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PhD Thesis

Seroepidemiology of Polyomaviruses, Herpesviruses and Helicobacter pylori in early life and associations with health outcomes in childhood, the RHEA mother child cohort in Crete, Greece

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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Ανίχνευση του οροεπιπολασμού των Polyomaviruses, Herpesviruses & Helicobacter pylori σε πρώιμες ηλικίες και η συσχέτισή με επιπτώσεις στην υγεία των παιδιών - Προοπτική μελέτη Μητέρας- Παιδιού Κρήτης, Μελέτη ΡΕΑ

Ηράκλειο 2017

Μαριάννα Καραχάλιου

Ιατρός

Στο αγοράκι μας...που έρχεται!

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"η Ιθάκη σε έδωσε το ωραίο ταξίδι

χωρίς αυτήν δεν θα έβγαινες στο δρόμο

άλλα, δεν έχει να σε εδώσει πια"

Κ.Π. Καβάφης

Abstract in Greek

Εισαγωγή

Τα πρώτα χρόνια της ζωής, τα παιδιά εκτίθενται συνεχώς σε λοιμώξεις. Οι λοιμώξεις που συνοδεύονται από κλινικές εκδηλώσεις έχουν μελετηθεί συστηματικά και έχουν οδηγήσει στην ανάπτυξη αποτελεσματικών μέτρων πρόληψης, όπως ο εμβολιασμός. Ωστόσο, ο ρόλος των κοινών λοιμώξεων στην υγεία και τη νόσο έχει ελάχιστα μελετηθεί. Υπάρχουν υποθέσεις που υποστηρίζουν ότι μια φυσιολογική πορεία έκθεσης σε λοιμώξεις στην παιδική ηλικία αποτελεί προστατευτικό παράγοντα για την ανάπτυξη της παιδικής λευχαιμίας (η υπόθεση του Greaves) καθώς και για την ανάπτυξη χρόνιων φλεγμονώδων νοσημάτων (η υπόθεση της υγιεινής). Παρόλα αυτά οι υποθέσεις αυτές βασίζονται σε μελέτες που η έκθεση σε κοινές λοιμώξεις εκφράζεται με μεταβλητές όπως η ηλικία έναρξης του σχολείου. Ελάχιστες προοπτικές μελέτες υπάρχουν που να περιγράφουν τη φυσική πορεία έκθεσης σε πολλές κοινές λοιμώξεις ταυτόχρονα τα πρώτα χρόνια της ζωής, χρησιμοποιώντας βιολογικούς δείκτες, για παράδειγμα μετρώντας την ανοσολογική απάντηση.

Αντικείμενο της παρούσας διατριβής αποτέλεσε αρχικά η περιγραφή της έκθεση στην παιδική ηλικία στους Polyomaviruses και Herpesviruses. Οι Polyomaviruses αποτελούν μια ομάδα DNA ιων που προσβάλουν τον άνθρωπο (δεκατέσσερις γνωστοί μέχρι σήμερα) και φαίνεται να αποκτώνται νωρίς στη ζωή, προκαλώντας υποκλινική νόσο. Μελέτες σε ενήλικες έχουν δείξει πολύ υψηλά ποσοστά οροεπιπολασμού ωστόσο δεν είναι γνωστή η συνήθης ηλικία ορομετατροπής. Οι Herpesviruses αποκτώνται συνήθως στην παιδική ηλικία με εξαίρεση τον HSV-2 που αποκτάται αργότερα στη νεαρή ενήλικο ζωή με την έναρξη της σεξουαλικής δραστηριότητας. Νεότερα δεδομένα δείχνουν ότι η επιδημιολογία των Herpesviruses έχει αλλάξει σημαντικά και η ορομετατροπή έχει μετατεθεί σε μεγαλύτερες ηλικίες, πιθανά λόγω αλλαγών που έχουν επισυμβεί στον τρόπο ζωής όπως η σύνθεση της οικογένειας και οι τρόποι ανατροφής των παιδιών. Τα παραπάνω υποσημαίνουν την αξία μελέτης του οροεπιπολασμού των Polyomaviruses και Herpesviruses τα πρώτα χρόνια ζωής στο γενικό πληθυσμό.

Επόμενο σημαντικό ερώτημα της παρούσας διατριβής αποτέλεσε κατά πόσο οι Polyomaviruses και Herpesviruses σχετίζονται με την νευροανάπτυξη και την σωματική ανάπτυξη στα πρώτα χρόνια ζωής στο γενικό πληθυσμό. Το

αναπτυσσόμενο νευρικό σύστημα είναι ιδιαίτερα ευαίσθητο στην φλεγμονή που προκαλείται απο ιογενείς λοιμώξεις. Από τους Polyomaviruses, ο JCPyV είναι νευροτρόπος ιός και ευθύνεται για την προοδευτική πολυεστιακή λευκοεγκεφαλοπάθεια σε ανοσοκατεσταλμένα άτομα. Επίσης σε μία μελέτη ασθενών-μαρτύρων, απομονώθηκαν οι BKPyV, JCPyV και SV40 σε εγκεφαλικό ιστό ατόμων με αυτισμό. Από τους Herpesviruses που μελετήσαμε, οι EBV, CMV και HSV-1 είναι δυνητικά νευροτρόποι ιοί και κάτω από ορισμένες συνθήκες μπορούν να οδηγήσουν σε εγκεφαλίτιδα/μηνιγγίτιδα καθώς και μακροπρόσθεσμες νευρολογικές επιπτώσεις, για παράδειγμα σε νευροαισθητήρια απώλεια της ακοής μετά από συγγενή λοίμωξη με CMV. Η πιθανή σχέση των Polyomaviruses και Herpesviruses με την σωματική ανάπτυξη, την παχυσαρκία και καρδιαγγειακούς δείκτες στην παιδική ηλικία είναι λιγότερο προφανής, ωστόσο στηρίζεται σε μια νεότερη υπόθεση ότι οι λοιμώξεις μπορεί να οδηγούν σε παχυσαρκία μέσω διαφόρων μηχανισμών που υποδεικνύονται από μελέτες σε ζώα. Συγκεκριμένα, ο αδενοϊός 36, που αποκτάται στην παιδική ηλικία, έχει συσχετιστεί με την παχυσαρκία σε μελέτες σε ανθρώπους και ζώα. Επίσης υπάρχουν αναφορές για τη σχέση των CMV, HSV-1, HSV-2 με καρδιαγγειακούς δείκτες ωστόσο τα δεδομένα αυτά είναι περιορισμένα. Τα τελευταία χρόνια αυξάνονται επίσης οι αναφορές για τη σχέση του αριθμού των λοιμώξεων που έχει εκτεθεί ο άνθρωπος (pathogen burden) με την παχυσαρκία και άλλους καρδιαγγειακούς δείκτες, ωστόσο δεν υπάρχουν δεδομένα στην παιδική ηλικία.

Τέλος, αντικείμενο της παρούσας διατριβής αποτέλεσε ο καθορισμός του οροεπιπολασμού του Helicobacter pylori, ενός κοινού παθογόνου για τον άνθρωπο που σχετίζεται με αυξημένο κίνδυνο για γαστρικό καρκίνο. Ιδιαίτερο ενδιαφέρον παρουσιάζουν οι αυξανόμενες αναφορές για τη σχέση του Helicobacter pylori με εξωγαστρικές εκδηλώσεις όπως η ιδιοπαθής θρομβοπενική πορφύρα, η σιδηροπενική αναιμία και η B12 ανεπάρκεια. Παράλληλα, υπάρχουν αναφορές σε ενήλικες που συσχετίζουν το Helicobacter pylori με νευροψυχιατρικά νοσήματα όπως η νόσος Alzheimer και Parkinson. Πιθανά παρόμοια συσχέτιση να υπάρχει και στην παιδική και εφηβική ηλικία ωστόσο δεν υπάρχουν σχετικές αναφορές. Υποθέσαμε ότι η λοίμωξη από Helicobacter pylori νωρις στη ζωή, ακόμα και κατά την ενδομήτριο ζωή, μπορεί να οδηγήσει σε δυσμενή νευροαναπτυξιακή εξέλιξη, μέσω μικροθρεπτικών ελλείψεων (π.χ. σε φυλλικό, B12) που φαίνεται να προκαλεί η λοίμωξη από Helicobacter pylori. Στην παρούσα διατριβή μελετήθηκε λοιπόν

συγκεκριμένα η σχέση της οροθετικότητας στο Helicobacter pylori στις μητέρες στο τέλος της κύησης και των παιδιών στα 4 έτη με τη νευροαναπτυξη των παιδιών στα 4 έτη.

Ειδικοί Στόχοι

- Να περιγράψουμε τον οροεπιπολασμό των Polyomaviruses, Herpesviruses και Helicobacter pylori νωρίς στη ζωή, χρησιμοποιώντας επαναλαμβανόμενα δείγματα αίματος που συλλέχθησαν στη γέννηση (ομφαλικό αίμα), στα 3 έτη και στα 4 έτη.
- Να περιγράψουμε τους παράγοντες- κοινωνικο-οικονομικούς, περιγεννητικούς, κοινωνικών επαφών, τρόπου ζωής, προσωπικής υγιεινής- που επιδρούν στην ορομετατροπή στους Polyomaviruses και Herpesviruses εως την ηλικία των 4 ετών.
- Να μελετήσουμε τη σχέση της έκθεσης σε ένα ή και περισσότερους Polyomaviruses και Herpesviruses εως την ηλικία των 4 ετών με τη νευροαναπτυξη των παιδιών στην ίδια ηλικία.
- Να μελετήσουμε τη σχέση της λοίμωξης με Helicobacter pylori νωρίς στη ζωή, συμπεριλαμβανομένου της ενδομήτριου ζωής, με τη νευροανάπτυξη των παιδιών στην ηλικία των 4 ετών.
- Να μελετήσουμε τη σχέση της έκθεσης σε ένα ή και περισσότερους Polyomaviruses και Herpesviruses εως την ηλικία των 4 ετών με την παχυσαρκία και καρδιαγγειακούς δείκτες στα 4 και 6 έτη ζωής.

Μεθοδολογία

Ο πληθυσμός της παρούσας διατριβής προέρχεται από τη μελέτη Μητέρας Παιδιού Κρήτης, Μελέτη ΡΕΑ (www.rhea.gr) και περιλαμβάνει ένα δείγμα περίπου 1,000 εγκύων γυναικών (Ελληνίδων και αλλοδαπών) και των παιδιών τους για τις οποίες ο τοκετός πραγματοποιήθηκε σε ένα από τα νοσοκομεία του νομού Ηρακλείου (δημόσια & ιδιωτικά) κατά τη χρονική περίοδο ενός έτους (Μάρτιος 2007-Φεβρουάριος 2008). Οι γυναίκες αυτές προσεγγίστηκαν τη 12η -14η και την 28η -32η εβδομάδα κύησης και τον τοκετό και ακολούθησε παρακολούθηση των παιδιών με συνάντηση στους 6 μήνες ζωής, στα 4 χρόνια και στα 6 χρόνια. Σε ένα τυχαίο δείγμα του πληθυσμού μας έγινε συνάντηση και στα 3 χρόνια ζωής για τους σκοπούς της παρούσας ορολογικής μελέτης.

Δείγματα αίματος που συλλέχθηκαν στη γέννηση (ομφαλικό αίμα) (n=626), 3 χρόνια (n=81) και 4 χρόνια (n=690) και είχαν αποθηκευθεί στους -80˚C, στάλθηκαν για ορολογική ανάλυση στο εργαστήριο, Molecular Diagnostics of Oncogenic Infections Department, Infection and Cancer Program, German Cancer Research Center (DKFZ) της Γερμανίας. Η IgG οροαντιδαστικότητα που μετρήθηκε αφορούσε το αντιγόνο VP1 για τους 12 Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV, MCPyV, HPyV9 and HPyV10), τέσσερα EBV αντιγόνα (ZEBRA, EBNA-1, EA-D, VCA p18), πέντε CMV αντιγόνα (pp52, pp65, pp150, pp28, CM2), ένα HSV-1 αντιγόνο (gG), ένα HSV-2 αντιγόνο (mgGunique), τέσσερα HHV-8 αντιγόνα (LANA, V-CYCLIN, K8.1, ORF-65) και δώδεκα Helicobacter pylori αντιγόνα (GroEL, UreA, HP0231, NapA, HP0305, HpaA, CagA, HyuA, catalase, VacA, HcpC, Omp). Σημειώνεται ότι η οροαντιδραστικότητα στα δείγματα ομφαλικού αίματος αντιπροσωπεύει την οροαντιδραστικότητα των μητέρων στο τέλος της κύησης. Η οροθετικότητα για κάθε αντιγόνο ορίστηκε χρησιμοποιώντας συγκεκριμένα όρια που στην περίπτωση των Polyomaviruses και Herpesviruses ορίστηκαν στον πληθυσμό των παιδιών στα 3 έτη, ενώ για το Helicobacter pylori χρησιμοποιήθηκαν ήδη ορισμένα όρια. Η συλλογή των δεδομένων σε σχέση με τους παράγοντες - κοινωνικο-οικονομικούς, περιγεννητικούς, κοινωνικών επαφών, τρόπου ζωής, προσωπικής υγιεινής - που επιδρούν στην απόκτηση των Polyomaviruses και Herpesviruses εως την ηλικία των 4 ετών πραγματοποιήθηκε με τη χρήση ερωτηματολογίων διαχρονικά από τη γέννηση εως τα 4 έτη.

Σε ότι αφορά την ψυχοκινητική ανάπτυξη των παιδιών, ειδικά εκπαιδευμένοι ψυχολόγοι την αξιολόγησαν χρησιμοποιώντας τις Κλίμακες Εκτίμησης Παιδικών Δεξιοτήτων (McCarthy Scales of Children's Abilities, MSCA) στην ηλικία των 4 ετών. Στην ίδια ηλικία έγινε η αξιολόγηση ελλειμματικής προσοχής-υπερκινητικότητας των παιδιών με κλίμακα που συμπληρώθηκε από τους γονείς αυτών (Gilliam J. E., 1995; Maniadaki K & Kakouros E, 2002). Ταυτόχρονα οι γονείς συμπλήρωσαν ερωτηματολόγιο σχετικά με τις δυνατότητες και τις δυσκολίες των παιδιών τους (Goodman, 1997; Mpipou-Nakou I, Stogiannidou A, & Kisseoglou G, 2001).

Σε οτι αφορα την σωματική ανάπτυξη των παιδιών, ειδικά εκπαιδευμένοι μελετητές πεδίου μέτρησαν στη συνάντηση των 4 και 6 ετών, το βάρος, ύψος, την περίμετρο μέσης και τέσσερις δερματικές πτυχές (τρικέφαλου, υπο-ωμοπλατιαία, υπερ-λαγόνια,

μηρού) ακολουθώντας συγκεκριμένα πρωτοκολλα. Επιπλέον στα 6 έτη, το ποσοστό λίπους μετρήθηκε με τη μέθοδο της βιοηλεκτρικής αντίστασης (Bodystat 1500). Επίσης μετρήθηκαν δείκτες που σχετίζονται με την καρδιαγγειακή υγεία και συγκεκριμένα η ολική και η HDL-χοληστερόλη (4 και 6 έτη) καθώς και η λεπτίνη και η αδιπονεκτίνη (4 έτη μόνο).

Στις αναλύσεις που αναφέρθηκαν, περιγραφικές μεθόδοι στατιστικής και πολυπαραγοντικά μοντέλα γραμμικής, Poisson και λογιστικής παλινδρόμησης χρησιμοποιήθηκαν ανάλογα για τη διερεύνηση των σχέσεων.

Αποτελέσματα

1) Ο οροεπιπολασμός των Polyomaviruses κυμάνθηκε μεταξύ 38.5% και 99.8% στα δείγματα ομφαλικού αίματος και μεταξύ 20.9% και 82.3% στα 4 έτη. Ο οροεπιπολασμός των Herpesviruses και συγκεκριμένα για τον EBV, CMV, HSV-1, HSV-2, HHV-8 ήταν 99.4%, 74.9%, 26.2%, 8.0% και 1.6% στα δείγματα ομφαλικού αίματος και 52.5%, 25.8%, 3.6%, 1.4% και 0% στα 4 ετη αντίστοιχα. Οι κύριοι παράγοντες που βρέθηκαν να επηρεάζουν τον οροεπιπολασμό στα 4 έτη ήταν η οροθετικοτητα του αντίστοιχου ομφαλικού δείγματος (JCPyV, HPyV7, HPyV10, CMV) που αντικατοπτρίζει τα επίπεδα της μητέρας, ο φυσιολογικός τοκετός (HPyV10), ο θηλασμός (CMV), η έναρξη παιδικού σταθμού σε μικρότερη ηλικία (BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, HPyV9, EBV, CMV) και η χρήση πισίνας (BKPyV, KIPyV, WUPyV, HPyV10). Αντιστρόφως ανάλογη ήταν η σχέση μεταξύ της διάρκειας τηλεθέασης, του γονεϊκού στρες και των πρακτικών υγιεινής με τον οροεπιπολασμό των Polyomaviruses και Herpesviruses στα 4 έτη.

2) Χρησιμοποιώντας συγχρονικά δεδομένα στα 4 έτη, μελετήσαμε τη σχέση της οροθετικότητας σε ένα ή περισσότερους Polyomaviruses και Herpesviruses με την νευροανάπτυξη και τη συμπεριφορά σε πληθυσμό 674 παιδιών. Η οροθετικότητα στον BKPyV σχετίστηκε με περισσότερα συμπτωματα ελλειμματικής προσοχής στην κλιμακα ADHDT. Επίσης, η οροθετικότητα σε ≥8 Polyomaviruses σε σχέση με την οροθετικότητα σε ≤3 Polyomaviruses σχετίστηκε με λιγότερα συμπτώματα ελλειμματικής προσοχής στην κλίμακα ADHDT και υπερκινητικότητας-ελειμματικής προσοχής στην κλίμακα SDQ.

3) Σε σχέση με το Helicobacter pylori, περιγράψαμε οροεπιπολασμό 41.5% στα δείγματα ομφαλικού αίματος, που αντιπροσωπεύουν το προφίλ των μητέρων, και

6.5% στα παιδιά στα 4 έτη. Η οροθετικότητα στα δείγματα ομφαλικού αίματος για Helicobacter pylori σχετίστηκε με χαμηλότερες επιδόσεις σε όλες τις Κλίμακες Εκτίμησης Παιδικών Δεξιοτήτων (MCSA) εκτός της κινητικής στα παιδιά ηλικίας 4 ετών. Συγκεκριμένα η οροθετικότητα σε 2 από τα 12 αντιγόνα του Helicobacter pylori που μελετήθηκαν, GroEl and NapA, σχετίστηκε με χαμηλότερες επιδόσεις στα νευροαναπτυξιακά τεστ. Η οροθετικότητα στο Helicobacter pylori στα 4 ετη σχετίτηκε επίσης με χαμηλότερες επιδόσεις στις Κλίμακες Εκτίμησης Παιδικών Δεξιοτήτων (MCSA) στην ίδια ηλικία, ωστόσο οι σχέσεις δεν ήταν στατιστικά σημαντικές.

4) Τέλος, χρησιμοποιήσαμε συγχρονικά δεδομένα από 674 παιδιά στα 4 ετη και προοπτικά δεδομένα για 440 παιδιά στα 6 ετη, για τον έλεγχο της συσχέτιση της οροθετικότητας σε Polyomaviruses και Herpesviruses εως τα 4 έτη με την παχυσαρκία και τους καρδιαγγειακούς δείκτες στις αντίστοιχες ηλικίες. Η οροθετικότητα στον BKPyV σχετίστηκε με χαμηλότερο δείκτη μάζας σώματος, περίμετρο μέσης, δερματικών πτυχών και λεπτίνης στα 4 έτη και χαμηλότερο δείκτη μάζας σώματος, περίμετρο μέσης και % σωματικού λίπους στα 6 έτη. Αντίθετα, η οροθετικότητα στον CMV σχετίστηκε με υψηλότερο δείκτη μάζας σώματος τόσο στα 4 όσο και στα 6 έτη και υψηλότερο άθροισμα δερματικών πτυχών στα 6 έτη. Επιπλέον, η οροθετικότητα σε “2-3 Herpesviruses” έναντι “0 Herpesviruses”, σχετίστηκε με υψηλότερο δείκτη μάζας σώματος, περίμετρο μέσης και άθροισμα δερματικών πτυχών στα 4 έτη.

Συμπεράσματα

Συνοψίζοντας, η παρούσα μελέτη περιγράφει την φυσική πορεία απόκτησης δέκα Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV, MCPyV, HPyV9 και HPyV10), πέντε Herpesviruses (EBV, CMV, HSV-1, HSV-2, HHV-8) και του Helicobacter pylori σε ένα πληθυσμό υγιών παιδιών που παρακολουθούνται προοπτικά στη μελέτη Μητέρας Παιδιού Κρήτης, Μελέτη ΡΕΑ. Στην υπάρχουσα βιβλιογραφία δεν υπάρχει ανάλογη λεπτομερής περιγραφή του οροεπιπολασμού αυτών των κοινών λοιμώξεων σε διαδοχικές χρονικές στιγμές νωρίς στη ζωή. Περιγράψαμε ότι τα παιδιά στην ηλικία των 4 ετών, ήταν συχνά οροθετικά στους Polyomaviruses και Herpesviruses με εξαίρεση τον HHV-8, HSV-1 και HSV-2. Ο οροεπιπολασμός στο Helicobacter pylori στα 4 έτη ήταν χαμηλός και σε αντιστοιχία με τα αποτελέσματα από άλλες δυτικές κοινωνίες. Η παρούσα ανάλυση

προσφέρει σημαντικές πληροφορίες για τους παράγοντες που επιδρούν στην απόκτηση αυτών των κοινών λοιμώξεων έως τα 4 έτη. Για παράδειγμα, περιγράψαμε την ισχυρή σχέση της ηλικίας έναρξης του σχολείου με τον οροεπιπολασμό των BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, HPyV9, EBV και CMV καθώς και την ισχυρή κοινωνικο-οικονομική διαστρωμάτωση του οροεπιπολασμού των Polyomaviruses στα 4 έτη. Τέτοιες συσχετίσεις θα βοηθήσουν στον χαρακτηρισμό των τρόπων μετάδοσης ειδικά των Polyomaviruses που παραμένουν ακόμα κατά πολύ αδιευκρίνιστοι.

Ιδιαίτερο ενδιαφέρον παρουσιάζουν τα αποτελέσματα των αναλύσεων που διερευνούν τη σχέση των Polyomaviruses και Herpesviruses με την νευροαναπτυξιακή εξέλιξη των παιδιών. Η αντίστροφη σχέση του αριθμού των Polyomaviruses με τα συμπτώματα ελλειμματικής προσοχής υπερκινητικότητας υποδεικνύει όχι μόνο πιθανούς μηχανισμούς αλληλεπίδρασης του ανοσοποιητικού συστήματος με το αναπτυσσόμενο κεντρικό νευρικό σύστημα αλλά και ένα διαφορετικό τρόπο απόκτησης αυτών των κοινών λοιμώξεων στα παιδιά με σχετικές συμπεριφορές. Σημαντικό από την πλευρά της πρόληψης είναι το εύρημα ότι τα παιδιά των οροθετικών στο Helicobacter pylori μητέρων είχαν δυσμενέστερη νευροανάπτυξη στα 4 έτη, δημιουργώντας νέα ερωτήματα για μελλοντική έρευνα για το κατά πόσο η θεραπεία των Helicobacter pylori θετικών μητέρων θα μπορούσε να αναστρέψει ένα τέτοιο αποτέλεσμα. Τέλος, διερευνώντας την πιθανή συσχέτιση των Polyomaviruses και Herpesviruses με τη σωματική ανάπτυξη και τους καρδιαγγειακούς δείκτες στα παιδιά, αναδείχθηκαν πολύ ισχυρά αποτελέσματα για τους BKPyV και CMV. Παρόμοια αποτελέσματα δεν έχουν αναφερθεί εως τώρα στα παιδιά και χρήζουν επιβεβαίωσης σε άλλους πληθυσμούς προκειμένου να ισχυροποιηθούν τα παραπάνω συμπεράσματα.

Abstract in English

Introduction

During the first years of life, children are constantly confronted to a number of infections. Clinically evident infections have been a subject of research for many years, enabling development of successful prevention programs (e.g., vaccination programs). Nonetheless, children subclinically acquire a number of other infections whose role in health and disease has been little examined. It has been suggested that a normal course of infections in early life protects against the development of childhood leukemia (the Greaves hypothesis) and of chronic inflammatory disorders (the hygiene or "old friends" hypothesis). Nonetheless, these hypotheses are based on epidemiological studies which use proxy variables such as age of first school attendance, to denote exposure to common infections. Very limited prospective data exist on characterizing this repertoire of common infections in childhood by use of biological measures.

The primary objective of this study has been the description of the seroepidemiology of Polyomaviruses and Herpesviruses in early childhood. Polyomaviruses are DNA viruses (fourteen known so far), which are largely acquired subclinically in early life. Studies in adults show high seroprevalence rates but the exact age of acquisition is not known. Herpesviruses are also largely acquired in childhood with the exception of HSV-2, which is predominantly acquired through sexual contact later in life. Emerging data suggest that recent changes in family structure, childrearing, and hygiene practices have modified the epidemiology of Herpesviruses. This might have important public health implications as the timing of exposure has a critical role in the outcome of infection with Herpesviruses. These facts highlight the importance of studying the seroepidemiology of Polyomaviruses and Herpesviruses in early life in the general population.

The next objective has been to explore the role of Polyomaviruses and Herpesviruses infections in child's neurodevelopment and growth. The developing brain is extremely sensitive to the neuroinflammation induced by viruses. Among Polyomaviruses, JCPyV is known to be neurotropic and is the etiologic agent of progressive multifocal leukoencephalopathy, a fatal demyelinating disease of the central nervous sytem occurring mainly in immonocompromised individuals. Moreover, Lintas et al. found

enhanced frequencies of Polyomaviruses (BKPyV, JCPyV, SV40) in postmortem neocortical tissues of autistic patients, raising questions for an association between Polyomaviruses and autism. EBV, CMV and HSV-1, members of the Herpesviruses family, are also neurotropic viruses and under certain circumstances can infect the central nervous system and cause acute encephalitis/meningitis and long-term neurological sequelae (e.g., sensorineural hearing loss due to congenital CMV infection). The potential association of Polyomaviruses and Herpesviruses with child's growth, obesity and cardiometabolic traits is less evident but is based on an emerging hypothesis of an infectious origin of obesity. In particular, adenovirus 36 has been associated with obesity both in animal and human studies. Moreover a metabolic dysfunction has been shown to accompany some viral infections such as CMV, HSV-1 and HSV-2. Interestingly, an increasing number of studies report a link between pathogen burden, obesity, cardiometabolic traits and disease, but no evidence exists in childhood.

Lastly, an objective of the present study has been to describe the seroepidemiology of Helicobacter pylori, a very common chronic bacterial infection in humans which is associated with increased risk for gastric cancer. A wealth of evidence strongly implicates Helicobacter pylori infection in the pathogenesis of extragastric diseases such as idiopathic thrombocytopenic purpura, unexplained iron-deficiency anemia and vitamin B12 deficiency. Moreover, an increasing number of studies report an association between Helicobacter pylori and neuropsychiatric diseases such as Parkinon's and Alzheimer. It is likely that an association with neurodevelopment is evident already in early life, but there are no data. Thus, we hypothesized that Helicobacter pylori infection during the first years of life but also maternal Helicobacter pylori infection during pregnancy may unfavorably affect offspring's neurodevelopment through micronutrients deficiencies (e.g folate, B12). Fetal brain is extremely vulnerable to such micronutrients defects. To the best of our knowledge, no study has examined differences in neurodevelopment between children of Helicobacter pylori seropositive versus seronegative mothers.

Specific Objectives

- To describe the seroprevalence of Polyomaviruses, Herpesviruses and Helicobacter pylori in early life using repeated samples collected at birth (cord blood), three years and four years of age.

- To identify the factors – sociodemographic, perinatal, variables denoting social interactions, lifestyle, hygiene practices- that determine the acquisition of Polyomaviruses and Herpesviruses up to four years of age.
- To explore the association between seropositivity to single and multiple Polyomaviruses and Herpesviruses up to age four with neurodevelopmental assessment of children at the same age.
- To explore the association between Helicobacter pylori infection early in life including fetal life with neurodevelopment of children at age four.
- To explore the association between seropositivity to single and to multiple Polyomaviruses and Herpesviruses up to age four with obesity and cardiometabolic traits at age four and six.

Methods

This study uses data from the Rhea study a birth cohort that recruited 1,606 pregnant women from February 2007 to January 2008 (www.rhea.gr). The inclusion criteria were confirmed pregnancy, residency within the study area, aged 16 years or older, and good understanding of the Greek language. Recruitment occurred mostly before 15 weeks of gestation. Mothers were contacted again at 24 weeks of gestation, at birth, and for child's follow-up at 9th month, 18th month, 4 years, and 6 years of age. When members of the cohort were 3 years of age, we invited a randomly selected subsample for a serological assessment (n = 109).

Blood samples collected at birth (cord blood) and at 3 and 4 years of age were processed following standard procedures and then stored at −80°C. Serum aliquots of 100 μL were shipped on dry ice to the German Cancer Research Center, Heidelberg, Germany, for serological analysis. Immunoglobulin G seroreactivity against the viral capsid protein 1 of ten Polyomaviruses (BKPyV, JCPyV, KIPyV,WUPyV,MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, HPyV10), four EBV antigens (ZEBRA, EBNA-1, EA-D, VCA p18), five CMV antigens (pp52, pp65, pp150, pp28, CM2), one HSV-1 antigen (gG), one HSV-2 antigen (mgGunique), four HHV-8 antigens (LANA, v-cyclin, K8.1, ORF-65) and twelve H.pylori proteins (GroEL, UreA, HP0231, NapA, HP0305, HpaA, CagA, HyuA, catalase, VacA, HcpC, Omp) was measured by fluorescent bead-based multiplex serology (1:1,000 dilution). Of note, immunoglobulin G levels in cord blood reflect the mother's immunoglobulin G levels. Seropositivity for a given antigen was defined as median fluorescence intensity values

greater than the antigen- specific cut off. EBV and CMV seropositivity was defined as seropositivity for at least two virus specific antigens. Seropositivity for a given Helicobacter pylori protein was defined as seroreactivity greater than the protein-specific cut-off which was re-evaluated using the published reference panel. The aggregate number of different Polyomaviruses that the children were seropositive to was referred as Polyomaviruses burden. Similarly, we calculated the Herpesviruses burden.

Information on potential determinants of seroprevalence of polyomaviruses and herpesviruses is based on repeated questionnaires from birth to 4 years of age. Children's cognitive and motor development was assessed by two trained psychologists with the McCarthy Scales of Children's Abilities (MSCA). Additional information on children's behavior was obtained via maternal report on standardized child behavior scales, which were administered at the 4 years of age follow-up. The Attention-Deficit/Hyperactivity Disorder Test (ADHDT) is designed to identify and evaluate ADHD in ages 3–23 years. The parent version of the Strengths and Difficulties Questionnaire (SDQ) is a behavioral screening instrument designed to assess strengths and difficulties of children aged 3–16 years. We measured body mass index, waist circumference and skinfold thickness at four anatomical sites at four and six years of age. At age six hand-to-leg bioelectrical impendence was also measured using a Bodystat 1500 machine. Data on metabolic traits including serum lipids, leptin and adiponectin levels were also available.

Descriptive analyses of the study population characteristics, exposures and outcomes were conducted. Multiple linear, logistic and Poisson regression models were used to explore the associations accordingly.

Results

1) Seroprevalence of Polyomaviruses ranged from 38.5% to 99.8% in cord blood and from 20.9% to 82.3% at age 4. Seroprevalence of EBV, CMV, HSV-1, HSV-2 and HHV-8 was 99.4%, 74.9%, 26.2%, 8.0%, and 1.6% in cord blood and 52.5%, 25.8%, 3.6%, 1.4%, and 0% at age 4, respectively. Determinants of seropositivity at age 4 were cord seropositivity (JCPyV, HPyV7, HPyV10, CMV), vaginal delivery (HPyV10), breastfeeding (CMV), younger age at day-care entry (BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, HPyV9, EBV, CMV), and swimming pool attendance (BKPyV, KIPyV, WUPyV, HPyV10). Television

viewing, parental stress, and hygiene practices were inversely associated with the seroprevalence of Polyomaviruses and Herpesviruses.

2) Based on crosss-ectional data at age four, we investigated the association between seropositivity to single or multiple Polyomaviruses and Herpesviruses with child's neurodevelopment and behavior among 674 children. Seropositivity to BKPyV, a potential neurotropic virus, was associated with higher score in ADHDT inattention subscale. Moreover, children seropositive to ≥8 polyomaviruses had lower score in ADHDT inattention subscale and lower score in SDQ hyperactivity–inattention subscale versus children seropositive to ≤3 Polyomaviruses.
3) H.pylori seroprevalence in cord blood, representing maternal status, was 41.5% and in children four years of age was 6.5%. Children of Helicobacter pylori seropositive mothers compared to those of seronegative mothers, had lower score in all scales of the MCSA excluding motor scale. In addition, seropositivity in cord blood specifically to GroEl and NapA – two of the 12 Helicobacter pylori proteins investigated – was associated with statistically significant lower scores in almost all scales of MCSA. At age four, Helicobacter pylori seropositive children performed worst in neurodevelopment assessment compared to their seronegative counterparts although no association reached statistically significant level.
4) Based on cross-sectional data of 674 children participating at the 4 years of age follow-up and prospective data of 440 children at age six, we investigated the association of seropositivity to single or multiple Polyomaviruses and Herpesviruses with child's obesity and cardiometabolic traits at the corresponding ages. BKPyV seropositivity was associated with lower BMI, waist circumference, sum of skinfolds and lower leptin levels at age four and with lower BMI, waist circumference and %body fat at age six. On the other hand, CMV seropositivity was associated with higher BMI at age four and six and higher sum of skinfolds at age six. Moreover, children with "2-3 Herpesviruses infections" versus those with "0 Herpesviruses infections" had higher BMI SD-score, waist circumference and sum of skinfolds at age four.

Conclusions

In summary, findings of the present thesis describe the natural history of acquisition of ten Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV, MCPyV, HPyV9 and HPyV10), five Herpesviruses (EBV, CMV, HSV-1, HSV-2, HHV-8) and Helicobacter pylori in a population of young healthy children which are prospectively followed in the Rhea mother-child cohort in Crete. To the best of our knowledge, there is no previous report gathering such detailed information on seroprevalence to those common infections. We have shown that, by age 4, children were commonly seropositive to Polyomaviruses and Herpesviruses, with the exceptions of HHV-8, HSV-1 and HSV-2. Seroprevalence to Helicobacter pylori was low and in accordance with reports from other western populations. The resent analysis provides important insights into the factors that determine the acquisition of such common infections by age four. For example, younger age at day-care entry importantly contributed in the epidemiology of BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, HPyV9,EBV, and CMV while there was as strong socioeconomic position patterning of Polyomaviruses seroprevalence at age four. Such findings are important allowing inference on potential routes of transmission for those viruses among young children.

Intriguing are the findings regarding the association of Polyomaviruses and Herpesviruses infections with child's neurodevelopment. The inverse association between the number of Polyomaviruses infections acquired up to age four with symptoms of inattention and hyperactivity might indicate not only potential interaction between the immune system and the developing brain but also a different pattern of acquisition of such common infections among children with such behaviours. From a public health perspective, it is particularly important the finding that children of Helicobacter pylori seropositive mothers had worst neurodevelopmental performance at age four, raising questions on whether treatment for Helicobacter pylori could prevent such an outcome. Finally, we observed very strong associations for BKPyV and CMV with obesity and cardiometabolic traits in childhood. Similar findings have not been reported in children and should be replicated in other populations.

PhD thesis publications

The current thesis consists of a compilation of four scientific publications: three of which have been published and one is currently under review.

1. **Marianna Karachaliou,** Tim Waterboer, Delphine Casabonne, Georgia Chalkiadaki, Theano Roumeliotaki, Angelika Michel, Eftichia Stiakaki, Leda Chatzi, Michael Pawlita, Manolis Kogevinas, and Silvia de Sanjose. The Natural History of Human Polyomaviruses and Herpesviruses in Early Life—The Rhea Birth Cohort in Greece, Am J Epidemiol. 2016;183(7):671–679

2. **Marianna Karachaliou,** Leda Chatzi, Theano Roumeliotaki, Mariza Kampouri, Andriani Kyriklaki, Katerina Koutra, Georgia Chalkiadaki, Angelika Michel, Eftichia Stiakaki, Manolis Kogevinas, Michael Pawlita, Tim Waterboer and Silvia de Sanjose, Common infections with polyomaviruses and herpesviruses and neuropsychological development at 4 years of age, the Rhea birth cohort in Crete, Greece, J Child Psychol Psychiatry. 2016 Nov;57(11):1268-1276

3. **Marianna Karachaliou,** Leda Chatzi, Angelika Michel, Andriani Kyriklaki, Mariza Kampouri, Katerina Koutra, Theano Roumeliotaki, Georgia Chalkiadaki, Eftichia Stiakaki, Michael Pawlita, Tim Waterboer, Manolis Kogevinas and Silvia de Sanjose. Helicobacter pylori infection and childhood neurodevelopment, the Rhea birth cohort in Crete, Greece. Paediatric and Perinatal Epidemiology, 2017

4. **Marianna Karachaliou,** Silvia de Sanjose, Tim Waterboer, Theano Roumeliotaki, Maria Vassilaki, Katerina Sarri, Vasiliki Leventakou, Marina Vafeiadi, Georgia Chalkiadaki, Eftichia Stiakaki, Angelika Michel, Michael Pawlita, Manolis Kogevinas, Leda Chatzi Is early life exposure to polyomaviruses and herpesviruses associated with obesity indices and metabolic traits in childhood? Under review at the International Journal of Obesity.

