Original Contribution

Common Childhood Viruses and Pubertal Timing: The LEGACY Girls Study

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Earlier pubertal development is only partially explained by childhood body mass index; the role of other factors, such as childhood infections, is less understood. Using data from the LEGACY Girls Study (North America, 2011–2016), we prospectively examined the associations between childhood viral infections (cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV) 1, HSV2) and pubertal timing. We measured exposures based on seropositivity in premenarcheal girls ($n = 490$). Breast and pubic hair development were classified based on mother-reported Tanner Stage (TS) (TS2⁺ compared with TS1), adjusting for age, body mass index, and sociodemographic factors. The average age at first blood draw was 9.8 years (standard deviation, 1.9 years). The prevalences were 31% CMV⁺, 37% EBV⁺, 14% HSV1⁺, 0.4% HSV2⁺, and 16% for both CMV⁺/EBV⁺ coinfection. CMV⁺ infection without coinfection was associated with developing breasts an average of 7 months earlier (hazard ratio (HR) = 2.12, 95% confidence interval (CI): 1.32, 3.40). CMV infection without coinfection and HSV1 and/or HSV2 infection were associated with developing pubic hair 9 months later (HR = 0.41, 95% CI: 0.24, 0.71, and HR = 0.42, 95% CI: 0.22, 0.81, respectively). Infection was not associated with menarche. If replicated in larger cohorts with blood collection prior to any breast development, this study supports the hypothesis that childhood infections might play a role in altering pubertal timing.

breast development; menarche; puberty; pubic hair; viral infection

Abbreviations: BMI, body mass index; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HR, hazard ratio; HSV, herpes simplex virus; IgG, immunoglobulin G; IgM, immunoglobulin M; TR, time ratio; TS, Tanner Stage.

The age at onset of pubertal development has been declining in the United States and globally (1, 2). Earlier age at pubertal timing in girls is associated with long-term health risks, including an increased breast cancer risk (3, 4). Studies have found that higher childhood body mass index (BMI) is associated with earlier pubertal development (5–8). Increasing childhood BMI partially explains earlier puberty (9); however, early puberty is still seen in areas of the world without a childhood obesity epidemic (10). Age at menarche had declined for 7 decades before the childhood obesity epidemic, suggesting that other factors might contribute to the earlier age at menarche, which might also be true for other pubertal markers, and we hypothesized that changes to childhood infections could be one such factor (4). Specifically, the last few decades have seen a decline in

the prevalence and a later age at acquiring many childhood infections, including cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (4, 11).

Two contradictory theories are proposed related to childhood infections and pubertal timing. First, it is possible that lower exposure to childhood infections might result in an earlier age at puberty onset based on the life-history theory that growth and reproduction can be prioritized over fighting infections (12, 13). Second, an alternative hypothesis is that childhood viral exposures are obesogenic (14–18) and therefore might result in an earlier age at pubertal onset via increased obesity. For example, in a sample of 11,476 US adults, selected infections in childhood, but not adulthood, were associated with higher central adiposity in women (14). To our knowledge, our study is the first prospective

study with detailed data on body size, pubertal timing, and serological markers to measure the following infections: CMV, EBV, herpes simplex virus (HSV) 1, and HSV2. With the exception of HSV2, we focused on these commonly contracted viruses based on a pilot study we conducted (19). Serological evidence is essential, given that some of these viruses (e.g., EBV and CMV) are asymptomatic; however, measurement is possible because antibodies for these viruses persist indefinitely in the host, making it feasible to assess past exposures (20). Last, CMV and EBV have been implicated in disease pathology, such as breast carcinogenesis (21–25), with recent implications in triple-negative breast cancer (26).

METHODS

Study population

The LEGACY Girls Study is a prospective cohort study of 1,068 girls, primarily aged 6–13 years, with 1,040 girls recruited from 2011–2013 and 28 siblings recruited after 2013 across 5 study sites in North America (New York, New York; Philadelphia, Pennsylvania; Salt Lake City, Utah; San Francisco Bay Area, California; Toronto, Ontario). Details have been published previously (27–29). All participating institutions obtained institutional review board approval to conduct the study. The cohort study was designed to examine growth and development in girls (baseline ages, 6–13 years); 81% were premenarcheal, and half of the girls had a first- or second-degree breast cancer family history at baseline. Of the 1,068 girls, 490 had an available blood sample and had not reported menses at the time of the blood sample.

Daughter demographic measures

Mother/guardian-reported questionnaire data at baseline included daughter's race and ethnicity (Hispanic, Black non-Hispanic, White non-Hispanic, Asian non-Hispanic, or other race/ethnicity), maternal educational status (some college/vocational/technical schooling or less, bachelor's degree, or graduate degree), and breast cancer family history. Anthropometric measurements (height and weight) were taken by trained study staff at the time of the blood draw (27) to calculate BMI-for-age percentile based on the Centers for Disease Control and Prevention growth charts (30).

