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Διδακτορική Διατριβή

Ο ρόλος της φλεγμονής και της πρώιμης έκθεσης σε επίμονους οργανικούς ρύπους στη νευροανάπτυξη παιδιών στην ηλικία των 4, 6 και 11 ετών - Μελέτη Μητέρας Παιδιού Κρήτης, Μελέτη ΡΕΑ

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The role of inflammation and early exposure to Persistent Organic Pollutants in offspring neurodevelopment at the age of 4, 6 and 11 years - the "Rhea" mother-child cohort study, in Crete

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Περίληψη / Abstract in Greek

Εισαγωγή

Οι Επίμονοι Οργανικοί Ρύποι (ΕΟΡ) θεωρούνται νευροτοξικοί, επηρεάζοντας τη σύνθεση και τη δραστηριότητα των νευροδιαβιβαστών και την οργάνωση του αναπτυσσόμενου εγκεφάλου μέσω μεταβολών στις βασικές διαδικασίες κυτταρικής σηματοδότησης και στην λειτουργία του ενδοκρινικού συστήματος. Μελέτες σε ανθρώπους σε διαχρονικό και/ή συγχρονικό επίπεδο έχουν συσχετίσει την έκθεση σε ΕΟΡ εντός της μήτρας και κατά την πρώιμη παιδική ηλικία με δυσμενείς νευροαναπτυξιακές εκβάσεις, όπως μειωμένη νοημοσύνη, ΔΕΠ-Υ, μειωμένη μνημονική επίδοση, διαταραχές του φάσματος του αυτισμού και άλλα συμπεριφορικά προβλήματα.

Η φλεγμονή είναι ένας σύνθετος φυσικός αμυντικός μηχανισμός των ιστών του σώματος ως απόκριση σε επιβλαβή ερεθίσματα. Αυτή η απόκριση μπορεί να πάψει να είναι προστατευτική για τον οργανισμό και να γίνει επιβλαβής εάν σημειώνεται σε χρόνιο επίπεδο. Οι δείκτες φλεγμονής, όπως οι κυτοκίνες, είναι πρωτεΐνες που εμπλέκονται σε φυσιολογικές πτυχές της νευροανάπτυξης και υπάρχουν ολοένα και αυξανόμενα στοιχεία που τους συνδέουν με σύνθετες, ανώτερης τάξης νευρολογικές λειτουργίες, όπως η γνωστική λειτουργία και η μνήμη. Η μη ισορροπημένη παραγωγή, σηματοδότηση και ρύθμιση των κυτοκινών μπορεί να επιφέρει ποικίλες νευρολογικές επιπτώσεις. Ένα σύνολο ερευνών έχει εξελιχθεί γύρω από το ρόλο των προγεννητικών κυτοκινών ως δεικτών κινδύνου για γνωστική δυσλειτουργία σε ειδικούς και ευάλωτους πληθυσμούς και εκτεταμένα ερευνητικά ευρήματα αναφέρουν ότι τα επίπεδα κυτοκινών στο πλάσμα και/ή τους ορούς μεταβάλλονται στις νευροαναπτυξιακές διαταραχές, π.χ. Διαταραχές Αυτιστικού Φάσματος.

Οι ειδικοί στόχοι της παρούσας διατριβής είναι οι εξής:

• Η διερεύνηση του ρόλου των προγεννητικά υψηλών επιπέδων συγκέντρωσης της έκθεσης σε HCB, DDE και PCBs στη γνωστική ανάπτυξη των παιδιών σε ηλικία 4, 6 και 11 ετών.

• Η διερεύνηση του ρόλου των προγεννητικά υψηλών επιπέδων συγκέντρωσης της έκθεσης σε HCB, DDE και PCBs στις συμπεριφορικές και συναισθηματικές δυσκολίες των παιδιών σε ηλικία 4, 6 και 11 ετών.

• Η διερεύνηση του ρόλου των υψηλών επιπέδων συγκέντρωσης δεικτών φλεγμονής (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) οι οποίοι μετρώνται στον ορό των παιδιών στην ηλικία των 4 ετών στη γνωστική τους ανάπτυξη σε ηλικία 4, 6 και 11 ετών.