1. General introduction

Children are exposed to a great number of infections. Clinically evident infections have been a subject of research for many years, enabling development of successful prevention programs. Thus, due to improvements in sanitation, antibiotics and vaccines, smallpox has now been eradicated, polio is on the brink of extinction and many other infectious diseases are on the decline. Nonetheless, children subclinically acquire a number of other infections whose role in health and disease has been little examined. A normal course of infections in early life has been hypothesized to confer protection against the development of leukemia, allergies and some autoimmune diseases. It is possible that these divergent pathologies (leukaemia, allergic disorders, autoimmune diseases) reflect the same underlying mismatch between evolutionary programming of the immune system and contemporary patterns of infection in early life. Moreover, accumulating evidence suggests that infectious agents may cause or interact in causing chronic diseases such as atherosclerosis and neuropsychiatric diseases, opening new areas of research in infectious diseases epidemiology.

1.1. Hypotheses on infectious origin of non-communicable diseases

On the aetiology of childhood leukemia, two infection-based hypotheses have been proposed: Kinlen's "population mixing" hypothesis and Greaves' "delayed infection" hypothesis (1,2). In brief, in 1988 Kinlen suggested that the apparent clusters of childhood leukemia and non-Hodgkin lymphoma recorded in certain communities (e.g. Seascale village in England and Thurso town in Scotland), might have infectious origins that were due to the unusual population mixing that occurred when these isolated communities were enlarged to accommodate the influx of migrant professional workers (1). Subsequent studies provided further evidence to suggest that some childhood leukemia clusters might be an unusual outcome of a common but relatively non-pathological infection arising in individuals who were non-immune and following contact of "population mixing" with carriers (3,4). Greaves' or "delayed-infection" hypothesis proposes that infectious insulation, a feature of modern or more affluent "hygienic" societies, might predispose the immune system to aberrant or pathological responses following subsequent or "delayed" exposure. This response is predicted to precipitate acute lymphoblastic leukemia through proliferative or

apoptotic stress. Moreover, in the minimal "two-hit" model of common acute lymphoblastic leukemia, delayed infection was postulated to have a crucial role in promoting, through the immune response, the second or postnatal "hit" (5). Epidemiological evidence overall is supportive of both population mixing and delayed infection in infancy hypotheses. The most considerable difficulty for epidemiological studies to explore these hypotheses is that the relevant infections, with respect to leukemia, may not result in documented diagnosis or significant clinical symptoms. Thus well-recognized proxies for infectious exposure in early life are used. For example, results from the UK case-control study and the Northern California case-control study of childhood leukemia demonstrate that social contacts in infancy, as indicated in attendance at day care, can reduce the risk of childhood acute lymphoblastic leukemia (6,7). Conflicting results are derived from studies using recorded infections, which suppose that infectious exposures will elicit overt symptoms or pathology that prompt general practitioner visits. Thus, we need proxies or even better biological measures of innocuous or "invisible" common infections to study further and better these hypotheses (8).

An essentially similar immunological argument is embodied in the "hygiene" hypothesis for childhood allergies, which postulates that better hygiene, resulting in decreased microbial exposure, leads to an increase in allergic diseases (asthma, allergic rhinitis, eczema) (9,10). It is proposed that the worldwide rising trends in allergic diseases is in some way linked to the adoption of the modern Westernized lifestyle characterized by better hygiene and reduced stimulation of the immune system by early life infections. Epidemiological studies so far, has shown that some infections are associated with the development of allergic diseases (e.g. respiratory syncytial virus, rhinovirus and lower respiratory tract infections with asthma), whereas other infections are protective (varicella zoster virus with eczema, Helicobacter pylori with asthma, frequent simple infantile colds independent of virus type and asthma) (11,12). There is an obvious difficulty in showing cause and effect in such observational studies but viral infections appear attractive targets for primary prevention efforts(12). Rook has argued that to be a candidate for the hygiene hypothesis any such agent should satisfy two criteria (13): (i) It must be something that has always been present throughout the evolution of the mammalian immune system. Only then will it become encoded in the genome as "knowledge" of the

environment; as something that has become a physiological necessity and (ii) it must be something that has been progressively depleted from the environment of developed countries during the last 2 or 3 decades. He argued that childhood virus infections satisfy neither of these criteria. But, it is hard to say how long viruses have co-evolved with human hosts given that many viruses show evidence of prolonged co-evolution in their genome.

Moreover, Rook has suggested that increases in chronic inflammatory diseases in developed countries are partly attributable to diminishing exposure to organisms that were part of mammalian evolutionary history (14). These organisms ("old friends") are depleted from the modern urban environment which may lead to a failure to develop adequate immunoregulatory pathways. Thus, the hygiene hypothesis can also provide the missing immunoregulatory environmental factor that is needed to explain the recent increases in autoimmune diseases such as type 1 diabetes, multiple sclerosis and inflammatory bowel disease. The incidence of childhood type 1 (insulin-dependent) diabetes mellitus has risen in parallel with that of childhood asthma and similar environmental factors are important in both conditions (15). Type 1 diabetes and asthma are mediated by the immune system and in both dysfunctional immune responses become apparent soon after birth. The relationship is to some extent reciprocal, as children with diabetes and their siblings are partially protected against atopic disorders, possibly because these represent opposing and mutually exclusive forms of immune deviation. Autoimmune disorders are characterised by a Th1 skew in T lymphocyte responses to stimulation, whereas Th2 responses dominate in the atopic disorders. We are therefore confronted by the apparent paradox that autoimmunity and allergy share many epidemiological features yet represent opposite ends of a spectrum of immune response (16).

Importantly the “hygiene hypothesis” may have trans-generational effects because it may affect the antibody repertoire of the mother, which also influences the susceptibility of her child to infectious agents and autoimmune diseases (17,18). Maternal antibodies, reflecting maternal accumulated immunologic experience, are passively transferred to the fetus through the placenta in the end of pregnancy. These antibodies do not only protect the child while its own immune system matures, but also permit microbial agents to immunize the child under optimal conditions. As discussed before, the high standards of hygiene in the developed world has decreased

the level of exposure to common infectious agents during childhood to the extent that many infections now occur only after maternal antibodies have waned. The outcome of an infection may differ depending on whether it occurs early in life or later after maternal protection has disappeared evoking responses related to autoimmune diseases (e.g. coxsackievirus B and type 1 diabetes) (19). Moreover, hygienic conditions may hamper the induction and maintenance of protective maternal antibodies before pregnancy, which might have disadvantages also for threatening infections of pregnancy (e.g. cytomegalovirus, toxoplasma) (20).

Lastly, accumulating evidence suggests that certain infectious agents are also implicated in the aetiology of a number of chronic conditions (21). These include the associations of Helicobacter pylori with peptic ulcer and gastric cancer, Hepatitis B virus or Hepatitis C virus with liver cirrhosis and cancer, and Human Papilloma virus with cervical cancer. Conversely, data on other chronic conditions indicate an infectious aetiology but a definite causative infectious agent has eluded detection. For many other chronic conditions, the evidence of an infectious aetiology is weak or even conflicting, e.g., the role of Chlamydia pneumoniae in the aetiology of atherosclerosis (22). When chronic disease stems from infectious disease, the situation is even more complex, because it may be difficult to ascertain the precise timing of infection (which may have happened well in the past) or the exact nature of the pathogen. Acute, chronic, latent, or recurrent infections may be involved in pathogenesis, and coinfections may play a critical role in disease manifestation. Thus it is important to understand the interaction of candidate pathogens with developmental processes starting in early life that are associated with later disease manifestations.

The aforementioned hypotheses are based on epidemiological studies, which use proxy variables such as age of first school attendance, to denote exposure to common infections. Very limited prospective data exist on characterizing the repertoire of common infections in childhood by use of biological measures. At the same time we need a good characterization of chronic diseases starting from early life to examine disease associations over the lifecourse. Population-based prospective studies offer an important opportunity for filling this gap.

Motivated by these hypotheses, in the present thesis we use serological markers of infection to Polyomaviruses, Herpesviruses and Helicobacter pylori to study their

natural history and their association with health and disease in early life, in the Rhea mother-child cohort in Crete, Greece.

1.2. Polyomaviruses

A. Discovery

Advances in sequencing technologies have boosted the discovery of "new" human viruses of unknown ordinarily clinical importance. Such a group of viruses are Polyomaviruses which have been detected in vertebrate hosts including rodents, cattle, birds, monkeys and primates. Fourteen human Polyomaviruses are described so far; BKPyV (1971), JCPyV (1971), KIPyV (2007), WUPyV (2007), MCPyV (2008), HPyV6 (2010), HPyV7 (2010), TSPyV (2010), HPyV9 (2011), HPyV10 (2012), STLPyV (2013), HPyV12 (2013), HPyV13 (2014) and LIPyV (2017) (23–26). The closest known relatives of Polyomaviruses are Papillomaviruses with which they share some similarities in morphology, genomic properties (early and late region, minichromosome structure), and cell transforming activities of viral proteins.

Polyomavirus infections were accidentally discovered in the 1950s when characterizing a transmissible agent causing multiple tumors in rodents, hence providing the name (Greek poly- many, multiple; -oma, tumors). The first two human Polyomaviruses BKPyV and JCPyV, were named after the initials of the patients from whom they were first isolated in 1971. The third and fourth Polyomaviruses, KIPyV and WUPyV, were named after the respective institutions e.g. Karolinska Institute (Stockholm, Sweden) and Washington University (St. Louis, USA). The fifth Polyomavirus, MCPyV, was named due to its detection in Merkel cell carcinoma. Likewise, the eight Polyomavirus, TSPyV was also named according to its identification in trichodysplasia spinulosa, a rare skin disease. HPyV6 and HPyV7, identified in skin and HPyV9 detected in serum and skin are named in order of appearance, while the tenth Polyomavirus, MWPyV or HPyV10 found in stools and skin was named according to the first geographical location where it was found (Malawi). STLPyV was present in multiple human stool samples and HPyV12 was initially identified in resected human liver tissue. HPyV13 (or New Jersey Polyomavirus) was identified in an organ transplant recipient with systemic vasculitis, myositis, and retinal blindness. The 14th and most recent human Polyomavirus to be described is named Lyon IARC Polyomavirus (LIPyV) and was isolated in human

skin using a sensitive degenerate PCR protocol combined with next-generation sequencing.

B. Natural history

Human Polyomaviruses are worldwide spread with high levels of seropositivity among adults while there is limited evidence on patterns of acquisition of infection in early childhood and on the factors that determine spread of infection . Studies show that there are substantial differences in their epidemiology depending on the virus and geographic area. Serological studies for the newly discovered Polyomaviruses (HPyV12, HPyV13 and LIPyV) are still lacking, therefore we do not know whether these are circulating in the human population. Understanding social, behavioral and environmental heterogeneities in Polyomaviruses natural history will help to elucidate aspects of their biology and pathogenic behavior.

Serology can provide us with reliable data on the prevalence of Polyomaviruses infections, since primary infection is asymptomatic/non-specific. Antibodies targeting the major viral capsid proteins (VP) are markers of infection. VP1 production is associated with active viral replication. The choice for VP1 as antigens of interest was governed by results of studies on BKPyV and JCPyV which showed that VP1 is immunodominant. However the less immunogenic large T antigens may also be useful in discriminating active from latent infection and be useful in the study of disease associations due to Polyomavirus reactivation (27). However, the cross-reactivity mainly observed between Polyomavirus capsids but also nonstructural proteins is challenging and serological data have to be interpreted with caution (28,29).

No symptoms or diseases have been linked to any Polyomavirus infection in childhood. It is generally believed that Polyomaviruses cause an asymptomatic primary infection leading to a primary viremia, following which virus is able to enter a state of latency and avoid the attention of the immune system (latent infection). Alternatively, it may persist in a state of active both asymptomatic replication occurring at a low level or episodically (persistent infection). Emergence of the virus from a latent or persistent state to one of active replication, which causes a pathological condition, is defined as reactivation of the virus and is a rare outcome. This likely reflects the long co-evolutionary history of Polyomaviruses and humans

and their delicate equilibrium of mutual benefit . Although not fully characterized for all Polyomaviruses, sites of latency in the body are the urinary tract (BKPyV, JCPyV), skin (MCPyV, TSPyV, HPyV6, HPyV7, HPyV9, LIPyV?), respiratory tract (KIPyV, WUPyV) and gastrointestinal tract (HPyV10, STLPyV, HPyV12?) (23).

C. Polyomaviruses and non-malignant health diseases

Given the increasing numbers of Polyomavirues, the interest in their role in disease has been revived. In contrast to the high frequency of Polyomaviruses infections, associated diseases are rare and immunosuppression is an important co-factor (30–32). From a public health perspective, the increasing use of immunosuppressive treatments due to transplantation and acquired or primary immune deficiency, may result in reactivation of these normally commensal viruses and in new disease syndromes. It is suggested that due to the long co-evolutionary history of Polyomaviruses with humans this relationship was initially not meant to be detrimental to the host, but Polyomaviruses "accidentally" undewent a shift in their lifecycle towards pathohenicity (33). Briefly, three different non-malignant pathology patterns have been described (28):

- Cytopathic Polyomavirus pathology pattern is characterized by the loss of infected cells through high-level virus replication, but without significant inflammation. This pathology pattern is seen in progressive multifocal leukoencephalopathy.
- A dominant inflammatory response to abundant Polyomavirus antigen, typically following a brisk recovery of the cellular immune response. This pathology pattern is seen in BKPyV-associated hemorrhagic cystitis after allogenic stem cell transplantation.
- Cytopathic-inflammatory Polyomavirus pathology is characterized by high-level virus replication and a significant inflammatory response due to cytopathic lysis, necrosis, with infiltration of granulocytes and lymphocytes. This pathology pattern is seen in Polyomavirus associated nephropathy.

To date, four clinical diseases have been associated to Polyomaviruses:

- BKPyV and BKPyV-induced hemorrhagic cystitis . Is a serious BKPyV-associated complication characterized by dysouria and varying degrees of hematuria that affects up to 10% of allogeneic haematopoietic stem cell transplant

recipients. Typically, BKPyV-induced hemorrhagic cystitis occurs more than 10 days post-transplant and although not usually life-threatening it is associated with significant morbidity.

- BKPyV and Polyomavirus-associated nephropathy (34). BK polyomavirus-associated nephropathy in kidney transplant recipients is the most serious infectious cause of renal allograft dysfunction and graft loss and is characterized by high-rate lytic virus replication in the transplanted kidney. BKPyV viruria and viremia due to reactivation are found in up to 80% of renal transplant patients and 10% of patients progress to Polyomavirus-associated nephropathy, resulting in allograft loss 90% of the time. The incidence of Polyomavirus-associated nephropathy has been rising with the introduction of new and more potent immunosuppressive regimens. Although Polyomavirus-associated nephropathy in kidney transplant recipients is caused by BKPyV in > 95% of the cases, < 5% are attributed to JCPyV.

- JCPyV and progressive multifocal leukoencephalopathy (31). This is a fatal (within 3-6 months) demyelinating disease of the central nervous system occurring mainly in immunocompromised individuals (in patients with advanced AIDS, bone marrow and solid organ transplant patients and natalizumab-treated multiple sclerosis patients). It is characterized by cognitive impairment, motoric dysfunction and visual deficits. At the cellular level, there is a cytolytic infection of oligodendrocytes resulting in cell death and development of lesions in the cerebrum, cerebellum and brain stem, particularly along the junction of the gray and white matter. It is hypothesized that JCPyV persists in brain long before onset of illness. Another model proposes that under immunosuppression JCPyV is reactivated from other sites of persistence and crosses the blood-brain barrier directly or associated with B-cells. Structural changes in the non coding control region but probably also in the outer capsid proteins are considered to be responsible for JCPyV neurotropism.

- TSPyV and trichodysplasia spinulosa (35). This is a very rare (30 cases worldwide) skin disease called Trichodysplasia spinulosa seen exclusively in severely immunocompromised hosts characterized by follicular distention and keratotic spine formation especially on the face. It is also associated with variable degrees of nonscarring alopecia, most severely affecting the eyelashes and

eyebrows. A recent study demonstrated that TSPyV occurs during primary infection of the immunosuppressed host and is not a result of virus reactivation (35).

D. Polyomaviruses and cancer

Enormous resources and efforts have been spent in searching for Polyomavirus-induced tumors (36). Two virus-detected mechanisms may explain the transforming potentials of polyomaviruses: viral DNA integration into the host cell genome or/and the action of viral proteins (37). Polyomaviruses DNA encodes T antigens which are potential oncoproteins based on their conserved tumor suppressor–targeting domains.

Among the Polyomaviruses, only MCPyV has been established to cause cancer in humans. Oncogenic effects of other Polyomaviruses have been suggested but not yet proven. The widespread presence of Polyomaviruses in a healthy population is one of the main difficulties when studying the potential association of these viruses with human cancer. Moreover, the etiologic contribution of Polyomaviruses is suggested to represent mechanistically a "driver" role to a given cancer, but this can be challenged in three ways (28):

- by the "hit and run" mechanism, where Polyomavirus infection contributed at an early stage to oncogenic progression e.g. by chromosomal instability, but Polyomavirus is no longer detectable after full progression to malignancy when diagnosis is made.
- by the "passenger" Polyomavirus that finds favorable conditions in an already transformed cell, but is neither necessary for, nor contributing to, its oncogenic characteristics.
- by the "by-stander" Polyomavirus that infects neighboring cells or is detectable in anatomically connected compartments, but is unrelated to the malignancy.

MCPyV has been classified by the International Agency for Research in Cancer, as probably carcinogenic to humans (Group 2A) (36). MCPyV has been implicated in the aetiology of Merkell Cell Carcinoma, which is a neuroectodermal tumour that originates from Merkel cells (38). Two aetiologies are currently discussed for Merkell Cell Carcinoma tumourigenesis: activity of MCPyV-encoded early transforming genes in MCPyV-positive tumours and accumulation of ultra violet-induced mutations

in the MCPyV negative Merkell Cell Carcinomas. Approximately 80% of Merkell Cell Carcinoma tumours are positive for clonally integrated MCPyV. Viral oncogenesis is mediated by the large and small T antigens of MCPyV, and MCPyV-positive Merkell Cell Carcinoma cell lines depend on viral oncogene expression for cell proliferation and survival.

Oncogenic effects of other Polyomaviruses have been suggested but not yet proven (23). Based on inadequate evidence in humans and sufficient evidence in experimental animals the International Agency for Research in Cancer classified BKPyV and JCPyV as possibly carcinogenic to humans (Group 2B) (36). JCPyV and BKPyV, have been detected in a variety of gastrointestinal and genitourinary cancers. We should note that a simian polyomavirus virus (SV40), discovered in 1960 as a contaminant of poliovirus and adenovirus vaccines, has been used as an experimental model of a DNA tumor virus and cell transformation. Despite human exposure to SV40 through contaminated attenuated poliovirus and adenovirus vaccines in the past, and possibly in wildlife reserves and animal facilities, serologic data do not support the independent circulation of SV40 in humans. Its possible role in human diseases including cancer has been controversial (39,40). SV40 is closely related to to BKPyV and JCPyV.

Attempts to link the remaining Polyomaviruses to clinical disease have focused on older individuals, in whom immunity is waning, or in the immunosuppressed. So far, KIPyV, WUPyV, HPyV6, HPyV7, HPyV9, HPyV10, STLPyV, HPyV12, HPyV13 and LIPyV have not yet been associated with any specific disease phenotype.

1.3. Herpesviruses

Similarly to Polyomaviruses, Herpesviruses have a long evolutionary history of co-evolution with human hosts while they can be a serious threat to the immunocompromised host. Human populations are infected with eight Herpesviruses, including herpes simplex virus 1 (HSV-1) and 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr Virus (EBV), cytomegalovirus (CMV), human Herpesvirus 6 (HHV-6) and 7 (HHV-7), and Kaposi sarcoma–associated herpesvirus (KSV or HHV-8). Herpesviruses are highly adapted to lifelong infection of their human hosts and thus can be considered a component of the human ‘‘microbiome’’ in addition to their role in illness triggered by primary infection (41). It has been suggested that recent

changes in family structure, childrearing, and hygiene practices have modified the epidemiology of Herpesviruses (42–45). This might have important public health implications as the timing of exposure has a critical role in the outcome of infection with Herpesviruses. In the present project we focused on five out of eight Herpesviruses; EBV, CMV, HSV-1, HSV-2 and HHV-8. For VZV there is uninersal immunization in Greece whereas for HHV-6 and HHV-7 there was not available multiplex serology developed at the time of serological testing.

The infection with Herpesviruses results in two distinct immune responses. The cellular and the humoral immune response. IgG antibodies can be used as markers for determination of post infection status. The Herpesviruses marker antigens leading to strong antibody responses consist of structural and non-structural viral proteins. Especially the glycoproteins (such as glycoprotein B, D and G) embedded in the virion envelope or nucleocapsid proteins (e.g. EBV VCAp18) are prominent targets for human IgG. Non-structural proteins such as viral transcription activators (Epstein-Barr virus nuclear antigen-1 (EBNA-1)) also serve as antigens for serological determination of the Herpesvirus infection status (46,47).

A. Epstein-Barr Virus

EBV is acquired silently early in life and carried thereafter as a lifelong asymptomatic infection in the B lymphoid system. Seroepidemiologic studies indicate that >90% of adults worldwide are infected with EBV. However, there is substantial variation in the age at primary infection, likely due to socio-demographic factors controlling exposure to the virus (48,49). The virus-host balance can be disturbed in various ways, and one of a range of virus-associated diseases may then ensue (50). For example, primary EBV infection can present as infectious mononucleosis if the virus is acquired during adolescence; as its fatal counterpart, X-linked lymphoproliferative disease, in boys with a particular immunodeficiency; or as a chronic active infection with severe hemophagocytosis when the infection spreads to other lymphoid lineages. Certain autoimmune conditions, particularly multiple sclerosis, may also be rare accidents of long-term virus carriage. Most importantly, EBV is aetiologically linked to a wide range of human tumors (51); these include several B cell malignancies, notably Burkitt's lymphoma, Hodgkin's lymphoma, and posttransplant lymphoproliferative disease; an extranodal lymphoma of T or natural killer cell origin; undifferentiated nasopharyngeal carcinoma; a smooth muscle cell sarcoma; and a distinct subset of

gastric carcinomas. Since age of primary infection has an important role in the outcome of EBV infection, current patterns of transmission in the general population are needed (52).

B. Cytomegalovirus

CMV is also an ubiquitous infectious agent (53). CMV can be transmitted i) congenitally, even among CMV seropositive mothers (54), ii) from mother-to-infant through exposure to the virus in genital secretions during birth or postnatally via breastfeeding and iii) later in childhood via exposure to body fluids from other infected individuals (55). Most overt CMV-related disease occurs following transmission during pregnancy, manifesting as congenital CMV disease in children, affecting 0.2–2.2% of all live births (56). Congenital CMV infection is the leading non-genetic cause of neurological defects, including mental retardation, cerebral palsy, and hearing impairment. Postnatal CMV infection is associated with severe disease, including respiratory distress syndrome and sepsis, in very premature babies (57). In healthy adults and children, CMV infection may cause a mild infectious mononucleosis syndrome, but is usually asymptomatic. These observations indicate that the control of CMV replication is limited during early life as compared to adult life and that this reduced viral control is associated with an increased risk of symptomatic infection in the fetus and in the very premature newborn. Despite, CMV infection has been associated with long-term consequences, including earlier immunosenescence during aging, atherosclerosis and cancer, areas of active research (58–60).

C. Herpes simplex virus 1 and 2

HSV-1 and HSV-2 are also considered common infections worldwide (61). For each virus, the primary mode of transmission is different and there is a tendency to infect different anatomical sites, causing a wide variety of mucocutaneous infections. HSV-1 inoculates usually the mucosal surfaces or skin sites of the lips and mouth leading to vesicular lesions of the oral mucosa commonly referred to as "cold sores". HSV-2 is more frequently associated with anogenital lesions, and is transmitted sexually. However, both HSV-1 and HSV-2 can cause genital herpes, and infection of the same anatomic site by both viruses has been documented. HSV-2 can be transmitted during delivery from primary genital infection and can result in significant morbidity to the

newborn child. The risk of vertical transmission is substantially greater among women who acquire a primary HSV-2 infection late in pregnancy as compared with women with reactivated infections (50% vs <1%), since there is not adequate time to develop maternal antibodies to suppress viral replication before labour (62). HSV-1 has been usually acquired in childhood whereas HSV-2 later in life as adolescents and young adults become sexually active. Despite, there has been a documented decline in the seropositivity rate of HSV-1 among adolescents in some developed countries, probably due to improved standards of living (63). Consequently, a substantial proportion of adolescents may be HSV-1 naive at sexual debut, and possibly due to changes in sexual behavior (increased rates of oral-genital contact), there have been increases in the proportion of genital HSV infection attributable to HSV-1. The impact of an increasing number of genital HSV-1 infections on transmission of HSV to the neonate remains unknown (64). A study shows that when HSV-1 is present in the genital tract at delivery, it appears to be more readily transmissible to the neonate than HSV-2 (65). Moreover, persons who develop genital HSV-2 infection but lack antibody to HSV-1 may have more prominent symptoms. This is because antibody to HSV-1 seems to attenuate the severity of HSV-2 disease in some cases, although it does not prevent infection with HSV-2. Current data on HSV epidemiology in Greece are lacking.

D. Human Herpesvirus 8

HHV-8, also known as Kaposi's sarcoma-associated herpesvirus, was declared as Group 1 carcinogenic agent by the International Agency for Research on Cancer in 2010 (36). HHV-8 is necessary for Kaposi sarcoma to develop but it is not a sufficient cause, and other co-factors such as immunosuppression are needed. Thus, once the HIV/AIDS epidemic began, the incidence of Kaposi sarcoma increased dramatically. HHV-8 is also linked to other proliferative diseases, including multicentric Castleman disease and primary effusion lymphoma (66,67).

HHV-8 is not restricted to Kaposi sarcoma patients and has varying infection rates depending on geographic location (68). Thus HHV-8 is very common in sub-Saharan Africa with seropositivity rates of >50%; moderately prevalent in Mediterranean countries (20- 30%) but much less common (<10%) in most of Europe, Asia and the US. Moreover, Whitby et al. documented regional differences in Italy; a low

prevalence was reported for the northern part, whereas a prevalence of 35% was detected in Sicily in parallel with earlier reports of high incidence rates of classic Kaposi sarcoma in this region (69). Outside HHV-8 endemic regions, high HHV-8 seroprevalence has been described in men who have sex with men and in migrants from African regions. Both sexual and non-sexual modes of transmission have been suggested but their relative importance may vary with HHV-8 endemicity (70). Thus, HHV-8 occurs in children in Italy, sub-Saharan Africa and South American Amerindian populations (71). Children are most likely to be infected if they have an infected mother or other family member. Primary HHV-8 infection may be associated with fever and a maculopapular rash in immunocompetent children. Little information is available about HHV-8 in Greece, an area considered endemic for "classic" Kaposi sarcoma. Existing studies indicate a 10% HHV-8 prevalence in adults, but these are based only on small sample sizes (72). No data on HHV-8 seroprevalence in Greek children exist.

1.4. Helicobacter pylori

A. Prevalence and determinants of Helicobacter pylori infection

Helicobacter pylori (H. pylori) is a spiral shaped, gram negative bacterium and is the most common chronic bacterial infection in humans. There is now considerable evidence that H.pylori has colonized the stomach of humans for more than 100 000 years. H.pylori has been demonstrated worldwide and in individuals of all ages. Natural colonization is restricted to humans and possibly several other primates. The stomach is the major habitat of H.pylori and when present the H.pylori usually is the numerically dominant gastric microorganism (73).

H.pylori is generally acquired in childhood probably through an oral–oral or a fecal–oral route. Infection is more frequent and acquired at an earlier age in developing countries compared with developed countries. Cross-sectional studies from northern European and North American populations, demonstrate an increasing prevalence of infection with age, which was first thought to represent a continuing rate of bacterial acquisition thoughout one’s lifetime (74). Epidemiologic evidence now suggests that this age difference represents a birth cohort effect due to the decreasing rate of H.pylori colonization during childhood years in next generations, rather than a cumulating risk of infection with age (75). Thus the lower prevalence of infection in

the younger generations suggests a further decline in H. pylori prevalence in the community over the coming decades. This decline is expected to be associated with a decline in the H.pylori associated diseases as seen in Portugal although contradictory results are derived from Sweden (76,77).

Seroprevalence data in Greece are limited and are largely based in symptomatic individuals. According to a study carried out in the early '90s, seroprevalence was 39.4% amog children aged 1-10 years, 67.1% among recruits (20-27 years) and 70% among blood donors (20-50 years) (78). A more recent study conducted among medical and nonmedical staff of a hospital demonstrated a seroprevalence of 40.6% for those aged 23-40 years and of 57.5% for those aged 41-60 years (79). Apostolopoulos et al identified a significant decrease in seroprevalence of H.pylori during a ten year period; from 59.5% in serum samples collected in 1987 to 49.2% in serum samples collected in 1997 (80).

As already mentioned, H.pylori is becoming less common in human populations with socioeconomic development (74). Contributing factors include improved sanitation, smaller family sizes and the frequent use of antibiotics during childhood (81,82). Because acquisition largely takes place in childhood the risk of acquiring H.pylori infection is related particularly to socioeconomic status and living conditions early in life (83). Factors such as density of housing, overcrowding, number of siblings, sharing a bed, and lack of running water have all been linked to a higher acquisition rate of H.pylori infection in childhood. Whether these or other factors are important in current settings of low H.pylori seroprevalence is not known.

B. Disease associations

H.pylori is highly adapted to the gastric environment where it lives within or beneath the gastric mucous layer. The bacterium generally does not invade gastroduodenal tissue. Instead, it renders the underlying mucosa more vulnerable to acid peptic damage by disrupting the mucous layer, liberating enzymes and toxins, and adhering to the gastric epithelium. In addition, the host immune response to H.pylori incites an inflammatory reaction which further perpetuates tissue injury. Through its pro-inflammatory effects, H.pylori modulates immunological, endocrine and physiological functions in the stomach with both local and systemic manifestations.

These interactions with the host might change the complex immune response with age and might be reflected in specific antibody patterns.

All clinical isolates of H. pylori produce a number of virulence factors that are essential for the colonization of the stomach and survival in the host(84). The best-known virulence factors are the urease, which is supposed to play an important role in the neutralization of gastric acid secretion; the flagella, which are essential for swimming through the mucous layer. Adhesins, such as Outer inflammatory protein and Sialic acid-binding adhesin, facilitate bacterial attechment to the host epithelium and often induce its inflammatory response. In addition to the above factors that are produced by all strains, some factors are produced by only a subset of clinical isolates: these are the CagA and the VacA which are more pathogenic. H.pylori strains carrying CagA have been associated with duodenal ulcer and gastric cancer. VacA is a proteic pore-forming toxin crucial to promote and maintain bacterial colonization. Combined seropositivity to both CagA and VacA correlates with elevated morbidity. Less pathogenic strains are those expressing the GroEL which is a protein that belongs to the chaperone family and promotes refolding of misfolded proteins especially under stress conditions. It was also reported to be associated with the adhesion of H. pylori to human gastric epithelial cells and the induction of inflammatory responses. Catalase is part of the antioxidant defence mechanisms of H. pylori upon infection and initial host inflammatory response and necessary for long-term colonization. NapA activates neutrophils, antagonizes oxidative stress, and mediates the binding of H. pylori to the host cell and stomach mucus. UreA plays a major role in the stress response and acid survival of H. pylori in the gastric habitat. HcpC belongs to the family of Helicobacter cysteine-rich proteins that induce proinflammatory cytokines.

Once acquired, infection persists and may or may not produce gastroduodenal disease. The majority of infections are asymptomatic, however infected people are at higher risk for peptic ulcers, chronic gastritis, gastric adenocarinoma and for gastric mucosa associated lymphoid tissue lymphoma. Pathogenicity is likely dependent on strain-specific factors, host factors, environmental factors and other yet unidentified factors (85). Emerging evidence suggests that H. pylori is also implicated in the pathogenesis of many extragastric diseases (86). Thus the role of the bacterium in idiopathic thrombocytopenic purpura, unexplained iron-deficiency anaemia and vitamin B12

deficiency has been fully validated and included in the current guidelines (87–89). Moreover, there is a rising interest in the role of H. pylori in cardiovascular diseases, neurodegenerative diseases, diabetes mellitus, metabolic syndrome and hepatobilliary diseases. Indeed, this bacterium produces a low grade inflammatory state, induces molecular mimicry mechanisms, and interferes with the absorbance of nutrients and drugs, possibly influencing the occurrence and/or the evolution of many diseases (88).

The idea that H. pylori might actually confer benefits to humans has engendered considerably controversy among investigators (90,91). There are inverse associations between the presence of H. pylori (especially cagA+ strains) and disorders such as gastroesophageal reflux disease, Barrett's esophagus and esophageal adenocarcinoma, suggesting a protective role of H. pylori (92,93). One potential mechanism for this effect could be that H. pylori colonization diminishes gastric acidity. Moreover, the absence of H. pylori is associated with an increased risk for allergies (94–96); this inverse association is specific for childhood-onset, but not later-onset, asthma, and is most pronounced for cagA+ H. pylori strains. If H.pylori is protective for some diseases, its elimination might cause unexpected problems.

C. Helicobacter pylori infection in special populations

Pregnant women

Studies suggest an increased susceptibility to H.pylori infection during pregnancy (97). Seroprevalence in pregnant women varies according to geographic area, socioeconomic conditions and method used to detect H. pylori infection similar to differences observed in non-pregnant population. H.pylori is now investigated in gastric and extra-gastric diseases during pregnancy (98). Evidence suggests that H.pylori infection is associated with higher risk of hyperemesis gravidarum (99), a form of vomiting which is severe or protracted or is accompanied by weight loss, dehydration, electrolyte disturbances, or hospitalization. Hyperemesis Gravidarum complicates 0.3-0.2% of pregnancies but mild to moderate dyspepsia associated with nausea and vomiting may complicate even 50% of pregnancies. A large study conducted among 5549 pregnant women in the Netherlands showed that H.pylori was a risk factor for vomiting in pregnancy and in women with daily vomiting. H.pylori was also associated with low maternal weight gain, reduced birth weight and small for gestational age offsprings (100).

The research of H. pylori implication in extra-gastric pregnancy related disorders is mainly focused on:

- pre-eclampsia with several epidemiological studies reporting a link with H.pylori. Interestingly, Simone et al showed in a case-control study for the first time, an association between H.pylori infection and preeclampsia with abnormal uterine arteries Doppler velocimetry, suggesting a role for the bacterium in impairing placental development and increasing in this way the risk of preeclampsia (101).
- micronutrient deficiencies (iron, folate, B12) (102,103) and associated complications such as iron deficiency anemia. An important aspect of micronutrient deficiencies associated with H.pylori is that they are relatively resistant to supplement administration.
- fetal malformations (e.g. neural tube defects). Two case-control studies, report that maternal H.pylori infection could play a role in neural tube defect causation by reducing folate and vitamin B12 concentration (104,105).

Several mechanisms have been suggested to contribute to the pathogenesis of H. pylori pregnancy related disorders including i) depletion of micronutrients (iron and vitamin B12) in maternal anemia and fetal neural tube defects; ii) local or systemic induction of pro-inflammatory cytokines release and oxidative stress in gastrointestinal disorders and pre-eclampsia; iii) cross-reaction between specific H.pylori antibodies and antigens localized in placental tissue and endothelial cells (preeclampsia, fetal growth restriction, miscarriage). An important aspect of H.pylori infection during pregnancy is the physiological immunosuppression of pregnancy, which could activate a latent H.pylori infection and complicate the pregnancy and perinatal period.

It seems interesting to investigate the possible association of H.pylori infection with other pregnancy outcomes (e.g. gestational diabetes, preterm birth) and offspring health (e.g. child's growth and neurodevelopment) over the long term. Since H. pylori infection is treatable, the demonstration of its causative role in pregnancy-related disorders will have important public health implications.

Children

Helicobacter pylori infection is acquired in childhood. According to limited studies using repeated samples in early childhood, children may be infected transiently or

persistently with significant seroresponses observed in persistent infections (106). Although H pylori infection is always associated with microscopic gastric inflammation, the overwhelming majority of H pylori–infected children are asymptomatic. Studies in children do not support a role for H pylori infection in functional disorders such as recurrent abdominal pain. In comparison with adults, children and adolescents, infrequently develop complications of infection (peptic ulcer, gastric cancer). Furthermore H. pylori infection in early childhood may be associated with immunologic benefits later in life. Therefore in children the decision to investigate and treat the infection should be supported by a clear benefit for the individual child (107).

Various studies have investigated hypotheses pertaining to H. pylori infection as a cause of a wide variety of extragastric diseases in children such as iron deficiency, otitis media, upper respiratory tract infections, periodontal disease, food allergies, sudden infant death syndrome, idiopathic thrombocytopenic purpura, and short stature (81). A systematic review in 2013 concluded that there is insufficient evidence about health benefits from treating H. pylori infection in pediatric populations (108).

1.5. Aims of the present thesis

A. Research gaps

Although, advances in sequencing technologies have boosted the discovery of "new" human viruses, such as Polyomaviruses, their role in human's health and disease remains largely unknown. A first step in the study of infectious diseases is to define the landscape of population immunity. Serological surveys are potentially the most direct and informative technique available to infer the dynamics of a population's susceptibility and level of immunity. This is particularly relevant when considering common infections such as Polyomaviruses which cause subclinical or non-specific disease symptoms. Moreover studies in early childhood, a period of high acquisition of new infections, are appropriate because not everyone is yet exposed. Lastly there is a significant gap in the literature regarding seroepidemiology of Polyomaviruses in early childhood with most studies assessing only a small number of samples, derived from hospital-based subjects and exploring only a few Polyomaviruses (Table 1).