Puberty measures

Growth and Development Questionnaires assessed pubertal markers and were administered every 6 months, with 75% of cohort continuing collection through 42 months (27). We used the pubertal maturation data that were collected at the time of the blood draw and all subsequent data until the pubertal outcome was achieved. Mothers used the standard Marshall and Tanner Staging images for breast and pubic hair development (31) and reported the age (in year and half-year) when their daughter started menses. Marshall and Tanner Staging (hereafter referred to as Tanner Staging (TS)) consists of 5 images ranging from no development (TS1)

to full development (TS5), where TS2 is considered the beginning of pubertal development. Mother's report is the primary outcome method; we have previously shown that it is a validated measure compared with clinical measures of TS (32), and it was available for the whole cohort. We also used clinician-assessed breast development that was administered every 6 months at the New York and Utah LEGACY sites (details published previously (28)).

Viral serology

We screened for viral antibodies in the first available serum sample for girls who had not started menses. Commercially available enzyme-linked immunosorbent assay (ELISA) kits had an interassay reliability coefficient of variation (CV%) that ranged from 2.4 to 10.3 and an intra-assay reliability CV% that ranged from 8.1–14.9. The following ELISA kits were used: CMV immunoglobulin G (IgG), CMV immunoglobulin M (IgM), EBV IgG, and EBV IgM (SeraQuest; Quest International, Inc., Miami, Florida; supplied by Grifols, Emeryville, California) and HSV1 and HSV2 (HerpeSelect; 1 IgG and 2 IgG Focus Diagnostics; DiaSorin Molecular LLC, Cypress, California). We screened for IgG antibodies that persist indefinitely in the host and can be used independently to confirm a CMV infection and for IgM antibodies, which are often considered a marker of "recent primary infection" or "reactivation of infection" (33, 34). Given the young age of the cohort, we considered IgM detection a marker of a recent primary infection. By screening for IgG and IgM, both past and recent infection exposure are included. We expected low prevalence of HSV2 given that it is considered an adolescent infection and not a childhood infection.

We used the manufacturer's cutoff to determine whether a girl's sample was seropositive, seronegative, or ambiguous (0.4–2% ambiguous). Ambiguous results were considered seronegative in the main analyses and excluded in separate sensitivity analyses (see statistical analysis section below). For CMV and EBV serology, if a sample was categorized as seropositive for either IgG or IgM antibodies, the sample was categorized as seropositive. If a sample was categorized as seronegative for IgG and IgM antibodies, the sample was categorized as seronegative. Viral infections can affect host biology differently; therefore, we generated a categorical infection measure: no infection, CMV⁺ only, CMV⁺ and EBV⁺ coinfection only, EBV⁺ only, HSV1⁺ and/or HSV2⁺ infection(s) only, and all other infection combinations. Throughout, reference to "only" infections will be in this context; individuals with coinfections are considered as separate categories.

Statistical analysis

We compared descriptive statistics of the 490 girls who had an available blood sample and had not reported menses at the time of the blood sample with the remaining 578 girls within the LEGACY Girls' Cohort (Web Table 1, available at <https://doi.org/10.1093/aje/kwaa240>). Among those who had a blood sample, we assessed whether their infection variables differed by the daughters' demographic variables.

Figure 1. Comparison of the distribution density of age at first pubertal event according to seropositivity status determined by model 2 in Table 3, LEGACY Girls Study, North America, 2011–2016. Age at first breast development (A), pubic hair development (B), and menses (C). Infection categories were as follows: no infection (blue), cytomegalovirus only (maroon), cytomegalovirus/Epstein-Barr virus (green), Epstein-Barr virus only (orange), herpes simplex virus 1/2 (grayish-blue), and all other infection combinations (red). Compared with girls with no infection, girls with a cytomegalovirus infection only developed breast 6.8 months earlier and girls with an Epstein-Barr virus infection only developed breast 5.9 months earlier ($P < 0.05$). Compared with girls with no infection, girls with a cytomegalovirus infection only developed pubic hair 9.1 months later and girls with a herpes simplex virus 1 and/or 2 infection developed pubic hair 8.9 months later ($P < 0.05$).

Figure 2. Comparison of the predicted median age of puberty onset according to seropositivity status determined by model 2 in Table 3, LEGACY Girls Study, North America, 2011–2016. Median age at first breast development (A), pubic hair development (B), and menses (C). Compared with girls with no infection, having a cytomegalovirus (CMV) infection only and an Epstein-Barr virus (EBV) infection only were associated with an earlier age at breast development, with differences significant at $P < 0.05$. Compared with girls with no infection, having a CMV infection only and a herpes simplex virus (HSV) 1 and/or 2 infection were associated with a later age at pubic hair development, with differences significant at $P < 0.05$. Others, all other infection combinations.

