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A toolkit for the application of placental-fetal molecular biomarkers in epidemiologic studies of the fetal origins of chronic disease

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Abstract

Purpose of review: In this review, we provide essential background knowledge and an analytical framework for the application of placental-fetal molecular biomarkers in fetal origins chronic disease epidemiology. The widely available and highly quantitative placental hormone human chorionic gonadotropin (hCG) is used as an example. hCG is currently used for diagnosing

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fetal genetic disorders; yet it can and should be expanded to understanding the fetal origins of chronic diseases. We provide justification and methods to do this.

Recent findings: Ten papers published in the last 5 years were identified with supportive findings relevant to the application of biomarkers of hCG in epidemiologic studies on the developmental origins of health and disease (DOHaD).

Summary: There is increasing and consistent evidence that placental-fetal biomarkers may be highly informative in observational studies, as exemplified by hCG, with the correct approaches for measurement and data analysis.

Keywords

hCG; human chorionic gonadotropin; fetal origins; epidemiology; placenta; directed acyclic graphs

Introduction.

The developmental origins of health and disease (DOHaD) is a biologic theory that is increasingly recognized as a public health challenge. The complex and chronic health disorders obesity (18–26%), asthma (8–16), autism (1.7%), and polycystic ovarian syndrome (12%) are suspected to originate in the prenatal period.[1] The body of empirical evidence to elucidate mechanism and support causality in these associations is nascent.

Because randomization of exposure in pregnant women as a strategy to assess causality is not an option for ethical reasons, one alternative is to apply diverse and creative approaches within the framework of observational birth cohort studies. For example, changes can be measured at the molecular level early during the period of fetal organogenesis (<12 weeks gestation). Molecules in maternal blood can be measured that are informative of the health and well-being of the early embryo, the fetus, and/or the placenta. These data can then be used to estimate and illuminate developmental time- and sex-specific associations between maternal exposures and fetal and child outcomes.

During the first trimester, the placenta generates necessary cytokines, growth factors, and hormones to support the pregnancy and the fetus. Many of these are secreted into maternal blood and circulate at fairly high concentrations. The placenta is of particular interest in the paradigm being presented here. Even though commonly assumed to be maternal tissue, the placenta is fetal, carrying the same genotype and the same sex as the baby. Given the exceedingly low and difficult to measure levels of molecules being secreted by the early embryo and fetus, the placenta can be an alternative and rich source of information. It is large in size and surface area relative to the early embryo, it is in more direct contact with maternal blood, and a variety of proteins are produced by the placenta in higher volumes and can be more easily measured in maternal circulation than fetal proteins.[8, 9]

Measuring placental molecules in maternal circulation is already in widespread use for the sake of prenatal screening for fetal genetic defects. Serum screening is commonly used in prenatal care to measure fetal well-being within developmental windows in the late first trimester (10–14 weeks) and early second trimester (15–20 weeks) to estimate the risk of

fetal Down's Syndrome, trisomy 18, and neural tube defects.[10–12] Five analytes most commonly examined are human chorionic gonadotropin (hCG), pregnancy associated plasma protein isoform A (PAPP-A), Inhibin-A (INHA), unconjugated estriol (uE3), and alpha fetoprotein (AFP). Since the initial implementation of these tests, follow-up studies have demonstrated their utility in estimating risks of obstetric outcomes including preeclampsia [13], preterm labor[14] and fetal growth restriction.[15]

The topic of this review is to understand how existing serum screening data can be utilized in observational studies that investigate the developmental origins of health and disease. The data exist and can be used generally at no additional cost. There is a biologically plausible rationale to apply these data as biomarkers of fetal developmental milestones in utero that can have lasting consequences. There are caveats as well as technical and analytical issues to be considered when analyzing these data in the context of fetal origins epidemiology, which will be outlined.

In this short review, the example of hCG will be the focus, given the large accumulation of knowledge of this particular hormone. The following will be addressed: 1) relevant background on hCG, including mechanisms by which hCG offers information on fetal development; 2) recently published epidemiologic findings on associations between maternal hCG levels and child health outcomes as well as environmental exposures; and 3) analytical approaches to apply these biomarkers to epidemiologic studies. Finally, we will extend important lessons and concepts learned from studies of hCG to other types of placental circulating biomarkers and to a checklist to guide future applications of placental-fetal biomarkers in DOHaD research.