At the same time recent changes in family structure, childrearing, and hygiene practices have likely modified the epidemiology of other common human pathogens

such as Herpesviruses and H.pylori. Our current understanding of the epidemiology of those childhood infections is limited by reliance on community-based data from decades ago using low-sensitivity diagnostic methods, and recent studies that primarily focus on severe, hospital-managed disease. Particularly, seroprevalence data in Greece are lacking. While foreseeing the development of a EBV, CMV and H.pylori vaccine, serological data are essential for evaluating the need for and planning of an effective vaccination programme. Serology could be an important tool for describing the changing landscape of population's immunity for Herpesviruses and H.pylori. Detailed longitudinal serological data are needed with regard to at what time and who is susceptible or immune.

Table 1. Published seroprevalence data for BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, MCPyV, TSPyV, HPyV9, HPyV10 in early childhood.

Study	BKPyV	JCPyV	KIPyV	WUPyV	HPyV6	HPyV7	MCPyV	TSPyV	HPyV9	HPyV10
R. Dei et al 1982 (109)	1-2 years 18.2% 2-3 years 38.4% 3-4 years 45.4% 4-5 years 62.7%									
W.A. Knowles et al 2003 (110)	1-4 years 64% 5-9 years 91%	1-4 years 11% 5-9 years 14%								
A. Stolt et al, 2003 (111)	1-3 years 20% 3-5 years 63%	1-3 years 16% 3-5 years 27%								
J.M.Kean et al 2009 (112)	1-5 years 38%	1-5 years 16%	1-5 years 45%	1-5 years 45%			1-5 years 12%/21%			
N.L. Nguyen et al 2009 (113)			1 year 40% 2 years 43% 3 years 50% 4 years 73% 5 years 93%	1 year 60% 2 years 60% 3 years 56% 4 years 67% 5 years 87%						
R.P.Viscidi et al, 2011 (114)	1-10 years 62%	1-10 years 9%					1-10 years 45%			
T. Chen et al, 2011(115)								0-3 years 8% 1-4 years 5% 4-7 years 34%		
T. Chen et al, 2011(116)							1-2 years 9% 2-4 years 10% 4-13 years 35%			
E. Meijden et al, 2011 (117)	<10 years 80%							<10 years 41%		
E. Meijden et al, 2013 (118)					1-2 years 16% 2-3 years 26% 3-4 years 42% 4-5 years 36%	1-2 years 23% 2-3 years 9% 3-4 years 29% 4-5 years 24%	1-2 years 14% 2-3 years 23% 3-4 years 39% 4-5 years 32%	1-2 years 11% 2-3 years 16% 3-4 years 29% 4-5 years 44%	1-2 years 7% 2-3 years 6% 3-4 years 35% 4-5 years 24%	
C. Martel-Jantin et al. 2013 (119)							1-4years 53%			
V. Sroller et al, 2014 (120)	6-9 years 73%	6-9 years 48%					6-9 years 59%			
H. Fukumoto et al, 2015 (121)								0-1 year 7% 2-4 years 24% 5-9 years 43%		
J.T.J Nicol et al, 2014(122)										1-2 years 27% 3-4 years 68% 5-9 years 65%
J.T.J Nicol et al, 2013 (123)					1-4 years 37%	1-4 years 10%	1-4 years 42%	1-4 years 31%	1-4 years 10%	

B. Which health outcomes?

It is likely that such common infections that have a long-term evolutionary relationship with their host species are good for the human and they can only exert their detrimental effects under certain conditions; e.g. depending on the age of exposure and presence or not of passively acquired maternal antibodies. Thus a challenge is obviously, which health outcomes are relevant to investigate. In the present thesis we decided to investigate the association of Polyomaviruses, Herpesviruses and H. pylori with childhood neurodevelopment and growth, which constitute major health outcomes in early life. A large part of the study of disease associations of Polyomaviruses, Herpesviruses and H.pylori in this thesis in considered exploratory. Despite there is substantial evidence that common infections - not directly Polyomaviruses, Herpesviruses and H.pylori- may associate with childhood neurodevelopment and growth. We refer to the specific hypotheses behind such associations in the results section for each association investigated in order to facilitate the reading of this thesis.

C. Do birth cohort studies assess common infections using biological measures?

Studies of infectious disease epidemiology are often cross-sectional and hospital-based in design, which present a number of limitations including a focus on more clinically apparent cases of infection or deaths, an inability to estimate who is actually susceptible or immune, an inability to provide data on asymptomatic infections, and a lack of information on pre-infection risk factors. Cohort studies offer a unique opportunity to monitor the longitudinal infectious disease experience of the population.

A number of large birth cohort studies have been established in Europe and North America over the last 25 years that focus on environmental exposures, child health and development of asthma, allergies and obesity in children (124). Infections consist also an important exposure in childhood. However, very few of those studies have systematically examined infectious agents including common infections using biological measures, in relation to health outcomes in children. A summary of published studies are presented in table 2. We noticed that most of these analyses are only recently conducted, mostly assess infections during pregnancy, are focused on certain infectious agents and examine very specific disease outcomes.

Table 2. Birth cohort studies in Europe and North America with available seroprevalence data on common infections and disease associations.

Birth cohort	Pathogen	Seroprevalence data	Time point	Disease associations	Ref
Generation R	EBV, CMV, HSV-1, VZV	Yes (2015)	Children 2 & 6 years of age	T cell subsets (2015)	(125)
	Helicobacter pylori	Yes (2016)	Children 6 years of age	Asthma in childhood (2016)	(95)
	Helicobacter pylori	Yes (2013)	18-25 week of pregnancy	Pregnancy outcomes (2017)	(83,100)
Born in Bradford (BiB)	EBV, CMV, VZV	Yes (2013)	24-28 week of pregnancy	No	(126)
	EBV, CMV, VZV	Yes (2017)	children 1 and 2 years old	No	(127)
Norwegian Mother and Child Cohort Study (MoBa)	CMV	Yes (2013)	17-18 week of pregnancy	Pre-eclampsia (2012)	(128)
Autism Birth Cohort within the MoBA	CMV, HSV-1, HSV-2, rubella, Toxoplasma gondii		18th week of pregnancy & after delivery	Autism spectrum disorders (2017)	(129)
Prevention and Incidence of Asthma and Mite Allergy (PIAMA)	Helicobacter pylori	Yes (2011)	Children 7-9 years of age	Atopic disorders in childhood (2012)	(94)
Assessment of Lifestyle and Allergic Disease During Infancy (ALADDIN)	EBV, CMV, HHV-6, HHV-7	Yes (2013)	Children 1 & 2 years of age	IgE sensitization at 2 years of age (2013)	(130)
Avon Longitudinal Study of Parents and Children (ALSPAC)	EBV	Yes (2014)	Children 4 years of age	Childhood IQ and psychotic experiences (2014)	(131)
Danish National Birth Cohort (DNBC)	HSV-2		Birth (dried blood spot samples)	Schizophrenia in the offspring (2010)	(132)
	CMV, HSV-1, HSV-2		18-36 week of pregnancy	Epilepsy in the offspring (2013)	(133)
Finnish Maternity Cohort	EBV, CMV, HSV-1, HSV-2		Early pregnancy	Gastroschisis in the offspring (2016)	(134)
KOALA	Rota & Noro-virus		Children 1 year of age	Atopic manifestations in infancy (2009)	(135)
Maternal–Infant Research on Environmental Chemicals (MIREC)	Rubella	Yes (2017)	Midpregnancy		(136)

D. Objectives

Given these gaps in research, the overall aim of the present thesis is to describe the seroepidemiology of common infections including ten Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV, MCPyV, HPyV9 and HPyV10), five Herpesviruses (EBV, CMV, HSV-1, HSV-2, HHV-8), and H.pylori and explore their association with disease outcomes within the only Greek mother-child birth cohort study to date, the Rhea study.

The specific objectives are:

- To provide seroprevalence data of Polyomaviruses, Herpesviruses and H.pylori in early life using repeated samples collected at birth (cord blood), three years and four years of age.
- To identify the factors – sociodemographic, perinatal, variables denoting social interactions, lifestyle, hygiene practices- that determine the acquisition of Polyomaviruses and Herpesviruses up to four years of age.
- To explore the association between seropositivity to single and multiple Polyomaviruses and Herpesviruses up to age four with neurodevelopmental assessment of children at the same age.
- To explore the association between H.pylori infection early in life including fetal life with neurodevelopment of children at age four.
- To explore the association between seropositivity to single and to multiple Polyomaviruses and Herpesviruses up to age four with obesity and metabolic traits at age four and six.

2. Methods

2.1. The Rhea mother-child birth cohort study

This thesis uses data from the "Rhea" birth cohort which is a prospective study that started in February 2007 in Crete, Greece (www.rhea.gr) (137). Pregnant women (Greek and immigrants) were recruited at the time of the first comprehensive ultrasound examination, around week 12 of gestation, from four prenatal clinics (two public and two private) in Heraklion city, during a twelve-month period from February 2007 until February 2008. The inclusion criteria for study participants were: residents in the study area; pregnant women aged >16 years; 1st prenatal visit in hospitals or private clinics at Heraklion district; no communication handicap.

Pregnant women were first contacted at the first trimester of pregnancy at the time of the first routinely scheduled major ultrasound test (median 12 weeks of gestation). After the clinical visit was completed, specially trained midwifes met privately with interested women to describe the study in greater detail, then obtained written informed consent, and completed in person a detailed questionnaire on diet, environmental exposures, socio-demographic and lifestyle characteristics. Midwifes also measured height, weight, and blood pressure and collected blood and urine samples from pregnant women. Pregnant women were contacted again at the third trimester of pregnancy (median 32 weeks of gestation) and during birth admission (median 38 weeks of gestation). In-person clinical visits with mothers and children were completed during infancy (median age 9 and 18 months), early childhood (median 4.2 years) and mid-childhood (median 6.5 years). When members of the cohort were three years of age, we invited a randomly selected subsample for a serological assessment. In short, child's visit included a detailed clinical examination (anthropometry, blood pressure measurement), spirometry, neurodevelopmental assessment and biological sample collection (blood, urine and hair samples). Serological assesment has been performed in samples collected at birth, three and four years of age follow-up (Figure 1). Parents were asked to provide information regarding their child's health, lifestyle characteristics, environmental exposures, dietary patterns and housing conditions via computer-assisted interviews. The majority of clinical visits were conducted at the University Hospital of Heraklion in Greece, but for families living in rural areas, we have also conducted visits at four

Rural Health Services in Crete, Greece. At each visit, we obtained written informed consent from the mothers and, the Ethical Committee of the University Hospital in Heraklion, Greece approved the study protocols.

Major efforts have been made by the study staff to keep the families involved in the study and minimize the loss to follow up including periodic newsletters, the web page and personal and regular contact with the families by our research assistants. Women, who attended the follow-ups during childhood tended to be older, married, of higher education and Greek origin. Attrition at follow-ups were mainly due to withdrawal, difficulty in keeping track of changed addresses and telephone numbers, emigration, children's severe health illness, and mothers' unwillingness to attend the in person visits. Attrition bias is an issue in most prospective longitudinal studies and is a big challenge as participating families should be engaged for long-term follow-up.

Figure 1. *Overall study design of the Rhea study and the serology assessment at birth, three and four years of age.*

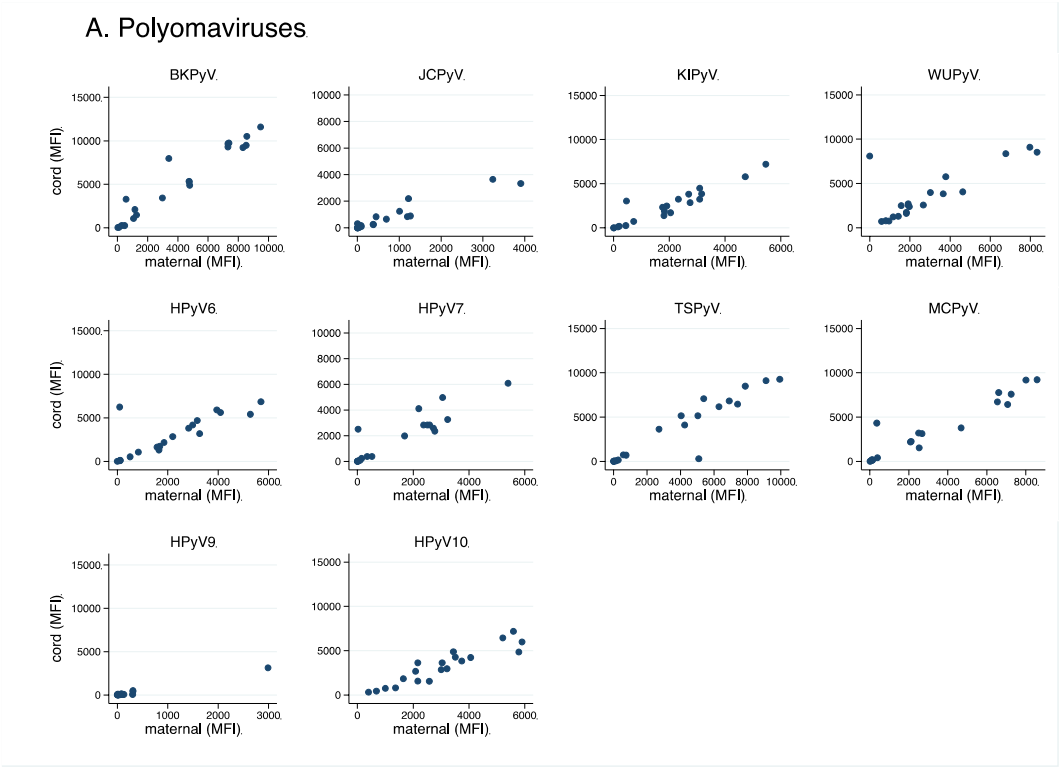
2.2. Serology

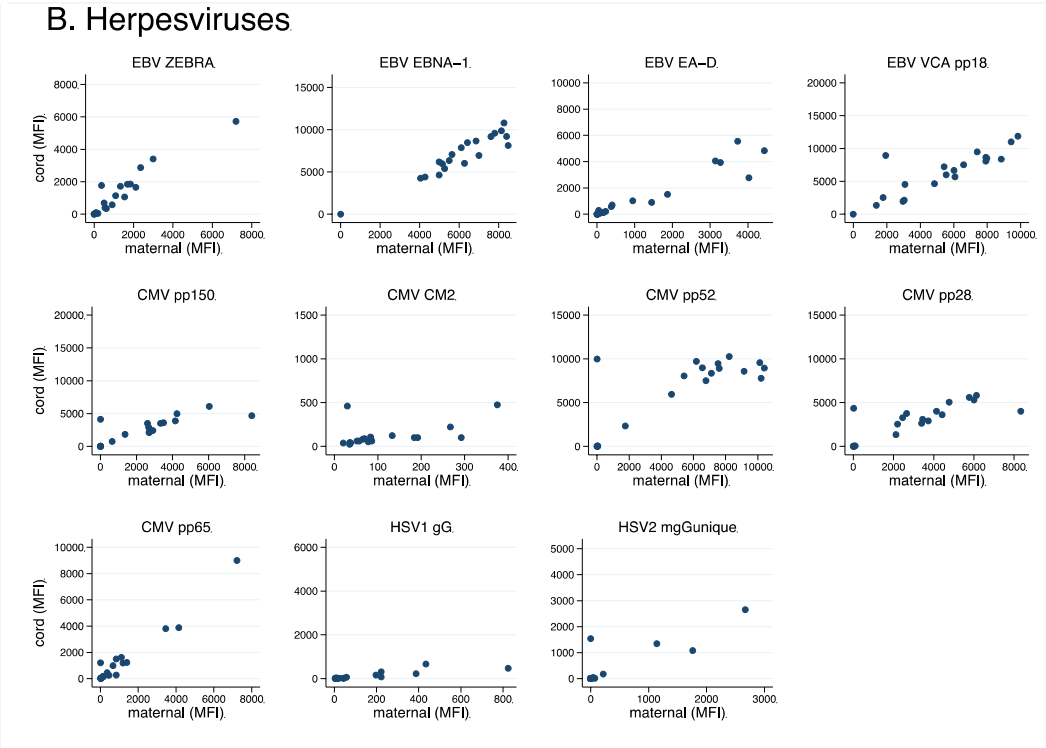
Blood samples collected at birth (cord blood) (n=626), at three (n=81) and four (n=690) years of age were processed following standard procedures and then stored at −80°C. Serum aliquots of 100 μL were shipped on dry ice to the German Cancer Research Center, Heidelberg, Germany, for serological analysis. Immunoglobulin G seroreactivity against the viral capsid protein 1 of ten polyomaviruses (BKPyV, JCPyV, KIPyV,WUPyV,MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, HPyV10), four EBV antigens (ZEBRA, EBNA-1, EA-D, VCA p18), five CMV antigens (pp52, pp65, pp150, pp28, CM2), one HSV-1 antigen (gG), one HSV-2 antigen (mgGunique), four HHV-8 antigens (LANA, v-cyclin, K8.1, ORF-65) and twelve H.pylori proteins [Chaperonin GroEL (GroEL), Urease alpha subunit (UreA), HP0231, Neutrophil-activating protein (NapA), HP0305, H. pylori adhesion A (HpaA), Cag pathogenicity island protein A (CagA), hydantoin utilization protein A (HyuA), catalase, vacuolating cytotoxin A (VacA), Helicobacter cysterine-rich protein C (HcpC) and outer membrane protein (Omp)] was measured by fluorescent bead-based multiplex serology (1:1,000 dilution) (table 1) (138). Results are median fluorescence intensity (MFI). MFI values reflect antibody affinity, titer, and reactivity determined by dilution series. Thus an advantage of this technology is that it provides not just a measure of positive versus negative but also a quantitative assessment. Immunoglobulin G denotes previous exposure to infection without being able to inform on the exact time of acquisition of the infection. At the time of serological testing, multiplex serology was not available for the most recently discovered human polyomaviruses (STLPyV, HPyV12, LIPyV) and for three herpesviruses (HHV-6, HHV-7, VZV). We should note that immunoglobulin G levels in cord blood reflect the mother's immunoglobulin G levels (139). To validate that in our population we measured the seroreactivity in a group of 21 cord blood samples alongside the respective mother's serum during pregnancy and we found very high correlations (Figure 2). These passively acquired maternal immunoglobulin G seroreactivities usually disappear by 9 months of age, by which time the infant's own immunoglobulin G synthesis is well established.

Table 1. Pathogens and corresponding targeted antigens assessed by multiplex serology.

Pathogen	Antigens					
Polyomaviruses*	VP1					
Herpesviruses						
EBV	ZEBRA	EBNA-1	EA-D	VCA p18		
CMV	pp52	pp65	pp150	pp28	CM2	
HSV-1	gG					
HSV-2	mgGunique					
Helicobacter pylori	GroEL	UreA	HP0231	NapA	HP0305	HpaA
	CagA	HyuA	Catalase	VacA	HcpC	Omp

*BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, MCyV, TSPyV, HPyV9, HPyV10


A. Polyomaviruses
BKPyV
JCPyV
KIPyV
WUPyV
HPyV6
HPyV7
TSPyV
MCPyV
HPyV9
HPyV10
cord (MFI)
maternal (MFI)


B. Herpesviruses
EBV ZEBRA
EBV EBNA−1
EBV EA−D
EBV VCA pp18
CMV pp150
CMV CM2
CMV pp52
CMV pp28
CMV pp65
HSV1 gG
HSV2 mgGunique
cord (MFI)
maternal (MFI)

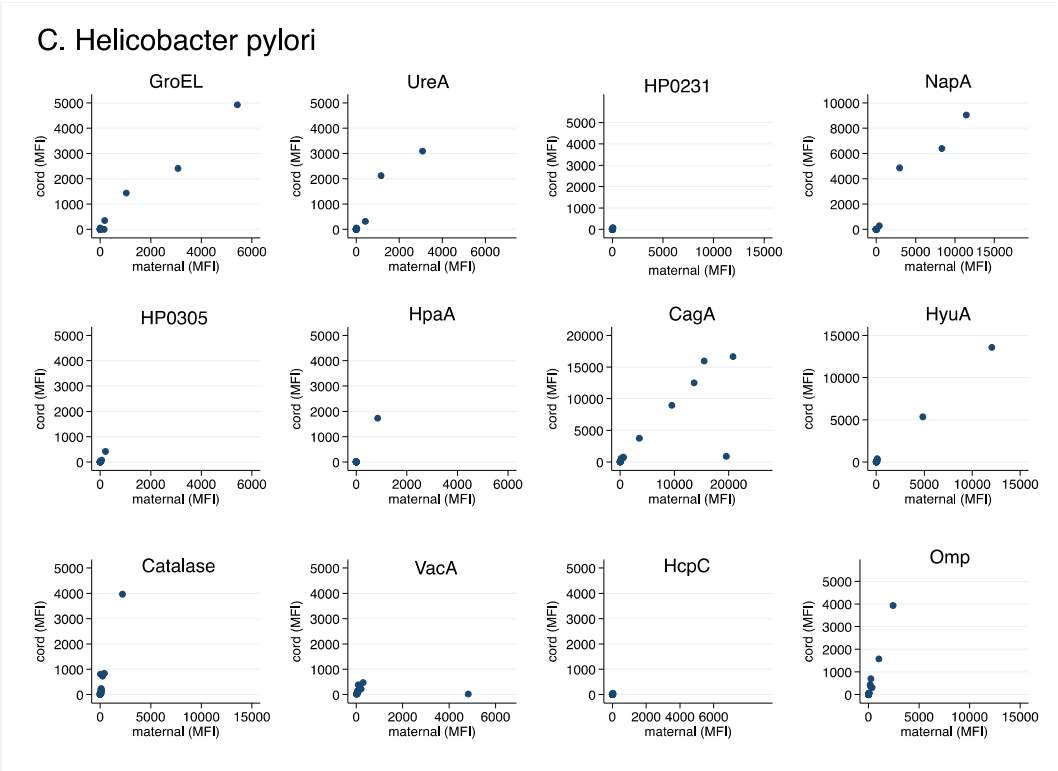


Figure 2. Scatterplots of maternal and cord seroreactivity to polyomaviruses (A), herpesviruses (B) and Helicobacter pylori (C) in 21 mother-child pairs. Correlations between maternal and cord seroreactivities were for BKPyV, 0.9534; for JCPyV, 0.9075; for KIPyV, 0.9236; for WUPyV, 0.7699; for HPyV6, 0.7534; for HPyV7, 0.8884; for TSPyV, 0.9515; for MCPyV, 0.9357; for HPyV9, 0.8206; for HPyV10, 0.9252; for EBV ZEBRA, 0.9154; for EBV EBNA, 0.9038; for EBV EA-D, 0.9697; for EBV VCA, 0.8150; for CMV pp150, 0.7759; for CMV CM2, 0.6689; for CMV pp52, 0.5656; for CMV pp28, 0.8256; for CMV, pp65 0.8149; for HSV-1 gG, 0.8237; and for HSV-2 mgGunique, 0.7940. HHV-8 seroreactivities were very low revealing seronegative results both in cord and maternal samples. Maternal samples were collected < 24th week of gestation.

The multiplex antibody detection approach is based on a glutathione S-transferase capture enzyme-linked immunosorbent assay method in combination with fluorescent bead technology (Luminex Corporation, Austin, Texas). The Glutathione-S-Transferase (GST)-capture ELISA developed by Sehr et al. uses glutathione coupled to casein as capture protein which is bound to microtiter plates (140). The coating of carriers (e.g. ELISA plates) with antigen-specific antibodies or molecules is advantageous, because it enables the immobilisation of the antigen avoiding its partly

denaturation by directly binding to the carrier and thereby preserves conformational epitopes. The Luminex system, in contrast to the ELISA, uses polystyrene beads instead of microtiter plates as carriers (Figure 3). These beads have a diameter of about 5.6 µm and are filled with different ratios of two fluorescent dyes leading to 100 differently coloured bead sorts which are commercially available. Superficial carboxyl groups residing on the beads can be coupled to amino groups of a target protein, which is glutathione-casein in this case. The procedure is quite similar to the standard ELISA as the incubation steps of the beads with the antigens, sera and detection solutions are also performed in microtiter plates. The antigen loaded beads as well as the reporter dye of the detection system is measured by a flow cytometer-like analyzer (xMAP; Luminex Corp). This device has two laser beams, the first excites the bead fluorescence to identify the bead sort and thereby the loaded antigen, the second excites the fluorescent reporter dye and therefore gives rise to the amount of secondary antibody and thus to the antigen-directed antibodies in the serum.

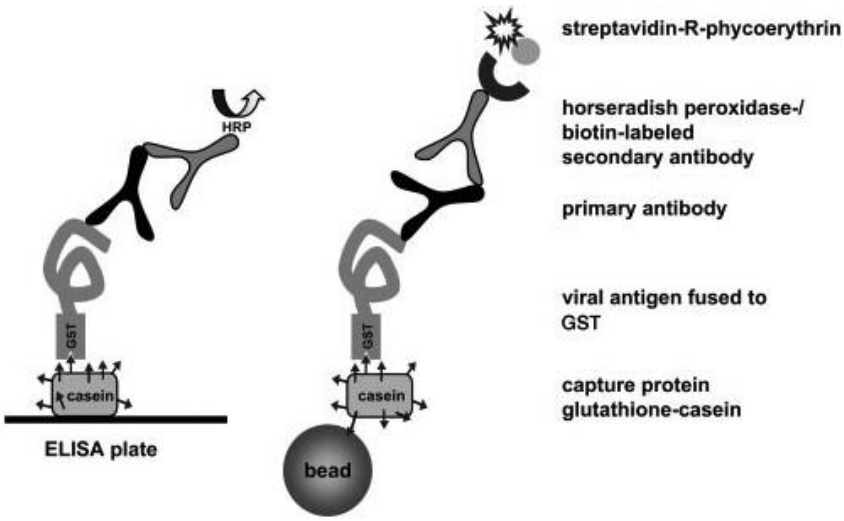


Figure 3 Schematic comparison of GST capture ELISA (left) and bead-based multiplex serology (right) (with permission from Tim Waterboer)

The Luminex method supersedes the GST-capture ELISA in many aspects. Because of the availability of 100 distinct bead sorts, antibody reactivities against up to 100 different antigens can be measured simultaneously in one well of a microtiter plate i.e. for one serum. In addition only a small amount of each serum (2 µl) is needed for the measurement. Furthermore, more than 1,000 sera can be measured on a single day. The Luminex™ method is more sensitive and more specific in its detection compared

to the standard ELISA as shown for weak antibody responses to HPV by Waterboer et al (138).

We defined the cut off values for seropositivity on the basis of the distribution of virus-specific seroresponses of the children three years of age. Following previous methodology (118), we performed a frequency distribution analysis with a bin width of 150 median fluorescence intensity on the seroresponses of the 81 children for each antigen tested. Samples falling within bins with a frequency percentage above 10% constituted the seronegative population. Cut off values were the mean seroreactivity of the seronegatives plus three times the standard deviation (Figure 4). EBV, CMV, and HHV-8 seropositivity was defined as seropositivity for at least two virus-specific antigens. We also calculated cut off values following different methodology used elsewhere (141). Cut off values for seropositivity were chosen for the MFI of each antigen tested by visual inspection of frequency distribution curves (percentile plots). This resulted in similar seroprevalence estimates as the standard cut off values described above. The cut-offs generated with the first approach are used in this thesis. The aggregate number of different Polyomaviruses to which the children were seropositive was referred to as Polyomaviruses burden. Similarly we calculated Herpesviruses burden. Seropositivity for a given H.pylori protein was defined as seroreactivity greater than the protein-specific cut-off which was re-evaluated using the published reference panel. H. pylori seropositivity was defined as reactivity to at least three out of the twelve proteins yielding a sensitivity of 90% and a specificity of 79% (142).

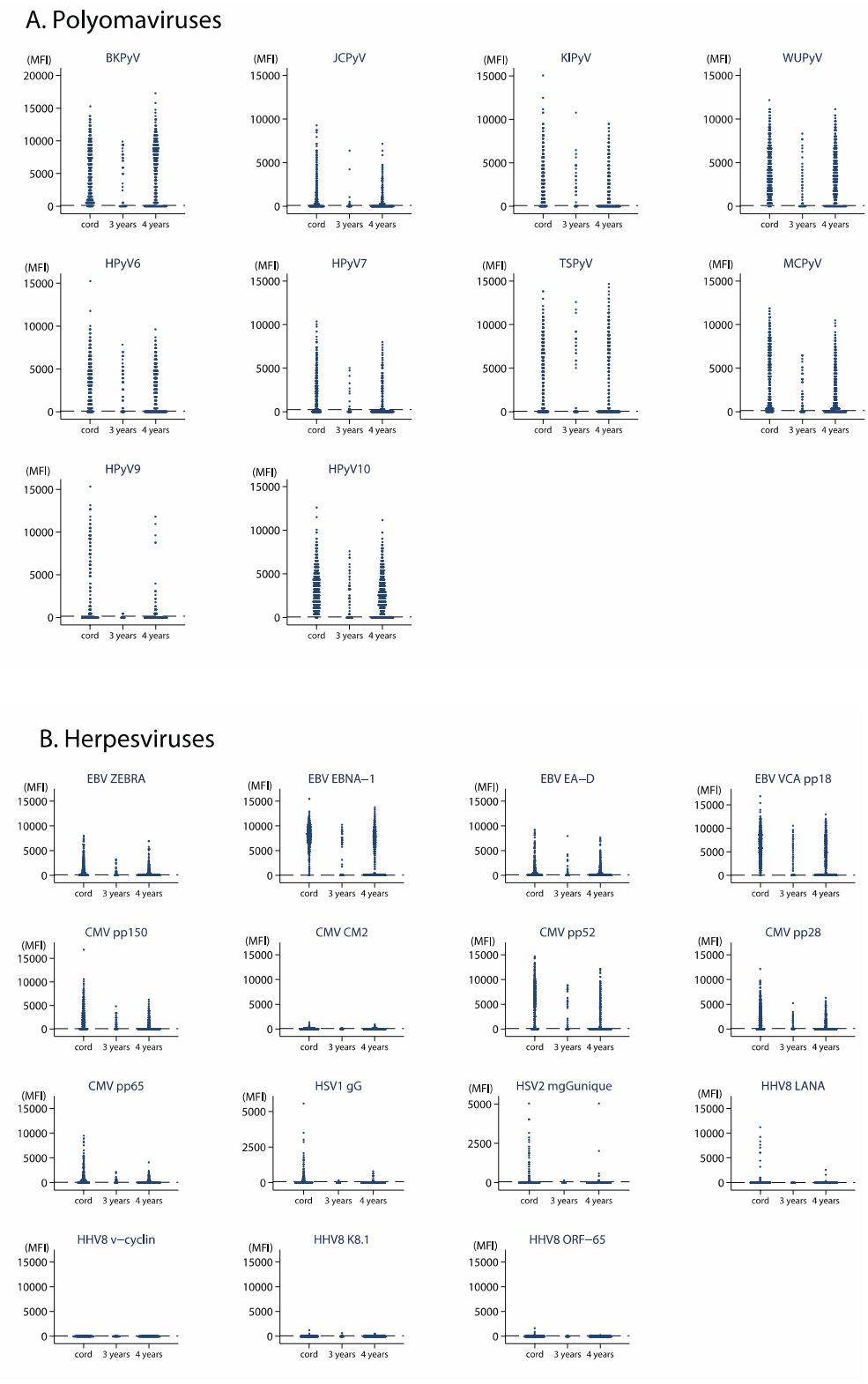


Figure 4. *Overall seroreactivity to human Polyomaviruses (A) and Herpesviruses (B) in cord (n=626), 3 years (n=81) and 4 years (n=690) of age samples with the cut-off values indicated by the horizontal dashed lines, the Rhea birth cohort in Crete, Greece. Cut-off for BKPyV was 118 MFI units, for JCPyV 100 MFI units, for KIPyV*

80 MFI units, for WUPyV 76 MFI units, for HPyV6 78 MFI units, for HPyV7 245 MFI units, for TSPyV 70 MFI units, for MCPyV 141 MFI units, for HPyV9 125 MFI units, for HPyV10 47 MFI units, for EBV ZEBRA 125 MFI units, for EBV EBNA-1 48 MFI units, for EBV EA-D 119 MFI units, for EBV VCA p18 63 MFI units, for CMV pp150 71 MFI units, for CMV CM2 102 MFI units, for CMV pp52 89 MFI units, for CMV pp28 114 MFI units, for CMV pp65 65 MFI units, for HSV1 gG 61 MFI units, for HSV2 mgGunique 50 MFI units, for HHV8 LANA 15 MFI units, for HHV8 v-cyclin 28 MFI units, HHV8 K8.1 56 MFI units and HHV8 ORF-65 50 MFI units.

2.3. Determinants

Aiming to identify the determinants of seroprevalence for Polyomaviruses and Herpesviruses in childhood, we focused this analysis in our study sample at four years of age. We explored the contribution of sociodemographic characteristics (parents' educational level, maternal origin, cars in the family, household ownership, financial status of the family, maternal working status), perinatal and early life characteristics (maternal antibodies, delivery type, preterm, breastfeeding), variables denoting social interactions (older siblings and ages, house crowding, age at daycare entry, swimming pool attendance), lifestyle variables (TV viewing, parental stress scale score), hygiene practices (frequency of house cleaning, frequency of handwashing, frequency of bodywashing, frequency of washing face) and home environment (passive smoking, pets in the house). Information on the sources and methods of assessment for each potential determinant is presented in Table 2. Sociodemographic variables were highly correlated (Figure 5); therefore, we present results regarding only the parents' educational level. The level of completeness for this variable was very high (96%). Variables of personal hygiene (frequency of washing hands and face and having a shower/bath) were also highly correlated (Figure 5); therefore, we decided to use only the variable "frequency of handwashing", which is the more relevant variable to the spread of infections.

Table 2. Definitions, Sources of Data and Methods of Assessment of Potential Determinants of Human Polyomaviruses and Herpesviruses Seroprevalence at Four Years of Age, the Rhea Birth Cohort in Crete, Greece.

Variable name	Sources of data	Specify measure, if not standard
A. Sociodemographic characteristics		
Parents' educational level High Medium Low Completeness: 96%	Highest achieved educational level by mother and the father reported at the questionnaire on recruitment in the Rhea study by the mother (personal interview). For 19 subjects of the present study information was missing and was derived from an alternative source. The alternative source was a questionnaire completed again by the mother (phone interview) as part of the 4 years of age follow-up. The relevant questions and coding were the same.	Highest achieved educational level of the mother and father was categorized as "low", <9 years of education; "medium", >9 years up to attending post-secondary school education; and "high", attending university or having a university/technical college degree. We took into account the educational level of both the mother and the father in order to construct a new variable of parents educational level characterizing the educational background of the child. Parents educational level was defined as "high" if at least one parent was of high level, "medium" if both parents were of medium level and "low" if parents were of low/medium or low/low level.
Maternal origin Greek Non-Greek Completeness: 100%	Maternal report at the questionnaire on recruitment in the Rhea study (personal interview). For 17 subjects of the present study information was missing and was based on the full name of the mother to define the Greek or non-Greek origin.	Classified as Greek, non-Greek. Mothers of non-Greek origin are mostly from Balkan countries (Albania, Bulgaria, Serbian). There are single cases from Lebanon, Romania, Russia, Lithuania, Letonia, Netherlands and Great Britain.
Cars in the family None or one Two or more Completeness: 89.0%	Maternal report at the questionnaire on recruitment in the Rhea study (personal interview).	Initial variable was continuous.
Owning the house Yes No Completeness: 87.7%	Maternal report at the questionnaire on recruitment in the Rhea study (personal interview).	

Financial status of the family Good or very good Average or bad Completeness: 57.3%	Maternal report on the question "how would you describe the economic situation of your family?" at the 18th month of life questionnaire (personal interview).	Initial variable had 4 levels "good", "very good", "average", "bad" and was then recoded accordingly.
Maternal working status, 4 years Working Non working Completeness: 99.6%	Maternal report on her current occupational status at the 4th year of life questionnaires.	Based on a 9 levels response recoded to express "working" and "non- working".
B. Perinatal and early life characteristics		
Maternal antibodies (cord) Seropositive Seronegative Completeness 41.7%	See methods section	Cut-offs: see methods section
Maternal antibodies (cord)-seroreactivity levels (only among seropositives) 1st tertile 2nd tertile 3rd tertile Completeness 41.7%		Among those considered to be seropositive, we analyzed seroreactivity in categories defined by tertiles.
Delivery type Vaginal Cesaric Completeness: 98.3%	Recorded at delivery or reported by the mother after delivery.	
Preterm Yes No Completeness: 97.6%	Gestational age on the interval between last menstrual period and date of delivery. When the menstrual estimate of gestational age was inconsistent by ≥7 days within the ultrasound measurement taken in the first trimester of pregnancy a quadratic regression formula describing the relationship between crown-rump	Preterm children were those born before completing the 37th week of gestation.

	length and gestational age was used instead.	
Breastfeeding never 1-3 months 4-6 months >6 months Completeness: 93.3%	Maternal report on i) initiation of any breastfeeding and ii) duration of breastfeeding. First recorded at the 9th month questionnaire (telephone interview) and updated at 18th month (personal interview) of life questionnaire using the same questions and coding.	Duration in completed months was recoded accordingly.
C. Social interactions		
Older siblings Yes No Completeness: 99.4%	Maternal report on the number of children before and after the index child on the 9th month (telephone interview) and 4th year of life (personal interview) questionnaires.	
Older siblings and ages Yes ≥6 years Yes <6 years No Completeness: 91.0%	Information on the ages of the older siblings (older or younger than 6 years) was only obtained on the 9th month of life questionnaire.	Based on the presence of older siblings and their ages. Children were categorized as "yes <6 years" if at least one sibling in the household was less than 6 years of age, as "yes, ≥6 years" if all siblings in the household were 6 years of age or more and as "no" if they had no older siblings.
House crowding, infancy ≥1 persons per room <1 persons per room Completeness: 99.3%	Maternal report on the number of people living in the same house with the child and the number of rooms of the house, excluding kitchen and bathroom at the 9th month of life questionnaire.	Total number of persons living in the house divided by the total number of rooms.
Age at daycare entry never after 3 years 2-3 years before 2 years Completeness: 93.3%	Maternal report on age at daycare entry up to the time of questionnaire. Recorded at the 9th month (phone interview), 18th month (personal interview) and 4th year of life (self-completed) questionnaires.	
Swimming pool attendance Yes No Completeness: 99.3%	Maternal report on the 4th year of life questionnaire (personal interview).	