Depending on the nature of the infection and demographic variables, different tests were used to determine their statistical significance. Chi square tests or analysis of variance were used for the categorical measures, while *t* tests or linear regression were used for the continuous measures. We did not model HSV2 independently because of the small sample size (0.4%). We excluded girls identified as “other” race/ethnicity from our analyses due to small sample size ($n = 12$), as well as girls whose age at pubertal event was missing (Web Figure 1).

We used a proportional hazards model to estimate hazard ratios and accelerated failure time models to estimate time ratios (TRs). In both models, we assumed the baseline hazard follows a Weibull distribution. Hazard ratios of >1.0 indicate an earlier pubertal event and <1.0 a later pubertal event. The TRs compare the median ages at the onset of the pubertal event between girls with and without the exposure (35). Consider a median age of 10 years at the first breast development and a given TR of 1.05 for the exposure of interest ($10.5/10 = \text{TR } 1.05$); this 5% difference translates to a 6-month-later age at first breast development ($5\% \times 12$ months) (36).

We compared the following nested Cox models: Model 1 is an age-adjusted “purely prospective” model that includes 457 premenarcheal girls, of whom 279 had not started breast development and 305 had not started pubic hair development, starting follow-up time at birth (Web Figure 1). The “purely prospective” models excluded left-censored girls; for example, when modeling for breast TS2 or higher (TS2⁺), girls were excluded if they were TS2 or higher at blood draw. We allowed age-varying effects because we assessed individuals’ serology at different ages and assume that the effect of infection might depend on age. Model 2 is model 1 with further adjustment for BMI and maternal education and race/ethnicity, with the most parsimonious model presented. Using these parsimonious models, we estimated associations without BMI adjustment (model 2a) and in girls below the 85th BMI-for-age-percentile (model 2b) (Web Table 2). Model 2c used clinician-assessed breast development ($n = 93$ girls) (27, 28). Model 3 assessed the same covariates as model 2 but starting follow-up time at blood draw. Model 4 included the left-censored, using retrospective puberty onset information, and included girls whose age at serology screening equaled age at pubertal event (also see Web Figure 1 and Web Table 3). Additional supplemental analyses included models that examined the associations between EBV-any and CMV-any and the onset of puberty. We also observed the same inferences when girls with ambiguous infection results were excluded from analyses. Analyses were conducted using STATA, versions 14.2 and 15.1 (StataCorp, College Station, Texas).

RESULTS

Descriptive statistics

The 490 girls who had blood samples available prior to menarche tended to be younger, have a lower BMI percentile, and include fewer girls who identified as Hispanic (Web Table 1). Table 1 summarizes the individual viral

Table 1. Prevalence of Individual Viral Infections and Serological Infection Burden ($n = 490$), LEGACY Girls Study, North America, 2011–2016

Viral Serology Measure	No.	%
CMV		
CMV IgG		
Negative	340	69.4
Positive	145	29.6
Ambiguous	5	1.0
CMV IgM		
Negative	469	95.7
Positive	13	2.7
Ambiguous	8	1.6
CMV seropositivity ^a		
Negative	330	67.3
Positive	152	31.0
Ambiguous	8	1.6
EBV		
EBV IgG		
Negative	299	61.0
Positive	180	36.7
Ambiguous	11	2.2
EBV IgM		
Negative	487	99.4
Positive	0	0.0
Ambiguous	3	0.6
EBV seropositivity ^a		
Negative	298	60.8
Positive	180	36.7
Ambiguous	12	2.4
HSV1		
Negative	416	84.9
Positive	68	13.9
Ambiguous	6	1.2
HSV2		
Negative	486	99.2
Positive	2	0.4
Ambiguous	2	0.4
Serological infection burden		
0	206	42.0
1	183	37.4
2	84	17.1
3	17	3.5
4	0	0.0

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV1, herpes simplex virus 1; HSV2, herpes simplex virus 2; IgG, immunoglobulin G; IgM, immunoglobulin M.

^a For CMV and EBV serology, if a sample was categorized as seropositive for IgG or IgM antibodies, the sample was categorized as seropositive.