Biologic Background

Human chorionic gonadotropin (hCG) biology.—hCG is a glycoprotein hormone. The intact hCG molecule is a heterodimer that consists of alpha and beta subunits. The alpha and beta subunits are encoded by distinct mRNAs. chorionic gonadotropin alpha (*CGA*) and chorionic gonadotropin beta (*CGB*), respectively. *CGB* has 6 coding and noncoding isoforms which are located on Chromosome 19q13.3.[16] The *CGA* gene is located on Chromosome 6q14.3 and is identical to the alpha subunit of other glycoprotein hormones including thyroid stimulating hormone (*TSHA*), luteinizing hormone (*LHA*), and follicle stimulating hormone (*FSHA*). The beta subunit confers biologic specificity.[17]

hCG tissue source.—In pregnancy, hCG is produced predominantly by the differentiated placental cell type called the trophoblast.[17, 18] There is also minor production by undifferentiated trophoblasts, stem cells, and the fetal adrenals.[19, 17, 20–23] The difference in maternal circulating levels of hCG corresponds to differences in the expression of hCG mRNAs and proteins in the placental tissue.[24, 9, 25]

hCG general function.—hCG has been implicated in almost every physiologic process in pregnancy including uterine quiescence, immune tolerance, angiogenesis, and parturition. [26, 27] These processes are indirectly relevant to fetal development by way of changing canonical placental functions. Canonical placental functions refer here to maternal-placental blood flow, delivery of nutrients to the fetus, protection from infection, and the timing of

delivery.[28] These types of processes might explain the well-reported association of maternal hCG levels and birthweight.[29, 30]

Fetal vs. placental vs. maternal concentrations.—One reason a placental protein is particularly informative relates to the ratio of maternal serum levels of that hormone relative to levels within the fetus and the placenta. The fetus and the placenta are the locations where the biologic action of interest is taking place, from the perspective of fetal development. Measuring and comparing levels of placental and fetal hormones in maternal serum informs us about the following processes: hormone synthesis, secretion, transport across the placental and fetal membranes, or metabolism. Such comparison has been thoroughly investigated for hCG, in a study where hCG concentrations were measured in the first trimester and compared between maternal and fetal compartments, via maternal serum and coelomic fluid, from the cavity which surrounds the amniotic sac and houses the embryo.[8] The coelomic fluid concentrations of hCG-α were 160 fold higher than those in maternal serum, hCG-β levels were 32-fold higher, and intact hCG was 1.3-fold higher.[8] These types of data confirm that hCG production is highest in close proximity to the developing fetus and embryo. On this basis, we theorize that variability in hCG levels in maternal serum likely reflect meaningful variability in fetal and placental levels.

hCG and fetal development.—hCG has been selected as a valuable prenatal screening analyte because of the difference in the distribution of hCG in women carrying euploid (normal) fetuses vs. women carrying fetuses with trisomy 12 (Down's Syndrome).[12, 31] Down's Syndrome children characteristically have intellectual disabilities; yet a mechanistic connection between abnormal hCG and fetal brain development has not been established. There are published hypotheses that have not yet been pursued in epidemiologic studies. One idea is that placental hCG, as part of a larger pathway, contributes to the development of the fetal neocortex and explains language development.[32] Experimental models also show that embryonic neurons can alter hCG secretion.[33] It is also possible that the hCG and Down's Syndrome association is non-causal and hCG is simply a reporter molecule of the genetic defect that is common the fetus and the placenta.

hCG also plays a direct role in the development of the fetal reproductive tract. It was demonstrated in the 1970s that hCG can bind the luteinizing hormone chorionic gonadotropin receptor (LHCGR) in the male fetal gonad and stimulate the onset of steroidogenesis.[34] In cases where this binding did not occur due to mutations in the LHCGR, males were born with hypogonadism and pseudohermaphrotism.[35, 36] In fetal organs collected from first trimester terminations, hCG levels were markedly higher in fetal gonads as compared to other tissues.[37]

Epidemiologic Findings

Literature of the last 5 years was reviewed for the purposes of identifying papers that give insight into the concept and the practice of applying hCG in epidemiologic studies to understand the fetal origins of chronic disease. The results of this search are summarized in Table 1, which was structured to include the associations examined, hCG measures utilized, and analytical approach. Three of the 10 papers identified are from the Generation R Study,

a large population-based cohort in Rotterdam (the Netherlands) which has measures of hCG throughout pregnancy. The Generation R papers both confirm previous knowledge of hCG in the context of a modern day European cohort, and offer novel insight into sources of effect modification of hCG associations with outcomes such as fetal growth and maternal thyroid function. The general categories of papers summarized in Table 1 are: 1) associations between hCG and child health outcomes; 2) associations with environmental exposures. Below, we summarize these findings, highlight sources of biologic and technical variability in hCG measurements that deserve careful handling in epidemiologic studies, and note some important considerations for future work.