D. Lifestyle and parental stress		
TV viewing (daily) Never or <30 min 1 hour ≥ 2 hours Completeness: 99.3%	Maternal report on the 4th year of life questionnaire (personal interview).	Initial coding was "never", "<30 minutes", "1 hour", 2 hours", "3-4 hours", ">5 hours". Was recoded due to the low frequency of the categories "never", "3-4 hours" and ">5 hours".
Parental stress scale score ≥75th percentile <75th percentile Completeness: 82.6%	Self-completed questionnaire by the mother at 4 years of age follow-up. The Parental Stress Scale is a self-reported scale that contains 18 items representing pleasure or positive themes of parenthood (emotional benefits, self-enrichment, personal development) and negative components (demands on resources, opportunity costs and restrictions).	The 75th percentile of the study cohort distribution was used to define a group of mothers with high score.
E. Hygiene practices		
Frequency of house cleaning, 4 years >1 per week ≤1 per week Completeness: 99.3%	Maternal report on the 4th year of life questionnaire (personal interview).	Initial coding: "less than once", "once", "more than once per week". Recoded as a binary variable: "once or less", "more than once per week" due to the low frequency of "less than once".
Frequency of handwashing, 4 years ≥5 times per day <5 times per day Completeness: 99.4%	Maternal report on the 4th year of life questionnaire (personal interview).	Initial variable as continuous. The median value in our sample was used to create the binary variable.
Frequency of bodywashing 9th month <7 times/week ≥7 times/week Completeness: 91.0%	Maternal report on the 9th month (telephone interview) and 4th year of life questionnaire (personal interview) to the same question.	Both the frequency of having a bath and/or a shower per week were considered. The median value in our sample was used to create the binary variable.
Frequency of bodywashing 4 years <5 times/week ≥5 times/week Completeness: 97.4%	Maternal report on the 4th year of life questionnaire (personal interview).	Both the frequency of having a bath and/or a shower per week were considered. The median value in our sample was used to create the binary variable.

Frequency of washing face 4 years <3times/day ≥3 times/day Completeness:99.1%	Maternal report on the 4th year of life questionnaire (personal interview).	Initial variable was continuous. The median value in our sample was used to create the binary variable.
F. Home environment		
Passive smoking (home) exposed non-exposed Completeness: 98.7%	Maternal report on the 4th year of life questionnaire (personal interview).	The variable is based on the responses (yes or no) on whether the mother, the father or any other person was smoking inside the home. We defined children exposed to passive smoking if at least one person was smoking in the house and as non exposed if none was smoking in the house.
Pets in the house, 4 years Yes No Completeness: 99.3%	Maternal report on the 4th year of life questionnaire (personal interview).	

Completeness is calculated based on the 4 years of age population (n=690).

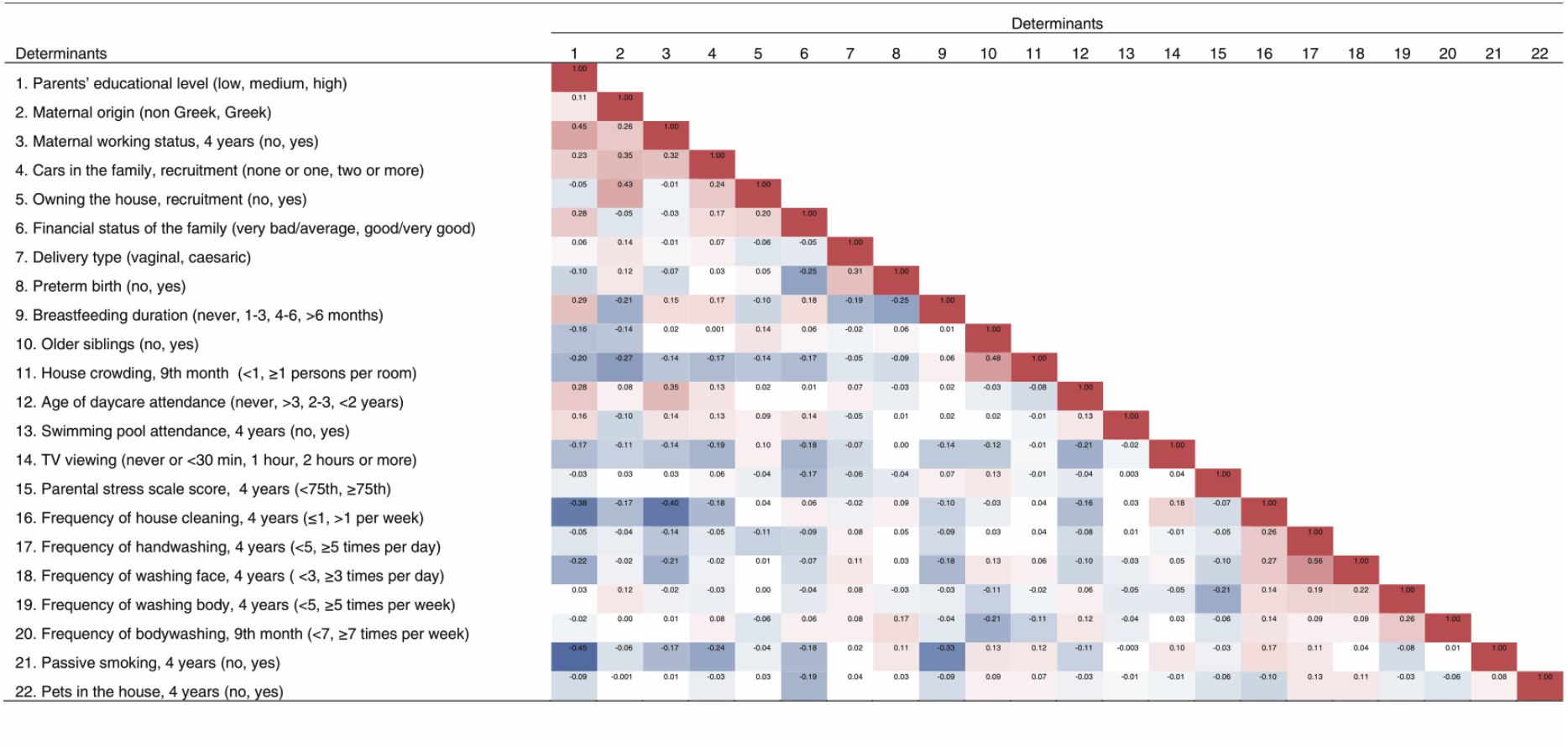


Figure 5. Correlation matrix of potential determinants of polyomaviruses and herpesviruses seroprevalence at 4 years of age, the Rhea birth cohort in Crete, Greece. (n=543-659) Values are polychoric correlations. The red color indicates a positive association and the blue color a negative association.

2.4. Neurodevelopmental assessment

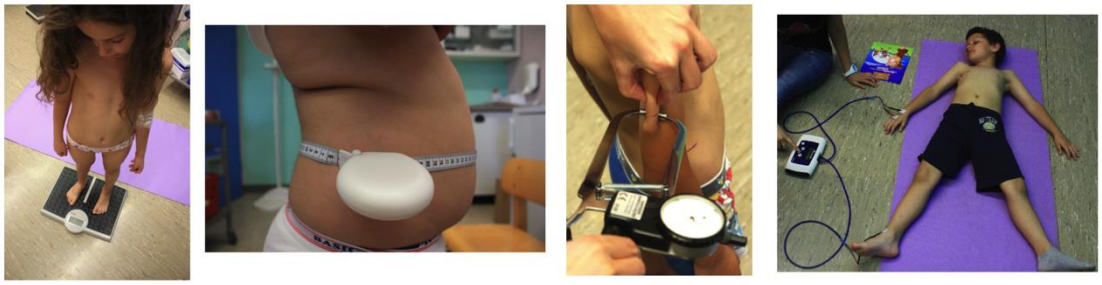
At age four, children's cognitive and motor development was assessed by two trained psychologists with the age appropriate instrument McCarthy Scales of Children's Abilities (MSCA), developed for children aged 2½ - 8½ (143). The MSCA include five conventional subscales (verbal, quantitative, memory, perceptual performance, and motor) and a general cognitive scale, which is a composite scale of verbal, perceptual performance, and quantitative subscales. MSCA raw scores were standardized for child's age and homogenized with a mean of 100 points and a standard deviation (SD) of 15 (144) (Royston & Wright, 1998). Scores were treated as continuous variables with higher scores representing better performance. Children were assigned to the two psychologists at random. The interobserver variability was <1%.

Additional information on children's behaviour was obtained via maternal report on standardized child behaviour scales, which were administered at the 4 years of age follow-up. The Attention-Deficit/Hyperactivity Disorder Test (ADHDT) is designed to identify and evaluate ADHD in ages 3–23 years and which is based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for ADHD (145,146). The ADHDT was translated and adapted to the Greek population (145,146). It is composed of 36 items in three subscales; (a) hyperactivity, (b) inattention, and (c) impulsivity. All 36 items are summed to generate an index for total ADHD difficulties (possible range, 0–72). Higher scores indicate higher and more severe ADHD-related symptomatology. The parent version of the Strengths and Difficulties Questionnaire (SDQ) is a behavioural screening instrument designed to assess strengths and difficulties of children aged 3–16 years (147,148). The SDQ was translated and adapted to the Greek population (147,148). It consists of five subscales generating scores for emotional symptoms, conduct problems, hyperactivity–

inattention, peer relations problems, and prosocial behavior; all but the last one are summed to generate a total difficulties score (possible range, 0–40). Scores in each scale of ADHDT and SDQ questionnaire were treated as continuous variables. These scores delineate symptoms and their perceived severity and not a clinical disorder.

2.5. Anthropometry and metabolic traits

At four and six years of age follow-up visits, trained research assistants performed anthropometric measurements according to standard operating procedures. We measured weight (Seca Corporation, Hanover, MD) and height (Seca 213; Seca Corporation) on light clothing without shoes. We calculated body mass index (BMI) and converted raw values into sex- and age-specific standard deviation scores (SD scores) by using internally generated growth reference curves. We applied the International Obesity Task Force classification system to define normal weight/underweight, overweight and obesity based on child's BMI, age and sex (149). We measured waist circumference in duplicate to the nearest 0.1 cm in a standing position, at the high point of the iliac crest at the end of a gentle expiration, with the use of a measuring tape (Seca 201; Seca Corporation). Skinfold thickness was measured at four anatomic sites (triceps, subscapular, suprailiac, and thigh) on the right side of the body in triplicate to the nearest 0.1 mm with a calibrated caliper (Harpenden HSK- BI, CE-0120; Baty International, West Sussex, UK). We then calculated the sum of these four skinfolds as a general index of subcutaneous fatness. Intraobserver and interobserver reliability assessments were undertaken as suggested elsewhere (150). Intraobserver reliability was >0.98, and interobserver reliability was >0.82 for all anthropometric measurements. Also, at age six hand-to-leg bioelectric impedance analysis was measured using a Bodystat 1500 machine. The child had been asked to empty his/her bladder and remove any heavy clothing and metal jewellery. Four electrodes were placed as specified by the manufacturer on the right wrist and right ankle. Children were tested in a supine position with legs abducted to 45º, after 5 minutes rest. Impedance was recorded to the nearest ohm. Trained research assistants recorded from the Body Manager report of every child, the body fat and percentage of body fat estimations to the clinical datasheet. The machine was calibrated daily using the resistor provided by the manufacturer.



At age four and six, total cholesterol and HDL-cholesterol in serum collected at the end of child's follow-up visits, were measured with standard enzymatic methods (Medicon Hellas SA, Gerakas, Greece). Leptin and adiponectin (Invitrogen, Carlsbad, CA) were measured only at age four by an enzyme-linked immunosorbent assay. Inter- and intraassay coefficients of variation were < 5%.

2.6. Statistical analysis

We calculated crude seroprevalences with 95% confidence intervals at each follow-up (birth, three and four years of age). For 61 children participating in follow-up at 3 and 4 years of age, we tested differences in seroprevalence between follow-ups by the McNemar exact test. We estimated associations between potential determinants at 4 years of age by polychoric correlations (a measure of association for ordinal variables) (151). Associations between seropositivity of individual infections were measured using tetrachoric correlations with pairwise deletion and exact two sided significance tests. We used Poisson regression models with robust variance to estimate the association of each potential determinant with individual virus seroprevalence at age four, as well as with the burden of Polyomaviruses (152). Results are presented as prevalence ratios with 95% confidence intervals and count ratios with 95% confidence intervals, respectively. Models were adjusted for the same a priori confounders: child' s age and sex; maternal origin; and parents' educational level.

Descriptive analyses of the study population characteristics, exposures, and outcomes were conducted for each individual analysis. Briefly, bivariate associations between normally distributed continuous variables and categorical variables were studied using either Student t-test or ANOVA. Bivariate associations between non-normally continuous variables were studied using non-parametric statistical methods (Mann-

Whitney, Kruskal-Wallis), whereas associations of categorical variables were tested using Pearson's Chi-square test.

Linearity of the associations between Polyomaviruses burden and MSCA, ADHDT, and SDQ scores as well as obesity outcomes and metabolic traits were assessed in the original dataset using generalized additive models (data not shown). There was no evidence of nonlinearity for any model (p-gain defined as the difference in normalized deviance between the generalized additive model and the linear model for the same predictor were always above >.10). Nonetheless, for ease of interpretation, we examined Polyomaviruses burden in categories of '≤3 Polyomaviruses infections', '4–7 Polyomaviruses infections', and '≥8 Polyomaviruses infections' based on the frequency distribution of this variable in our population (approximately defining the lowest and highest 15%). These categories were not predefined. Our intention by creating these groups was to examine differences between children with different levels of acquisition of Polyomaviruses. The Herpesviruses burden was examined as '0 viruses', '1 virus', and '2–3 viruses'. The last category was a combination because few children had three viral seropositivities (n = 6, 0.9%). We estimated the effect (b, 95% CI) of single infections (seropositive vs. seronegative), Polyomaviruses burden ('≤3 polyomaviruses infections' as the reference group) and Herpesviruses burden ('0 viruses' as the reference group) on outcomes using multiple regression models. Tests for trend were conducted by including an ordinal variable in the regression model in place of the categorical variable (Polyomaviruses and Herpesviruses burden). Regarding the outcomes- lipids, leptin and adiponectin- these were log transformed to normalize their distributions. The resultant regression coefficients were exponentiated to give a ratio of geometric means per change in exposure. To assess differences in BMI trajectories from four to six years of age follow-up for serological status to single infections or infectious burden, we constructed mixed effects linear regression models using BMI SD-scores of our population. We used a two-step approach (144,153); first, we identified the best fitting fractional polynomials of age and constructed sex-and age- specific BMI growth curves. Then we used mixed-effects linear regression models, including an interaction term of exposure under study with age along with previously described covariates, a random intercept for child and a random age slope. We estimated the association (β, 95% CI) of H.pylori seropositivity and each specific H.pylori protein seropositivity with standardized MCSA scores

using multivariable linear regression models. Seropositivity to each of the twelve H.pylori proteins was examined irrespective of the overall H.pylori serostatus.

For each analysis, potential confounders were selected based on previously described risk factors of our exposure and outcome variables. We then used directed acyclic graphs (DAGs) to identify variables associated with both the exposure and outcome (Figure 6-8). Causal diagrams are a useful way to summarize, clarify, and communicate one' s qualitative beliefs about the causal structure. The use of causal diagrams in epidemiology has been proposed by Greenland et al.(154). DAGs have been used in epidemiology to represent causal relations among variables, and they have been used extensively to determine which variables it is necessary to condition on in order to control for confounding. A DAG is composed of variables (nodes) and arrows between nodes (directed edges) such that the graph is acyclic—that is, it is not possible to start at any node, follow the directed edges in the arrowhead direction, and end up back at the same node. A causal DAG is one in which the arrows can be interpreted as causal relations and in which all common causes of any pair of variables on the graph are also included on the graph.

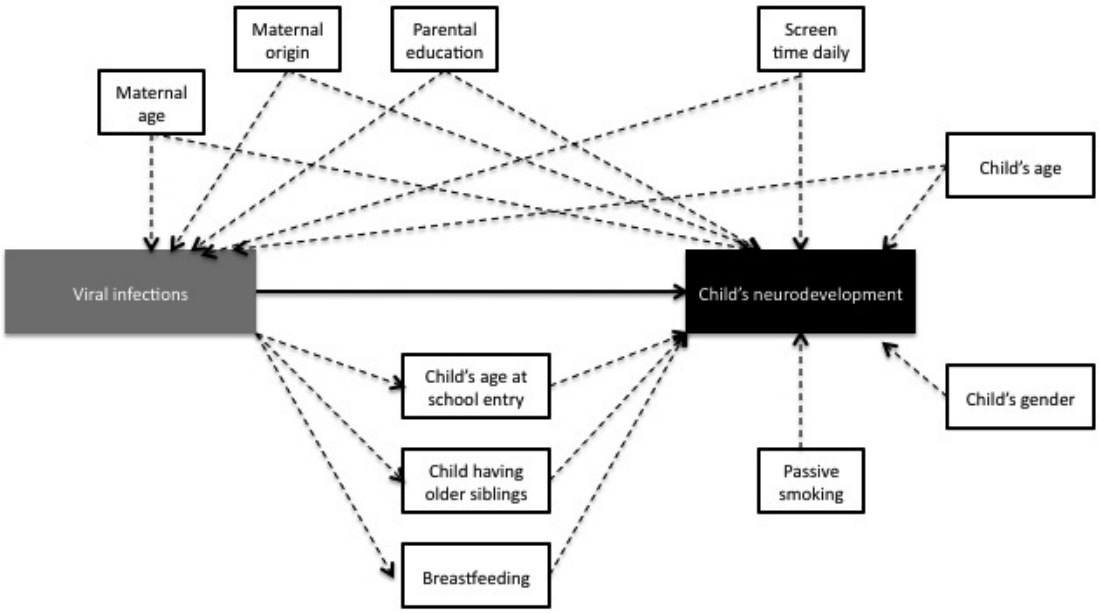


Figure 6. *Directed acyclic graphs (DAGs) to identify potential confounders of the association between Polyomaviruses and Herpesviruses infections at age four and child's neurodevelopment at the same age.*

Figure 6 depicts the DAG on the the association between viral infections and neurodevelopmental outcomes. Thus, these models were adjusted for child's age,

gender, parental education, maternal origin, age at school entry, presence of older siblings, and for duration of breastfeeding (only models of CMV infection). Models of MSCA scores were also adjusted for quality of assessment and examiner.

In this analysis we also had to deal with missing data. There were missing values in 1-17 items of the ADHDT questionnaire for 114 (18.4%) subjects and in 1-9 items of the SDQ questionnaire for 67 (10.8%) subjects. Missing items were imputed based on other item responses in each questionnaire to minimize the impact of lack of data and the scores for the subscales were then calculated based on imputed data. We applied chained equations to multiply impute missing values and 10 imputed data sets were generated. Regarding confounders, 72 of 674 participants (10.7%) had missing data on one or more confounders. We used multiple imputation with chained equations to impute missing data in confounders (155). Ten imputation datasets were generated. We used imputation models that were more general than the analyses models and included all the different polyomaviruses and herpesviruses, all MCSA, ADHDT and SDQ scales and auxiliary variables: mother's and father's age, mother's and father's employment status (yes, no), prematurity (yes, no), mother's tobacco smoking habits during pregnancy (never, quit during pregnancy, smoked during pregnancy), hours per day spent with mother and father as recorded at 9th month of life and 4th year of life questionnaires and hours spent watching TV as recorded at 4th year of life questionnaire (never or less than 30 minutes, 1 hour, 2 hours or more). The main analysis results are obtained by averaging across the results from each of these 10 data sets using Rubin's rules, which ensure that the standard errors for any regression coefficients take account of uncertainty in the imputations as well as uncertainty in the estimation (155).

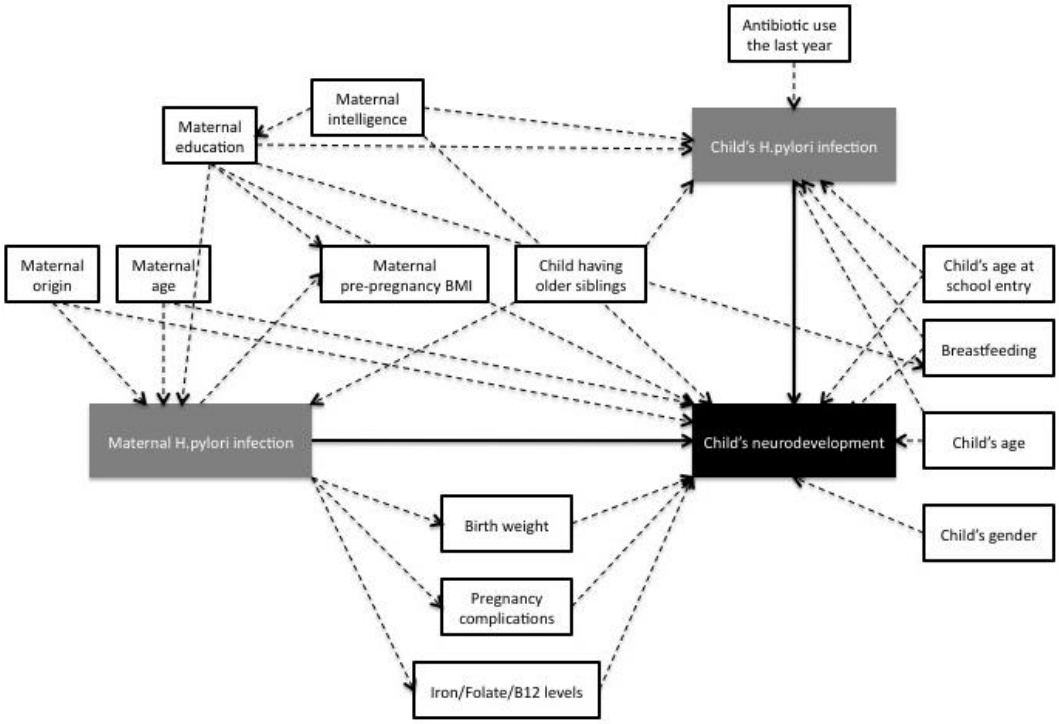


Figure 7*. Directed acyclic graphs (DAGs) to identify potential confounders of the association between maternal Helicobacter pylori infection based on seroreactivity in cord blood samples or child's Helicobacter pylori infection and child's neurodevelopment at age four.*

Figure 7 depicts the DAG on the association between H.pylori infection and neurodevelopmental outcomes. The initial model contained the variables child's age, gender, quality of assessment and examiner. The final model was also adjusted for mother's education, origin, age, child having older sibling and child's age at day-care entry (only for models of 4 years of age samples). Level of completeness for confounders was always above 93.4%. Sensitivity analysis was carried out and included adjustment for variables with limited number of data (maternal intelligence and child's H.pylori seropositivity) that could impact statistical power.

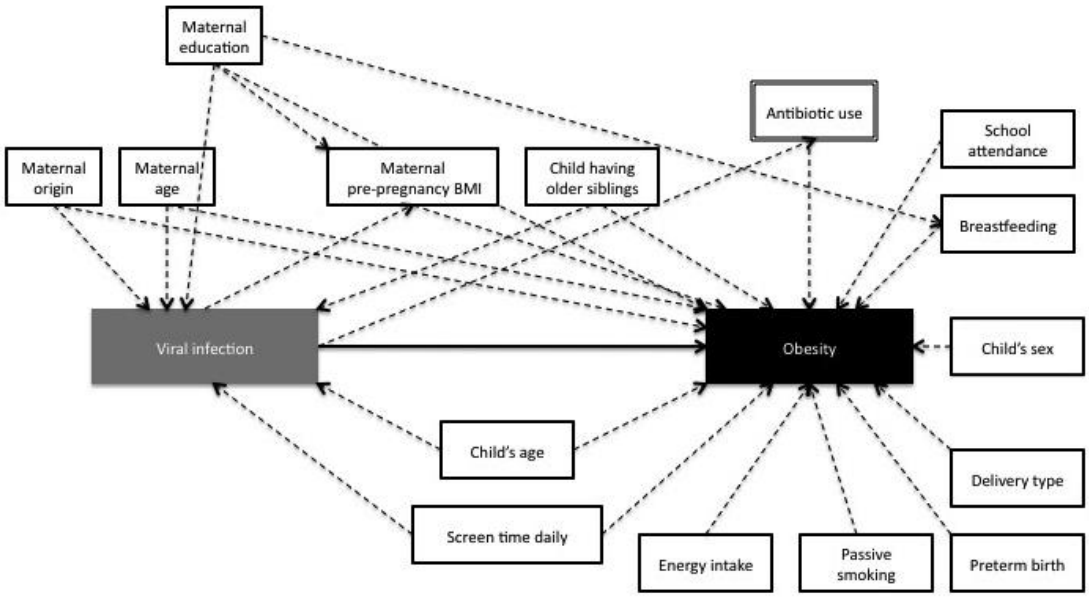


***Figure 8**. Directed acyclic graphs (DAGs) to identify potential confounders of the association between viral infections (Polyomaviruses and Herpesviruses) and obesity.*

Figure 8 depicts the DAG on the association between viral infections and obesity or metabolic traits. Models were adjusted for maternal age at delivery, origin, educational level at recruitment, pre-pregnancy BMI, child's sex, age at outcome assessment, breastfeeding initiation and duration, child having older siblings, child attending school and screen-time spent per day. Level of completeness for covariates included in the main models was > 94%. Antibiotic use during the 4th year of life (never, 1-2 times, ≥3 times; based on mother's responses at the 4th of life questionnaires) was on the causal pathway between the exposure and outcome and was included in sensitivity analyses. Antibiotic use has been considered a risk factor for childhood obesity based on the hypothesis that it is likely to affect the intestinal microbiome, although it has been recently suggested that the observed associations are likely attributable to failure to control for underlying infections (156). Child's BMI SD-score was also included in sensitivity analyses for models that used metabolic traits as outcomes in order to explore remaining confounding. To assess the potential modifying effect of child sex (male, female) (157), breastfeeding duration (never, 1–6 months, >6 months) and maternal education (low, medium and high) (158) we included appropriate interaction terms in regression models and stratified the sample.

3. Results

3.1. Seroprevalence and seroreactivity to Polyomaviruses and Herpesviruses in repeated samples from birth to four years of age and determinants of acquisition of infection up to age four.

Publication 1: Marianna Karachaliou, Tim Waterboer, Delphine Casabonne, Georgia Chalkiadaki, Theano Roumeliotaki, Angelika Michel, Eftichia Stiakaki, Leda Chatzi, Michael Pawlita, Manolis Kogevinas, and Silvia de Sanjose. The Natural History of Human Polyomaviruses and Herpesviruses in Early Life—The Rhea Birth Cohort in Greece, Am J Epidemiol. 2016;183(7):671–679

Aims

In this study we use serum samples available at birth, three years and four years of age to describe the natural history of Polyomaviruses and Herpesviruses in the Rhea birth cohort. We provide antigen specific seroprevalence data and seroreactivity levels among seropositive individuals for each pathogen studied. Also we used prospectively collected data from the Rhea birth cohort, in order to identify potential determinants of Polyomaviruses, EBV and CMV acquisition up to the four years of age. HSV-1 and HSV-2 showed very low seroprevalence rates at age four and thus were not included in the analysis of determinants.

Results

Study population

Of 1,363 singleton live births of the Rhea cohort, 1,041 children were included in this study with samples available at birth and/or three years and/or four years of age (Figure 1).

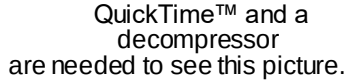


Figure 1. *Flowchart of the study population.*

In total, we analyzed 626 cord samples and 81 samples and 690 samples from follow-ups at three and four years of age, respectively. Paired samples at birth and four years of age were available for 288 children, while most of the children with a sample at three years of age had an available sample at four years of age as well (n = 61, 75%). Reasons for lacking a sample included the following: parent/child denying a blood collection, unsuccessful collection, or collection of inadequate blood volume. Reasons of nonparticipation in follow-up included unavailability at the study period, moved to another area, loss of interest, and serious medical/behavioral problems with the child. One child was excluded because of documented immunosuppression by medical history. Children with cord and four year samples compared with those without a four year follow-up sample were more likely to be of Greek mothers (93.6% vs. 84.8%; P value < 0.001). Children with a three year sample versus those without were less likely to be HPyV7 seropositive at four years of age (9.8% vs. 24.01%; P value = 0.01) and more likely to be CMV seropositive (37.7% vs. 24.6%; P value = 0.03).

Descriptive characteristics of the study population

Among the children aged four years who were tested for polyomaviruses and herpesviruses, 47.4% were female, 93.3% had a Greek mother, 39.2% were of parents with a high educational level, 49.4% were delivered by a cesarean section, 22.4% were breastfed for more than 6 months, and 21.4% entered day care before 2 years of age. HPyV6 and HPyV9 were more prevalent in girls than in boys at 65.4% versus 58.1% (P value = 0.0) and 24.8% versus 17.4% (P value = 0.01), respectively.

Seroprevalence data for Polyomaviruses and Herpesviruses

Table 1 presents the follow-up specific seroprevalences for the ten Polyomaviruses and the five Herpesviruses. Figure 2 and 3 present the Polyomaviruses and Herpesviruses burden by follow-up correspondingly. Seroprevalences of Polyomaviruses in cord blood were very high (>80%), aside from HPyV7 (65.3%), JCPyV (53.5%), and HPyV9 (38.5%). All cord samples were seropositive to at least four out of the ten Polyomaviruses tested while seropositive to all of the ten Polyomaviruses were 12% (Figure 2A). Most frequently, cord blood samples were seropositive to eight Polyomaviruses. At three years of age [mean=3.0 (standard deviation, 0.29) years], the seroprevalence of Polyomaviruses ranged from 6.2% for HPyV9 to 55.6% for HPyV10. Only 2.5% (n=2) of samples were seronegative to all

of the ten Polyomaviruses, while none of the children at age three was seropositive to either nine or ten Polyomaviruses (Figure 2B). Most frequently, children 3 years of age were seropositive to two or three Polyomaviruses. At four years of age [mean =4.3 (standard deviation, 0.23) years], BKPyV, WUPyV, and HPyV10 were the most prevalent (>75.5%), while the least prevalent were JCPyV, HPyV7, and HPyV9 (<34.1%). The majority of children were seropositive to at least one Polyomavirus up to the age of four years, as only four samples were seronegative to all of the ten. Approximately 28% of the children were seropositive to at least seven Polyomaviruses at age four (Figure 2C). Most frequently, children 4 years of age were seropositive to five Polyomaviruses. Among the 61 children with samples at three and four years of age, the seroprevalence increased significantly during the lag period of one year (range, 0.5– 2.0 years) for most Polyomaviruses (Figure 4). Only HPyV6, KIPyV, HPyV9, and HPyV7 did not show significant variation over time.

EBV and CMV were highly prevalent in cord blood (99.4% and 74.9%) and much less at age four years (52.5% and 25.8%) (Table 1). In cord blood, HSV-1 was more prevalent than HSV-2 (26.2% and 8.0%). HSV-1 and HSV-2 were not common among children. HHV-8 seroprevalence was negligible in our population. In cord blood, only one sample was seronegative to all Herpesviruses studied, 17.4% were seropositive to at least one Herpesvirus and 26% were seropositive to at least three or four Herpesviruses (Figure 3A). No significant differences in the distribution of Herpesviruses burden between three and four years of age were observed (Figure 3B & 3C).

Table 1. Seroprevalence (95% CI) of Polyomaviruses and Herpesviruses by Follow-up, the Rhea Birth Cohort in Crete, Greece, 2007-2012.

Virus	Cord blood (n=626)		Three years of age follow-up (n=81)		Four years of age follow-up (n=690)	
	%	95% CI	%	95% CI	%	95% CI
Polyomaviruses						
BKPyV	95.8	94.0, 97.3	38.3	27.7, 49.7	76.2	72.9, 79.4
JCPyV	53.5	49.5, 57.5	12.3	6.1, 21.5	34.1	30.5, 37.7
KIPyV	83.4	80.2, 86.2	37.0	26.6, 48.5	44.2	40.5, 48.0
WUPyV	98.1	96.7, 99.0	39.5	28.8, 51.0	75.5	72.1, 78.7
MCPyV	89.6	87.0, 91.9	50.6	39.3, 61.9	68.4	64.7, 71.8
HPyV6	92.0	89.6, 94.0	53.1	41.7, 64.3	61.6	57.8, 65.2
HPyV7	65.3	61.5, 69.1	13.6	7.0, 23.0	22.8	19.7, 26.1
TSPyV	80.2	76.9, 83.2	30.9	21.1, 42.1	51.3	47.5, 55.1
HPyV9	38.5	34.7, 42.4	6.2	2.0, 13.8	20.9	17.9, 24.1
HPyV10	99.8	99.1, 99.9	55.6	44.1, 66.6	82.3	79.3, 85.1
Herpesviruses						
EBV	99.4	98.4, 99.8	42.0	31.1, 53.5	52.5	48.7, 56.2
VCA p18	99.0	97.9, 99.6	45.7	34.6, 57.1	53.0	49.2, 56.8
EBNA-1	99.0	97.9, 99.6	42.0	31.1, 53.5	53.0	49.2, 56.8
ZEBRA	69.8	66.0, 73.4	24.7	15.8, 35.5	31.0	27.6, 34.6
EA-D	56.1	52.1, 60.0	32.1	22.2, 43.4	38.1	34.5, 41.9
CMV	74.9	71.3, 78.3	30.9	21.1, 42.1	25.8	22.6, 29.2
pp52	77.0	73.5, 80.2	33.3	23.2, 44.7	31.7	28.3, 35.4
pp28	74.1	70.5, 77.5	34.6	24.3, 46.0	28.4	25.1, 31.9
pp150	75.1	71.5, 78.4	30.9	21.1, 42.1	25.8	22.6, 29.2
pp65	64.2	60.3, 68.0	27.2	17.9, 38.2	22.9	19.8, 26.2
CM2	23.6	20.4, 27.2	1.2	0.03, 6.7	4.9	3.4, 6.8
HSV-1	26.2	22.8, 29.8	3.7	0.8, 10.4	3.6	2.4, 5.3
HSV-2	8.0	6.0, 10.4	1.2	0.03, 6.7	1.4	0.7, 2.6
HHV-8	1.6	0.8, 2.9	0.0	0.0, 4.5[a]	0.0	0.0, 0.5[a]
LANA	5.1	3.5, 7.1	1.2	0.03, 6.7	2.0	1.1, 3.4
v-cyclin	1.3	0.6, 2.5	2.5	0.3, 8.6	1.2	0.5, 2.3
K8.1	0.7	0.4, 2.1	2.5	0.3, 8.6	1.0	0.4, 2.1
ORF-65	2.1	1.1, 3.5	2.5	0.3, 8.6	1.0	0.4, 2.1

Abbreviations: CI, confidence intervals; CMV, cytomegalovirus; EBV, Epstein Barr virus; HHV-8, human herpesvirus 8; HSV-1, herpes simplex virus 1;HSV-2, herpes simplex virus 2
[a]one-sided, 97.5% confidence interval

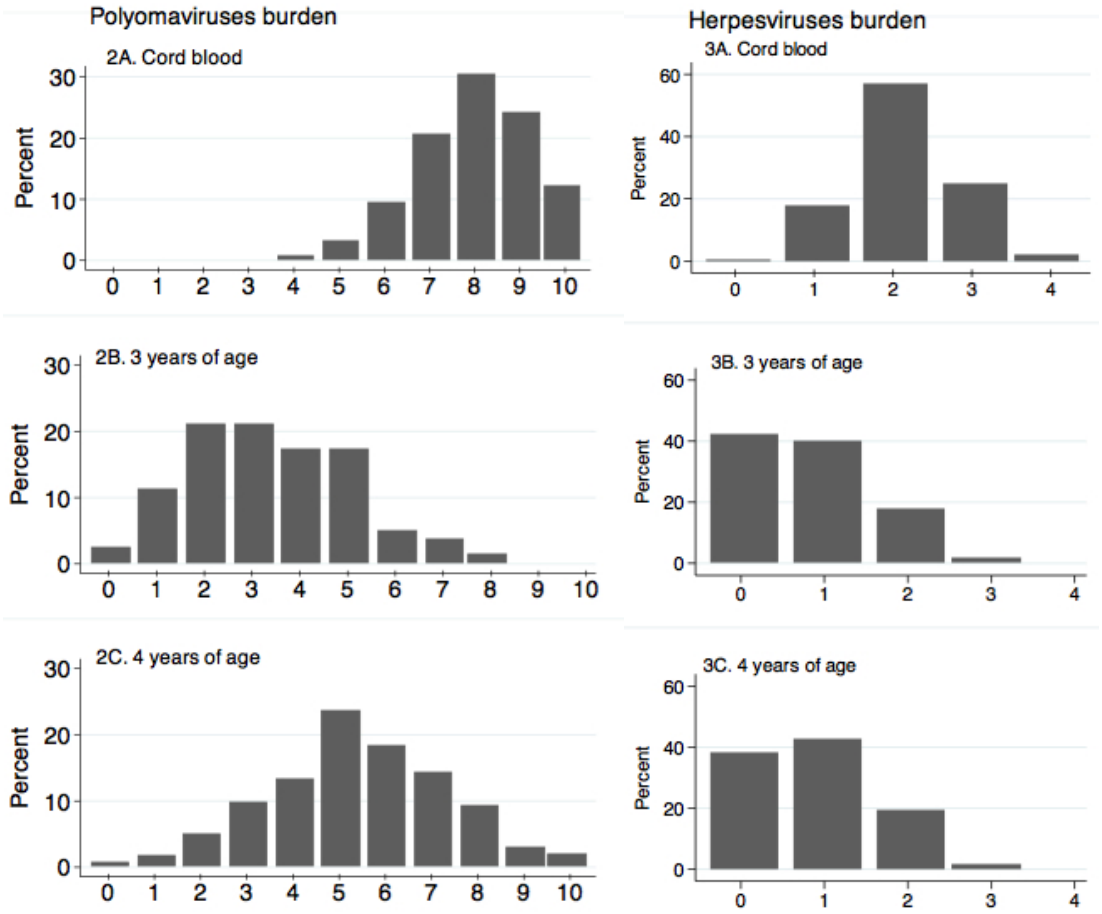


Figure 2 & 3. Polyomaviruses and herpesviruses burden among cord blood (2A & 3A) ,3 years (2B & 3B) and 4 years (2C & 3C) of age samples.