infections and serological infection burden in the cohort. Table 2 summarizes the daughters' demographic measures in the cohort overall and by the categorical serology infection measure. We observed no statistically significant differences by EBV-any, CMV-any, or HSV1-any variables by age, breast cancer family history at the time of the blood draw, or study site (data not shown). However, girls with an EBV⁺-any infection had a larger body size (BMI percentile ≥ 85 th vs. 85%: 21% EBV⁺-any vs. 12% EBV⁻-any) and were more likely to have a mother with lower educational attainment (attainment of some college or less: 33% of EBV⁺-any versus 22% of EBV⁻-any). Girls with CMV⁺-any, or EBV⁺-any, or HSV1⁺-any infection were significantly more likely to be non-White or Hispanic ($P = 0.02$, $P < 0.0001$, and $P = 0.004$, respectively). In a linear regression model where the outcome was the number of infections, we observed that there was a larger number of infections in non-White or Hispanic girls compared with White non-Hispanic girls (for Hispanic, $\beta = 0.49$, 95% confidence interval (CI): 0.28, 0.70; Black, $\beta = 0.65$, 95% CI: 0.35, 0.95; Asian, $\beta = 0.30$, 95% CI: 0.03, 0.57) and in girls whose mothers had the lowest educational attainment level (compared with graduate degree, some college or less, $\beta = 0.29$, 95% CI: 0.10, 0.48; bachelor's degree, $\beta = 0.10$, 95% CI: -0.07, 0.27) (data not shown in tables). The categorical infection measure also differed by race/ethnicity and maternal education (Table 2).

Age at breast development

For breast development we present the "purely prospective" model findings in Table 3 and Figures 1 and 2. Compared with having no infections, CMV⁺ only, EBV⁺ only, and "all other infection combinations" were significantly associated with earlier age at first breast development (model 1, Table 3). Model 2 further adjusted for BMI-for-age percentile and the interaction between BMI percentile and age found CMV⁺-only girls had a 7-months-earlier age at first breast development (hazard ratio (HR) = 2.12, 95% CI: 1.32, 3.40) and EBV⁺-only girls had a 6-months-earlier age at first breast development (HR = 1.90, 95% CI: 1.15, 3.13) (Figures 1 and 2). Associations were slightly attenuated and still significant when analyses excluded BMI adjustment (model 2a) and when restricted to girls under the 85th BMI percentile (model 2b) (Web Table 3); suggesting being above the 85th BMI percentile might be associated with even earlier breast development. In model 2c we used clinician-assessed breast development as the outcome, and inferences were in the same direction but of lower magnitude; however, due to the limited sample size of girls with clinical measurements ($n = 93$), the estimates were imprecise (CMV⁺ only, HR = 1.44, 95% CI: 0.78, 2.65; and EBV⁺ only, HR = 1.07, 95% CI: 0.56, 2.02) (data not shown in tables). In model 3, when follow-up time started at blood draw, overall inferences for CMV⁺ only and EBV⁺ only were in the same direction but with attenuated magnitudes (Table 4), where CMV⁺ only remained significantly associated. Model 4, the hybrid "prospective + left-censored" model, suggests that our findings might be sensitive to selection bias. While none of

the estimates remained significantly associated with breast development, CMV⁺ only remained positively associated (HR = 1.11, 95% CI: 0.82, 1.50) (Table 4).

Pubic hair development

For pubic hair development we present the "purely prospective" model findings in Table 3 and Figures 1 and 2. Compared with having no infections, CMV⁺-only and HSV1⁺ and/or HSV2⁺ infections were both independently associated with a 9-month-later age at first pubic hair development (HR = 0.41, 95% CI: 0.24, 0.71 and HR = 0.42, 95% CI: 0.22, 0.81, respectively) (model 2, Figures 1 and 2).

"All other infection combinations" was also associated with a 7-month-later age at pubic hair development; however, the TR estimates were borderline significant (TR = 1.06, 95% CI: 1.00, 1.11) (Table 3). Associations remained when analyses excluded BMI adjustment (model 2a) and when restricted to girls under the 85th BMI percentile (model 2b) (Web Table 2). Overall inferences remained similar when follow-up time started at blood draw (model 3) and when assessing selection bias (model 4), with one exception where CMV⁺-only estimates were attenuated in model 4 (HR = 0.69, 95% CI: 0.49, 0.98) (Table 4).

Age at menarche

Overall there were no associations between serological markers and age at menarche, except for CMV⁺-any infection and earlier age at menarche.

DISCUSSION

This is, to our knowledge, the first prospective study to provide evidence that commonly acquired childhood infections are associated with age at pubertal development, independent of childhood body size. We measured the associations between infection and pubertal outcomes using multiple models and scenarios to consider the potential role of different types of measurement and selection biases that could affect the findings. We observed that girls with a CMV⁺-only infection developed breasts 7 months earlier; however, this finding was observed only in the "purely prospective" models where we collected blood prior to the onset of breast development and not when we included the girls who already had breast development at the time of selection into the cohort. We also observed that girls with a CMV⁺ infection or a HSV1⁺ and/or HSV2⁺ infection developed pubic hair 9 months later, and these findings were robust to different assumptions about measurement and selection. The estimated size of effects was similar in magnitude to that in the growing literature on other environmental factors, including endocrine disruptors, which have been in the range of 4 to 9 months earlier or later age of onset of puberty (37–40). For further context, 2 factors consistently associated with accelerated breast development, larger body size and Black versus White race, are in the range of 12 months (41) and 6 months earlier, respectively (42, 43). The magnitude of the delay in pubic hair onset is similar to that in a previous study where the authors observed that greater