Associations with child health outcomes.—Several studies characterized hCG associations with child outcomes related to reproductive tract development. At this time, the only reports are on prenatal hCG and neonatal anogenital distance (AGD) and the risk of hypospadias and undescended testes (UDT) at birth. AGD is an anthropometric marker of masculinization whereby shorter AGD indicates anti-androgenic actions *in utero* and longer AGD indicates pro-androgenic actions.[38] AGD is considered a sensitive and specific marker of fetal endocrine disruption.[39–41] Research prior to our 5-year window reported that mothers who gave birth to boys with UDT had 20% lower hCG in the second trimester as compared to women who gave birth to non-cryptorchid boys. [42] In summary, results of the literature review demonstrated that hCG was correlated with developmental outcomes in boys; there was a stronger role for hCG in the first trimester vs. later in pregnancy; hCG associations are dynamic and require specialized techniques to address gestational age variation; hCG levels are correlated with maternal thyroid function, and also with outcomes in 2 domains of development: fetal brain and reproductive tract development. In future analyses, hCG may have value as an indicator of fetal reproductive tract development that can be exploited in birth cohort studies where there are opportunities to follow children through puberty and to the point at which fertility can be evaluated.

One study examined associations of hCG with child outcomes related to brain development. hCG levels were measured in women who gave birth to children with cerebral palsy, diagnosed before the age of 5, and compared to levels in women who did not (Table 1, Paper 8). Maternal thyroid hormone is critically important to normal fetal brain development in this early period before the fetus begins producing its own thyroid hormone. hCG is considered thyrotropic (can bind to thyroid receptors and stimulate thyroid function).[43, 44] The Generation R study demonstrated a strong association of hCG with maternal thyroid hormones in the first trimester (Table 1, Paper 6), and also showed that first trimester thyroid hormone was associated with child cognition and brain morphology at 8 years of age.[45] However, in that paper, they did not report the association of hCG and brain development and only state that it was evaluated as a potential confounder.

Outcomes which deserve further investigation in this field include: brain development, childhood obesity or fat mass, asthma, diabetes, pediatric cancer, outcomes during the pubertal transition, polycystic ovarian syndrome, and fertility by way of sperm counts or ovarian reserve later into early adulthood.

Associations with environmental exposures.—Conveniently, hCG is used *in vitro* as a standard endpoint indicative of toxic effects on trophoblast differentiation and function. Hence, toxicology studies can be a tool to look for evidence of dose-response relationships of diverse exposures and hCG. Experimental studies have the advantage of randomized exposure and can therefore make statements on causality. They provide an important and useful basis from which to justify human biomarker studies. It cannot be assumed, however, that these dose response relationships translate directly to human pregnancy. Generally, in vitro studies lack the same contextual signals of human pregnancy, namely fetal and maternal paracrine and endocrine signals. Tissue culture conditions are designed to mimic human physiology but nonetheless differ in important ways.

Endocrine disrupting compounds which altered hCG in placental cells cultured *in vitro* include: chlorpyrifos, a commonly used organophosphate pesticide that has also been associated with child brain development [46, 47]; triclosan, an antimicrobial agent used in pharmaceutical and personal care products [48]; bifenthrin, a pyrethroid pesticide [49]; tetrabromobisphenol A, a brominated flame retardant [50]; phthalates, components of plastic and personal care products [51, 52]; bisphenol A, an epoxy used in plastics and food packaging [53, 54]; the surfactant p-Nonylphenol [55, 56]; cigarette smoke [57]; polycyclic aromatic hydrocarbons which are components of smoke [58]; and diethylstilbesterol, the teratogenic xenoestrogen prescribed to pregnant women from 1938–1971.[59] Placental cells in vitro dosed with stress hormones (oxytocin, arginine-vasopressin, prolactin) also cause higher release of hCG.[60]

Across these studies, the toxic substances both caused increases and decreases in hCG. In some cases the doses used were non-comparable to human exposure. It is likely the hCG mechanisms at work also differed by exposure. Therefore, it is not possible to collapse this knowledge to a prediction of an overall direction of expected effect, or even a clear distinction between adverse and non-adverse effects. However, this literature is presented here is sufficiently robust to support hCG as a fruitful biomarker in human studies investigating the consequences of exposure to endocrine disrupting compounds.