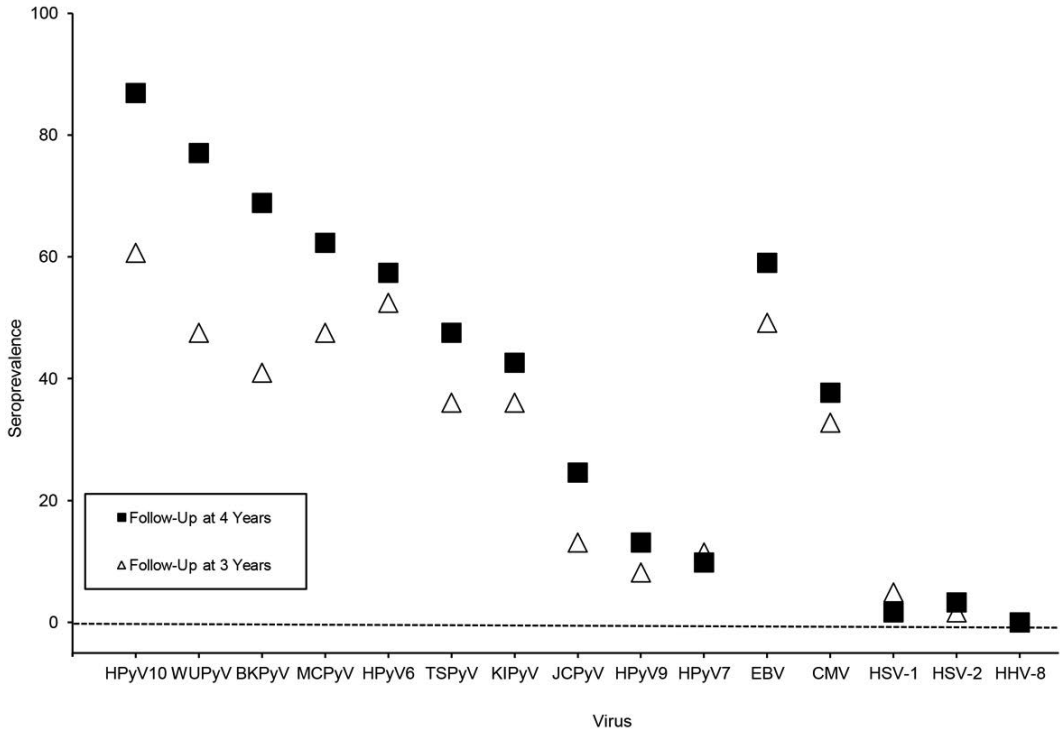


Figure 4. Crude seroprevalences of Polyomaviruses and Herpesviruses in 61 children followed up at 3 and 4 years of age, the Rhea birth cohort in Crete, Greece, 2007–2012. Statistically significant differences in seroprevalence between follow-ups based on the McNemar exact test were observed for HPyV10 and WUPyV ($P < 0.001$); BKPyV and MCPyV ($P < 0.01$); and TSPyV, JCPyV, and Epstein-Barr virus (EBV) ($P < 0.05$). CMV, cytomegalovirus; HHV-8, human herpesvirus 8; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2.

Correlation between Polyomaviruse and Herpesviruses

We explored the correlation between seropositivity to each Polyomavirus and Herpesvirus separately in cord blood samples, reflecting maternal status, and in four years of age samples. Correlations among individual infections are shown in Table 2. We use the correlations between different infections and at different ages - among women of childbearing age as reflected in cord samples and children four years of age- to gain a better understanding of how infections are transmitted taking into account that transmission vary with age (159). Interpetation of the observed patterns is deferred to the discussion.

Overall, due to the high seroprevalence rates in cord blood samples for most Polyomaviruses and EBV, there was expected less heterogeneity. In cord blood samples, the strongest corelations were among HPyV6 and HPyV7 (0.82), JCPyV and WUPyV (0.40), TSPyV and HPyV9 (0.37), JCPyV and HPyV9 (0.31). In four years of age samples, we also observed strong correlations among HPyV6 and HPyV7 (0.79), TSPyV and HPyV9 (0.64), JCPyV and HPyV9 (0.46). Other strong correlations in four years of age samples but not in cord blood samples were among MCPyV and HPyV9 (0.52), BKPyV and JCPyV (0.46), HPyV9 and BKPyV (0.44). There was a negative correlation between EBV and JCPyV (-0.14) and HPyV9 (-0.17).

Table 2. Tetrachoric correlations of seroprevalence status for ten polyomaviruses and four herpesviruses in cord blood (n=626) and 4 years of age samples (n=690)

	BKPyV	JCPyV	KIPyV	WUPyV	HPyV6	HPyV7	TSPyV	MCPyV	HPyV9	HPyV10	EBV	CMV	HSV-1	HSV-2
Cord blood														
BKPyV	1.00													
JCPyV	0.09	1.00												
KIPyV	0.11	-0.07	1.00											
WUPyV	-1.00	0.40*	0.23	1.00										
HPyV6	-0.16	-0.09	-0.16	-1.00	1.00									
HPyV7	-0.05	0.11	-0.09	-0.01	0.82*	1.00								
TSPyV	-0.01	0.21*	-0.06	0.17	0.11	0.18*	1.00							
MCPyV	0.25*	0.04	-0.07	0.25	0.15	0.06	0.18*	1.00						
HPyV9	0.15	0.31*	0.03	0.26	-0.02	-0.04	0.37*	0.22*	1.00					
HPyV10	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	1.00	1.00	1.00				
EBV	-1.00	0.03	-1.00	0.53	-1.00	-0.10	-1.00	-1.00	-0.10	-1.00	1.00			
CMV	0.08	-0.08	0.13	0.00	0.08	0.13	0.03	0.10	-0.01	1.00	0.00	1.00		
HSV-1	-0.06	0.06	0.11	0.29	0.18	0.00	0.04	0.09	0.02	1.00	0.01	0.00	1.00	
HSV-2	-0.10	0.03	-0.07	1.00	0.00	0.01	0.04	-0.05	0.13	1.00	-0.27	0.09	-0.04	1.00
4 years														
BKPyV	1.00													
JCPyV	0.46*	1.00												
KIPyV	0.16*	0.03	1.00											
WUPyV	0.43*	0.12	0.33*	1.00										
HPyV6	0.18*	0.10	-0.05	0.02	1.00									
HPyV7	0.01	0.07	0.01	0.01	0.79*	1.00								
TSPyV	0.29*	0.24*	0.06	0.31*	-0.11	-0.02	1.00							
MCPyV	0.18*	0.19*	-0.04	0.13	0.01	0.11	0.14*	1.00						
HPyV9	0.44*	0.46*	0.04	0.17*	-0.07	0.05	0.64*	0.52*	1.00					
HPyV10	0.32*	-0.02	0.30*	0.31*	-0.07	0.01	0.37*	0.08	0.25*	1.00				
EBV	-0.02	-0.14*	0.15*	0.10	-0.08	-0.09	0.10	-0.02	-0.17*	0.18*	1.00			
CMV	-0.07	0.06	0.03	0.11	-0.09	-0.05	0.08	-0.03	0.03	0.23*	0.25*	1.00		
HSV-1	-0.21	-0.13	0.00	0.01	0.18	0.02	0.01	-0.20	-0.08	-0.04	0.28*	-0.02	1.00	
HSV-2	-0.18	0.16	0.26	-0.07	-0.12	-0.04	-0.01	0.30	0.11	-0.03	0.18	0.05	-1.00	1.00

*P-values<0.05

Seroreactivity levels for Polyomaviruses and Herpesviruses

In table 3, we present the seroreactivity levels among seropositive individuals by follow-up. Among Polyomaviruses, the lowest seroreactivity levels were observed for JCPyV and HPyV9. Among EBV antigens, the lowest seroreactivity levels were for EAD while among CMV antigens were for cm2.

Table 3. Seroreactivity levels (median, IQR) of Polyomaviruses and Herpesviruses among seropositives by Follow-up, the Rhea Birth Cohort in Crete, Greece, 2007-2012.

	Cord blood (n=626)			Three years of age follow-up (n=81)			Four years of age follow-up (n=690)		
Virus	n	median	IQR	n	median	IQR	n	median	IQR
Polyomaviruses									
BKPyV	600	5130	1563-8349	31	6136	471-8053	526	7413	4063-9263
JCPyV	335	788	264-2364	10	332.5	317-1155	235	364	169-1184
KIPyV	522	2736	1224-4555	30	3160.5	1904-4732	305	2611	904-4526
WUPyV	614	3716.5	1840-6153	32	2755	1042-4653	521	3946	2169-5970
HPyV6	576	3566	1610-5132	43	3895	2461-4936	425	3620	2054-4898
HPyV7	409	2767	1575-4327	11	2510	473-4232	157	2528	627-3961
TSPyV	502	5381	1984-7637	25	7513	5880-8480	354	6945	2418-8519
MCPyV	561	4847	1213-7343	41	2084	737-4268	471	2372	678-4521
HPyV9	241	796	251-7376	5	349	285-404	144	263.5	178.5-697
HPyV10	625	3208	1656-4781	45	3229	1720-4310	568	2869	1639-4489
Herpesviruses									
EBV									
VCA	620	6270	4020-8498	34	5892	3609-7603	359	5733	3782-7693
EBNA	620	8133	6677-9409	32	7445.5	6695-8591	351	7649	6218-8887
ZEBRA	437	1018	440-2204	20	676	284-1551	211	564	248-1420
EAD	351	697	308-1581	24	552.5	268-2341	262	623	285-1825
CMV									
pp150	466	2481	1340-4048	24	1448	715-2399	171	1386	718-2332
cm2	147	147	118-235	1			32	141	121-238.5
pp52	469	6781	4877-8636	25	5355	2242-7978	175	4882	2774-6859
pp28	461	2252	1264-3759	24	1810	956-2564	173	1517	819-2602
pp65	401	474	205-1167	20	445.5	269-714	155	351	180-610
HSV1	164	237	114.5-472.5	3	70	64-133	25	163	111-328
HSV2	50	589	179-1848	1			10	126	95-612

Values are median fluorescent intensity values
IQR, interquartile range;
Seroreactivities for EBV and CMV anitgens is among seropositives to the virus but also seropositive for the respective antigen.

In table 4, we present seroreactivity levels among children who were stable seropositive at both three and four years of age follow-up for each Polyomavirus examined. Overall we observed an increase in seroreactivity levels from three to four years of age follow-up. In the 24 children who were stable seropositive to BKPyV, the mean difference in the MFI value beween the two follow-ups (from three to four years of age follow-up) was 1117 and the highest increase observed was 9404 MFI values. Also marked increase in MFI values between follow-ups was observed for WUPyV (mean difference 1037, max 6620). In the 37 children who were stable seropositive to HPyV10 there seem to be a decrease in MFI values (mean difference -691) between follow-ups.

Table 4. Difference in seroreactivity levels between MFI values at four and three years of age in children stable seropositive at both follow-ups.

	n	Mean	SD	Min	Max
BKPyV	24	1117	2751	-2226	9404
JCPyV	7	-350	661	-1806	71
KIPyV	22	-17	1543	-2695	3516
WUPyV	29	1037	2303	-3339	6620
HPyV6	32	299	1557	-2519	4481
HPyV7	6	652	1001	-351	2227
TSPyV	21	449	1852	-3801	3741
MCPyV	29	88	1424	-3424	3533
HPyV9	1	248	(NA)	(NA)	(NA)
HPyV10	37	-691	1736	-4684	4819

MFI, median fluorescent intensity values

Determinants of Polyomaviruses, EBV and CMV seroprevalence at age four

Table 5 presents the association of each determinant with Polyomaviruses burden and seropositivity to each Polyomavirus, EBV, and CMV after adjusting for child' s age and sex, maternal origin, and parents' educational level. Data on HSV-1, HSV-2, and HHV-8 were not analyzed because of the low seroprevalence in this age group. The presence of maternal antibodies to JCPyV and HPyV7 and higher levels of maternal antibodies to HPyV10 in cord blood were associated with higher seroprevalence to the corresponding viruses in children aged four. Children delivered vaginally had a higher seroprevalence to HPyV10 compared with those delivered by cesarean section (prevalence ratio = 1.09, 95% confidence interval (CI): 1.02, 1.17). CMV seropositivity in cord blood and longer breastfeeding duration were significantly associated with higher seroprevalence to CMV at age four (prevalence ratio = 3.09, 95% CI: 1.48, 6.45 and prevalence ratio = 1.25, 95% CI: 1.09, 1.43, respectively).

Variables denoting social interactions showed heterogeneous results. BKPyV and HPyV10 were more prevalent in children with older siblings than in firstborn children (prevalence ratio = 1.13, 95% CI: 1.04, 1.23 and prevalence ratio = 1.12, 95% CI: 1.04, 1.20, respectively). These associations remained only for siblings that were closer in age (<6-year difference in age) (data not shown). On the other hand, MCPyV was much less prevalent in children with older siblings than in firstborn children (prevalence ratio = 0.86, 95% CI: 0.78, 0.95). Younger age at day-care entry was associated with a higher burden of Polyomaviruses (count ratio = 1.15, 95% CI: 1.11, 1.19). This was driven by the positive associations with BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, and HPyV9. Swimming pool attendance conveyed a substantial increase in the seroprevalence of BKPyV, KIPyV, WUPyV, and HPyV10. EBV and CMV were also more prevalent among children entering day care at a younger age (prevalence ratio = 1.12, 95% CI: 1.02, 1.22 and prevalence ratio = 1.30, 95% CI: 1.11, 1.52, respectively). Also, CMV was more prevalent in children raised in more crowded houses (prevalence ratio = 1.45, 95% CI: 1.01, 2.08).

Television viewing was inversely associated with the burden of Polyomaviruses (count ratio = 0.92, 95% CI: 0.89, 0.96) and with the seroprevalence of BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, and HPyV9. Children of mothers with a high score on the parental stress scale (≥ 75th percentile) had a lower burden of polyomaviruses compared with the reference group (count ratio = 0.91, 95% CI: 0.85,

0.98). Living in houses that were cleaned more frequently was associated with a lower burden of Polyomaviruses (count ratio = 0.93, 95% CI: 0.88, 0.98) and lower seroprevalence to HPyV10 (prevalence ratio = 0.89, 95% CI: 0.83, 0.96). Similarly, frequent handwashing was associated with a lower burden of polyomaviruses (count ratio = 0.94, 95% CI: 0.89, 1.00) and lower seroprevalence to CMV (prevalence ratio =0.74, 95% CI: 0.56, 1.00). Prematurity, passive smoking, and pets in the house did not show any significant association with polyomaviruses, EBV, or CMV.

We further assessed the associations above for residual confounding on the basis of the correlation structure of the variables in our cohort (data not shown). In detail, we tested confounding by the following: 1) associations of delivery type and breastfeeding and vice versa; 2) associations of age at day-care entry and older siblings with television viewing; 3) associations of frequency of cleaning the house and older siblings with parental stress; and 4) associations of house crowding with frequency of house cleaning. Neither the direction nor the magnitude of associations changed remarkably from those presented in Table 5.

Table 5. Determinants of Polyomaviruses burden (count ratios, CR), Polyomaviruses and Herpesviruses seropositivity (prevalence ratios, PR) in children at 4 years of age follow-up.

		Polyomaviruses										Herpesviruses	
		Burden	BKPyV	KIPyV	WUPyV	TSPyV	HPyV10	HPyV9	JCPyV	HPyV6	HPyV7	MCPyV	EBV	CMV
Determinants	n	CR	PR	PR	PR	PR	PR	PR	PR	PR	PR	PR	PR	PR
Cord seropositive vs seronegative	273	1.02	1.19	0.99	NA[a]	1.04	NA[a]	1.23	1.61[b]	1.15	1.86[b]	1.24	NA[a]	3.09[c]
Vaginal vs cesarean delivery	652	0.99	0.97	1.03	1.06	0.87	1.09[b]	0.79	0.95	1.00	0.99	0.99	0.93	1.22
Preterm birth, yes vs no	647	0.96	1.07	1.00	0.98	1.04	0.92	0.76	0.87	0.99	0.71	0.92	0.95	0.69
Breastfeeding duration	625	1.01	1.03	0.95	1.03	0.99	1.01	0.98	1.07	0.99	0.98	0.99	0.96	1.25[c]
House crowding, ≥1 vs <1 ppr	608	1.01	1.07	1.17	1.08	1.05	1.08	0.94	0.92	0.93	0.87	0.90[b]	1.13	1.45[b]
Older siblings, yes vs no	654	1.03	1.13[c]	1.12	1.05	1.08	1.12[c]	0.85	0.96	1.01	0.97	0.86[c]	1.03	1.09
Earlier age at daycare entry	616	1.15[d]	1.17[d]	1.41[d]	1.26[d]	1.37[d]	1.14[d]	1.21[b]	1.02	1.01	0.96	0.98	1.12[b]	1.30[c]
Swimming pool attendance, yes vs no	653	1.07[b]	1.12[c]	1.32[c]	1.12[b]	0.94	1.16[d]	1.00	1.06	0.93	0.98	1.02	1.09	1.09
TV viewing	653	0.92[d]	0.90[d]	0.81[d]	0.94[b]	0.86[c]	0.93[c]	0.74[c]	0.92	1.00	1.05	1.01	1.00	0.97
PSS score ≥75th percentile, yes vs no	543	0.91[c]	0.96	0.82	1.02	0.90	0.97	0.85	0.91	0.85	0.66	0.92	0.94	0.95
House cleaning, >1 vs ≤1 per week	653	0.93[c]	0.93	0.95	0.94	0.80[c]	0.89[c]	0.79	0.93	1.07	1.00	0.96	1.09	0.77
Handwashing, ≥5 vs <5 times per day	654	0.94[b]	0.93	1.02	0.95	1.01	0.96	0.76	0.83	1.00	0.96	0.91	0.90	0.74[b]
Passive smoking, yes vs no	649	1.03	1.05	0.95	1.01	1.08	1.02	1.12	0.96	1.02	1.22	1.02	1.15	0.89
Pets in the house, yes vs no	653	0.95	0.97	0.91	1.04	0.91	1.01	0.78	0.81	0.96	0.98	0.99	0.98	0.88

[a]NA, non-applicable as almost all cord samples were seropositive for the corresponding viruses. In such cases we used tertiles of seroreactivity among seropositives. Results per increasing tertile of maternal seroreactivity were for WUPyV PR: 1.03 (95% CI: 0.95, 1.12), for HPyV10 PR: 1.12 (95% CI :1.05, 1.19) and for EBV (VCA antigen) PR: 0.97 (95% CI: 0.84, 1.12).; [b]P<0.05 ; [c]P<0.01; [d]P<0.001. All models are Poisson regression models adjusted for child's age, gender, maternal origin and parents' educational level. For the binary variables CR/PR compares exposed vs unexposed. For the ordinal variables breastfeeding duration (never, 1-3 months, 4-6 months and >6 months), age of daycare attendance (never, > 3 years of age, 2-3 years of age, <2 years of age) and TV viewing (never or <30 min, 1 hour and ≥2 hours) CR/PR represents change per increasing category of the ordinal variable.

Differences by parents' education and origin

All the Polyomaviruses tested were more prevalent among children of higher socioeconomic position, as assessed by parents' educational level (data not shown). Figure 5, illustrates the predicted Polyomaviruses burden by age and parents' educational level among 710 children of the three and four year of age follow-up (children with two samples available, participate only with the three years of age sample). The Polyomaviruses burden was always higher in children of high parents' educational level compared to those of low educational level and increased in all groups with increasing child's age.

Table 6 shows the crude and adjusted associations between parents' education and the burden of Polyomaviruses. In the crude model, children of high compared with those of low parental education had a higher burden of Polyomaviruses (count ratio = 1.23, 95% CI: 1.15, 1.31). Adjusting for age at day-care entry and parental stress accounted for 7.3%and 4.0% reductions of the crude count ratio, respectively. In the full model, the burden of Polyomaviruses was still higher among children of high compared with those of low parental education (count ratio = 1.12, 95% CI: 1.04, 1.21). EBV and CMV did not differ by parental education but were more prevalent among children of non-Greek mothers than among children of Greek mothers (EBV: prevalence ratio = 1.46, 95% CI:1.20, 1.78; CMV: prevalence ratio = 2.21, 95% CI: 1.59, 3.08) adjusted for child' s age, sex, and parental educational level.

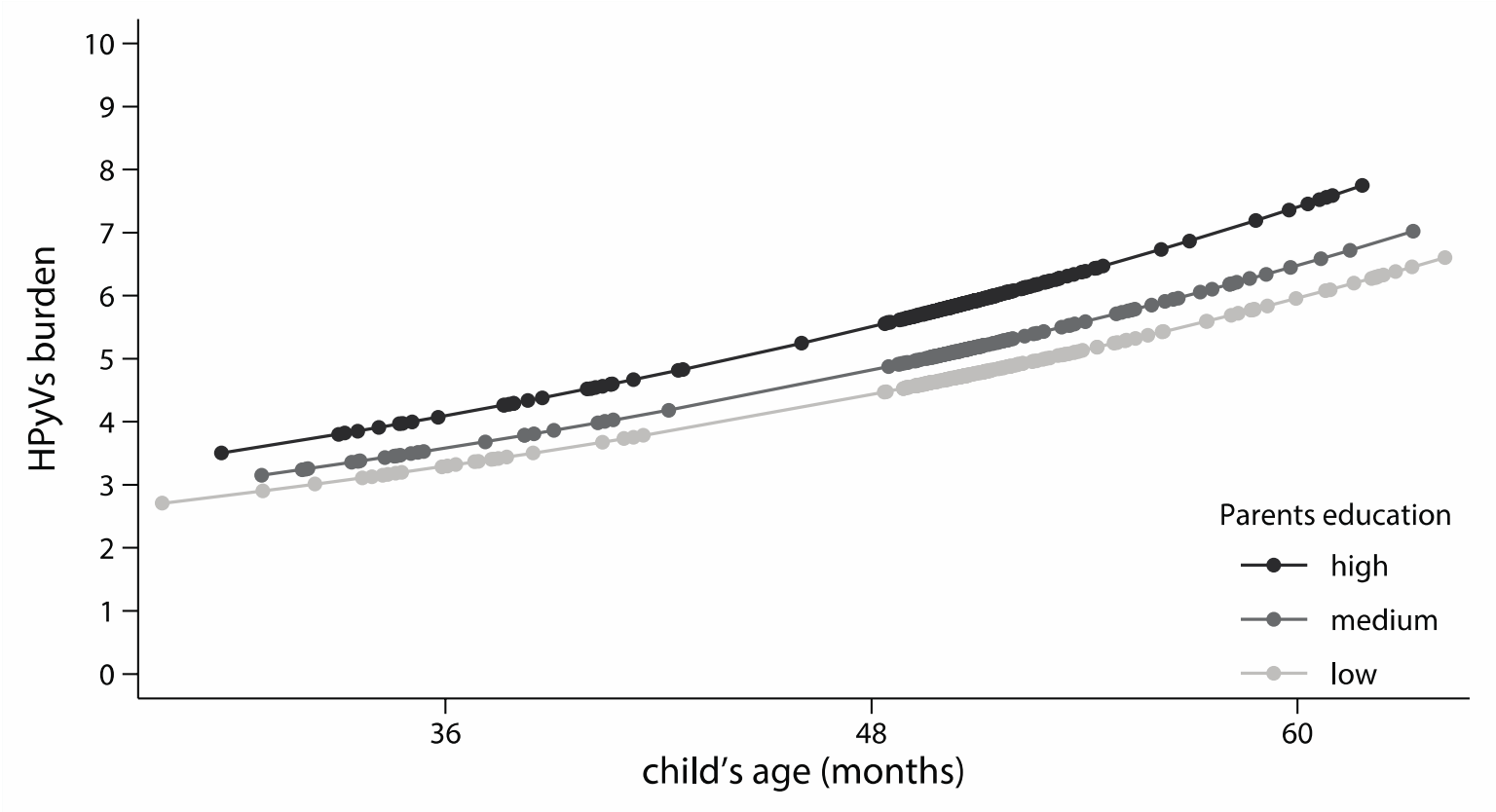


Figure 5. Predicted human Polyomaviruses (HPyVs) burden by child's age and parents education in 710 children participating at the 3 and 4 years of age follow-up (children with two samples available, participate only with the 3 years of age sample) using the prediction equation of the Poisson regression model, the Rhea birth cohort in Crete, Greece.

Table 6. Association of Parents' Educational Level With the Human Polyomaviruses Burden at Age Four Before (Crude Model) and After Adjustment for Potential Mediators, the Rhea Birth Cohort in Crete, Greece, 2007-2012.

		Parents' educational level						
		Low	Medium			High		
Models	n	Reference	CR	95% CI	% change[a]	CR	95% CI	% change[a]
Crude model	658	1.00	1.09[a]	1.01, 1.18	reference	1.23[d]	1.15, 1.31	reference
Mediators models								
Delivery type	652	1.00	1.09[b]	1.01, 1.18	0	1.23[d]	1.16, 1.32	0
Breastfeeding duration	625	1.00	1.09[b]	1.01, 1.17	0	1.23[d]	1.15, 1.31	0
Older siblings	654	1.00	1.09[b]	1.01, 1.18	0	1.23[d]	1.15, 1.32	0
Age at daycare attendance	616	1.00	1.04	0.96, 1.11	-5.5	1.14[d]	1.08, 1.22	-7.3
Swimming pool attendance	653	1.00	1.08	1.00, 1.16	-0.9	1.22[d]	1.14, 1.30	-0.8
Frequency of house cleaning	653	1.00	1.09[b]	1.01, 1.17	0	1.20[d]	1.13, 1.29	-2.4
PSS score ≥75th percentile	543	1.00	1.06	0.98,1.15	-2.7	1.18[d]	1.10, 1.27	-4.0
all of the above	487	1.00	1.04	0.96,1.13	-4.5	1.12[c]	1.04, 1.21	-8.9

Abbreviations: CI, confidence interval; CR, count ratio; PSS, parental stress scale

[a] % change in the count ratio (CR) of the corresponding mediation model compared to the crude model.

[b] P <0.05, [c] P <0.01 and [d] P <0.001. All models are Poisson regression with robust variance adjusted for child's age, gender and additional variables as indicated.

Discussion

Our four-year birth cohort study provides a comprehensive analysis on acquisition of Polyomaviruses and Herpesviruses in healthy young children. Overall, we have shown that, by age four, children were commonly seropositive to Polyomaviruses and Herpesviruses, with the exceptions of HHV-8 (which was negligible in our population) and HSV-1 and HSV-2 (which displayed very low seroprevalences) (63). We observed remarkably higher seroprevalences to HPyV6 and MCPyV at age four, compared to previous studies (116,118,123,160). Only one population-based study in Africa reported higher seroprevalence for MCPyV (80.0%) (119). Regarding EBV and CMV, seroprevalences were comparable to those reported in Europe and USA but much lower than those reported in non-developed countries (161,162). HSV-1 seroprevalence in our population is placed among the lowest in Europe (63). Most Polyomaviruses and EBV showed a significant increase in their seroprevalence from three to four years of age, but still a considerable proportion of the population was lacking exposure to such common infections by age four. For example, half of the children at age four were EBV seronegative and, although this proportion is comparable to those reported in Europe and the United States, it is much higher than that reported in nondeveloped countries at the same age (128). It has been suggested that this increase in the proportion of children who miss the opportunity to acquire EBV in early life is associated with an increase in the incidence of infectious mononucleosis in Western populations (43). Because exposure to those infections largely took place in early childhood throughout human evolution, it has been suggested that deviation from this norm is implicated in the etiology of immune-related disorders, such as allergy, asthma, and type 1 diabetes, as well as childhood leukemia and lymphomas (5,14,16,17,163–165). We need to follow children longer to confirm whether there is a long, persistent, delayed infection or whether acquisition will occur in the following years.

This is the first study characterizing determinants of Polyomaviruses, EBV and CMV seroprevalence in early life allowing inference on potential routes of transmission for those viruses among young children. We have shown that the mother' s status at baseline (cord blood) was significantly associated with the child' s status at four years for JCPyV, HPyV7, and HPyV10. In particular, this was the only significant determinant for JCPyV and HPyV7, highlighting within-family transmission for those

viruses. Vaginal delivery was probably related to the seroprevalence of HPyV10, and ingestion of the virus during delivery may explain this association given that HPyV10 resides in the gastrointestinal tract (166). Correspondingly, maternal serostatus was significantly associated with CMV seroprevalence at age four. It is described that early in life CMV infection generally results through exposure to the virus in genital secretions during birth or postnatally via breast milk, as our data also support (55,167).

When we explored the importance of different types of contacts inside and outside the household, viruses were grouped together on the basis of observed associations. Younger age at day-care entry importantly contributed in the epidemiology of BKPyV, KIPyV, WUPyV, TSPyV, HPyV10, HPyV9, EBV, and CMV and likely explained the increase in seroprevalence observed from three to four years of age for most of those viruses. In contrast, JCPyV, HPyV6, HPyV7, and MCPyV showed null association, indicating that transmission of those Polyomaviruses is less efficient and/or occurs by different routes. An intriguing finding was the association between swimming pool attendance and BKPyV, KIPyV, WUPyV and HPyV10 seroprevalence. Swimming pool attendance implies social interactions as well as ingestion of water contaminated with Polyomaviruses (168–170), although chlorine would probably inactivate any viruses if present (171). Living with older siblings, who may represent a major source of infection for toddlers, increased the seroprevalence of BKPyV, HPyV10, and CMV. The reverse association was observed for MCPyV, but it is difficult to explain such a finding given that the infection status of the siblings was not available in our study. More evident in this perspective are the results from a population-based study in Africa, showing a significant correlation between siblings and MCPyV infection, especially for siblings close together in age (119).

The negative associations of television viewing and maternal stress with seroprevalence of Polyomaviruses should be treated with caution. Television viewing could be regarded as a surrogate marker of child-rearing practices that can be in the pathway of the transmission of infections in early life by isolating the child from contact with other children and/or family. High levels of hygiene were shown to hamper children from acquiring Polyomaviruses and CMV, probably by inhibiting the spread of those infections. Likewise, a previous report (172) showed that adults who

bathed less frequently were more likely to be MCPyV positive. These findings are important under the hygiene hypothesis, which suggests that the rapid rise in both the autoimmune and allergic diseases in affluent populations is at least partly explained by the reduced exposure to infections due to an increase in hygiene practices. An intriguing finding was that Polyomaviruses were more prevalent among children whose parents' education was high compared with those whose parents' education was low. This disparity was not fully explained by potential mediators, indicating that less obvious pathways are implicated in shaping those differences.

Our study provides insights into the mechanisms of EBV transmission among very young children. Among adolescents and adults we know that kissing is the major route. Here we report that age at daycare entry is the predominant determinant for EBV acquisition up to the age of four. Time of EBV infection is probably an important characteristic of the viral pathogenesis due to the differences in infant's, child's and adult's immune response to primary EBV infection (173–176). Differences in EBV seroprevalence by maternal origin are probably indicating a combination of cultural characteristics and genetics (177). Consistent with reports from other studies we showed that mother's CMV serostatus, breastfeeding, overcrowding and earlier age of daycare attendance determined the CMV seroprevalence of the four year old children (55,167,178). Such information will aid in the design of vaccination programmes against CMV in an effort to prevent congenital CMV infections (179).

Although our analysis on determinants of Polyomaviruses, EBV and CMV seroprevalence at age four, provide important insights into the potential routes of transmission it is likely that certain individual behaviors are difficult to measure or define. For example, for HPyV6 we were unable to identify an individual determinant. Exploring the correlation of individual infections in a population at different ages may open a window on understanding shared major transmission routes for those infections(159) . For this reason, we explored the tetrachoric correlations between Polyomaviruses and Herpesviruses both among cord blood smaples, reflecting maternal status, and among children four years of age. We speculate that the stronger the association between two infections the more likely it is to be transmitted by the same route. Moreover, it is suggested that correlations in adulthood are route-specific whereas correlations in childhood stem from confoundingd of different transmission

routes. In our analysis we observed strong correlation between HPyV6 and HPyV7, JCPyV and WUPyV, TSPyV and HPyV9, JCPyV and HPyV9 among women of childbearing age. Based on the aforementioned assumptions, it is likely that these Polyomaviruses share a major transmission route. We should note that more sophisticated approaches exist for analyzing bivariate serological survey data and modeling age effects (159).

Strengths and limitations

Loss to follow-up and nonparticipation in blood sampling are potential sources of selection bias in our study, but they are not likely to be affected by the seroresponses tested here and therefore to affect the determinants-seroprevalence associations (180). Overestimation of seroprevalences is likely for the closely related viruses KIPyV/WUPyV and HPyV6/HPyV7 because of cross-reactivity, but it is expected to be small (29). Information on determinants relies mostly on questionnaires, and misreport is possible. Variables such as years of education, number of siblings, and age at day-care entry are usually very precisely reported. Lifestyle variables are more vulnerable to misreport, although even this type of misclassification can be expected to be small; information on lifestyle was mostly at the time of blood drawing and referred to the present or recent past. Finally, chance findings are always of concern when we perform multiple comparisons, but we reported the level of significance of associations and drew our conclusions on the basis of the consistency of results (181).

Conclusion

To summarize, in this large prospective study, we demonstrated that children in Crete commonly acquired polyomaviruses, EBV, and CMV during their first four years of life. For the first time, we described certain inside and outside family factors were related to their seroprevalence at age four.

3.2. Seroprevalence of Helicobacter pylori infection and determinants of acquisition of infection in pregnancy and early childhood.

Aims

In this study we use the serum samples available at birth, three years and four years of age to describe the natural history of H. pylori in the Rhea birth cohort. We provide antigen specific seroprevalence data and seroreactivity levels among seropositive individuals for each pathogen studied. We assessed differences in maternal and child characteristics by H.pylori serostatus in cord blood samples, reflecting maternal status in pregnancy, and in children four years of age. Additionally, we explored differences in pregnancy outcomes by maternal H.pylori status.

Results

Study population (as presented in section 3.1.)

Seroprevalence and seroreactivity levels for Helicobacter pylori antingens

H.pylori seroprevalence (95% CI) in cord blood, reflecting maternal status, was 44.7% (40.79%-48.72%). Among H.pylori seropositive cord samples, 58.2% were CagA seropositive. Mean (SD) age of the mothers at delivery was 29.7 (5.1) years. H.pylori protein-specific seroprevalence (95% CI) was highest for Omp [42.8% (38.9%- 46.79%)], NapA [33.2% (29.54%-37.07%)], CagA [32.9% (29.23%-36.74%)], lowest for HpaA [13.3% (10.7%-16.17%)], UreA [16.8% (13.9%-19.9%)] and VacA [17.9% (14.9%-21.1%)] and distributed in between for the other proteins (Table 1). H.pylori seroprevalence (95% CI) at age three was 4.9% (1.4%-12.2%) and at age four was 6.5% (4.8%-8.7%). All children that were tested seropositive at age three remained seropositive at age four. Among H.pylori seropositive samples, 25% and 57.8% were CagA seropostive at age three and four. H.pylori protein-specific seroprevalence (95% CI) at age four was highest for Omp [10.9% (8.65%-13.43%)] and lowest for NapA [2.9% (1.78%-4.44%)] (Table 1).

Seroreactivity levels among seropositives for each H.pylori specific antigen are presented in table 2. We did not include in this analysis samples from children three years of age because of the low seropositivity rates. In cord blood samples the highest seroreactivity levels were observed against NapA although extremely high MFI values were also observed for CagA. In children four years of age the highest seroreactivity levels were clearly observed against CagA.

Table 1. Seroprevalence (95% CI) of Helicobacter pylori and to twelve specific proteins by follow-up, the Rhea Birth Cohort in Crete, Greece, 2007-2012.