Table 2. Demographic Information Overall and According to Categorical Viral Serology^a, LEGACY Girls Study, North America, 2011–2016

Demographic Variable	Total (n = 490)		No Infection (n = 206)		CMV ⁺ (n = 66)		CMV ⁺ /EBV ⁺ (n = 61)		EBV ⁺ (n = 88)		HSV1 ⁺ and/or HSV2 ⁺ (n = 29)		Other Infection Combinations (n = 40)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Age at blood draw ^b , years	9.8 (1.9)		9.8 (1.9)		10.0 (1.8)		9.8 (2.0)		9.8 (2.0)		9.7 (1.8)		9.4 (2.1)	
BMI-for-age percentile at blood draw ^b	46.1 (31.4)		44.8 (31.0)		39.4 (29.9)		45.8 (31.6)		49.3 (33.6)		45.4 (27.8)		57.1 (30.7)	
Missing	11	2.2	5	2.4	3	4.6	1	1.6	1	1.1	0	0.0	1	2.5
BMI-for-age percentile at blood draw														
<85th percentile	404	82.5	175	85.0	57	86.4	51	83.6	68	77.3	26	89.7	27	67.5
≥85th percentile	75	15.3	26	12.6	6	9.1	9	14.8	19	21.6	3	10.3	12	30.0
Missing	11	2.2	5	2.4	3	4.55	1	1.6	1	1.1	0	0.0	1	2.5
Race/ethnicity														
Hispanic	72	14.7	20	9.7	6	9.1	11	18.0	17	19.3	4	13.8	14	35.0
Black non-Hispanic	31	6.3	8	3.9	2	3.0	8	13.1	6	6.8	1	3.5	6	15.0
White non-Hispanic	336	68.6	163	79.1	49	74.2	34	55.7	54	61.4	21	72.4	15	37.5
Asian non-Hispanic	39	8.0	12	5.8	7	10.6	6	9.8	7	8.0	3	10.3	4	10.0
Other race/ethnicity	12	2.4	3	1.5	2	3.0	2	3.3	4	4.6	0	0.0	1	2.5
Maternal education														
Some college/vocational/ technical/less	126	25.7	46	22.3	12	18.2	22	36.1	25	28.4	4	13.8	17	42.5
Bachelor's degree	173	35.3	69	33.5	26	39.4	16	26.3	34	38.6	14	48.3	14	35.0
Graduate degree	185	37.8	89	43.2	27	40.9	22	36.1	27	30.7	11	37.9	9	22.5
Missing	6	1.2	2	1.0	1	1.5	1	1.6	2	2.3	0	0.0	0	0.0
Breast cancer family history ^c														
None	240	49.0	92	44.7	31	47.0	36	59.0	45	51.1	16	55.1	20	50.0
Any	250	51.0	114	55.3	35	53.0	25	41.0	43	48.9	13	44.8	20	50.0

Abbreviations: BMI, body mass index; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV1, herpes simplex virus 1; HSV2, herpes simplex virus 2.

^a The categorical infection measure includes no infection, CMV⁺ only, EBV⁺ and CMV⁺ coinfection, EBV⁺ only, HSV1⁺ and/or HSV2⁺ infection(s) only, and all other infection combinations. All measures were assessed at the time of blood draw with the exception that race/ethnicity, maternal education, and breast cancer family history were assessed at baseline.^b Values are expressed as mean (standard deviation).^c We classified daughter's breast cancer family history as the daughter having a first- or second-degree relative diagnosed with breast cancer.

Table 3. Association Between Viral Serology and Onset of Pubertal Characteristics Using Weibull Longitudinal Models in Girls Without Breast or Pubic Hair Development or Menses When Infections Were Measured by Serology, LEGACY Girls Study, North America, 2011–2016