Despite this body of evidence, birth cohort studies have minimally reported associations of maternal endocrine disrupting exposures with hCG. In the last 5 years, two papers were published from our group on maternal phthalates and hCG. (Table 1, Papers 3 and 7) Previous to the review period, it was reported that a small number of women who reported diethylstilbesterol (DES) exposure in utero had a slower rise of urinary hCG in the first 7 days after implantation of their embryos.[61] Because the DES exposure of the subject's mother preceded the current pregnancy and the formation of her placenta, the finding here suggests that placental hCG expression could be determined partially through an intergenerational mechanism in the oocyte (exposed to DES at the time when it formed) turned into zygote (grand-daughter of the DES exposed).[62] This slower rise of hCG was reported in the same cohort to be associated with high vs. low monobenzyl phthalate levels measured in the same pregnancy.[63] Finally, a landmark study conducted at the population level demonstrated that aggregate levels of hCG may serve as indicators of aggregate-level economic stressors related to unemployment.[64]

Analytical issues

Appropriately identifying confounders and effect modifiers.—In the papers reviewed, there is a consistent strategy with regard to adjustment for confounders of hCG associations with child health outcomes and environmental exposures, such as race, body mass index, age, etc. In one paper the authors adjusted for 'placental function' by including in the model small for gestational age and preterm delivery.(Table 1, Paper 10). This and other factors such as gestational age at birth or birthweight or other pregnancy complications could potentially introduce bias by adjusting for either a potential intermediate variable or a condition that could be downstream of the hypothesized exposure effect on fetal or placental development. This strategy should be avoided when estimating effects of early hCG levels on outcomes at birth or after birth.

Gestational age at the time of the blood draw is a significant source of variability in hCG levels. hCG production begins within hours of conception and concentrations in maternal serum rise steadily from 0 to 10 weeks gestation.[65, 61] At 10 weeks, levels of intact hCG and hCG-β begin decreasing while hCG-α levels continue to rise in maternal serum until the end of pregnancy.[65, 66] The gestational age dependencies are not linear within the two periods in which prenatal screening takes place and these non-linearities should be properly accounted for in measurement and statistical modeling strategies.[67, 66, 68, 69] Gestational age at the time of blood draw is correlated with dynamic aspects of maternal physiology and also with fetal developmental processes. For this reason, it may operate as an important effect modifier.

Fetal sex is also an established source of variability in circulating levels of hCG. Levels are approximately 10% higher in women carrying female fetuses vs. women carrying male fetuses.[67] The difference first emerges at approximately 3 weeks after conception.[70] Female fetal levels were 17% higher than male levels at the beginning of the first trimester screening window. They decreased to be 2% higher at the end, and then the female levels increased steadily relative to the male levels to be 19% higher by 20 weeks or the end of the second trimester screening window.[67] This is a finding that might be illustrative of a developmental process in one sex or the other where hCG played a role. For this reason, fetal sex should also be considered as a potential confounder and/or effect modifier of exposure effects on hCG and hCG effects on child outcome.

Measurement and acquisition of data.—Maternal serum analytes including hCG are generally measured by high throughput, antibody-based methods. hCG can also be measured in small batches in maternal serum and urine by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. Laboratories that conduct the high-throughput analysis include clinical laboratories within the hospital, research laboratories with proper certification within the insitutition, or commercial laboratories outside of the institutions. In 4 U.S. states including California, these analyses are carried out in State Health Department Laboratories.[71] Absolute quantitation is achieved through the use of hCG standards and an absolute standard curve approach. In the case of hCG-β, the standards are maintained and distributed by the World Health Organization.[72, 73] The details of these methods are

described elsewhere.[74–76] The form of hCG which is measured for serum screening purposes might be intact or total hCG or hCG-β depending on which assay is purchased.

In order to obtain these data for research purposes, there is often no additional human subjects protection approval required if the original consent form asked permission to access the prenatal medical record. If not, a human subjects protection modification should be submitted with the request for waiver given that the identifiability risks are the same as with the data already in use The alternative would be to recontact subjects and ask for informed consent.