	Cord blood (n=626)		Three years of age follow-up (n=81)		Four years of age follow-up (n=690)	
	%	95% CI	%	95% CI	%	95% CI
Helicobacter pylori	44.7	40.79, 48.72	4.9	01.36, 12.16	6.5	4.79, 8.63
Helicobacter pylori proteins						
GroEL	31.0	27.38, 34.77	1.2	0.03, 6.69	3	1.89, 4.61
(% among HP positives)	65.7		0		35.6	
UreA	16.8	13.93, 19.93	1.2	0.03, 6.69	5.4	3.80, 7.31
(% among HP positives)	23.2		0		13.3	
HP0231	19.3	16.31, 22.64	7.4	2.77, 15.43	7.1	5.29, 9.28
(% among HP positives)	40.7		75.0		71.1	
NapA	33.2	29.54, 37.07	3.7	0.77, 10.44	2.9	1.78, 4.44
(% among HP positives)	69.3		50.0		31.1	
HP0305	25.4	22.03, 29.00	4.9	1.36, 12.16	4.2	2.83, 5.98
(% among HP positives)	55.0		75.0		53.3	
HpaA	13.3	10.70, 16.17	8.6	3.54, 16.99	9	6.96, 11.37
(% among HP positives)	26.8		100		66.7	
CagA	32.9	29.23, 36.74	2.5	0.30, 8.64	7	5.17, 9.11
(% among HP positives)	58.2		25		57.8	
HyuA	20.1	17.05, 23.49	6.2	2.03, 13.82	7	5.17, 9.11
(% among HP positives)	43.6		50		26.7	
Catalase	21.4	18.25, 24.83	7.4	2.76, 15.43	5.1	3.56, 6.98
(% among HP positives)	37.5		25		26.7	
VacA	17.9	14.96, 21.12	0	0, 4.45*	3.9	2.59, 5.64
(% among HP positives)	37.5		0		44.4	
HcpC	24.8	21.42, 28.33	3.7	0.77, 10.44	4.2	2.83, 5.98
(% among HP positives)	52.1		50		57.8	
Omp	42.8	38.90, 46.79	11.1	5.21, 20.05	10.9	8.65, 13.43
(% among HP positives)	80.4				80.0	

Abbreviations: CI, confidence intervals, HP, Helicobacter pylori

Table 2. Seroreactivity levels (median, IQR) of Helicobacter pylori proteins among seropositives by Follow-up, the Rhea Birth Cohort in Crete, Greece, 2007-2012.

	Cord blood (n=626)			Four years of age follow-up (n=690)		
Virus	n	median	IQR	n	median	IQR
Helicobacter pylori proteins						
GroEL	184	1796.5	461.5-2849	16	376	65.5-1451.5
UreA	65	698	203-2101	6	125.5	76-148
HP0231	114	177	69-1315	32	136	58.5-754
NapA	194	3446.5	214-7325	14	209	95-477
HP0305	154	194	88-463	24	168	75-321
HpaA	75	82	51-173	30	149.5	87-375
CagA	163	13454	6041-17861	26	16162.5	9408-18794
HyuA	122	663.5	119-5606	12	151.5	67-328.5
Catalase	105	3162	715-6456	12	742	614-1350
VacA	105	520	262-1489	20	541.5	307.5-2069.5
HcpC	146	166.5	57-1477	26	89.5	56-402
Omp	225	1070	511-2771	36	2820	1172.5-5786.5

IQR, interquartile range

Determinants of Helicobacter pylori serostatus in pregnancy and differences in pregnancy outcomes

Table 3 presents the characteristics of the study population by maternal H.pylori serostatus based on serology in cord blood samples. The H. pylori seropositivity rate was highest among women of non-Greek origin (75.93%), of low educational level (58.41%), with three or more siblings (63.89%) and with older children (51.67%). Moreover, H.pylori seroprevalence was highest among underweight women based on their pre-pregnancy BMI (70.0%). Interestingly, women who were drinking alcohol (48.45%) during their pregnancy were more likely to be H.pylori seropositive versus those who were not drinking alcohol (37.50%) whereas such differences were not observed regarding alcohol consumption before pregnancy.

Table 4 presents differences in pregnancy outcomes according to maternal H.pylori serostatus. A nuber of pregnancy outcomes were tested including birth outcomes. H.pylori seroprevalence was highest among those who gained inadequate gestational weight gain (48.91%) compared to those who gained adequate (46.90%) or excessive (33.71%). No other significant differences were observed.

Determinants of Helicobacter pylori seroprevalence in childhood

Table 5 presents the characteristics of the study population by H.pylori status in children four years of age. Slightly higher seroprevalence was observed among female (8.26%) versus male (4.96%) children. The H. pylori positivity rate was highest among children of mothers younger in age (9.57%), of non-Greek origin (15.22%) and who were also tested H.pylori seropositive in pregnancy (8.73%). Children born vaginally, non-preterm and those exposed to passive smoking in childhood were also more likely to be H.pylori seropositive at age four. Antibiotic use during the last year and age at school entry were not found to be associated with H.pylori seroprevalence at age four.

Table 3. Study population characteristics by Helicobacter pylori serostatus in cord blood, the Rhea birth cohort.

		Helicobacter pylori		
Characteristics	n	Seropositive	Seronegative	P-value
Age at delivery				0.251
16-25	161	67 (41.61%)	94 (58.39%)	
26-35	335	150 (44.78%)	185 (55.22%)	
36-46	49	27 (55.10%)	22 (44.90%)	
Greek origin				<0.001
yes	492	201 (40.85%)	291 (59.15%)	
no	54	41 (75.93%)	13 (24.07%)	
Educational level				0.001
high, n (%)	140	49 (35.0%)	91 (65.0%)	
medium, n (%)	287	124 (43.21%)	163 (56.79%)	
low, n (%)	113	66 (58.41%)	47 (41.59%)	
Area of residency				0.094
urban	385	167 (43.38%)	218 (56.62%)	
rural	121	63 (52.07%)	58 (47.93%)	
Siblings				<0.001
none or one	94	23 (24.47%)	71 (75.53%)	
two	70	33 (47.14%)	37 (52.86%)	
three or more	72	46 (63.89%)	26 (36.11%)	
Older children				<0.001
yes	206	170 (51.67%)	159 (48.33%)	
no	213	71 (33.33%)	142 (66.67%)	
Pre-pregnancy BMI status				0.04
obese	69	33 (47.83%)	36 (52.17%)	
overweight	124	46 (37.10%)	78 (62.90%)	
normal	327	146 (44.65%)	181 (55.35%)	
underweight	20	14 (70.0%)	6 (30.0%)	
Smoking before pregnancy				0.673
yes	215	96 (44.65%)	119 (55.35%)	
no	283	121 (42.76%)	162 (57.24%)	
Smoking during pregnancy				0.548
yes	457	205 (44.86%)	252 (55.14%)	
no	73	30 (41.40%)	43 (58.90%)	
Alcohol before pregnancy				0.57
yes	246	107 (43.50%)	139 (56.50%)	
no	153	71 (46.41%)	82 (53.59%)	
Alcohol during pregnancy				0.026
yes	258	125 (48.45%)	133 (51.55%)	
no	168	63 (37.50%)	105 (62.50%)	

P-values based on a chi-square test or Mc Nemar's exact test.
Abbreviations: BMI, body mass index;

Table 4. Pregnancy outcomes by Helicobacter pylori serostatus in cord blood, the Rhea birth cohort.

		Helicobacter pylori		
Pregnancy outcomes	n	Seropositive	Seronegative	P-value
Gestational weight gain				0.017
Excessive	175	59 (33.71%)	116 (66.29%)	
Adequate	145	68 (46.90%)	77 (53.10%)	
Inadequate	92	45 (48.91%)	47 (51.09%)	
Gestational diabetes mellitus				0.941
yes	40	18 (45.0%)	22 (55.0%)	
no	455	202 (44.4%)	253 (55.60%)	
Gestational hypertension				0.93
yes	21	9 (42.86%)	12 (57.14%)	
no	470	206 (43.83%)	264 (56.17%)	
Nausea early pregnancy				0.319
yes	273	115 (42.12%)	158 (57.88%)	
no	166	78 (46.99%)	88 (53.01%)	
Severity of nausea early pregnancy				0.204
no nausea	162	76 (46.91%)	86 (53.09%)	
nausea	92	33 (35.87%)	59 (64.13%)	
nausea & eating less	185	84 (45.41%)	101 (54.59%)	
Duration of nausea in early pregnancy				0.603
no nausea	166	78 (46.99%)	88 (53.01%)	
nausea lasting <=12 weeks	243	102 (41.98%)	141 (58.02%)	
nausea lasting >=13 weeks	30	13 (43.33%)	17 (56.67%)	
Vomiting early pregnancy				0.935
yes	163	71 (43.56%)	92 (56.44%)	
no	273	120 (43.96%)	153 (56.04%)	
Duration of vomiting in early pregnancy				0.935
no vomiting	273	120 (43.96%)	153 (56.04%)	
vomiting lasting ≤12 weeks	144	62 (43.06%)	82 (56.94%)	
Vomiting lasting >12 weeks	19	9 (47.37%)	10 (52.63%)	
Nausea late pregnancy				0.887
yes	53	23 (43.40%)	30 (56.60%)	
no	448	199 (44.42%)	249 (55.58%)	
Vomiting late pregnancy				0.743
yes	29	12 (41.38%)	17 (58.62%)	
no	472	210 (44.49%)	262 (55.51%)	
Delivery type, n (%)				0.939
vaginal	286	129 (45.10%)	157 (54.90%)	
planned caesarean section	162	72 (44.44%)	90 (55.56%)	
urgent caesarean section	77	33 (42.86%)	44 (57.14%)	
Preterm birth				0.821
yes, n (%)	61	28 (45.90%)	33 (54.10%)	
no, n (%)	489	217 (44.38%)	272 (55.62%)	

P-values based on a chi-square test or Mc Nemar's exact test.
Abbreviations: BMI, body mass index;

Table 5. Study population characteristics by Helicobacter pylori serostatus at age four.

		Helicobacter pylori		
Characteristics	n	Seropositive	Seronegative	P-value
Maternal age				0.006
<30 years	303	29 (9.57%)	274 (90.43%)	
>=30 years	375	16 (4.27%)	359 (95.73%)	
Maternal Greek origin				0.013
yes	644	38 (5.9%)	606 (94.1%)	
no	46	7 (15.22%)	39 (84.78%)	
Maternal education				0.806
high, n (%)	215	14 (6.51%)	201 (93.49%)	
medium, n (%)	342	21 (6.14%)	321 (93.86%)	
low, n (%)	114	9 (7.89%)	105 (92.1%)	
Maternal serostatus in pregnancy				0.038
seropositive	126	11 (8.73%)	115 (91.27%)	
seronegative	162	5 (3.09%)	157 (96.91%)	
Preterm birth				0.019
yes	74	0 (0%)	74 (100%)	
no	599	42 (7.01%)	557 (92.99%)	
Delivery type				0.009
vaginal	343	30 (8.75%)	313 (91.25%)	
caesarean section	335	13 (3.88%)	322 (96.12%)	
Older siblings				0.869
yes	374	24 (6.42%)	21 (6.73%)	
no	312	21 (6.73%)	291 (93.27%)	
Age at daycare entry				0.464
> 3years	249	17 (6.83%)	232 (93.17%)	
2-3 years	257	14 (5.45%)	243 (94.55%)	
< 2 years	138	12 (8.70%)	126 (91.30%)	
Child's BMI status at age four				0.678
underweight	15	2 (13%)	13 (87%)	
normal	529	35 (6.6%)	494 (93.4%)	
overweight	97	6 (6.2%)	91 (93.8%)	
obese	46	2 (4.4%)	44 (95.6%)	
Frequency of hand washing, 4 years				0.833
1-4 times	452	29 (6.42%)	423 (93.58%)	
5 times or more	234	16 (6.84%)	218 (93.16%)	
Passive smoking, 4 years				0.017
yes	264	25 (9.47%)	397 (95.20%)	
no	417	20 (4.80%)	397 (95.20%)	
Antibiotic use the last year, 4 years				0.787
never	150	11 (7.33%)	139 (92.67%)	
1-2 times	291	20 (6.87%)	271 (93.13%)	
3 times or more	245	14 (5.71%)	231 (94.29%)	

P-values based on a chi-square test or Mc Nemar's exact test.

Discussion

This study provides for the first time population based seroprevalence data for H.pylori infection in Greece. Among mothers of a mean age of 31 years old, seroprevalence was 45% while the proportion of CagA positive strains among those was 58%. Much lower was the seroprevalence among children 3 and 4 years of age; 5% and 6.5% correspondingly. The proportion of CagA positive strains among H.pylori positive children at age four was also 58%.

These estimates are comparable to those reported for pregnant women and women of child-bearing age in developed countries (74,182). In the European area, only studies in Portugal show much higher seroprevalence. In a population based study in Porto, seroprevalence among pregnant women was 73.6% and among their 4-5 years old children was 31% (77). In our study almost half of the seropositive population was CagA positive, whereas most recent studies conducted in North Europe report lower prevalence of H.pylori cagA (+) strains (183,184).

There was a remarked difference in H.pylori seroprevalence between mothers and their four year old children, in accordance with findings form other studies. Epidemiologic evidence suggests that this age difference in H.pylori prevalence is a birth cohort effect due to the decreasing rate of H.pylori colonization during childhood years in next generations, rather than a cumulating risk of infection with age (183,185). Currently, fewer than 10% of children born in Western countries are H. pylori positive. Because acquisition largely takes place in early childhood (186), it has been suggested that H.pylori seroprevalence estimates of a population in childhood years may serve as a predictor for the future incidence of H.pylori associated diseases, in particular peptic ulcer disease and gastric cancer (187).

The highest H.pylori seroprevalence among mothers of non-Greek origin and their children is in accordance with results form other populations which show that H pylori remains highly prevalent in migrant communities (82,83,188). As expected mothers of low educational level, those having more siblings and older children were more likely to be tested H.pylori positive. Interestingly, women who were drinking alcohol during their pregnancy were more likely to be H.pylori seropositive. This association could be confounded by socioeconomic position or other factors but it could also indicate that alcohol might reactivate a latent H.pylori infection especially

during pregnancy. Results on the association of alcohol with H.pylori infection show a reverse or no association but no studies exist among pregnant women (189). We observed that H.pylori infection was more frequent among underweight women and among those who gained inadequate weight during their pregnancy. It has been postulated that H.pylori could afect growth and appetite whereas a limited number of studies show that eradication of the bacterium is associated with weight gain (190). No other association between H.pylori infection and pregnancy outcomes were observed. Recent studies suggest an association between H.pylori and hyperemesis gravidarum, severe nausea and vomiting of pregnancy, preeclampsia and gestational diabetes mellitus (98,191,192).

In childhood, H.pylori infection was more frequent among children of mothers who were also seropositive, suggesting a mother to child transmission. Variables denoting social contacts such as age at school entry, child sharing a room and having other siblings were not found to be associated with H.pylori acquisition up to the age four. These findings are in contrast with those from previous studies (77). This might reflect the low seroprevalence rates that exist already in the population, which generate less chances of exposure to the pathogen independent of the frequency of social contacts. Thus, in settings of low seroprevalence, closer inside family contacts are more important in H.pylori transmission.

Conclusion

In conclusion, our results show that H.pylori is still common among women of childbearing age in Greece, especially among women of non-Greek origin which may constitute a target group for screening and eradication to prevent H. pylori associated diseases. On the other hand, acquisition of H.pylori in childhood was not common as only a small proportion of the children were tested seropositive at age four. Whether children will acquire H.pylori in the following years remains to be seen in future follow-up of our population.

3.3. Association of Polyomaviruses and Herpesviruses with neuropsychological development at four years of age.

Publication: *Common infections with polyomaviruses and herpesviruses and neuropsychological development at 4 years of age, the Rhea birth cohort in Crete, Greece. M Karachaliou, L Chatzi, T Roumeliotaki, M Kampouri, A Kyriklaki, K Koutra, G Chalkiadaki, A Michel, E Stiakaki, M Kogevinas, M Pawlita, T Waterboer, S de Sanjose; Journal of child psychology and psychiatry, 2016; doi: 10.1111/jcpp.12582*

Background-Hypothesis

The developing brain is extremely sensitive to the neuroinflammation induced by viruses (193). Previous studies report an increased occurrence of autism and attention-deficit/hyperactivity disorder (ADHD) in children who have experienced viral encephalitis/meningitis (194,195). A meta-analysis involving 1035 cases of schizophrenia and over 1.2 million controls showed that childhood central nervous system infections, and particularly viral infections, were associated with nearly two-fold increased risk of adult schizophrenia, suggesting that the detrimental effects may persist over the life course (196). Apart from viruses directly infecting the central nervous system, emerging evidence suggests that the brain is also sensitive to stimulus from peripheral infections (197). Several mechanisms have been suggested by which viral infections of childhood interact with developmental processes to engender schizophrenia, dementias and other neuropsychiatric diseases (198,199). Despite, studies assessing whether common viral infections are associated with impaired neurodevelopment already in childhood or adolescence are sparse and have not explored the role of novel viruses (200–203).

Thus, viral infections of the central nervous system may have detrimental effects for the developing brain, but the effects of less virulent common infections are unclear. Among Polyomaviruses, JCPyV is known to be neurotropic and is the etiologic agent of progressive multifocal leukoencephalopathy. Lintas et al found enhanced frequencies of polyomaviruses (BKPyV, JCPyV, SV40) in postmortem neocortical tissues of autistic patients, raising questions for an association between polyomaviruses and autism (85,86). Despite, none epidemiological study has explored the association of Polyomaviruses with neurodevelopment in childhood. EBV, CMV

and HSV-1, members of the Herpesviruses family, are also neurotropic viruses and under certain circumstances can infect the central nervous system and cause acute encephalitis/meningitis and long-term neurological sequelae (e.g. sensorineural hearing loss due to congenital CMV infection) (206). Little is known on the asscociation of Herpesviruses with neurodevelopment in the general pediatric population.

Aims

In this study we explore the association of seropositivity to ten Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, and HPyV10) and four Herpesviruses (EBV, CMV, HSV-1, HSV-2) with neuropsychological performance in various domains among children at age four in the Rhea birth cohort. We report the effects associated with exposure to single and to multiple Polyomaviruses and Herpesviruses infections.

Results

Study population

We used cross-sectional data from the four years of age follow-up in the Rhea birth cohort. In total, 879 singleton children attended the four years of age follow-up and 741 provided blood samples at the same time. Of the 690 children with serological data available, cognitive and motor abilities were evaluated in 685 using psychologist-based scales. Children with a diagnosed neurodevelopmental condition following intervention programmes were excluded (n=11). Thus, the study sample included 674 children. Additional data on symptoms related to ADHD and other behavioral problems as reported by mothers were available for 618 children (Figure 1).

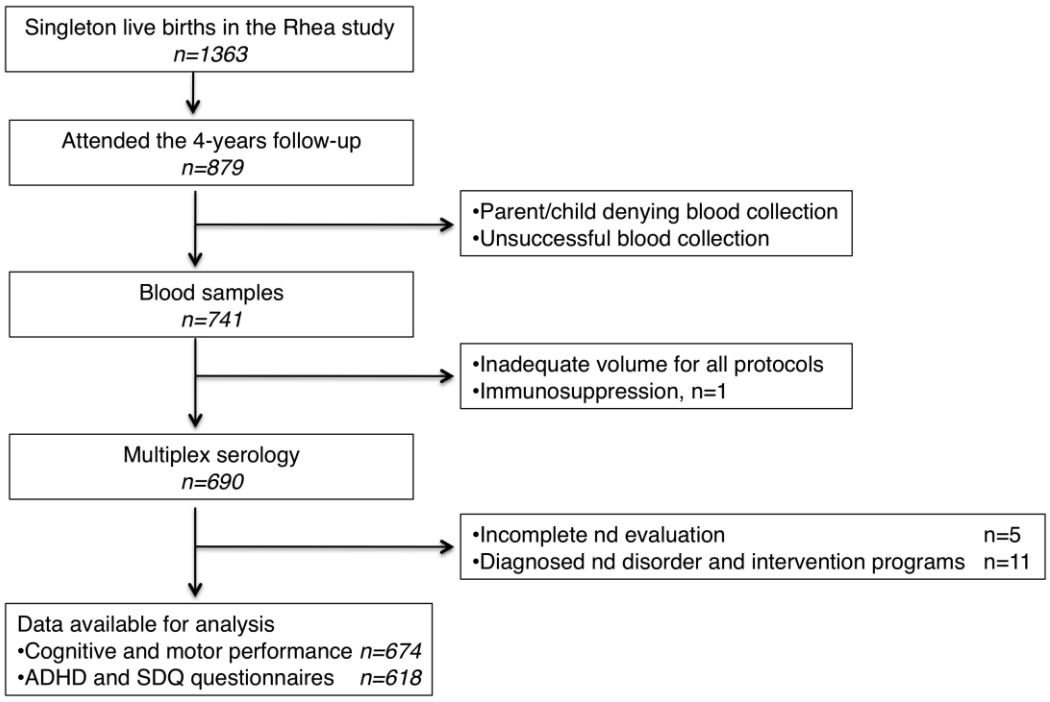


Figure 1. *Flowchart of the study population.*

Children included in this study, were of mean (SD) age 4.2 (0.2) years, 93.3% were of Greek mothers, 33.3% were of parents with low educational level, 13.0% were never breastfed, 54.8% had older siblings and 61.3% entered school before three years of age. Seroprevalence to Polyomaviruses was ranging from 21.1% for HPyV9 to 82.3% for HPyV10. Seroprevalence for EBV was 52.5%, for CMV 26.1% and for HSV-1 3.6%. HSV-2 showed very low seroprevalence (1.5%) and was not considered further in the analysis as single viral infection. Overall, 14.0% of the children were seropositive to more than eight Polyomaviruses and 20.3% to more than two Herpesviruses.

Cognitive and motor development

No significant associations were revealed between Polyomaviruses or Herpesviruses examined as single infections and scores in the McCarthy Scales of Children's Abilities (MSCA) (Table 1). Overall, seropositivity to Polyomaviruses tended to be associated with higher scores in MSCA, whereas seropositivity to Herpesviruses with lower scores in MSCA. For example,WUPyV seropositive versus seronegative children scored better in the general cognitive scale of MSCA [2.34 (95% CI: -0.35, 5.03)], whereas CMV seropositive children scored worst in the same scale of MSCA [-2.29 (95% CI: -4.72, 0.13)] compared to their seronegative counterparts

Table 2 presents associations between the Polyomaviruses and Herpesviruses burden and scores in the MSCA after adjusting for potential confounders. Although none of the associations reached statistical significance, a ≥8 Polyomaviruses burden versus ≤3 Polyomaviruses burden tended to be associated with higher scores in MSCA, whereas a 2–3 Herpesviruses burden versus 0 Herpesviruses burden tended to be associated with lower scores especially in the verbal scale.

Table 1. Adjusted associations (β, 95% CI) between serostatus to polyomaviruses and herpesviruses and scores in McCarthy Scales of Children's Abilities (n=674 children).

	Verbal		Perceptual performance		Quantitative		Memory		General cognitive		Motor skills	
Seopositivity	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI
Polyomaviruses												
BKPyV	0.97	[-1.70, 3.64]	0.38	[-2.26, 3.01]	-0.56	[-3.35, 2.22]	0.20	[-2.58, 2.98]	0.73	[-1.92, 3.37]	-0.94	[-3.77, 1.89]
JCPyV	0.35	[-1.91, 2.61]	-0.10	[-2.30, 2.11]	0.27	[-2.05, 2.60]	-0.16	[-2.50, 2.19]	0.18	[-2.04, 2.40]	-0.25	[-2.64, 2.13]
KIPyV	-1.43	[-3.60, 0.73]	1.00	[-1.12, 3.12]	-0.35	[-2.58, 1.89]	-1.45	[-3.70, 0.81]	-0.38	[-2.52, 1.75]	-0.70	[-3.00, 1.60]
WUPyV	2.20	[-0.52, 4.92]	2.48	[-0.20, 5.15]	0.64	[-2.18, 3.46]	0.89	[-1.95, 3.73]	2.34	[-0.35, 5.03]	-0.30	[-3.19, 2.58]
HPyV6	0.46	[-1.73, 2.65]	1.03	[-1.10, 3.17]	1.02	[-1.23, 3.27]	0.94	[-1.33, 3.21]	0.99	[-1.16, 3.14]	0.85	[-1.46, 3.17]
HPyV7	-0.66	[-3.19, 1.87]	0.09	[-2.38, 2.57]	-0.04	[-2.65, 2.57]	0.48	[-2.15, 3.11]	-0.26	[-2.75, 2.23]	-0.98	[-3.66, 1.70]
TSPyV	1.43	[-0.76, 3.62]	-1.36	[-3.50, 0.79]	-0.06	[-2.31, 2.20]	1.54	[-0.73, 3.82]	0.20	[-1.96, 2.36]	-0.29	[-2.62, 2.04]
MCPyV	1.87	[-0.45, 4.18]	0.41	[-1.87, 2.68]	0.63	[-1.77, 3.02]	1.51	[-0.90, 3.92]	1.26	[-1.02, 3.55]	2.18	[-0.28, 4.63]
HPyV9	1.60	[-1.01, 4.22]	-0.37	[-2.93, 2.19]	0.19	[-2.51, 2.89]	0.57	[-2.15, 3.30]	0.58	[-1.99, 3.16]	0.47	[-2.30, 3.24]
HPyV10	1.80	[-1.08, 4.68]	0.14	[-2.70, 2.98]	0.53	[-2.46, 3.53]	1.32	[-1.68, 4.31]	1.31	[-1.54, 4.16]	-0.98	[-4.03, 2.07]
Herpesviruses												
EBV	-1.32	[-3.45, 0.81]	-0.37	[-2.46, 1.71]	-0.33	[-2.53, 1.86]	0.01	[-2.20, 2.23]	-0.98	[-3.07, 1.12]	0.16	[-2.09, 2.42]
CMV	-2.09	[-4.56, 0.39]	-1.74	[-4.16, 0.67]	-1.63	[-4.18, 0.92]	-1.76	[-4.33, 0.80]	-2.29	[-4.72, 0.13]	-0.52	[-3.15, 2.11]
HSV-1	-2.47	[-8.20, 3.26]	2.51	[-3.11, 8.12]	-3.94	[-9.85, 1.97]	-1.63	[-7.59, 4.33]	-1.28	[-6.92, 4.37]	0.70	[-5.38, 6.78]

Abbbreviations: CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV-1, herpes simplex virus 1

All models are linear regression adjusted for examiner, quality at assessment, child's age, sex, parent's education, mother's origin, having older siblings and age at school entry.

Table 2. Adjusted associations (β, 95% CI) between the number of polyomaviruses and herpesviruses infections and scores in the McCarthy Scales of Children's Abilities (n=674).

	Polyomaviruses burden[a]						Herpesviruses burden[b]					
	4-7 polyomaviruses infections			≥8 polyomaviruses infections			1 herpesvirus infection			2-3 herpesviruses infections		
MCSA scores	β	95% CI	P-value	β	95% CI	P-value	β	95% CI	P-value	β	95% CI	P-value
Verbal	1.46	[-1.60, 4.53]	0.35	2.93	[-1.14, 7.00]	0.16	0.35	[-2.02, 2.73]	0.77	-2.70	[-5.65, 0.26]	0.07
Perceptual	0.50	[-2.53, 3.53]	0.75	2.38	[-1.61, 6.37]	0.24	-0.42	[-2.75, 1.91]	0.72	-0.45	[-3.35, 2.46]	0.76
Quantitative	0.25	[-2.96, 3.45]	0.88	1.25	[-2.97, 5.47]	0.56	0.14	[-2.31, 2.59]	0.91	-1.40	[-4.46, 1.66]	0.37
Memory	0.51	[-2.68, 3.70]	0.75	1.76	[-2.47, 6.00]	0.41	0.17	[-2.30, 2.65]	0.89	-0.85	[-3.93, 2.24]	0.59
General cognitive	0.98	[-2.06, 4.01]	0.53	2.88	[-1.13, 6.89]	0.16	-0.03	[-2.37, 2.31]	0.98	-2.07	[-4.98, 0.85]	0.17
Motor skills	-0.88	[-4.13, 2.36]	0.59	0.14	[-4.17, 4.45]	0.95	0.70	[-1.82, 3.22]	0.59	0.39	[-2.76, 3.54]	0.81

Abbreviations: CI, confidence interval; MCSA, McCarthy Scales of Children's Abilities.

All models are linear regression adjusted for examiner, quality at assessment, child's age, sex, parent's education, mother's origin, having older siblings and age at school entry.

[a] Reference group is that of ≤3 polyomaviruses infections.

[b] Reference group is that of 0 herpesviruses infections.

Behavioral outcomes

Results regarding the association of single Polyomaviruses and Herpesviruses infections with behavioral outcomes are presented in Tables 3 and 4. Seropositivity to most Polyomaviruses was associated with lower scores in the ADHDT and SDQ with some of the associations reaching statistical significance. In particular, seropositivity to WUPyV was associated with lower total score in the ADHDT [-2.76 (95% CI: -5.19, -0.34)] as well as in the hyperactivity [-1.23 (95% CI: -2.29, -0.18)] and inattention subscale [-0.89 (95% CI: -1.76, -0.02)]. Seropositivity to MCPyV was associated with lower score in the impulsivity subscale [-0.78 (95% CI: -1.49, -0.06)] of ADHDT. Only BKPyV seropositive children were observed to have higher total score in the ADHDT [2.05 (95% CI: -0.31, 4.41)] and, in particular, higher score in the inattention subscale [0.87 (95% CI: 0.03, 1.71)] (Table 3). Analysis of the SDQ questionnaire revealed that HPyV6 seropositivity was associated with lower total score in the SDQ [-0.75 (95% CI: -1.50, -0.002)], MCPyV seropositivity with lower score in the hyperactivity–inattention subscale [-0.39 (95% CI: -0.74, -0.04)], and TSPyV seropositivity with lower score in the peer relationships subscale [-0.24 (95% CI: -0.47, -0.02)] (Table 4). Herpesviruses were not shown to associate with ADHDT nor with SDQ scores.

Table 5 presents the associations of Polyomaviruses and Herpesviruses burden with scores in the ADHDT and SDQ questionnaires. After adjusting for potential confounders, children with '≥8 polyomaviruses infections' versus those of '≤3 polyomaviruses infections' had lower total ADHDT score [-3.13, 95% CI (-6.73, 0.46)] and, in particular, lower score in the inattention subscale [-1.28 (95% CI: -2.56, -0.00)]. Moreover, children with '≥8 polyomaviruses infections' had significantly lower total SDQ score [-1.98, 95% CI (-3.39, -0.57)] and, in particular, lower score in the hyperactivity–inattention subscale [-0.99 (95% CI: -1.60, -0.37)] compared to children with '≤3 polyomaviruses infections'. The number of Herpesviruses infections was not shown to associate with ADHDT nor with SDQ scores.

Table 3. Adjusted associations (β, 95% CI) between serostatus to Polyomaviruses and Herpesviruses and scores in the subscales of the Attention Deficit Hyperactivity Disorder Test (n=618 children).

	Total ADHDT			Hyperactivity			Impulsivity			Inattention		
Seropositivity	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value
Polyomaviruses												
BKPyV	2.05	[-0.31, 4.41]	0.09	0.52	[-0.50, 1.55]	0.31	0.65	[-0.18, 1.49]	0.12	0.87	[0.03, 1.71]	0.04
JCPyV	0.84	[-1.15, 2.82]	0.40	0.35	[-0.51, 1.22]	0.42	0.41	[-0.29, 1.11]	0.25	0.08	[-0.63, 0.78]	0.83
KIPyV	0.00	[-1.91, 1.90]	0.99	0.05	[-0.78, 0.88]	0.91	0.35	[-0.32, 1.02]	0.30	-0.40	[-1.08, 0.27]	0.24
WUPyV	-2.76	[-5.19, -0.34]	0.03	-1.23	[-2.29, -0.18]	0.02	-0.65	[-1.51, 0.21]	0.14	-0.89	[-1.76, -0.02]	0.04
HPyV6	-1.45	[-3.36, 0.46]	0.13	-0.32	[-1.16, 0.51]	0.44	-0.64	[-1.31, 0.04]	0.06	-0.48	[-1.17, 0.20]	0.16
HPyV7	-1.03	[-3.25, 1.19]	0.36	-0.27	[-1.24, 0.70]	0.59	-0.39	[-1.18, 0.39]	0.32	-0.37	[-1.16, 0.42]	0.35
TSPyV	0.42	[-1.49, 2.34]	0.66	0.47	[-0.36, 1.31]	0.26	0.10	[-0.58, 0.78]	0.77	-0.15	[-0.83, 0.54]	0.67
MCPyV	-1.88	[-3.91, 0.15]	0.07	-0.82	[-1.71, 0.06]	0.07	-0.78	[-1.49, -0.06]	0.03	-0.28	[-1.01, 0.45]	0.45
HPyV9	0.72	[-1.60, 3.04]	0.54	0.24	[-0.77, 1.25]	0.64	0.56	[-0.26, 1.38]	0.18	-0.07	[-0.90, 0.75]	0.85
HPyV10	-1.35	[-3.93, 1.23]	0.30	-0.50	[-1.63, 0.62]	0.38	-0.24	[-1.15, 0.68]	0.61	-0.61	[-1.53, 0.31]	0.19
Herpesviruses												
EBV	0.20	[-1.67, 2.06]	0.83	-0.09	[-0.90, 0.73]	0.83	0.15	[-0.51, 0.81]	0.65	0.14	[-0.53, 0.80]	0.68
CMV	-0.28	[-2.49, 1.94]	0.80	-0.19	[-1.16, 0.78]	0.70	-0.04	[-0.82, 0.74]	0.91	-0.05	[-0.84, 0.75]	0.90
HSV-1	1.51	[-3.41, 6.44]	0.54	1.07	[-1.08, 3.22]	0.32	0.02	[-1.72, 1.76]	0.98	0.42	[-1.33, 2.18]	0.63

Abbreviations: ADHDT, Attention Deficit Hyperactivity Disorder Test; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV-1, herpes simplex virus 1

All models are linear regressions adjusted for child's age and sex, for parent's education, mother's origin, breastfeeding (only models of CMV), having older siblings and age at school entry.

Table 4. Adjusted associations (β, 95% CI) between serostatus to Polyomaviruses and Herpesviruses and scores in the Strengths and Difficulties Questionnaire (n=618 children).

	Total SDQ		Emotional problems		Conduct problems		Hyperactivity-Inattention		Peer relationships		Prosocial behavior	
Seropositivity	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI
Polyomaviruses												
BKPyV	-0.03	[-0.96, 0.89]	-0.06	[-0.39, 0.27]	0.07	[-0.25, 0.39]	0.09	[-0.32, 0.49]	-0.13	[-0.41, 0.15]	-0.11	[-0.48, 0.25]
JCPyV	-0.26	[-1.05, 0.52]	0.00	[-0.28, 0.28]	-0.01	[-0.28, 0.26]	-0.06	[-0.40, 0.28]	-0.20	[-0.43, 0.04]	0.03	[-0.27, 0.34]
KIPyV	-0.34	[-1.09, 0.41]	-0.07	[-0.33, 0.20]	0.10	[-0.16, 0.36]	-0.18	[-0.51, 0.15]	-0.19	[-0.41, 0.04]	0.03	[-0.26, 0.33]
WUPyV	-0.69	[-1.65, 0.27]	-0.09	[-0.43, 0.26]	-0.02	[-0.35, 0.31]	-0.39	[-0.81, 0.03]	-0.19	[-0.48, 0.10]	0.19	[-0.19, 0.57]
HPyV6	-0.75	[-1.50, -0.002]	-0.09	[-0.36, 0.17]	-0.16	[-0.42, 0.10]	-0.32	[-0.65, 0.01]	-0.18	[-0.40, 0.05]	0.03	[-0.26, 0.33]
HPyV7	-0.50	[-1.37, 0.37]	0.00	[-0.30, 0.31]	-0.19	[-0.49, 0.11]	-0.23	[-0.61, 0.16]	-0.09	[-0.35, 0.17]	-0.15	[-0.49, 0.19]
TSPyV	-0.19	[-0.95, 0.57]	0.13	[-0.14, 0.39]	0.00	[-0.26, 0.26]	-0.08	[-0.41, 0.26]	-0.24	[-0.47, -0.02]	0.25	[-0.05, 0.55]
MCPyV	-0.64	[-1.44, 0.16]	-0.05	[-0.33, 0.23]	-0.24	[-0.51, 0.03]	-0.39	[-0.74, -0.04]	0.04	[-0.20, 0.28]	0.30	[-0.01, 0.61]
HPyV9	-0.16	[-1.07, 0.75]	0.08	[-0.24, 0.40]	0.02	[-0.29, 0.34]	-0.12	[-0.52, 0.28]	-0.13	[-0.41, 0.14]	0.23	[-0.13, 0.59]
HPyV10	-0.66	[-1.67, 0.36]	-0.27	[-0.63, 0.08]	-0.12	[-0.47, 0.23]	-0.24	[-0.68, 0.21]	-0.03	[-0.33, 0.28]	0.00	[-0.40, 0.40]
Herpesviruses												
EBV	-0.27	[-1.01, 0.46]	-0.02	[-0.28, 0.24]	-0.17	[-0.42, 0.08]	-0.07	[-0.39, 0.25]	-0.01	[-0.23, 0.21]	0.08	[-0.21, 0.36]
CMV	0.24	[-0.63, 1.10]	-0.01	[-0.31, 0.30]	0.09	[-0.20, 0.39]	0.13	[-0.25, 0.50]	0.03	[-0.23, 0.29]	0.01	[-0.33, 0.35]
HSV-1	0.40	[-1.55, 2.36]	0.08	[-0.61, 0.77]	-0.01	[-0.68, 0.66]	0.31	[-0.54, 1.16]	0.02	[-0.57, 0.61]	0.38	[-0.38, 1.14]

Abbreviations: SDQ, Strengths and Difficulties Questionnaire; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV-1, herpes simplex virus 1

All models are linear regressions adjusted for child's age and sex, for parent's education, mother's origin, breastfeeding (only models of CMV), having older siblings and age at school entry.

Table 5. Fully adjusted associations (β, 95% CI) between the number of polyomaviruses and herpesviruses infections and scores in the Attention Deficit Hyperactivity Disorder Test and Strengths and Difficulties Questionnaire (n=618).