• Η διερεύνηση του ρόλου των υψηλών επιπέδων συγκέντρωσης δεικτών φλεγμονής (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) που μετρώνται στον ορό των παιδιών στην ηλικία των 4 ετών στις συμπεριφορικές και συναισθηματικές δυσκολίες τους σε ηλικία 4, 6 και 11 ετών.

Μέθοδος

Η παρούσα μελέτη χρησιμοποιεί δεδομένα από τη Μελέτη Ρέα, μια προοπτική μελέτη κοόρτης μητέρας-παιδιού που ξεκίνησε το 2007 στην Κρήτη. Στη μελέτη προσεγγίστηκαν έγκυες γυναίκες κατά την επίσκεψή τους για την πρώτη εξέταση υπερήχου, περίπου κατά την 12η εβδομάδα κύησης, σε δύο δημόσιες και δύο ιδιωτικές μαιευτικές κλινικές του Ηρακλείου, κατά τη διάρκεια δωδεκάμηνης περιόδου, από 02/2007 έως 02/2008. Εκπαιδευμένες μαίες περιέγραψαν λεπτομερώς τη μελέτη στις έγκυες γυναίκες, έλαβαν γραπτή συγκατάθεση, μέτρησαν ύψος, βάρος και αρτηριακή πίεση, συνέλεξαν δείγματα ούρων και αίματος και συμπλήρωσαν ένα περιεκτικό ερωτηματολόγιο σχετικά με τη διατροφή των συμμετεχουσών, τα κοινωνικοδημογραφικά χαρακτηριστικά και τα χαρακτηριστικά του τρόπου ζωής και την έκθεση σε ποικίλους περιβαλλοντικούς παράγοντες. Τα ζεύγη μητέρας-παιδιού προσκλήθηκαν να συμμετάσχουν σε αξιολογήσεις παρακολούθησης της ανάπτυξης των παιδιών όταν εκείνα ήταν 18 μηνών, 4, 6 και 11 ετών.

POPs: Δείγματα μητρικού ορού κατά την πρώτη προγεννητική επίσκεψη γύρω στον 3ο και 4ο μήνα της εγκυμοσύνης συνελέχθησαν, σε φιαλίδια των 10 ml με γέλη σιλικόνης για διαχωρισμό (Becton Dickinson, UK). Τα φιαλίδια φυγοκεντρήθηκαν εντός 2 ωρών από τη συλλογή αίματος στα 2500 rpm για 10 λεπτά και στη συνέχεια αποθηκεύτηκαν σε δείγματα στους -80°C μέχρι την ανάλυσή τους. Οι αναλύσεις των ΕΟΡ πραγματοποιήθηκαν στο National Institute for Health and Welfare, Chemicals and Health Unit, στο Kuopio της Φινλανδίας με φασματογράφο μάζας τριπλού τετραπόλου αερίου χρωματογράφου Agilent 7000B (GC-MS/MS). Προσδιορίστηκαν οι συγκεντρώσεις στον ορό έξι μεμονωμένων ισομερών PCBs (IUPAC numbers: 118, 138, 153, 156, 170 και 180), HCB και DDE. Όλα τα αποτελέσματα περιγράφηκαν σε ολικό βάρος και εκφράστηκαν σε pg/ml ορού, ενώ στα δείγματα κάτω από το όριο

ποσοτικοποίησης (LOQ) αποδόθηκε η τιμή 0,5×LOQ. Το LOQ ήταν 6 pg/ml για τα PCB118 και PCB156, 10 pg/ml για HCB, DDE, PCB138, PCB153, PCB170, PCB180. Επιλέξαμε να χρησιμοποιήσουμε επίπεδα νωπού βάρους για τους EOP, αλλά προσαρμοσμένα για τα τριγλυκερίδια και τη χοληστερόλη του μητρικού ορού ως συνεχείς μεταβλητές σε όλα τα πολυπαραγοντικά μοντέλα προκειμένου να ελαχιστοποιήσουμε πιθανά σφάλματα μεροληψίας που σχετίζονται με την αυτόματη προσαρμογή των λιπιδίων. Οι EOP αντιμετωπίστηκαν ως κατηγορικές μεταβλητές. Υπολογίσαμε τις συνολικές συγκεντρώσεις PCB αθροίζοντας τις συγκεντρώσεις των 6 μεμονωμένων PCB ισομερών και μελετήσαμε τις σχέσεις που μας ενδιαφέρουν για το άθροισμα των PCBs.

