefore, the exact origin of steroid production cannot be determined. As shown in Figure 2, P4 increased almost linearly while E2 elevated more rapidly from 6 to 8 weeks' gestation. We decided to define statistical significance as the time when 90% CIs of the calculated RI limits (2.5th or 97.5th) were decreased or increased compared with the non-pregnant equivalents. Therefore, our results support the notion of increased sex

steroid production around week 6 (P4 97.5th and 2.5th significantly increased by 4.5 and 5.9 weeks' gestation, respectively), probably attributed by the placental onset and seemingly more pronounced in E2 (E2 97.5th and 2.5th were significantly increased by 4.5 and 4.7 weeks' gestation, respectively).

CA125 is usually associated with ovarian cancer diagnostics. In pregnancy, CA125 is expressed by the decidual cells and secreted to circulation in the first trimester due to decidual invasion by the trophoblast during placentation [34]. Therefore, CA125 becomes a marker of placentation also seen in Figure 2, as the values from 4 to 8 weeks' gestation exceeds the ovarian cancer diagnostic cutoff of 35 U/mL. Interestingly, the upper limit was significantly increased already at 4.5 weeks' but returned to non-pregnant limits after 11 weeks. This finding warrants clinical vigilance towards interpretation of CA125 in the early first trimester and support the previously shown potential of CA125 as a first trimester biomarker [35]. As expected, the theoretical lower limit of 0 in the CA125 non-pregnant RIs was exceeded in all observations.

Renal

Pregnancy has a major impact on kidney function and fluid/salt balance and the biomarkers Na, K, URIC, CREA and BUN reflect these physiologically changes in the pregnant body throughout the first trimester. Following progesterone and relaxin-mediated decreased systemic vascular resistance from the corpus luteum, also renal vasculature is relaxed and allows increased blood flow and glomerular filtration rate (GFR) [36, 37]. Consequently, as seen in Figure 2 and Supplementary Figure 1, CREA (97.5th and 2.5th percentile significantly decreased throughout the observation and by 4.5 weeks' gestation, respectively), URIC and BUN started at a low value and decreased with gestation [8–10]. Furthermore, the hypervolemic-hypoosmolar-characteristic state of pregnancy [36, 37] was seen by the stable and unchanged RIs of sodium and potassium – both with at least the 2.5th percentile at a lower level compared with the non-pregnant references.

Liver

There was a steady increase in serum lipids throughout the first trimester. As the fetus develops, the triglycerides increase as metabolic changes prioritize the development of fatty stores during the first trimester to satisfy maternal energy needs later in pregnancy. Additionally,

the increased production of sex steroids necessitates increased CHOL, HDL and LDL levels as precursor substrates [37, 38]. Compared with conventional limits the differences were marginal. Nonetheless, our data clearly illustrate that the well-known gestational changes already can be visualized from the beginning of the pregnancy. Also evident at this stage, was the well-known reduction in albumin during the first trimester [9, 10]. The albumin RIs were initially within conventional limits [39], but already from about 5.5 weeks' gestation the 2.5th percentile was lower than the conventional equivalent (Figure 2) and significantly different at 6.8 weeks. This decrease continued throughout the first trimester before ending up in a range >12 weeks (31–42 g/L) comparable to the work by Klajnbard et al. [8] who reported an RI of 32–43 g/L by 13–20 weeks' gestation. Although their work constitutes the most thorough investigation of the Danish population and serves as the reference for pregnant women in most Danish obstetric consultations, the authors were not able to sample individuals from the first trimester and an RI is therefore unavailable. In another report from the Scandinavian population, Larsson et al. [9] also found ALB values from 7 to 17 weeks' gestation of 32–43 g/L. We argue, that this 10-week period probably contained a higher and non-pregnant-like ALB RI in the earliest part of the first trimester and illustrate the added precision that may arise from scrutinizing references in smaller intervals.

Multiple mechanisms such as hemodilution [9], increased catabolism [8] and maximized pregnancy-dependent reprogrammed liver synthesis [40] have been suggested to cause the decrease in albumin concentration with gestation. Our early declining values support the theory of albumin being increasingly catabolized for the development of the fetal unit at a rate that exceeds the normal capacity for liver synthesis. From gestational week 5.5 some hemodilution is possible, although this traditionally has not been observed before at least 6 weeks' gestation [41]. Additionally, bigger molecules such as HDL are unchanged or slightly increasing within the period as also shown by Klajnbard et al. [8]. Supporting the view that these changes are caused by reprogramming of liver synthesis during pregnancy is the fact that liver cell turnover, as evaluated by the ALT, AST and LDH, was stable and unaffected (Supplementary Figure 1). Conjugation and excretion of bilirubin also seemed unaffected (Supplementary Figure 1). The lower 2.5th percentile reference limit of AST, ALP and BIL was interpreted as a difference between the age of our study population (22–38 years) and the conventional value population. These mostly originated from the Nordic Reference Interval Project (NORIP) [39] providing

comparable non-pregnant intervals specified by female gender, ethnicity and societal factors such as the dietary intake in the Nordic countries, but pooled values from participants from 18 to 49 years of age.

Inflammation

CRP has been intensively researched in pregnancy with a general tendency of increased values by gestation [8, 42, 43]. Supported by our findings, values vary immensely, and any meaningful interpretation needs to be closely correlated to clinical development of symptoms.

Strengths and limitations

The study population was a well-characterized and homogenous cohort of Caucasian women with complete follow-up until delivery. Hence, the reported RIs give a solid base for clinical application in populations of this demography. As seen in Table 2, compared with the Danish birth cohort of 2017, our sample represents the nationwide numbers rather accurately with minor differences of negligible significance. All reported RIs follow the recommendations of the IFCC [11, 21], albeit with few samples in the extreme <6 and >12 weeks' gestational intervals. These values should therefore be cautiously interpreted, while they simultaneously provide a rare view at naturally conceived pregnancies <6 weeks' gestation. The prospective design allowed for CRL-determined gestational age even at the earliest available samples. All analyzed samples were handled by the same investigator and followed the directions of the UK biobank [44], optimizing the integrity and precision of the reported values. Ideally, we would have provided a complete first trimester material of all parameters investigated by Klajnbard et al. [8] Unfortunately, our laboratory setup was unable to do full-blood analyses, for example, hematological analytes. Future research might focus on procuring these missing quantities in the early part of the first trimester.

Conclusions

Collectively, this study reports gestational age-specific RIs from 4 to 12 weeks' gestation in 29 commonly used analytes applicable to clinicians caring for pregnant women. Our findings confirm the well-known fact that pregnancy alter non-pregnant RI. This should be considered already in the first trimester.

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