Viral Serology Measure	Model 1 ^a		Model 2 ^b		Model 2 ^c		Age at Median Development ^d , years	Difference ^e , months
	HR	95% CI	HR	95% CI	TR	95% CI		
Onset of Breast Development								
Categorical viral serology								
No infection	1.00	Referent	1.00	Referent	1.00	Referent	11.04	Referent
CMV ⁺ only	1.84 ^f	1.08, 3.14 ^f	2.12 ^f	1.32, 3.40 ^f	0.95 ^f	0.92, 0.98 ^f	10.47 ^f	6.84 ^f
CMV ⁺ /EBV ⁺ coinfection only	1.20	0.70, 2.04	1.04	0.63, 1.72	1.00	0.96, 1.03	11.01	0.36
EBV ⁺ only	1.68 ^f	1.02, 2.77 ^f	1.90 ^f	1.15, 3.13 ^f	0.96 ^f	0.92, 0.99 ^f	10.55 ^f	5.88 ^f
HSV1 ⁺ and/or HSV2 ⁺	1.55	0.83, 2.89	1.14	0.61, 2.13	0.99	0.95, 1.04	10.94	1.20
All other infection combinations	1.93 ^f	1.03, 3.63 ^f	1.64	0.97, 2.75	0.97	0.93, 1.00	10.66	4.56
CMV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	10.91	Referent
Positive	1.40	1.00, 1.95	1.24	0.86, 1.80	0.98	0.96, 1.01	10.73	2.16
EBV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	10.92	Referent
Positive	1.52 ^f	1.09, 2.11 ^f	1.28	0.89, 1.82	0.98	0.96, 1.01	10.72	2.40
Onset of Pubic Hair Development								
Categorical viral serology								
No infection	1.00	Referent	1.00	Referent	1.00	Referent	11.08	Referent
CMV ⁺ only	0.67	0.41, 1.08	0.41 ^f	0.24, 0.71 ^f	1.07 ^f	1.03, 1.11 ^f	11.84 ^f	−9.12 ^f
CMV ⁺ /EBV ⁺ coinfection only	1.15	0.68, 1.94	0.84	0.48, 1.45	1.01	0.97, 1.06	11.23	−1.80
EBV ⁺ only	0.85	0.53, 1.35	0.76	0.49, 1.18	1.02	0.99, 1.06	11.31	−2.76
HSV1 ⁺ and/or HSV2 ⁺	0.54	0.26, 1.11	0.42 ^f	0.22, 0.81 ^f	1.07 ^f	1.02, 1.12 ^f	11.82 ^f	−8.88 ^f
All other infection combinations	1.32	0.68, 2.55	0.49 ^f	0.24, 0.98 ^f	1.06	1.00, 1.11	11.69	−7.32
CMV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	11.30	Referent
Positive	1.24	0.88, 1.75	0.96	0.65, 1.42	1.00	0.97, 1.03	11.34	−0.48
EBV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	11.35	Referent
Positive	1.34	0.96, 1.89	1.13	0.78, 1.64	0.99	0.97, 1.02	11.29	0.72
Onset of Menses								
Categorical viral serology								
No infection	1.00	Referent	1.00	Referent	1.00	Referent	13.11	Referent
CMV ⁺ only	1.65	0.99, 2.77	1.49	0.87, 2.57	0.97	0.94, 1.01	12.78	3.96
CMV ⁺ /EBV ⁺ coinfection only	1.65	0.98, 2.77	1.45	0.90, 2.33	0.98	0.95, 1.01	12.81	3.60
EBV ⁺ only	1.70 ^f	1.04, 2.78 ^f	0.82	0.51, 1.32	1.01	0.98, 1.04	13.28	−2.04
HSV1 ⁺ and/or HSV2 ⁺	1.22	0.49, 3.02	0.96	0.53, 1.76	1.00	0.96, 1.04	13.14	−0.36
All other infection combinations	2.33 ^f	1.12, 4.87 ^f	0.65	0.28, 1.52	1.03	0.97, 1.09	13.48	−4.44
CMV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	13.16	Referent
Positive	1.61 ^f	1.13, 2.28 ^f	1.65 ^f	1.15, 2.35 ^f	0.97 ^f	0.95, 0.99 ^f	12.76 ^f	4.80 ^f

Table continues

Table 3. Continued

Viral Serology Measure	Model 1 ^a		Model 2 ^b		Model 2 ^c		Age at Median Development ^d , years	Difference ^e , months
	HR	95% CI	HR	95% CI	TR	95% CI		
EBV any								
Negative	1.00	Referent	1.00	Referent	1.00	Referent	13.19	Referent
Positive	1.70 ^f	1.19, 2.43 ^f	1.42	0.97, 2.07	0.98	0.95, 1.00	12.89	3.60

Abbreviations: BMI, body mass index; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HR, hazard ratio; HSV1, herpes simplex virus 1; HSV2, herpes simplex virus 2; TR, time ratio.

^a Model 1 HRs adjusted for age as the underlying time scale and the interaction between each viral measure as an indicator variable and the daughter's age at the time of serology assessment. HRs above 1.0 indicate the rate of an earlier pubertal event. HRs below 1.0 indicate the rate of a later pubertal event.

^b Model 2 HRs were adjusted for covariates in model 1, and BMI percentile at the age of serology assessment, and the interaction between BMI percentile and the daughter's age at the time of serology assessment. We present the most parsimonious models, excluding variables with $P > 0.05$. Model 2 hazard ratios were additionally adjusted for race/ethnicity.

^c Model 2 TRs comparing median time to the age at pubertal event for the given exposure, in years, with median time for the reference group.

^d Predicted median time to the age at pubertal event for the given exposure, in years.

^e Difference between exposure and reference group, where positive values indicate an earlier age of onset of pubertal event compared with reference group and negative values indicate a later age of onset of pubertal event compared with reference group.