	Polyomaviruses burden[a]						Herpesviruses burden[b]					
	4-7 polyomaviruses infections			≥8 polyomaviruses infections			1 herpesvirus infection			2-3 herpesviruses infections		
Questionnaire-based scores	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value
ADHDT												
Total	-2.13	[-4.82, 0.56]	0.12	-3.13	[-6.73, 0.46]	0.09	0.24	[-1.84, 2.31]	0.82	0.13	[-2.53, 2.79]	0.92
Hyperactivity	-0.85	[-2.02, 0.32]	0.15	-1.15	[-2.72, 0.41]	0.15	-0.11	[-1.01, 0.79]	0.81	-0.12	[-1.28, 1.04]	0.84
Impulsivity	-0.70	[-1.66, 0.25]	0.15	-0.70	[-1.97, 0.57]	0.28	0.18	[-0.56, 0.91]	0.64	0.10	[-0.83, 1.04]	0.83
Inattention	-0.58	[-1.53, 0.38]	0.24	-1.28	[-2.56, 0.00]	0.05	0.17	[-0.57, 0.91]	0.65	0.14	[-0.80, 1.09]	0.77
SDQ												
Total	-0.83	[-1.89, 0.22]	0.12	-1.98	[-3.39, -0.57]	0.01	-0.22	[-1.04, 0.59]	0.59	0.01	[-1.04, 1.06]	0.98
Hyperactivity/Inattention	-0.31	[-0.77, 0.15]	0.19	-0.99	[-1.60, -0.37]	0.00	0.02	[-0.33, 0.38]	0.89	0.02	[-0.44, 0.48]	0.93
Emotional problems	-0.21	[-0.59, 0.16]	0.26	-0.22	[-0.71, 0.28]	0.39	0.00	[-0.29, 0.29]	0.99	0.03	[-0.34, 0.40]	0.88
Conduct problems	-0.33	[-0.69, 0.03]	0.07	-0.45	[-0.93, 0.04]	0.07	-0.15	[-0.43, 0.13]	0.30	-0.11	[-0.47, 0.25]	0.54
Peer problems	0.02	[-0.29, 0.34]	0.89	-0.33	[-0.75, 0.10]	0.13	-0.10	[-0.34, 0.14]	0.42	0.07	[-0.24, 0.39]	0.65
Prosocial behavior	-0.16	[-0.57, 0.26]	0.46	0.24	[-0.31, 0.80]	0.39	0.00	[-0.32, 0.32]	1.00	0.11	[-0.30, 0.52]	0.60

Abbreviations: CI, confidence interval; ADHDT Attention-Deficit/Hyperactivity Disorder Test; SDQ, Strengths and Difficulties Questionnaire

All models are linear regression adjusted for child's age, sex, parent's education, mother's origin, having older siblings and age at school entry.

[a] Reference group is that of ≤3 Polyomaviruses infections

[b] Reference group is that of 0 Herpesviruses infection

Discussion

To our knowledge this is the first study exploring associations between Polyomaviruses and Herpesviruses infections and neuropsychological performance in a population of very young healthy children. We did not find any significant association between a particular infectious agent and cognitive or motor performance. Polyomaviruses tended to be positively associated with less behavioural problems at age four, with the only exception being seropositivity to BKPyV which was associated specifically with more inattention difficulties. Moreover, we observed that children who have acquired more Polyomaviruses up to age four presented fewer symptoms of inattention and hyperactivity at the same age. These novel data suggest that infections with common and less virulent viruses may interact with neurodevelopmental processes during the first years of life.

Silva et al have previously shown that infection-related hospitalizations, including intestinal infections, bacterial and viral infections, pyrexia of unknown origin, acute respiratory infections, ear and tonsillar infections, were significantly associated with being diagnosed and treated for ADHD (207). In contrast, we observed that children seropositive to ≥8 polyomaviruses presented less ADHD-related symptomatology at 4 years of age compared to children seropositive to ≤3 polyomaviruses. Polyomaviruses do not cause severe infections but are rather "silently" acquired in childhood. Thus it is likely that such common and less virulent infections compared to severe clinical infections affect differently neurodevelopmental outcomes. Experience of common infections in childhood is a natural process that shapes the immune system and regulates inflammatory responses (208,209). Increasing evidence suggests that common infective stimuli outside the central nervous systme modify also the responses of the brain's innate immune cells, microglia, which have a central role in many processes of neural development including cell proliferation, neurogenesis, synaptic formation and pruning, and programmed cell death (197). Unfortunately, we cannot test in our analysis whether an immune's system dysregulation mechanistically explains the aforementioned association. This is an area in need of further research. Due to the cross-sectional design of our study we cannot exclude reverse causality. Thus it could be that children presenting more ADHD-related symptomatology are less likely to be exposed to Polyomaviruses in early life. We speculate that such an altered pattern of acquisition of common infections in children with ADHD could

mirror causal pathways responsible for the strong relation between ADHD and atopic diseases reported by several epidemiological studies (210,211).

Interestingly, we found that only BKPyV seropositivity was associated with more inattention symptoms suggesting virus-specific mechanisms for this association. BKPyV is increasingly recognized as an opportunistic infectious agent of the central nervous system in children and adults (212–214). Potentially, infiltration of the brain by BKPyV could alter or disrupt the development of neural pathways important for attention (215). However, BKPyV seroprevalence was close to 76% in our population and by adult life it would be close to 100% (216). No previous studies have examined the role of BKPyV or other Polyomaviruses infections on brain development and behavioral outcomes in the general population. Apparently, more studies are needed on Polyomaviruses role as plausible candidates for neurodevelopmental insult with a focus on pathogenic mechanisms.

Few studies have evaluated the association between Herpesviruses and cognitive function in children and adolescence in the absence of severe infections (e.g. congenital CMV infection or HSV meningoencephalitis). Tarter et al showed that HSV-1 - but not CMV - seropositivity among children 6-16 years old, was associated with worst scores in the reading and block design examination (202). Similarly, Jonker et al found that HSV-1 seropositive children at 16 years of age had lower memory function at 18 years of age, whereas HSV-2, EBV and CMV infections were not associated with cognitive functioning (200). In ALSPAC study, EBV seropositivity at 4 years of age was not associated with IQ measures at 9 years of age (201). Similarly we found no association between EBV and neuropsychological performance at 4 years of age. In contrast with previous studies (200,202), we did not detect an association between HSV-1 seropositivity and cognitive performance. Power to detect statistically significant associations may have been limited due to the low seroprevalence of HSV-1 in our population. Moreover, HSV-1 seroprevalence has been shown to peak during adolescence and probably infections acquired at that age might contribute to the effects reported before (200,202).

Strengths and limitations

We used data from a birth cohort study, the serological analysis included multiple Polyomaviruses and Herpesviruses, we adjusted for a broad range of confounders and

we applied imputation techniques to reduce any selection bias due to missing data and retain sample size in multiple linear regression models. Because our study has been cross-sectional, we cannot make conclusions about the direction of the associations. However, IgG antibodies denote exposure at anytime in the past. Previous studies have shown that acquisition of Polyomaviruses starts as early as the first months of life (112,217). Misclassification of exposure estimates is always of concern but is not expected to appreciably impact our findings based on previous sensitivity analysis using different cut-off definitions. Although our hypothesis is intriguing, we lack of direct evidence regarding the neurotropism of most polyomaviruses. The analysis of multiple infections provides a more comprehensive approach to our data, despite we acknowledge that it does not take into account the specific combination of pathogens for which an individual is seropositive and that complex interactions at a virological and immunological may contribute to the observed effects. We did not examine associations with clinically diagnosed ADHD. However, using quantitative, dimensional traits related to ADHD implies reduced outcome misclassification and enhanced statistical power (218). ADHDT and SDQ are widely used and well validated instruments although their scores can not be considered objective (219). Due to the young age of the children, we can rely only on their parents as source of information about a child's symptoms and problems. We should comment that hyperactivity/inattention scores of SDQ and total SDQ scores were significantly associated with exposure to ≥8 Polyomaviruses, whereas only inattention scores of ADHDT and not hyperactivity scores or total ADHDT scores were significantly associated with exposure to >8 Polyomaviruses. Nonetheless, because the direction of the associations were similar, we consider results between ADHDT and relevant subscales of SDQ comparable. Comparison of participants versus non-participants revealed that children who were assessed by means of the MCSA test but had no serological data and therefore were not included in the present analysis were more likely to have lower scores in MCSA. No differences were observed in scores of ADHDT and SDQ questionnaires or in basic characteristics. Reasons for lacking serological data included parent/child denying a blood collection, unsuccessful blood collection or collection of inadequate blood volume. An explanation would be that children with worst neurodevelopment scores could not successfully complete protocols such as blood collection, leading to selection bias and most likely underestimation of the associations. It is also reasonable to suggest that child's mood

or other physical conditions may have affected performance in MCSA and blood collection at the time of evaluation. Chance findings are always of concern when multiple comparisons are performed but because our outcomes were not independent and our analysis was considered exploratory, correction for multiple testing could be very restrictive (181). Finally, generalizability of the study findings is strengthened by i) the population based study design, ii) the assessment of children during healthy visits, iii) the fact that seroprevalence estimates in our study were comparable to those reported in other populations and iv) the use of instruments to assess children's neurodevelopment which allow studying continuously distributed traits in the population.

Conclusion

To conclude, these results suggest that acquisition of Polyomaviruses in early life and neurodevelopmental processes related to traits of inattention and hyperactivity may closely interact during the first years of life. Interestingly, BKPyV, a virus potentially neurotropic, was associated with decrements in attention. These findings raise the possibility that Polyomaviruses may play a more important role in mental health and disease than previously understood and shows new directions for research in birth cohort studies (220). Future follow- up in the Rhea cohort will allow us to examine these associations prospectively and investigate effects over childhood.

3.4. Association of Helicobacter pylori seropositivity in cord blood and four years of age samples with child's neurodevelopment at four year of age

Publication: *Helicobacter pylori seropositivity and childhood neurodevelopment, the Rhea birth cohort in Crete, Greece. M. Karachaliou, L. Chatzi, A. Michel, A. Kyriklaki, M. Kampouri, K. Koutra, T. Roumeliotaki, G. Chalkiadaki, E Stiakaki, M. Pawlita, T. Waterboer, M. Kogevinas, S de Sanjose; Paediatric and Perinatal Epidemiology, 2017 doi: 10.1111/ppe.12374*

Background-Hypothesis

Research on modifiable risk factors for adverse neurodevelopmental outcomes over the life course, is seeking for novel exposures especially those occuring in the formative stages of life (221). Emerging evidence suggesting that H.pylori is implicated in the pathogenesis of neuropsychiatric diseases, may promise new prevention strategies. Kountouras et al showed that H.pylori eradication was associated with improved cognitive and functional status in patients with Alzheimer disease two years after treatment compared to age-matched controls (222) and to a lower five-year mortality risk (223). Data from 4,484 Parkinson cases and 22,416 population controls in Denmark showed that prescriptions for H.pylori eradication drugs and proton pump inhibitors five or more years prior to the diagnosis of Parkinson disease were associated with an increased disease risk (224). In a large cross-sectional study in Malaysia, H.pylori was also positively associated with Parkinson's disease motor severity (225). Further, H.pylori eradication was associated with a marked improvement at a three year follow-up in patients with idiopathic Parkinsonism in a randomized, placebo-controlled study (226). A recent systematic review and meta-analysis showed a significant positive association between H.pylori and dementia with a pooled odds ratio of 1.71 (95% CI 1.17-2.49) (227). In a small case-control study, histologically confirmed H.pylori infection was significantly more prevalent in cases with a prodromal phase of dementia than in controls (88.9% versus 48.6%) (228). A population-based study from NHANES III demonstrated that H.pylori seropositivity was associated with poorer performance on a verbal memory test among US adults 60-90 years of age (229).

Despite the aforementioned associations in adults and the consideration that neurodegenerative diseases such as Parkinson's and Alzheimer are late manifestations

of an underlying brain process which may have its origin in early life (230), only one study in childhood population exists. Muhsen et al showed a negative association between H.pylori and cognitive function among children 6-9 years of age from a high socioeconomic community of the Israeli Arab population (231). Thus, we conducted the present analysis and hypothesized that H.pylori during the first years of life but also maternal H.pylori seropositivity during pregnancy may unfavorably affect offspring's neurodevelopment. Our interest in the role of maternal H.pylori in child's neurodevelopment is founded on two case-control studies, reporting that maternal H.pylori infection could play a role in neural tube defect causation by reducing folate and vitamin B12 concentrations (104,105). During pregnancy, H.pylori has been associated with micronutrient deficiencies which are relative resistant to the indicated supplementation (232,233). Fetal brain is extremely vulnerable to such micronutrients defects (234). To the best of our knowledge, no study has examined differences in neurodevelopment between children of H.pylori seropositive versus seronegative mothers.

Aims

In this study, we used prospective data on 352 mother-child pairs and cross-sectional data on 674 children to assess the association of maternal and child's H. pylori seropositivity correspondingly on child's neurodevelopment at age four in the Rhea birth cohort in Crete, Greece. Furthermore, we specifically explored the association of seropositivity to each of the twelve H.pylori proteins measured with a newly developed multiplex serology method, with child's neurodevelopment.

Results

Study population

Of the 1363 singleton live births of the Rhea study, serological data on H.pylori were available for 626 children at birth (cord blood) and for 690 children at age four. Of those, 352 and 674 children correspondingly, were also assessed at age four in terms of neurodevelopment using psychologist-administered scales (Figure 1). Children with a diagnosed neurodevelopmental condition following intervention programs were excluded (n=11).

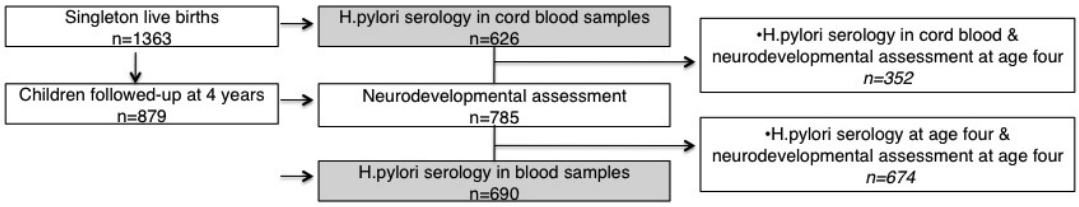


Figure 1. *Flowchart of the study population.*

In the study population, seroprevalence to H.pylori in cord blood was higher among non-Greek mothers, mothers who were defined as underweight before pregnancy, and those who had older children. Seroprevalence to H.pylori at age four was higher among children of non-Greek mothers, children of H.pylori seropositive mothers, children who were delivered vaginally and those of term pregnancies.

We used data on serum iron [mg/dl, n=234 maternal samples, mean (SD) gestational age of 13.2 (2.3) weeks], plasma vitamin B12 (pg/ml; n=97) and erythrocyte folate concentrations (ng/ml; n=34) in cord blood. Although these data were available only for a small subgroup of the population, we included this information in the present analysis in an effort to provide a more comprehensive approach to our hypothesis. The distribution of these micronutrients concentrations according to maternal H.pylori serostatus, based on seroresponses in cord blood smaples, is presented in Table 1. Lower erythrocyte folate concentrations were detected in cord blood samples of H.pylori seropositive mothers compared to seronegative mothers, although differences were not significant. No differences were observed regarding serum iron in maternal samples or plasma B12 levels in cord blood.

Table 1. Distribution of micrunutrients (iron, folate, B12) by Helicobacter pylori serostatus in cord blood.

	Seropositive			Seronegative			
	n	Mean (SD)	Median (IQR)	n	Mean (SD)	Median (IQR)	P-value
Serum iron, mg/dl	88	79.5 (105.6)	67 (40.5-99.5)	146	75.5 (83.3)	66 (40-93)	0.98
Erythrocyte folate, ng/ml	14	751.6 (245.6)	714.2 (617.9-945.8)	20	877.6 (373.3)	913.2 (585.9-1134.6)	0.42
Plasma B12, pg/ml	44	197.3 (137.6)	173.5 (130.9-232.9)	53	180.4 (125.7)	175.1 (103.6-227.1)	0.71

P-values based on a Mann-Whitney U-test

Abbreviations: SD, standard deviation; IQR, interquartile range

H.pylori seropositivity and MCSA scores

Table 2 shows the association between H.pylori seropositivity in cord blood and at age four and the scores in McCarthy Scales of Children's Abilities **(**MCSA) attained at age four. H.pylori seropositivity in cord blood, reflecting maternal status, was associated with lower scores in all scales of MCSA investigated, the one exception being motor scale (crude model, model 1). After adjusting for potential confounders the associations attenuated but mostly remained statistically significant. In detail, children of H.pylori seropositive mothers, as indicated in cord blood, had lower score in the general cognitive [-3.87 (95% CI: -7.02, -0.72)], verbal [-2.96 (95% CI: -6.08, 0.15)], perceptual performance [-3.37 (95% CI: -6.60, -0.15)], quantitative [-2.85 (95% CI:-6.28, 0.58)] and memory scale [-3.37 (95% CI: -6.67, -0.07)] compared to those of seronegative mothers (Table 2, model 2). H.pylori seropositivity at four years of age was also negatively associated with scores in MCSA although none of the associations reached statistical significance.

H.pylori protein-specific seropositivity and MCSA scores

Figure 2 presents the association between H.pylori protein-specific seropositivity in cord blood and at age four and scores in MCSA after adjusting for potential confounders. Seropositivity in cord blood specifically to GroEl and NapA – two of the twelve H.pylori proteins investigated – was associated with statistically significant lower scores in almost all scales of MCSA. Sparse findings were revealed for the remaining proteins; seropositivity to HyuA was associated with lower scores in the perceptual [-5.08 (95% CI: -9.06, -1.09)] and motor scale [-5.80 (95% CI: -9.99, -1.62)], seropositivity to Catalase with lower score in the memory scale [-5.45 (95% CI: -9.53, -1.37)] and seropositivity to HcpC with lower score in the verbal scale [-3.92 (95% CI: -7.52, -0.32)]. At age four, seropositivity to HP0305 was associated with lower score in the quantitative scale [-6.24 (95% CI: -11.97, -0.50)] and seropositivity to HpaA with lower score in the perceptual performance scale [-3.76 (95% CI:-7.48, -0.05)] in the fully adjusted model.

Sensitivity analyses

When we repeated analyses adjusting also for maternal intelligence, the direction of associations did not change but associations between maternal H.pylori seropositivity based on cord blood samples and MCSA scales were no more statistically significant

(Table 3). We also adjusted models of cord blood samples for child's H.pylori serostatus and results were very similar to those presented in the main analyses (Table 3).

Table 2. Helicobacter pylori seropositivity in cord blood and age four and performance in McCarthy Scales of Children's Abilities at four years of age.

	Cord blood			Four years of age		
	Crude model (n=352)	Model 1 (n=352)	Model 2 (n=335)	Crude model (n=674)	Model 1 (n=674)	Model 2 (n=602)
	coef [95% CI]	coef [95% CI]	coef [95% CI]	coef [95% CI]	coef [95% CI]	coef [95% CI]
General cognitive	-7.39 [-10.83, -3.95]	-6.06 [-9.27, -2.85]	-3.87 [-7.02, -0.72]	-4.00[-9.06, 1.05]	-3.90 [-8.46, 0.66]	-2.54 [-6.93, 1.85]
Verbal	-6.72 [-10.13, -3.31]	-5.63 [-8.82, -2.43]	-2.96 [-6.08, 0.15]	-3.98 [-8.97, 1.01]	-3.22 [7.77, 1.33]	-1.01 [-5.45, 3.43]
Perceptual	-5.55 [-8.92, -2.17]	-4.42 [-7.62, -1.22]	-3.37 [-6.60, -0.15]	-2.97 [-7.85, 1.90]	-3.64 [-8.13, 0.84]	-3.11 [-7.47, 1.25]
Quantitative	-5.58 [-9.10, -2.06]	-4.11 [-7.45, -0.76]	-2.85 [-6.28, 0.58]	-3.46 [-8.51, 1.57]	-3.55 [-8.21, 1.11]	-3.27 [-7.91, 1.38]
Memory	-6.98 [-10.46, -3.51]	-5.47 [-8.74, -2.19]	-3.37 [-6.67, -0.07]	-1.96 [-6.95, 3.01]	-1.95 [-6.55, 2.66]	-0.77 [-5.40, 3.86]
Motor	-3.01 [-6.61, 0.57]	-1.62 [-4.90, 1.67]	-1.85 [-5.25, 1.55]	-2.49 [-7.71, 2.72]	-2.91 [-7.60, 1.78]	-3.02 [-7.77, 1.73]

Model 1 is adjusted for child's age, sex, examiner and quality of assessment.

Model 2 is additionally adjusted for mother's age, education, origin, child having older siblings and child's age at school entry (models of 4 years samples).

Abbrevations: CI, confidence intervals

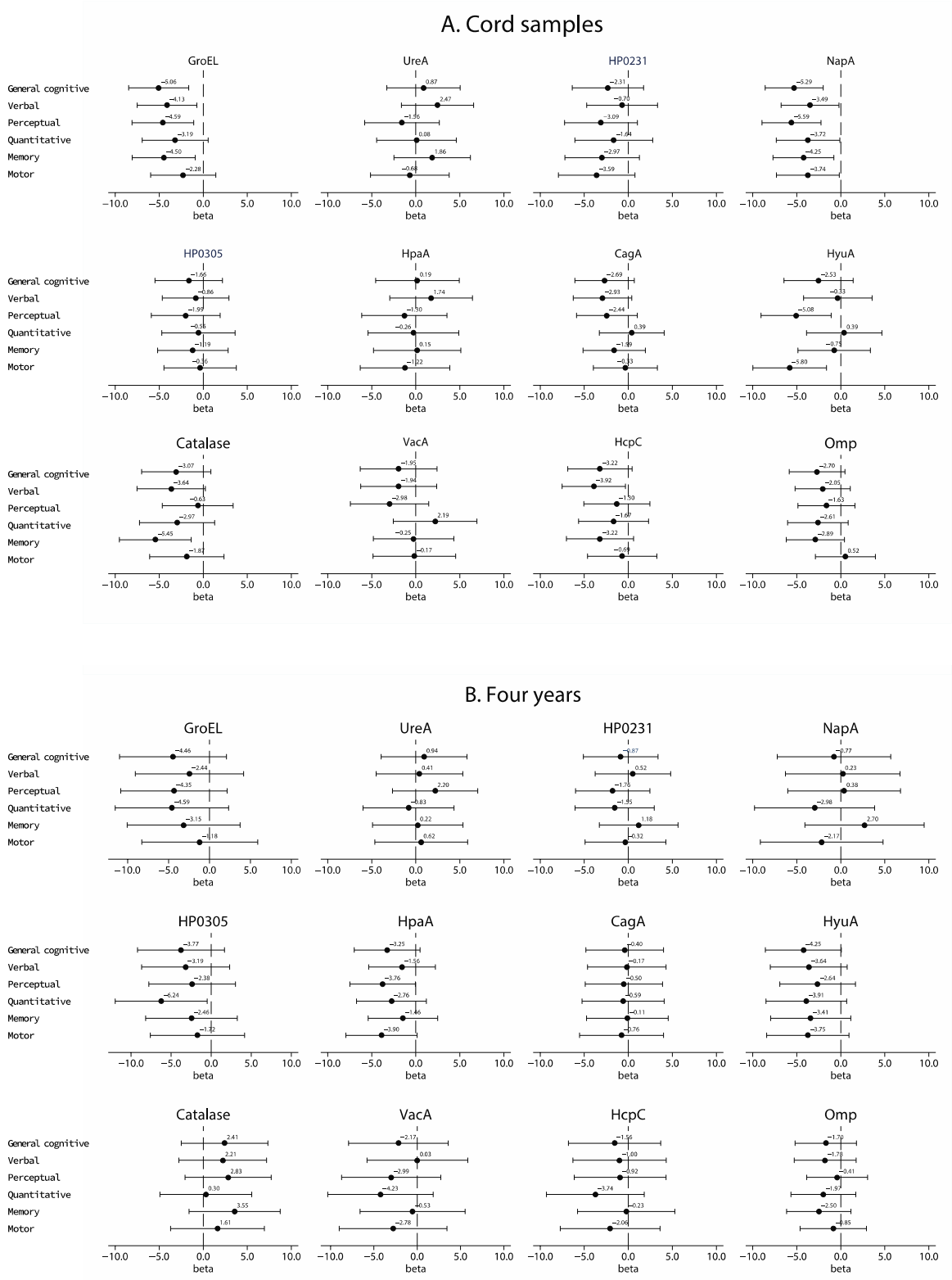


Figure 2. *Association (beta, 95% CI) between seropositivity to each Helicobacter pylori protein in cord blood (A) and age four (B) and performance in McCarthy Scales of Children's Abilities at 4 years of age after adjustment for child's age, sex, examiner, quality of assessment, mother's age, education, origin, child having older siblings, and child's age at school entry (models of 4 years samples).*

Table 3. Helicobacter pylori seropositivity in cord and 4 years of age blood samples and performance in McCarthy Scales of Children's Abilities at four years of age: confounding

	Model 1	Model 2: maternal intelligence	Model 3: child's serostatus
Cord blood	n=335 coef [95% CI]	n=162 coef [95% CI]	n=270 coef [95% CI]
General cognitive	-3.87 [-7.02, -0.72]	-2.20 [-6.92, 2.52]	-4.12 [-7.54, -0.69]
Verbal	-2.96 [-6.08, 0.15]	-2.07 [-6.81, 2.67]	-3.37 [-6.73, -0.01]
Perceptual	-3.37 [-6.60, -0.15]	-1.19 [-6.12, 3.73]	-3.40 [-6.90, 0.11]
Quantitative	-2.85 [-6.28, 0.58]	-1.91 [-6.78, 2.96]	-2.79 [-6.66, 1.09]
Memory	-3.37 [-6.67, -0.07]	-1.00 [-5.62, 3.62]	-3.34 [-7.04, 0.36]
Motor	-1.85 [-5.25, 1.55]	-1.37 [-6.53, 3.80]	-1.67 [-5.42, 2.08]
Four years	n=602 coef [95% CI]	n=292 coef [95% CI]	
General cognitive	-2.54 [-6.93, 1.85]	-0.89 [-7.49, 5.70]	(NA)
Verbal	-1.01 [-5.45, 3.43]	-0.18 [-6.87, 6.51]	(NA)
Perceptual	-3.11 [-7.47, 1.25]	-1.12 [-7.75, 5.51]	(NA)
Quantitative	-3.27 [-7.91, 1.38]	0.23 [-6.60, 7.07]	(NA)
Memory	-0.77 [-5.40, 3.86]	2.79 [-4.13, 9.72]	(NA)
Motor	-3.02 [-7.77, 1.73]	-2.57 [-10.09, 4.93]	(NA)

Model 1 is adjusted for child's age, sex, examiner, quality of assessment, mother's age, education, origin, child having older siblings and child's age at school entry (only for models of four years samples).

Model 2 is additionally adjusted for maternal intelligence.

Model 3 is additionally adjusted for child's Helicobacter pylori serostatus.

Abbreviations: CI, confidence intervals; BMI, body mass index; NA, non applicable

Discussion

This is the first study investigating the association between maternal H.pylori seropositivity during pregnancy and offspring's neurodevelopment. The exploratory analysis on antibodies to twelve H.pylori specific proteins, revealed two proteins as potential risk markers of maternal H.pylori seropositivity for poor child's neurodevelopment; GroEL and NapA. Acquisition of H.pylori infection during the first years of life was not common in our population as only 6.5% of the children at age four were seropositive. Interestingly, H.pylori seropositive children performed worst in neurodevelopmental assessment compared to their seronegative counterparts although none of the associations were statistically significant.

We have shown that maternal H.pylori seropositivity during pregnancy was associated with unfavorable neurodevelopmental outcomes in children at age four. Previous findings suggest that H.pylori is a risk factor for low folate, B12 and iron levels especially in high risk groups such as pregnant women (102,103). These micronutrients are considered essential for several neurodevelopmental processes such as myelination, dendritic arborization, synaptic connectivity and neurotransmitter synthesis (234,235). An important aspect of micronutrient deficiencies associated with H.pylori is that they are relatively resistant to supplement administration. Thus, suppression of the periconceptional micronutrient environment in H.pylori seropositive mothers might be a mechanism explaining the negative association with child's neurodevelopment in this study although most mothers (94%) received adequate fortification. In a very small sub-sample of this population we observed relatively lower folate levels in cord blood from children of seropositive versus seronegative mothers although difference was not statistically significant. Although this observation provides some evidence, more data are required in order to further support (or not) the aforementioned hypothesis in this population. Moreover, it has been suggested that antibodies and T-cells activated in response to current H.pylori infection may cross-react with host's proteins in the nervous system that are structurally similar to H.pylori epitopes (86). This phenomenon, called molecular mimicry, might induce adverse effects in the developing brain and has been previously suggested as a potential mechanism in the association of H.pylori with Henoch-Schonlein purpura, preeclampsia, ischaemic heart disease and neurodegenerative diseases among adults (86,236,237). In our study, H.pylori

infection is most likely acquired before pregnancy, thus we can speculate that a reactivation of latent H.pylori infection owing to the physiological immunosuppression of pregnancy (238), could also induce a molecular mimicry phenomenon.

Only one study has investigated the association of H.pylori with neurodevelopment in childhood. Muhsen et al showed a negative association between H.pylori infection and cognitive function among children 6-9 years of age from a high socioeconomic community of the Israeli Arab population (231). Similarly we observed a negative association at age four but associations were not statistically significant. Reasons for lacking to find a statistically significant association could be i) lack of power given that only 6.5% of children at age four were H.pylori seropositive and ii) timing of evaluation of both the exposure and the outcome.

The antigen-specific analysis revealed some intriguing findings. Children of mothers who were GroEL and NapA seropositive performed worst in cognitive and memory scales than those of seronegative mothers. GroEL belongs to the family of molecular chaperones and it has been identified to bind iron from the host (239). NapA among other functions, is involved in iron sequestering and storage by H.pylori (240). These data further suggest that a H.pylori induced depletion in iron stores during pregnancy might be a potential mechanism for poor neurodevelopmental outcome in the offspring. Interestingly, antibodies against the two major virulence proteins of H.pylori, VacA and CagA, were not significantly associated with child's neurodevelopment suggesting that other factors than those usually identified in the disease process of gastric diseases might be implicated in the pathogenesis of extragastric diseases (85).

Strengths and limitations

Although histology is considered the practical diagnostic gold standard of active H.pylori infection, we defined exposure based on serology because a histological method would be practically and ethically difficult to achieve during pregnancy or in early childhood. We evaluated the exposure based on the presence of immunoglobulin G antibodies therefore, we lack of knowledge on the exact time of acquisition of infection. It is likely that in most cases of maternal H.pylori seropositivity, infection was acquired before pregnancy because H.pylori is usually acquired during childhood

(73). Nonetheless, studies suggest an increased susceptibility to H.pylori infection in pregnancy (97). Moreover, we are unaware of whether H.pylori infection was ever diagnosed, treated or eradicated and as a result whether H.pylori seropositivity in our study reflects a primary untreated infection (raised antibody titres), an eradication failure (unchanged or slightly rising titres despite treatment) or a re-infection after successful treatment (raised antibody titres following normal titres achieved with previous treatment) (241–245). Studies show that immunoglobulin G antibodies produced in response to infection, remain present as long as infection is active, and quantitatively decrease to normal titers after infection is cured (242,245). This process might last two years (242). Despite, we speculate that most cases of seropositivity in this study represent a primary untreated infection because largely H.pylori infection remains asymptomatic and thus undiagnosed while re-infection is unusual (73,246). Moreover, we did not identify any prescriptions for H.pylori eradication drugs and proton pump inhibitors for mothers during pregnancy and children up to the age of four. Regarding the outcome, we have used standardized instruments to assess neurodevelopment at age four and several quality controls were introduced. Regarding confounders, we adjusted for a broad range of variables whereas the inclusion of maternal intelligence, although available for a subsample of our population, should be considered as an additional strength. We acknowledge that H.pylori could also be a surrogate of other factors that we are not able to identify with current knowledge. Unfortunately folate and B12 were measured only to a very small sub-sample of our population. We examined prospectively whether maternal H.pylori seropositivity during pregnancy was linked with child's neurodevelopment at age four allowing us to infer on causality of the association. At age four, association was examined cross-sectionally but future follow-up in our study will allow us to examine this association prospectively as well. Selection bias could be present in our study due to the fact that only 40% of the children that were assessed at age four had available cord blood samples. Children who were also assessed at age four tended to be of mothers with higher education and Greek origin. No other significant differences in baseline characteristics and in H.pylori seroprevalence in cord blood samples were revealed (data not shown). Moreover, comparison of participants versus non-participants at age four revealed that children, who were assessed by means of the MCSA test but had no serological data and therefore were not included in the present analysis, were more likely to have lower scores in MCSA. Reasons for lacking

serological data included parent/child denying a blood collection, unsuccessful blood collection or collection of inadequate blood volume. An explanation would be that children with worst neurodevelopment scores could not successfully complete protocols such as blood collection, leading to selection bias and most likely underestimation of the associations. It is also reasonable to suggest that child's mood or other physical conditions may have affected performance in MCSA and blood collection at the time of evaluation. Chance findings are always of concern when multiple comparisons are performed but because the outcomes were not independent and our analysis was considered exploratory, correction for multiple testing could be very restrictive (181).

Conclusion

To conclude, we have shown that children of H.pylori seropositive mothers performed worst in all domains of neurodevelopment at age four excluding motor development, compared to those of H.pylori seronegative mothers. We speculate that changes in the periconceptional micronutrient environment following a H.pylori infection might mediate, at least in part, such an association. These findings need replication from other studies. Whether screening for H.pylori infection during pregnancy and childhood and eradication of the bacterium could be effective in preventing the adverse neurodevelopmental outcome in the offspring remains to be seen.

3.5. Association of Polyomaviruses and Herpesviruses with obesity indices and metabolic traits in childhood.

Publication: *Is early life exposure to polyomaviruses and herpesviruses associated with obesity indices and metabolic traits in childhood? M Karachaliou, S de Sanjose, T Waterboer, T Roumeliotaki, M Vassilaki, K Sarri, V Leventakou, M Vafeiadi, G Chalkiadaki, E Stiakaki, A Michel, M Pawlita, M Kogevinas, L Chatzi; This paper has been submitted to the International Journal of Obesity (27 June 2015).*

Background-Hypothesis

Health policies to tackle obesity are based on conventional risk factors such as diet and lifestyle with little effect, forcing researchers to rethink on the multifactorial aetiology of obesity (247). In this direction, the emerging hypothesis of an infectious origin of obesity seems interesting because it may promise new prevention strategies (156), also regarding obesity related metabolic comorbidities (249). Several pathogens show an obesogenic effect in animal models by inducing hypothalamic damage, malfunctions in the neuroendocrine system and changes in the adipose tissue (250,251). In humans, most of the data concern adenovirus 36, which is a virus commonly acquired in childhood (252). Atkinson et al characteristically demonstrated in a study of 89 pairs of twins, 28 of which were discordant for adenovirus 36 infection, that the seropositive twin had a significantly higher body mass index (BMI) and more body fat than the seronegative sibling (253). Not all studies have demonstrated an association between adenovirus 36 and obesity, while associations seem more robust in children than adults (254). Paradoxically, adenovirus 36 seropositivity has been associated with lower levels of serum cholesterol and triglycerides (253). A metabolic dysfunction has been shown to accompany some viral infections such as CMV, HSV-1 and HSV-2 (255–258). An increasing number of studies report a link between pathogen burden, obesity, cardiometabolic traits and disease (259–264), but no such literature exists for childhood populations.

Further efforts to identify new pathogens that are linked with obesity may benefit from serological surveys in humans (265), especially when considering common infections which cause subclinical or non-specific disease symptoms. Li et al have shown that infections in infancy not treated with antibiotics and therefore more likely to be due to common viral infections, were associated with an increased risk of

childhood obesity compared with controls without infection, while a clear dose-response relation between infection episodes and risk of childhood obesity was observed (156).

Aims

In this study we seek to identify whether acquisition of ten Polyomaviruses (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV, MCPyV, HPyV9, HPyV10) and four Herpesviruses (EBV, CMV, HSV-1, HSV-2) up to four years of age is associated with obesity indices and metabolic traits at four and six years of age. We report the effects associated with exposure to single and to multiple polyomaviruses and herpesviruses infections.

Results

Study population

Of 1,363 singleton live births of the Rhea cohort, 879 children attended the four years of age follow-up and 607 the six years of age follow-up (Figure 1). At age four, blood samples from children were collected at the end of the health-visit and serological data were available for 690 children. Of those, 687 children had data at the same age on BMI, waist circumference, skinfold thickness at four anatomical sites and 440 children had data at age six on the same anthropometric measurements along with bioelectrical impedance analysis; these constitute the population of the present analyses. Data on metabolic traits including serum lipids, leptin and adiponectin levels were also available.