Δείκτες φλεγμονής: Στην αξιολόγηση παρακολούθησης των 4 ετών, συνελέχθησαν δείγματα αίματος με φλεβοκέντηση για κάθε παιδί (10 ml) σε φιαλίδια SST με γέλη σιλικόνης για διαχωρισμό (BD vacutainers, UK), μετά από γραπτή συγκατάθεση των γονέων. Για τη μείωση του πόνου και της δυσφορίας των παιδιών χρησιμοποιήθηκε αναισθητική κρέμα 5% EMLA με σύνθεση 2,5% λιδοκαΐνη και 2,5% πριλοκαΐνη (AstraZeneca, UK). Οι αναλύσεις έλαβαν χώρα στο Εργαστήριο Κλινικής Διατροφής και Επιδημιολογίας Νοσημάτων της Ιατρικής Σχολής του Πανεπιστημίου Κρήτης. Τα δείγματα αίματος φυγοκεντρήθηκαν (Kubota 4000, Ιαπωνία) στα 2500 rpm για 10 λεπτά εντός 2 ωρών μετά τη συλλογή και φυλάχθηκαν στους -80°C έως ότου αναλυθούν. Το Milliplex Map human high sensitivity T cell magnetic bead panel (Cat. # HSTCMAG-28SK) από το Millipore (Billerica, MA) χρησιμοποιήθηκε για τον ταυτόχρονο ποσοτικό προσδιορισμό των IFN-γ IL-1β, IL-6, IL-8, IL- 10, IL-17α, MIP-1α και TNF-α στα υπερκείμενα υγρά. Η αρχή της ανάλυσης βασίζεται στον ποσοτικό προσδιορισμό πολλαπλών βιομορίων χρησιμοποιώντας ταυτόχρονα μαγνητικά σφαιρίδια με κωδικοποίηση φθορισμού (μικροσφαιρίδια MagPlex-C). Τα μικροσφαιρίδια επωάστηκαν με τα δείγματα και στη συνέχεια αφέθηκαν να περάσουν γρήγορα μέσα από συστήματα λέιζερ τα οποία διακρίνουν τα διαφορετικά σύνολα μικροσφαιριδίων και τις φθορίζουσες βαφές στα βιομόρια αναφοράς. Η ευαισθησία της ανάλυσης για κάθε βιομόριο ήταν: 0,3 pg/ml IFN-γ, 0,1 pg/ml IL-1β, 0,1 pg/ml IL-6, 0,1 pg/ml IL-8, 0,6 pg/ml IL- 10, 0,3 pg/ml IL-17α, 0,9 pg/ml MIP-1α και 0,2 pg/ml TNP-α. Χρησιμοποιήσαμε ένα όριο 4 SD βάσει της στατιστικής συνθήκης ότι οι παρατηρήσεις 4 ή περισσότερων SD από τον αναμενόμενο μέσο όρο μπορούν να θεωρηθούν ως «έντονα ακραίες τιμές» και, επομένως, να εξαιρεθούν από τις

στατιστικές αναλύσεις. Η επαναληπτικότητα (%CV) για όλα τα βιομόρια ήταν <5%. Η αναπαραγωγιμότητα (%CV) για IFNγ, IL-6, IL-10 και IL-17α ήταν <20%, για IL-1β, IL-8, MIP-1α και TNF-α ήταν <15%. Οι παραπάνω αναλύσεις πραγματοποιήθηκαν σε έναν αυτοματοποιημένο αναλυτή Luminex 100 συνδεδεμένο με το λογισμικό Luminex xPONENT.