^f Associations are significant at $P < 0.05$.

phthalate exposure (fifth compared with the first quintile) was associated with a 6.5–9.5-month-later age at pubic hair development (44).

Our study suggests that CMV and EBV, primarily asymptomatic infections, result in earlier breast development, with stronger evidence for CMV, and EBV associations being sensitive to selection bias. Despite observing associations between CMV⁺-only and EBV⁺-only infection and puberty, we observed no association between a CMV⁺/EBV⁺ coinfection and puberty in the 12% of girls who were coinfecting. One reason for this discordance is that the coinfecting girls differed by BMI (see Table 2). Last, the results suggest that the viral-host response could vary by virome composition (45). Studies suggest that immune responses to the host's past infectious experiences can alter the outcome of a subsequent infection, such that the course of a herpesvirus infection can be influenced by previous pathogens (e.g., viruses, microflora) (46). This has been suggested for herpesviruses given that studies have shown that herpesviruses are able to modulate host immunity and might make the host more resistant or susceptible to subsequent infections (47–50). Future studies are needed to further examine the role of virome composition and the immune response on childhood growth and development.

Considering the opposing directions observed in breast and pubic hair development, prospective studies with repeated hormonal measures are needed. Although the bidirectionality between infection and hormones is established (51–53), there are a limited number of studies that examine the mechanistic association between herpesviruses and hormones. Studies have demonstrated hormones modulating herpesviruses (54–57), but limited studies examine herpesviruses modulating hormones. While the CMV findings for breast development might be more sensitive to exclusion of the left-censored girls, and therefore it is possible that

this type of selection bias explained the findings, additional studies could examine whether CMV infections have opposing roles for estrogens versus androgens, given that studies have suggested androgen-dependent CMV immune responses (58, 59). Although findings for pubic hair development were robust across all statistical models, we did not have a gold standard for measuring pubic hair development; therefore, we cannot discount the possibility that findings for pubic hair development might be spurious and due to measurement error.

Human immunodeficiency virus type 1 (HIV1) and hepatitis B infections have been associated with a later age at breast development (as reviewed in McDonald et al. (4)). In contrast to CMV, HIV and hepatitis B are symptomatic, triggering detectable host immune responses (60, 61). Therefore, a trade-off as suggested by life-history theory might not be needed in the case of asymptomatic infections, especially those infections that have been shown to protect against secondary infections to other pathogens to the advantage of the host (reviewed (20)). In contrast, HSV1/2 infections, which can be symptomatic, were associated with later pubic hair development, supporting childhood HIV studies (reviewed (4)). A prospective study suggests that greater HIV disease severity is associated with a 3–8-month-later age at pubic hair development (62). Little is known about signaling during the latent phase of herpesviruses. Evidence suggests that CMV latency might be coordinated by 2 viral genes that operate through an interaction with the epidermal growth factor receptor (EGFR) (63), which has several key cellular functions (64, 65), including being necessary for the development of the pubertal mammary gland (66, 67). EBV infections might promote proliferation of primary mammary epithelial cells (68).

Although the correlation between the age at thelarche and age at menarche was 0.66 ($P < 0.00001$), the lack of

Table 4. Model Comparisons on the Association Between Viral Serology and Pubertal Outcomes, LEGACY Girls Study, North America, 2011–2016