Normalization of hCG for the purpose of causal estimation.—When working with a raw data set, there may be sources of technical variability such as antibody batch, drift over time in laboratory assays, sample shipment and storage, and error in how gestational age at time of blood draw was determined. The normalization of hCG for these factors is standard practice when used to estimate risk of Down's Syndrome at the individual level. The normalization procedure results in a quantity called the multiple of the median (MoM). The MoM was originally proposed in 1977 for the serum prenatal screening analyte AFP as a method to use a population-level association to establish clinically meaningful cut-offs at the individual level.[77, 78] It is a stepwise algorithm that includes regression techniques, likelikehood ratio tests and the Bayes Theoroem. A detailed description is given elsewhere. [79] Despite the conceptual and statistical limitations of the MoM as have been pointed out, [79] it generally works well, in combination with other markers, to detect a large proportion of affected fetuses. It has recently been proposed for use in the interpretation of child hormone levels, [80] and could potentially be used for other placental biomarkers.

In addition to technical variability, the MoM is also used to normalize for biologic variability. The list of variables used in standard clinical algorithms to normalize hCG, generating the hCG MoM, include maternal race, weight or BMI, smoking status, pre-conception diabetes status, and ovum donor status.[81, 68] MoM algorithms might be invaluable in a clinical setting where the goal is to predict individual level risk of Down's Syndrome, but may work against the epidemiologist who would like to estimate population risks. Normalization of the biomarker for sources of biologic variability can result in overadjustment. [82]

In summary, normalization of placental biomarkers is necessary as part of the toolkit. However, normalization by the clinical variables currently used in the hCG MoM could create collider bias. The recommendation here is to base normalization on sources of technical variability and on gestational age at the time of the blood sample only, and to adjust for other types of variability as confounders.

Estimation of non-linear associations.—In the case of hormonally-mediated mechanisms, such as the role of hCG, linear assumptions may fall short for the simple reason that hormonally-mediated mechanisms characteristicially have a low dose effect (below the mean of exposure/mediator) that is distinct from the high dose effect (above the mean of exposure/mediator).[86, 87] Relying on the linear model in this case might result in model misspecification and consequently incorrect estimates and conclusions. Other

statistical techniques, such as splines, can and should be adopted to relax the linear assumption. [88] [89]

Estimation of direct vs. indirect pathways.—If hCG is being considered a component of the pathway between the exposure and the child outcome, then the analytical strategy could, in some fashion, include a comparison of the direct and indirect pathways. The direct pathway is assessing the effect of the exposure on the child outcome, not assuming that there are important intermediate variables. The indirect pathway in this case would assume that placental hCG is an important intermediate variable. Analysis of the indirect pathway assumes that hCG either interacted with the exposure to change the risk of the outcome, or operated as a mediator of the effect of the exposure on fetal development leading to child outcome. There are multiple approaches to assessing the indirect pathway.[90–92] The first step is to clearly articulate why we suspect that placental hCG mediates a pathway that is distinct from and independent of the direct effect of exposure on outcome. The second step could be to estimate the total effect of the exposure and hormone, and then to dissect the total effect into 4 components (i.e., the four-way decomposition method developed by VanderWeele). [91] This methodology can be easiliy implemented via the STATA package, Med4way.[94]

Future directions

hCG is a well-studied and well-validated protein biomarker. In order to increase the value of this review for investigators interested in other types of placental-fetal biomarkers, we have included Supplemental Table 1 with a review of diverse biomarkers representative of extracellular vesicles called exosomes, epigenetics, proteomics, and metabolomics. These are new areas where the analytical issues necessary to measure and apply these biomarkers in a high-throughput, highly quantitative framework such as a longitudinal birth cohort study have not been as well worked out as hCG. In this mini-review, the literature was identified that related these biomarkers to obstetric outcomes (preeclampsia, placenta previa, growth restriction, gestational diabetes), due to a dearth of studies relating them to child health outcomes.

Furthermore, as a resource for investigators interested in applying placental biomarkers in their DOHaD research, we developed a checklist modeled after the STROBE and STOBE-ME checklists (Supplemental Table 2).[95, 96] This covers essential steps in both developing a biomarker and also in applying an existing biomarker. This list can serve as a guide for journal reviewers and editors.