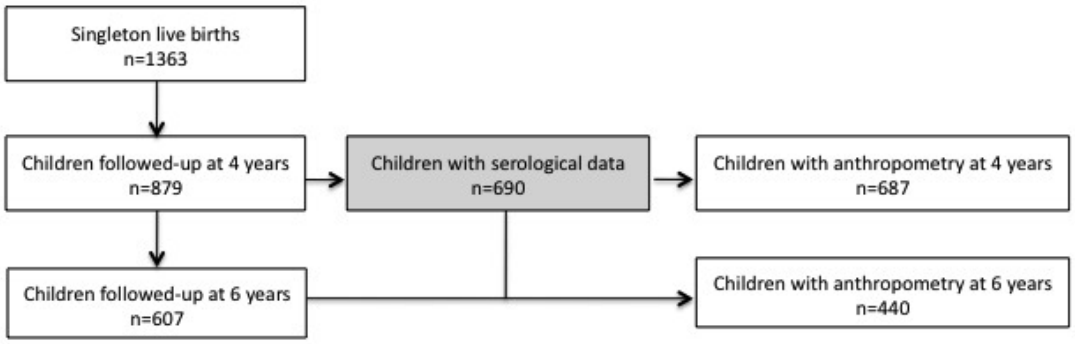


Figure 1. Flowchart of the study population, the Rhea birth cohort in Crete, Greece, 2007-2012

Descriptives

Mean (SD) age of children was 4.2 (0.2) and 6.6 (0.3) years old at the two consecutive follow-ups. Characteristics of the study population are presented in table 1. Nearly all children (93.6%) were of Greek mothers, 16.4% were of mothers with low educational level, 49.6% were born with caesarean section (a pattern typical in Greece), 13.5% were never breastfed, 55.8% had older siblings, 15.5% were not attending school at age four, 35.1% had screen times more than 2 hours daily and 45.3% were exposed to passive smoking. At age four, seroprevalence to Polyomaviruses was ranging from 20.8% for HPyV9 to 82.3% for HPyV10 whereas, 52.5%, 25.8% and 3.6% were EBV, CMV and HSV-1 seropositive respectively. HSV-2 showed very low seroprevalence (1.5%) and was not considered further in the analysis as single viral infection. The prevalence of overweight and obesity was 14.1% and 6.7% at four years and increased to 21.8% and 9.6% at six years, respectively.

Significant differences were observed in child's BMI status at age four and six by BKPyV serostatus (table 2); BKPyV seropositive children were less likely to be obese at age four and six (4.96% and 7.16%) versus seronegative children (12.27% and 17.14%). Also, KIPyV seropositive children were less likely to be obese (3.96%) than seronegative children (8.85%) at age four. On the other hand, CMV seropositive children were more likely to be overweight (19.77%) versus seronegative children (12.16%) at age four (table 2).

Table 1. Characteristics of the study population, the Rhea birth cohort.

Characteristic	N	%
Age four		
Maternal origin		
Greek	643	93.6
Non-Greek	44	6.4
Maternal educational level		
High	220	32.6
Medium	344	51.0
Low	111	16.4
Maternal pre-pregnancy BMI status		
Underweight	23	3.5
Normal	402	61.9
Overweight	141	21.7
Obese	84	12.9
Mode of delivery		
Caesarean section	335	49.6
Vaginal	340	50.4
Preterm birth		
Yes	74	11.0
No	596	89.0
Child's sex		
Female	327	47.6
Male	360	52.4
Breastfeeding duration		
>6 months	148	22.9
1-6 months	410	63.6
Never	87	13.5
Child having older siblings		
Yes	383	55.8
No	303	44.1
School attendance		
Yes	580	84.5
No	106	15.5
Area of residency		
Urban	479	69.7
Rural	208	30.3
Screen-time per day		
30min or less	191	28.0
1 hour	252	36.9
2 hours or more	240	35.1
Passive smoke exposure		
Yes	303	45.3
No	366	54.7
Polyomaviruses seropositivity		
BKPyV	526	76.3%
JCPyV	235	34.1%
KIPyV	304	44.1%
WUPyV	521	75.6%

MCPyV	470	68.3%
HPyV6	424	61.5%
HPyV7	157	22.8%
TSPyV	354	51.4%
HPyV9	144	20.9%
HPyV10	567	82.3%
Polyomaviruses burden		
≤ 3 polyomaviruses	117	17.0%
4-7 polyomaviruses	476	62.2%
≥ 8 polyomaviruses	95	13.8%
Herpesviruses seropositivity		
EBV	362	52.5%
CMV	178	25.8%
HSV-1	25	3.6%
HSV-2	10	1.5%
Herpesviruses burden		
0 herpesviruses	258	37.5%
1 herpesvirus	293	42.5%
2-3 herpesviruses	138	20.0%
Child's BMI status		
Obese	46	6.7%
Overweight	97	14.1%
Normal/Underweight	544	79.2%
Age six		
Child's BMI status		
Obese	42	9.6%
Overweight	96	21.8%
Normal/Underweight	302	68.6%
Screen-time per day		
30min or less	37	8.5%
1 hour	279	63.8%
2 hours or more	121	27.7%

Abbreviations: BMI, body mass index

Tabe 2. Child's BMI status at age four (n=687) and six (n=440) by Polyomaviruses and Herpesviruses serostatus at age four, the Rhea birth cohort.

	Age four				Age six			
	Normal Underweight	Overweight	Obese	P-value	Normal Underweight	Overweight	Obese	P-value
Polyomaviruses								
BKPyV +	80.53%	14.5%	4.96%	0.005	72.54%	20.30%	7.16%	0.002
-	74.85%	12.88%	12.27%		56.19%	26.67%	17.14%	
JCPyV +	79.9%	12.39%	7.69%	0.522	73.61%	19.44%	6.94%	0.242
-	78.81%	15.01%	6.18%		66.22%	22.97%	10.81%	
KIPyV +	80.86%	15.18%	3.96%	0.035	71.29%	21.78%	6.93%	0.217
-	77.86%	13.28%	8.85%		66.39%	21.85%	11.76%	
WUPyV+	79.19%	15.03%	5.78%	0.142	70.88%	20.29%	8.82%	0.173
-	79.17%	11.31%	9.52%		61.0%	27.0%	12.0%	
MCPyV+	80.34%	13.68%	5.98%	0.442	70.0%	20.0%	10.0%	0.371
-	76.61%	13.68%	5.98%		65.47%	25.90%	8.63%	
HPyV6 +	78.67%	14.22%	7.11%	0.851	65.67%	24.25%	10.07%	0.230
-	80.0%	13.96%	6.04%		73.26%	18.02%	8.72%	
HPyV7 +	76.92%	16.03%	7.05%	0.709	66.67%	20.59%	12.75%	0.451
-	79.85%	13.56%	6.59%		69.23%	22.19%	8.58%	
TSPyV +	77.27%	14.20%	8.52%	0.140	69.06%	20.63%	10.31%	0.746
-	81.19%	14.03%	4.78%		68.20%	23.04%	8.76%	
HPyV9 +	72.73%	19.58%	7.69%	0.081	63.16%	24.21%	12.63%	0.358
-	80.88%	12.68%	6.43%		70.14%	21.16%	8.70%	
HPyV10+	79.12%	14.87%	6.02%	0.178	69.53%	21.05%	9.42%	0.668
-	79.51%	14.87%	9.84%		64.56%	25.32%	10.13%	
Herpesviruses								
EBV +	77.50%	14.44%	8.06%	0.299	68.4%	23.38%	8.23%	0.488

	-	81.04%	13.76%	5.20%		68.90%	20.10%	11.0%	
CMV	+	74.01%	19.77%	6.21%	0.043	64.6%	23.89%	11.50%	0.529
	-	80.98%	12.16%	6.86%		70.03%	21.10%	8.87%	
HSV-1	+	76.0%	16.0%	8.0%	0.921	64.71%	23.53%	11.76%	0.926
	-	79.31%	14.05%	6.65%		68.79%	21.75%	9.46%	

P-values based on chi-squared test and Fisher's exact test for sparse data.

"+" denotes seropositivity, "-" denotes seronegativity

Obesity outcomes

Figure 2, depicts the association (β, 95% CI) of Polyomaviruses and Herpesviruses seropositivity at age four with BMI SD-score, waist circumference and sum of skinfolds at age four and six after adjusting for potential confounders. We also explored associations with body fat percentage at age six. BKPyV seropositive children had lower BMI SD-score at age four [-0.21 (95% CI:-0.39, -0.03)] and six [-0.27 (95% CI:-0.48, -0.05)] and had constantly lower BMI SD-score across the study period (Figure 3) compared with seronegative children. Moreover, BKPyV seropositivity was associated with lower waist circumference at age four [-1.12cm (95% CI:-2.10, -0.15)] and six [-1.73 cm (95% CI:-3.33, -0.12)], sum of skinfolds at age four [-2.97mm (95% CI:-5.70, -0.24)] and body fat percentage at age six [-1.85 (95% CI:-3.42, -0.28)]. On the other hand, CMV seropositivity was associated with higher BMI SD-score at age four [0.28 (95% CI: 0.11, 0.45)] and six [0.24 (95% CI: 0.03, 0.45)] as well as between follow-ups (Figure 3) and higher sum of skinfolds at age six [4.75mm (95% CI:0.67, 8.83)].

Table 3 presents associations between the Polyomaviruses and Herpesviruses burden at age four and BMI SD-score, waist circumference, sum of skinfolds and body fat percentage at age four and six. After adjusting for potential confounders, children with "2-3 Herpesviruses infections" versus those with "0 Herpesviruses infections" had higher BMI SD-score [0.32, (95% CI: 0.12, 0.53)], waist circumference [1.22cm, (95% CI: 0.13, 2.31)] and sum of skinfolds [3.26mm, (95% CI: 0.18, 6.35)] at age four. Moreover, children with "2-3 Herpesviruses infections" remained in higher BMI SD-scores from four to six years of age follow-up (Figure 3). We also identified a dose-response association between Herpesviruses burden and BMI SD-score (P value for trend:0.001), waist circumference (P value for trend:0.023) and sum of skinfolds (P value for trend:0.031) at age four. Although not statistically significant, similar associations were observed at age six. Because results regarding Herpesviruses burden might reflect the aforementioned association with CMV seropositivity, we repeated the analysis among CMV seronegative children and associations were similar (data not shown). Polyomaviruses burden was not found to associate with obesity indices.

Figure 2. *Adjusted associations (β, 95% CI) of Polyomaviruses and Herpesviruses seropositivity at age four with BMI SD-score, waist circumference and sum of skinfolds at age four and six, the Rhea birth cohort. All models are linear regressions adjusted for maternal age, origin, education, pre-pregnancy BMI, child having older siblings, breastfeeding, screen-time spent per day at outcome assessment, school attendance (only for models of age four), child's sex and age at outcome assessment.*

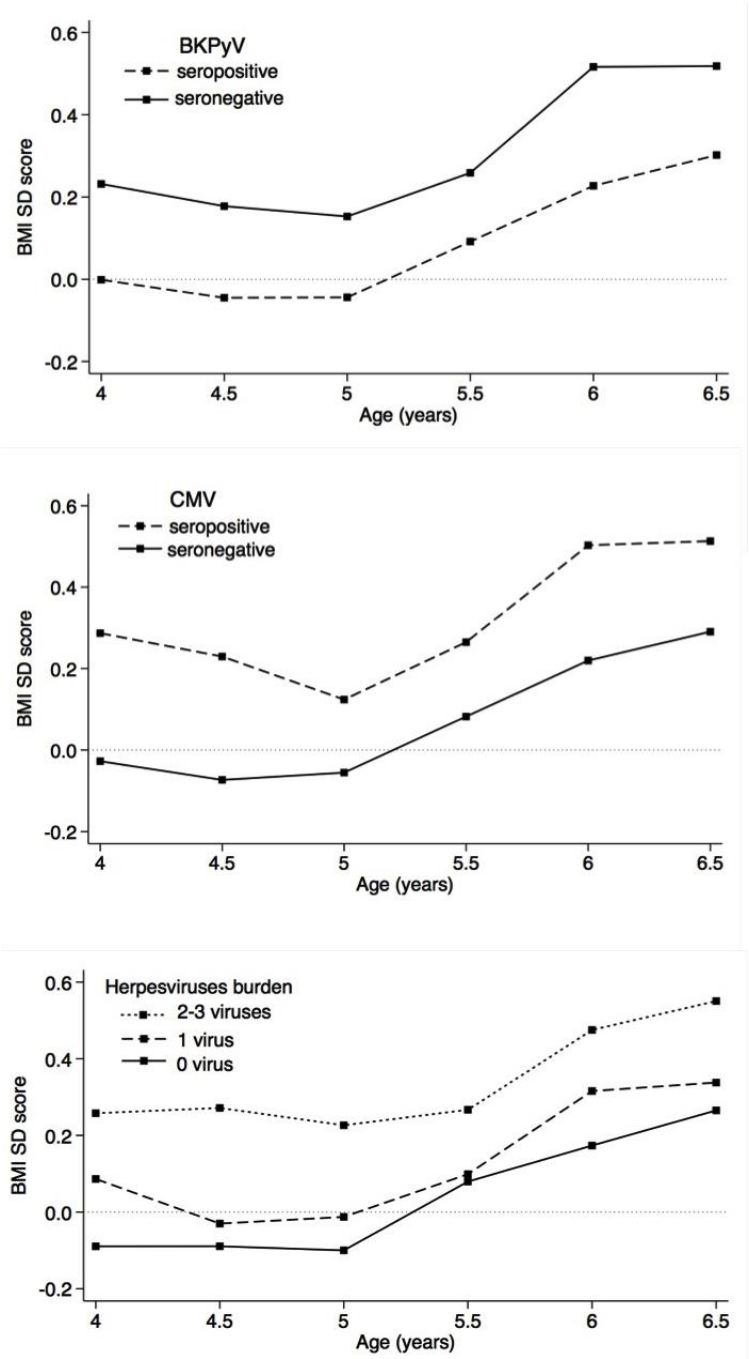


Figure 3*. Body mass index (BMI) trajectories from four to six years of age follow-up according to the serological status to BKPyV, cytomegalovirus (CMV) and each category of herpesviruses burden after adjusting for maternal age, origin, education, pre-pregnancy BMI, child having older siblings, breastfeeding and screen-time spent per day at outcome assessment.*

Table 3. Adjusted associations (β, 95% CI) between Polyomaviruses and Herpesviruses burden and obesity outcomes at four and six years of age, the Rhea birth cohort.

	Polyomaviruses burden[a]						Herpesviruses burden[b]					
	Medium (4-7 viruses)			High (≥8 viruses)			1 virus			2-3 viruses		
	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value	β	95%CI	P-value
BMI standard deviation score												
4 years	-0.16	[-0.37, 0.05]	0.128	-0.08	[0.35, 0.19]	0.582	0.16	[0.003, 0.32]	0.046	0.32	[0.12, 0.53]	0.002
6 years	-0.15	[-0.39, 0.09]	0.218	-0.17	[-0.49, 0.15]	0.301	0.07	[-0.13, 0.26]	0.514	0.19	[-0.05, 0.45]	0.121
Waist circumference, cm												
4 years	-0.29	[-1.39, 0.82]	0.614	0.02	[-1.46, 1.49]	0.984	0.65	[-0.21, 1.51]	0.141	1.22	[0.13, 2.31]	0.028
6 years	0.15	[-1.67, 1.97]	0.869	-0.39	[-2.79, 1.99]	0.746	0.17	[-1.30, 1.63]	0.82	0.44	[-1.43, 2.31]	0.642
Sum of skinfolds, mm												
4 years	0.21	[-2.89, 3.30]	0.895	-0.43	[-4.62, 3.75]	0.84	1.83	[-0.59, 4.26]	0.139	3.26	[0.18, 6.35]	0.038
6 years	-3.07	[-7.90, 1.76]	0.212	-5.25	[-11.68,1.17]	0.109	1.18	[-2.76, 5.13]	0.55	1.67	[-3.29, 6.64]	0.508
Body fat percentage,												
6 years	-1.14	[-2.91, 0.63]	0.206	-1.82	[-4.15, 0.51]	0.126	0.06	[-1.37, 1.50]	0.929	0.61	[-1.22, 2.45]	0.511

Abbreviations: CI, confidence interval; BMI, body mass index

All models are linear regression adjusted for maternal age, origin, education, pre-pregnancy BMI, child having older siblings, breastfeeding, screen-time spent per day at outcome asessment, school attendance (only for models of age four), child's sex and age at outcome assessment.

[a] Reference group is that of low polyomaviruses burden (seropositivity to ≤3 polyomaviruses)

[b] Reference group is that of 0 herpesviruses burden

Metabolic traits

Results regarding the association of single Polyomaviruses and Herpesviruses infections with metabolic traits are presented in table 4. At age four, BKPyV seropositivity was associated with lower leptin levels (ratio of geometric means, 0.83; 95% CI: 0.70, 0.98). Also, WUPyV seropositivity was associated with lower HDL (ratio of geometric means, 0.95; 95% CI: 0.91, 0.99) and adiponectin levels (ratio of geometric means 0.82; 95% CI: 0.73, 0.93) at age four. Neither Polyomaviruses nor Herpesviruses burden at age four were associated with metabolic outcomes at four and six years of age (data not shown).

Sensitivity analyses

When we repeated analyses adjusting also for antibiotic use during the 4th year of life, results were very similar to those presented in the main analysis. After adjusting for child's BMI, associations with metabolic traits were attenuated but remained statistically significant (data not shown). We saw no evidence for a multiplicative interaction of virus seropositivity with child sex, breastfeeding duration and maternal education.

Table 4. Adjusted associations (GM ratio, 95% CI) between seropositivity to polyomaviruses and herpesviruses and metabolic outcomes at four and six years of age, the Rhea birth cohort.

	Total cholesterol			HDL			Leptin			Adiponectin		
Seropositivity	GM ratio	95%CI	P-value	GM ratio	95%CI	P-value	GM ratio	95%CI	P-value	GM ratio	95%CI	P-value
Polyomaviruses												
BKPyV												
4 years	0.98	[0.95,1.02]	0.265	0.97	[0.92,1.01]	0.156	0.83	[0.70,0.98]	0.031	0.92	[0.81,1.04]	0.186
6 years	0.99	[0.96,1.03]	0.758	1.04	[0.99,1.09]	0.147						
JCPyV												
4 years	1.02	[0.99,1.05]	0.275	1.02	[0.98,1.06]	0.330	1.03	[0.89,1.19]	0.673	1.01	[0.91,1.12]	0.828
6 years	1.03	[1.00,1.06]	0.068	1.03	[0.99,1.08]	0.159						
KIPyV												
4 years	1.00	[0.97,1.03]	0.973	1.00	[0.96,1.04]	0.857	0.89	[0.78,1.02]	0.094	0.95	[0.86,1.05]	0.348
6 years	0.99	[0.96,1.02]	0.559	1.03	[0.99,1.07]	0.212						
WUPyV												
4 years	0.99	[0.95,1.02]	0.389	0.95	[0.91,0.99]	0.024	0.99	[0.84,1.16]	0.89	0.82	[0.73,0.93]	0.002
6 years	0.99	[0.95,1.03]	0.541	1.00	[0.95,1.05]	0.934						
HPyV6												
4 years	1.00	[0.97,1.03]	0.978	1.01	[0.97,1.05]	0.745	1.03	[0.89,1.18]	0.725	1.00	[0.90,1.10]	0.951
6 years	0.98	[0.95,1.01]	0.248	1.02	[0.97,1.06]	0.482						
HPyV7												
4 years	1.02	[0.99,1.06]	0.229	1.02	[0.97,1.06]	0.468	0.97	[0.83,1.14]	0.754	0.92	[0.82,1.04]	0.170
6 years	1.05	[1.01,1.08]	0.10	1.03	[0.98,1.08]	0.201						
TSPyV												
4 years	0.99	[0.96,1.02]	0.400	0.97	[0.93,1.00]	0.075	1.04	[0.90,1.19]	0.615	0.98	[0.88,1.08]	0.669
6 years	1.01	[0.98,1.04]	0.391	1.03	[0.99,1.07]	0.152						

MCPyV												
4 years	0.98	[0.95,1.01]	0.275	0.97	[0.93,1.01]	0.194	0.97	[0.84,1.13]	0.702	1.07	[0.96,1.20]	0.206
6 years	0.99	[0.96,1.03]	0.712	1.00	[0.95,1.04]	0.864						
HPyV9												
4 years	1.00	[0.97,1.04]	0.881	1.00	[0.95,1.05]	0.917	1.13	[0.96,1.34]	0.151	1.03	[0.91,1.17]	0.640
6 years	1.01	[0.97,1.04]	0.764	1.00	[0.95,1.06]	0.874						
HPyV10												
4 years	0.99	[0.96,1.03]	0.789	0.99	[0.94,1.04]	0.726	0.97	[0.81,1.16]	0.772	0.92	[0.80,1.05]	0.195
6 years	0.99	[0.95,1.03]	0.469	1.02	[0.96,1.07]	0.590						
Herpesviruses												
EBV												
4 years	1.00	[0.97,1.02]	0.760	1.02	[0.98,1.06]	0.412	1.05	[0.92,1.21]	0.438	0.95	[0.86,1.05]	0.275
6 years	0.99	[0.96,1.02]	0.393	1.02	[0.98,1.06]	0.383						
CMV												
4 years	0.99	[0.96,1.02]	0.615	0.99	[0.95,1.04]	0.789	1.11	[0.95,1.29]	0.195	0.93	[0.83,1.04]	0.193
6 years	0.98	[0.95,1.02]	0.264	1.00	[0.95,1.05]	0.986						
HSV-1												
4 years	1.01	[0.93,1.10]	0.822	1.03	[0.92,1.15]	0.588	0.97	[0.66,1.43]	0.870	0.96	[0.72,1.29]	0.800
6 years	1.02	[0.95,1.11]	0.551	1.09	[0.97,1.21]	0.139						

Abbreviations: GM ratio, ratio of geometric means; HDL, high density lipoprotein; CI, confidence interval

All models are linear regressions adjusted for maternal age, origin, education, pre-pregnancy BMI, child having older siblings, breastfeeding, screen-time spent per day at outcome asessment, child attending school (only for models of age 4), child's sex and age at outcome assessment.

Discussion

In this longitudinal population-based birth cohort study, we explored the association between common viral infections of early childhood with obesity indices and metabolic traits as assessed at two consecutive follow-ups. We observed that, CMV seropositive versus seronegative children had higher BMI and sum of skinfolds values. Moreover, a higher Herpesviruses burden -with CMV, EBV, HSV-1 and HSV-2 contributing to the score- was also associated with higher BMI, waist circumference and sum of skinfolds. On the other hand, BKPyV seropositivity was associated with lower obesity indices and leptin levels. These findings were robust against potential confounding, as shown by multiple sensitivity analyses, nonetheless inferences should be cautious given the exploratory character of this study.

This is the first study showing a positive association between CMV infection and obesity indices in children, including BMI and measures of subcutaneous fatness such as skinfolds. It has been suggested that adipose tissue may expand in response to certain infections due to its ability to act as an immunological tissue (265,266). In vitro and animal studies show that CMV is likely to induce obesity because it infects adipocytes and induces increased IL-6 production (267–269). Previous epidemiological studies have failed to show an association but these are limited, are exclusively based on adult population samples and mostly use BMI and no other obesity indices (270–273). It is likely that studies in childhood might be more appropriate to uncover such an association because other risk factors for obesity may have yet minimal effect whereas timing of exposure might also be relevant. Regarding metabolic traits, we did not detect an association with CMV. Our null findings are contrary to those of several prior studies in adults which report altered lipid and glucose metabolism and higher prevalence of cardiovascular diseases among participants with antibodies to CMV (162–166). A reason for this disparity could be that there is a need for long-chronic infection to induce these effects whereas in the present study, participants were young, follow-up time was relatively short and later effects are not yet explored.

Although no other Herpesvirus -apart from CMV- was individually associated with obesity indices, a higher Herpesviruses burden was associated with higher obesity indices. These findings were not driven by CMV because they were also evident among CMV seronegative children. For Polyomaviruses burden, null findings were revealed. Several studies in adults suggest that a higher pathogen burden -with

different pathogens contributing in each study to the score- is a risk factor for obesity and impaired metabolic health (259–263), but no such literature was identified in childhood. Interestingly, a large study in the USA provides evidence that childhood infectious burden (HSV-1, CMV and hepatitis A), and not adult infectious burden (HSV2, HHV8 and hepatitis B or C) predisposes females specifically to central adiposity in adulthood (264).

Among Polyomaviruses, BKPyV showed a negative association with obesity indices and metabolic traits. BKPyV is an ubiquitous virus -showing seroprevalence close to 100% in adult life (216)-, is acquired largely in early childhood, persists lifelong in the reno-urinary tract and only rarely causes disease (276). Existing knowledge does not allow us to suggest for a potential mechanism explaining why BKPyV seropositive children displayed lower BMI and other obesity related indices in early childhood. It could be that a common predisposing factor for both conditions may be invoked rather than a true causal association. Residual confounding is thus a plausible explanation for these associations. Adjustment included major potential confounders but there may be other unidentified factors not included in our models. For example, associations could be confounded by an immune dysfunction related to both immune response to infection and predisposition to obesity, as BKPyV infection was determined based on antibody detection.

Strengths and limitations

Strengths include the population-based prospective design and the detailed anthropometric and metabolic traits measured in early childhood. We were able to assess a number of confounding factors that included several sociodemographic characteristics and child lifestyle characteristics. Another strength is multiplex serology used to establish antibody profiles for multiple antigens in only a low serum volume, reducing selection bias. Misclassification of exposure estimates is always of concern, but it is not expected to appreciably impact our findings based on previous sensitivity analysis using different cut-off definitions. Although our analysis in intriguing, we lack of direct evidence regarding the obesogenic effect of most viruses assessed in the present study but we considered our analysis exploratory. While, as hypothesized in this manuscript, certain common infections may lead to obesity, an alternate theory suggests that obesity may impair host defences and predisposes to infection (190,277). This last theory is based largely on studies assessing hospital acquired infections (278) and serious complications of common infections (e.g.

H1N1) (279) which use cross-sectional data, thus the temporal direction of the relationship is unclear. Although our prospective design reduced the possibility of reverse-causality, inferences should be cautious. For example, we lack knowledge on how many children seroconverted from four to six years, which might have affected the observed associations at age six. Importantly, the analysis on repeated measures of BMI from four to six years of age provides evidence for effects on early life BMI trajectories and not only on specific time points. Selection bias due to loss to follow-up is always of concern in cohort studies. Among the children with available serology at age four, 63% had available outcome data at age six. Children who were also assessed at age six tended to be of mothers with higher education (37.6% versus 23.8%) and to reside in urban areas (72.5% versus 64.9%). No other significant differences in baseline characteristics, Polyomaviruses and Herpesviruses seroprevalence at age four were revealed (data not shown). This may limit the generalizability of our results, but we would not expect comparisons of polyomaviruses and herpesviruses seropositivity with obesity indices and metabolic traits to have been biased. Chance findings are always of concern when multiple comparisons are performed but because the outcomes were not independent and our analysis was considered exploratory, correction for multiple testing could be very restrictive (181). Nonetheless, after accounting for multiple testing by controlling the false discovery rate at 0.25 based on the method by Benjamini and Hochberg, we still observed statistically significant associations for CMV seropositivity and Herpesviruses burden with BMI at age four. Overall, we tried to draw our conclusions on the basis of the consistency of results.

Conclusion

In conclusion, this is the first population based cohort study exploring the association between Polyomaviruses and Herpesviruses seropositivity with obesity indices and metabolic traits in early childhood. While acknowledging several limitations, the data indicate that CMV is a candidate virus for inducing obesity in childhood. A higher Herpesviruses burden was also associated with higher obesity indices, suggesting obesity effects associated with this virus family. Interestingly, lower obesity indices were observed among BKPyV seropositive versus seronegative children, raising further questions for future research. Analyses should be repeated in later ages, in different populations and settings in order to further explore the observed associations.

4. General discussion

This thesis describes in detail acquisition patterns of Polyomaviruses, Herpesviruses and H.pylori, all of which represent common infections of childhood, and the drivers of their infectious dynamics. This is particularly important because acquisition of such common infections co-occurs and likely jointly affects health. An additional value of this thesis is the identification of novel associations between Polyomaviruses, Herpesviruses and H.pylori infection with health outcomes in childhood. Analysis of common infectious agents in early childhood is unique in the context of European birth cohorts. This section provides a summary of the thesis findings and a broader interpretation of the entire research study.

4.1. What this study adds

- Polyomaviruses are ubiquitous in our population. This is the first study with detailed seroepidemiological data on ten Polyomaviruses. We confirmed that primary infection with Polyomaviruses occurs largely in childhood but different infectious dynamics exist. Thus, BKPyV and HPyV10 displayed high seroposotivity rates at age four showing at the same time a significant force of infection between three and four years of age. On the other hand, HPyV7 and HPyV9 displayed much lower seropositivity rates which remained stable during three and four years of age. It is uncertain whether children will achieve in their adult life similar seropositivity rates as their mothers had, or changes in factors that determine infectious dynamics may result in lower seroprevalence rates.

- Although, it is unclear how the Polyomaviruses are spread throughout the population, we identified that seroprevalence of Polyomaviruses in childhood differ according to individual characteristics. For example, children with lower socioeconomic position were less likely to be Polyomaviruses seropositive. At the same time certain inside and outside family factors were differentially associated with seropositivity to each Polyomavirus. These factors were identified for the first time and can be used to infer on potential routes of transmission of Polyomaviruses which remains largely unknown. For example, vaginal delivery was associated with HPyV10 seroprevalence suggesting that ingestion of the virus during delivery may explain this association given that HPyV10 resides in the gastrointestinal tract.

- As expected, children by age four were commonly seropositive to EBV and CMV. Very low seroprevalences were observed for HSV-1 and HSV-2 while HHV-8 was negligible in our population. These data follow current seroprevalence estimates from other developed counties with the exception of HSV-1 which showed much lower seropositivity rates in our study compared to others. We confirmed that younger age of daycare attendance was the strongest predictor of EBV seroprevalence while maternal seropositivity, breastfeeding, house crowding and younger age of daycare attendance importantly contributed to CMV seroprevalence. These data are important in estimating disease burden related to those infections and identify groups most at risk.

- Children who have acquired more Polyomaviruses up to age four presented fewer symptoms of inattention and hyperactivity at the same age. Due to the cross-sectional design, it could also be that children presenting more ADHD-related symptomatology are less likely to be exposed to Polyomaviruses in early life. Only seropositivity to BKPyV was associated specifically with more inattention difficulties. Herpesviruses were not found to associate with neurodevelopmental outcomes. These data suggest that infections with common and less virulent viruses may interact with neurodevelopmental processes during the first years of life. Future studies examining the biological pathways by which viruses influence neurodevelopmental processes in early life are warranted.

- This is the first study indicating that CMV is a candidate virus for inducing obesity in childhood. In our study CMV seropositive versus seronegative children had higher BMI and sum of skinfolds values. A higher Herpesviruses burden -with CMV, EBV, HSV-1 and HSV-2 contributing to the score- was also associated with higher obesity indices suggesting obesity induced effects for this virus family. On the other hand, BKPyV seropositivity was associated with lower obesity indices and leptin levels. Further studies are needed to replicate these findings and examine potential underlying biological mechanisms.

- We provide current epidemiological data on H.pylori prevalence in preschool children and in pregnant women in Greece. We found that H.pylori is common among women of childbearing age in Greece, especially among women of non-Greek origin; these women may constitute a target group for screening and

eradication to prevent H. pylori associated diseases. On the other hand, only a small proportion of the children were tested seropositive at age four in accordance with studies in developed countries showing decreasing colonization rates in subsequent generations. The H.pylori seroprevalence in early childhood may serve as a predictor for the future incidence of H.pylori associated diseases in the population such as gastric cancer. For this reason, monitoring of the epidemiologic pattern of H. pylori prevalence is relevant both from clinical as well as from a public health perspective.

- We found that H.pylori seropositive mothers were more likely to be underweight according to their pre-pregnancy BMI and gain inadequate weight during their pregnancy. Although these are preliminary data and we should investigate strain specific factors associated with these effects, they are particularly important given the limited studies in the field. Studies in non-pregnant population show that eradication of the bacterium is associated with weight gain, but whether similar effects are observed in pregnant women remains to be seen.

- We reported for the first time that maternal H.pylori seropositivity during pregnancy was associated with unfavourable neurodevelopmental outcomes in children at age four. It is likely that changes in the periconceptional micronutrient environment following a H.pylori infection might mediate at least in part such an association. H.pylori seropositive children performed also worst in neurodevelopmental assessment compared to their seronegative counterparts although none of the associations were statistically significant. These findings open new opportunities for primary prevention of neurodevelopmental diseases in childhood through screening for a H. pylori infection before/during pregnancy and in childhood and eradication of the bacterium. Further studies are needed in this direction.

4.2. Strengths and Weaknesses

This section provides a general description of strengths and limitations of the present thesis that have been extensively discussed in the results section.

The main strength of this study is that is uses data from a population based sample of healthy children and their mothers during pregnancy from different socio-economic

background enrolled in the Rhea cohort. Due to the longitudinal design, we took advantage of repeated serum samples and epidemiological data collected from birth to childhood. In the Rhea cohort, clinical phenotyping assessment was conducted by trained and expert staff for the mother and for the child while standardized questionnaires and extensive biological collection were used to enrich the data collection. Importantly, we used multiplex serology which allowed us to simultaneously detect immune response to multiple pathogens in only a small blood volume in a few days under almost identical conditions for all samples. Thus we had available seroresponses to 37 antigens in 1418 samples.

Weaknesses include the loss to follow-up over time, which is an important issue in most prospective longitudinal studies. At the time of serological testing, multiplex serology was not available for the most recently discovered human Polyomaviruses (STLPyV, HPyV12, LIPyV) and the human herpesviruses 6, 7, and varicella-zoster virus. Despite our novel findings on the association of common infections with neurodevelopmental and obesity outcomes in childhood, our analyses were largely exploratory. Since we examined 37 different antibodies for association with health outcomes, some associations were expected to occur by chance and the findings were interpreted with caution. Lastly we lack of statistical power to study rare outcomes such as cancer.

4.3. Future research

Studying infectious disease epidemiology and infectious determinants of health is challenging. Our study represents a first step in this direction and shows new areas for research in birth cohort studies. Still there are many questions to be answered.

What will happen in later ages in our population?

This is an important question to be answered. Will acquisition occur in the following years? Which will be the determinants and sources of infection in later ages ? Can we identify which individuals lack exposure to such common infections until later in life? This is particularly important because EBV acquired during childhood is often subclinical but, when acquired in adolescent or adult years it is associated with higher risk for infectious mononucleosis. For HSV-1 transmission in childhood results in oral infections, but lack of exposure until adolescent/adult years is associated with an increased risk for genital infections. Thus, age of infection is important in the

outcome of common infections. Whether delayed infection to Polyomaviruses or Herpesviruses is associated with other heath outcomes such as levels of inflammatory markers is not known.

Are there differences in acquisition of common infections in other population and settings in Europe?

Acquisition of common infections of childhood may differ not only within a population but also between populations. Previous European studies have shown North to South and East to West differences in acquisition of H.pylori, HHV-8 and other Herpesviruses. Interestingly, in our population, we observed a strong socioeconomic position pattern in the acquisition of Polyomaviruses in early life. This was a unique finding and should be confirmed (or not) in other populations. There is a strong relationship between socioeconomic status and health outcomes. Exposure and susceptibility to infections may be one way socioeconomic status affects long-term health but social patterning of infections may not be present in all settings.

Is there an association between H.pylori infection and obesity?

Several cross-sectional associations of H.pylori with obesity are reported although some studies show null findings (190). Interestingly, a number of studies show an increase in weight after eradication of the bacterium. Because H.pylori affects gastric hormones that have a role in energy homeostasis, such as leptin and ghrelin, a link between its disappearance and the increasing prevalence of metabolic syndrome, type II diabetes and obesity has also been postulated (280,281). Moreover, H.pylori might be considered an "indicator organism" for changing human microecology and disease risk.

In the descriptive analysis of our data we obeseved a higher H.pylori seroprevalence among underweight mothers based on their pre-pregnancy BMI and among women who gained inadequate weight during their pregnancy. Similarly, H.pylori was more common among underweight children at age four but associations were not significant probably due to lack of statistical power. Our intention is to examine these associations in more detail using also the serological data of the twelve H.pylori specific proteins. For example, it is not known whether H.pylori infection is associated with particular patterns of gestational weight gain, with metabolic

disturbances during pregnancy and with maternal weight retention after pregnancy. Potential mediation effects by nausea and vomiting could also be tested.

Is there an association between Polyomaviruses, Herpesviruses and H.pylori with asthma and allergic conditions in childhood?

Conflicting results exist on the assciation of viral infections with asthma, atopic dermatitis and allergic rhinitis in childhood. Several studies report that viral infections of the respiratory tract (e.g. respiratory syncytial virus) may enhance the development of asthma. On the other hand the protective effect from early start in day-care has generally been interpreted as an effect of virus exposure. Viral infections other than lower respiratory tract infections early in life have been suspected to induce protective effects against asthma, atopic dermatitis and allergic rhinitis (11). Among Polyomaviruses, KIPyV and WUPyV have been indentified in the respiratory tract but they have not been linked with certain symptoms and the link between these Polyomaviruses and acute respiratory diseases remains speculative (282). Hypothesis that EBV and CMV play a role in the pathogenesis of allergic diseases in children has not been supported so far (283,284). Some studies have reported a negative association between H.pylori and asthma and allergic conditions, but data are inconsistent and there are only few studies in childhood populations (94,95).

Can we combine our efforts in birth cohorts?

The value of pooling data from two or more cohorts to address research questions on environmental or lifestyle exposures has been illustrated in previous studies. Pooling data from different cohorts will provide us the statistical power to study the effect of less common viral infections (e.g. HSV-1, HSV-2), explore rare categorical outcomes (e.g. preeclampsia) or extreme values for continuous traits as well as potential modifying effects.

Lastly, we should note that we are conducting an analysis of a project entitled "Seroprevalence of human Polyomaviruses, Herpesviruses and Helicobacter pylori in children at the time of tumour diagnosis in the paediatric haematology oncology department of the University Hospital in Crete". This study includes serological data and baseline information on 90 children which have been diagnosed with cancer (52 children with leukemia and 38 with solid tumors) during 2000 and 2013 and referred to the paediatric haematology oncology department of the University Hospital in

Crete. Results from this study aim to provide further insights into the oncogenic potential of Polyomaviruses, Herpesviruses and H.pylori.

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