Viral Serology Measure	Model 2 ^a : Starting Follow-up Time at Birth		Model 3 ^b : Starting Follow-up Time at Blood Draw		Model 4 ^c : Using the Entire Data Set “Prospective+Left-Censored”	
	HR	95% CI	HR	95% CI	HR	95% CI
<i>Onset of Breast Development</i>						
Categorical viral serology						
No Infection	1.00	Referent	1.00	Referent	1.00	Referent
CMV ⁺ only	2.12 ^d	1.32, 3.40 ^d	1.68 ^d	1.11, 2.52 ^d	1.11	0.82, 1.50
CMV ⁺ /EBV ⁺ coinfection only	1.04	0.63, 1.72	1.01	0.64, 1.58	0.84	0.60, 1.19
EBV ⁺ only	1.90 ^d	1.15, 3.13 ^d	1.51	0.98, 2.33	0.88	0.66, 1.16
HSV1 ⁺ and/or HSV2 ⁺	1.14	0.61, 2.13	1.23	0.74, 2.06	0.79	0.52, 1.18
All other infection combinations	1.64	0.97, 2.75	1.26	0.73, 2.18	0.59 ^d	0.38, 0.90 ^d
<i>Onset of Pubic Hair Development</i>						
Categorical viral serology						
No infection	1.00	Referent	1.00	Referent	1.00	Referent
CMV ⁺ only	0.41 ^d	0.24, 0.71 ^d	0.55 ^d	0.34, 0.88 ^d	0.69 ^d	0.49, 0.98 ^d
CMV ⁺ /EBV ⁺ coinfection only	0.84	0.48, 1.45	1.03	0.66, 1.59	0.88	0.63, 1.25
EBV ⁺ only	0.76	0.49, 1.18	0.64	0.41, 1.01	0.71 ^d	0.53, 0.96 ^d
HSV1 ⁺ and/or HSV2 ⁺	0.42 ^d	0.22, 0.81 ^d	0.47 ^d	0.25, 0.90 ^d	0.51 ^d	0.31, 0.81 ^d
All other infection combinations	0.49 ^d	0.24, 0.98 ^d	0.82	0.48, 1.42	0.69	0.44, 1.09
<i>Onset of Menses</i>						
Categorical viral serology						
No infection	1.00	Referent	1.00	Referent	1.00	Referent
CMV ⁺ only	1.49	0.87, 2.57	0.98	0.63, 1.52	1.63	0.93, 2.83
CMV ⁺ /EBV ⁺ coinfection only	1.45	0.90, 2.33	0.98	0.63, 1.53	1.58	0.97, 2.57
EBV ⁺ only	0.82	0.51, 1.32	1.19	0.78, 1.79	1.29	0.76, 2.17
HSV1 ⁺ and/or HSV2 ⁺	0.96	0.53, 1.76	0.86	0.44, 1.68	1.01	0.55, 1.86
All other infection combinations	0.65	0.28, 1.52	1.28	0.74, 2.23	1.71	0.80, 3.66

Abbreviations: BMI, body mass index; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HR, hazard ratio; HSV1, herpes simplex virus 1; HSV2, herpes simplex virus 2.

^a Model 2 is the “purely prospective” model from Table 3, which starts follow-up time at birth.

^b Model 3 is the “purely prospective” model from Table 3; however, the model starts follow-up time at blood draw.

^c Model 4 is the “prospective+left-censored” model using the entire data set, including girls whose age at serology equaled age at pubertal event. The model is adjusted for the same set of covariates described in the methods and we present the most parsimonious models, excluding variables with $P > 0.05$.

^d Associations are significant at $P < 0.05$.

association with age at menarche might be because 57% of the girls within our prospective analyses were right-censored for the outcome of menarche. We did observe that girls with any CMV⁺ infection developed breasts 5 months later compared with girls with no infections. However, while estimates were similar, results for girls with a CMV⁺-only infection never reached significance, which diminishes the significance of the CMV⁺-any finding.

Pubertal milestones were assessed by mothers/guardians and breast development specifically was compared with clinical breast TS assessment at the in-person visit, which we

have previously reported to have high accuracy (69). We did previously report differences between maternal breast TS and clinical TS by BMI; specifically, specificity was lower for mothers of overweight girls (≥ 85 th percentile) (73.7% vs. 97.0% for girls < 85 th percentile) (69). However, most of our cohort ($> 80\%$) had BMIs of < 85 th percentile, and our inferences for the onset of breast development remained stable when analyses were restricted to girls with BMIs of < 85 th percentile. Estimates for the onset of pubic hair development were also stable when analyses were restricted to BMI of < 85 th percentile. The “purely prospective” models

ensured temporality between the exposure and the outcomes; however, the exclusion of left-censored girls could introduce selection bias into the analyses, making findings less generalizable by excluding the girls with earlier breast development.

Serological markers were measured prior to the pubertal outcomes and with a high degree of reliability, but we do not know when seroconversion occurred, and the timing of seroconversion might influence the timing of pubertal development. While the seroprevalences for the screened viruses in our cohort are low from a global perspective, they are similar to other youth cohorts in other Westernized countries (11, 70, 71). We were unable to adjust for viruses commonly vaccinated for in early childhood, which might also influence the viral-host response. We did not assess additional environmental factors that might influence pubertal timing, including but not limited to household crowding, paternal absence, chronic stress, history of antibiotic use, and endocrine disruptor chemicals. However, given that childhood seroprevalence of infections is socially patterned (72–74), we assessed confounding by maternal education and race/ethnicity.

In conclusion, this is, to our knowledge, the first prospective study to demonstrate an association between common herpesviruses and pubertal development. The association between CMV and HSV1/2 and later age at pubic hair development were found even after considering key drivers of pubertal development (e.g., age, body size, sociodemographic factors), follow-up time, and the introduction of selection bias. These findings are timely given that the Institute of Medicine has identified CMV vaccination as a high priority, potentially becoming part of the routine childhood vaccination schedule (75–79), and additional vaccinations are under development for HSV (80), which supports the need for replication and further definitive research on the mechanistic relationship between childhood virome composition, asymptomatic infections, and childhood growth and development. If replicated in larger cohorts with serological results prior to the onset of any pubertal event, these findings support the hypothesis that select herpesviruses might alter pubertal timing.

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