Conclusion

Placental circulating hCG as a biomarker in epidemiology is well-established with respect to a diverse set of maternal exposures and also with respect to dynamic processes in pregnancy. It is less well-established as a biomarker of long-term child health. We report here recent findings on associations of placental hCG with fetal brain and reproductive tract development. Provided here are technical and analytic details on the measurement and utiliziation of hCG measurements in epidemiologic studies, with the goal of providing an investigator toolkit to apply to other circulating placental biomarkers. The Supplement

contains a mini-review of diverse types of placental biomarkers (exosomal, epigenetic, metabolome, proteome) that hold promise for this field of research. An investigator checklist is presented to help formalize and standardize these approaches.

In studies of the influence of prenatal exposures on child health outcomes, maternal-placental-fetal mechanisms play an important and causal role. Studies that report only the direct effects of the prenatal exposure on childhood outcome ignoring the intermediate variables are omitting important information. Additionally, there are opportunities in this type of analysis for novel insight into the basic biology of pregnancy, the placenta, and the fetus. There are also opportunities to strengthen causal inferences regarding pregnancy exposures as important determinants (or not) of child health. Lastly, placental biomarkers could be a missing and essential key to reproducibility in epidemiologic findings. For example, investigators who have measured the same exposures and the same child outcomes at multiple sites could apply hCG data and the methods outlined here to their analysis. In doing this, they may find that there is a common indirect pathway between exposure and outcome through a mechanism involving placental hCG. This may be true even in cases where the direct effect of the exposure on the outcome was null or inconsistent across studies and sites.

This review provides an outline for others to plug in the same type of information for the prenatal screening analytes not reviewed here, namely alpha fetoprotein, estriol, and inhibin-A which are also measured in prenatal serum screening programs. These could also serve as useful and valuable biomarkers of fetal endocrine disruption. Taken together, this would strengthen the toolkit for the epidemiologist who is developing specific and quantitative evidence of how exposures in early pregnancy can impact specific physiologic pathways related to fetal development. In some cases, one or more of these proteins may prove to be highly associated and therefore highly informative.

The ‘low hanging fruit’ has been plucked in terms identifying biomarkers of rare fetal genetic diseases, and now we need a different toolkit to assess the utility of these biomarkers in identifying the early origins of chronic disease outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Summary of published papers of the last 5 years in support of placental hCG as an informative biomarker in the epidemiology of fetal origins of chronic disease.

Paper	Year	First author (ref)		
1	2015	Koreevar [69]	**N**	8,195
			Population	Generation R, Rotterdam, Netherlands
			hCG, serum	analyte in IU/L, MoM, z-score
			Gestational window	<9 to >25 weeks
			Primary association	Gestational age at blood draw and circulating hCG
			Technical variability	Method of gestational age determination
			Biologic variability	Gestational age at time of blood draw, smoking, BMI, parity, ethnicity, maternal age, fetal sex, placental weight, reflux, nausea, vomiting, fertility, twins
			Effect modification	Pregnancy complication
			Estimation method	Linear regression, ANOVA, plotted using restricted cubic splines
			Main finding	Confirmation of hCG predictors in this population.
2	2015	Adibi [67]	**N**	1,088,668
			Population	Genetic Disease Screening Program, California, USA
			hCG, serum	analyte in IU/mL, MoM
			Gestational window	10–14, 15–20 weeks
			Primary association	Fetal sex and circulating hCG
			Biologic variability	Fetal sex
			Effect modification	Maternal weight, age, gestational age at time of blood draw
			Estimation method	Linear regression, additive interactions
			Main finding	hCG was 11% higher in women carrying females in the first trimester. There were small fluctuations in the female/male mean hCG ratio by maternal weight, race, and age, and larger fluctuations by gestational age.
3	2015	Adibi [97]	**N**	541
			Population	The Infant Development and Environment Study, 4 sites, US
			hCG, serum	z-score
			Gestational window	10–14, 15–20 weeks
			Primary association	Maternal urinary phthalates and neonatal anogenital distance (AGD), mediated by placental hCG
			Biologic variability	First trimester maternal urinary phthalate metabolites
			Effect modification	Fetal sex
			Estimation method	Linear regression, additive interactions, total and controlled direct effects (mediation analysis) by structural transformation.
			Main finding	Qualitative interactions by fetal sex with exposure, mediator, and outcome. Higher hCG was associated with longer AGD in females and shorter AGD in amles. 52% of the MnBP effect and 25% of the MEHP effect on male AGD was attributed to hCG mediation, and 78% of the MBzP effect in females. This is the only evidence to date that hCG was associated with size of female genitalia.
4	2016	Schneuer [98]	**N**	22,617
			Population	Two states in Australia: New South Wales, Western Australia

Paper	Year	First author (ref)		
			hCG, serum	hCG-beta, MoM
			Gestational window	10 and 13 weeks gestation
			Primary association	hCG and hypospadias and undescended testes
			Effect modification	Placental dysfunction, co-existing congenital anomalies
			Estimation method	Kruskal-Wallis test, chi-squared test, Wilcoxon rank sum, logistic regression
			Main finding	No association
5	2016	Barjaktarovic [30]	**N**	7,987
			Population	Generation R, Rotterdam, Netherlands
			hCG, serum	z-scores, normalized by gestational age at blood draw
			Gestational window	11–14 weeks
			Primary association	Circulating hCG and fetal growth (repeat measures from 18–25 weeks gestation), birthweight, small for gestational age (SGA)
			Effect modification	Fetal sex
			Estimation method	Linear fixed, and linear mixed effects, and logistic regression with splines for hCG, Markov Chain Monte Carlo imputation for missing data.
			Main finding	High hCG in the first trimester was associated with increased growth of female fetuses, and decreased growth of male fetuses.
6	2017	Korevaar [99]	**N**	5,435
			Population	Generation R, Rotterdam
			hCG, serum	analyte in IU/L
			Gestational window	9.6 – 17.6 weeks
			Primary association	hCG and maternal thyroid function
			Effect modification	Maternal thyroid disorders, thyroid autoimmunity, BMI, sex, parity
			Estimation method	Regression with restricted cubic splines with 3–4 knots, Markov Chain Monte Carlo imputation for missing data.
			Main finding	hCG was inversely associated with the risk of hypothyroxemia (low T4) and hyperthyroidism (high T4 of T3), but there was no association of hCG and hypothyroidism (high TSH, low T4). Thyroid peroxidase (TPOAb) did not modify the association.
7	2017	Adibi [100]	**N**	180
			Population	Columbia Children's Environmental Health Center
			hCG, placental tissue	*CGA* mRNA, *PPARG* mRNA (transcription factor that regulates mRNA), absolute quantitation
			Gestational window	Third trimester to birth
			Primary association	Maternal phthalates and placental gene expression
			Effect modification	Baby sex
			Estimation method	Linear regression, phthalates modeled as quartiles, additive interactions
			Main finding	Of 8 candidate genes, *CGA* was the most strongly associated with maternal phthalates and association differed in direction by sex.
8	2018	Eskild [101]	**N**	29,948
			Population	Norway, population study, 1992–1994
			hCG, serum	Analyte, IU/L
			Gestational window	4–12 weeks, 13–27 weeks, 28–40 weeks

Paper	Year	First author (ref)		
			Primary association	hCG and risk of cerebral palsy (CP) up to 5 years of age
			Effect modification	Trimester of pregnancy
			Estimation method	Student t tests, logistic regression
			Main finding	Women had 0.8 (95% CI 0.6, 1.1) lower odds of CP per log unit hCG in the first trimester, and a 1.4 (95% CI 0.9, 2.2) higher odds in the second trimester.
9	2019	Chen [102]	**N**	131
			Population	Prenatal Screening Center of Yuhang District Maternal and Child Health Hospital of Hangzhou City (China)
			hCG, serum	hCG-beta, MoM
			Gestational window	15 – 20 weeks and 5 days
			Primary association	hCG and hypospadias (individual risk prediction)
			Estimation method	t-test, ROC and Youden Index, life cycle-like risk calculation
			Main finding	Women who gave birth to boys with hypospadias had 1.5 higher hCG-beta than controls. There was higher sensitivity and specificity in detecting hypospadias when both hCG and alpha fetoprotein (AFP) were used.
10	2019	Peycelon [103]	**N**	1,269
			Population	Paris registry of Congenital Malformations
			hCG, serum	hCG-beta, MoM
			Gestational window	10 – 13 weeks gestation
			Primary association	hCG and hypospadias, proximal and distal
			Adjustment	Small for gestational age and prematurity (‘proxies for placental function’), maternal age
			Estimation method	Quantile regression
			Main finding	MoM was 0.5 (95% CI 0.2, 0.9) higher in women with proximal hypospadias vs. controls