Η αξιολόγηση της γνωστικής ικανότητας στα 4 έτη πραγματοποιήθηκε με την κλίμακα *McCarthy Scales of Children's Abilities (MSCA)*, η οποία αξιολογεί την ανάπτυξη σε 5 τομείς: τον λεκτικό, τον αντιληπτικό, τον αριθμητικό, το μνημονικό και τον κινητικό τομέα. Η αξιολόγηση της γνωστικής ανάπτυξης στα 6 έτη έγινε με την κλίμακα *Raven's Colored Progressive Matrices (RCPM)*, το οποίο εξετάζει τη μη-λεκτική γενική γνωστική ικανότητα, το *Finger Tapping Test (FTT)*, το οποίο μετράει τη κινητική ταχύτητα και το Trail Making Test μέρος Α & μέρος Β (*TMT-Part A* & *TMT-Part B*), που αξιολογούν την οπτική αναζήτηση, την ταχύτητα επεξεργασίας, τη νοητική ευελιξία και τις επιτελικές λειτουργίες. Η γνωστική ικανότητα στα 11 έτη πραγματοποιήθηκε με τη χρήση της κλίμακας *Wechsler Intelligence Scale for Children*, *πέμπτη έκδοση (WISC-V),* η οποία αξιολογεί τη διανοητική λειτουργία σε παιδιά σε πέντε *Κλίμακες Πρωτογενών Δεικτών* (συμπεριλαμβανομένου του Δείκτη Λεκτικής Κατανόησης, του Οπτικοχωρικού Δείκτη, το Δείκτη Ρέοντος Συλλογισμού, του Δείκτη Μνήμης Εργασίας και του Δείκτη Ταχύτητας Επεξεργασίας) και σε τέσσερις *Κλίμακες Βοηθητικών Δεικτών* (συμπεριλαμβανομένου του Δείκτη Ποσοτικού Συλλογισμού, του Μη Λεκτικού Δείκτη, του Δείκτη Γενικής Ικανότητας και του Δείκτη Γνωστικής Επάρκειας). Η συμπεριφορική και συναισθηματική ανάπτυξη αξιολογήθηκε γενικά με ερωτηματολόγια αναφοράς συμπτωμάτων από τους γονείς. Στα 4 έτη χρησιμοποιήθηκε το ερωτηματολόγιο *Attention Deficit Hyperactivity Disorder Test (ADHDT)* και το *Strengths and Difficulties Questionnaire (SDQ)*. Το *ADHDT* βασίζεται στα κριτήρια του DSM-IV και παρέχει 3 δείκτες που αξιολογούν την υπερκινητικότητα, τη διάσπαση προσοχής και την παρορμητικότητα και έναν δείκτη γενικής αξιολόγησης των δυσκολιών ΔΕΠ-Υ. Το *SDQ* αποτελεί ένα σύντομο ερωτηματολόγιο εντοπισμού συμπεριφορικών δεξιοτήτων και δυσκολιών και αξιολογεί τα συναισθηματικά συμπτώματα, τα προβλήματα διαγωγής, την υπερκινητικότητα και τη διάσπαση προσοχής, τα προβλήματα με τους συνομηλίκους και την προκοινωνική συμπεριφορά. Το SDQ παρέχει επίσης δύο σύνθετους δείκτες που αξιολογούν τα εσωτερικευμένα και εξωτερικευμένα προβλήματα. Τα συμπεριφορικά και συναισθηματικά προβλήματα στα 6 και 11 έτη αξιολογήθηκαν με

το ερωτηματολόγιο *Child Behaviour Checklist – Parent Report Form (CBCL)* και με το *Conner's Parent Rating Scale, Revised, Short Form (CPRS-R: S)*. Το *CBCL* περιλαμβάνει 6 κλίμακες που αξιολογούν διαφορετικές διαγνωστικές κατηγορίες του DSM-IV: συναισθηματικά προβλήματα, προβλήματα άγχους, σωματικά προβλήματα, διάσπαση προσοχής/υπερκινητικότητα, εναντιωματικά/προκλητικά προβλήματα, και προβλήματα διαγωγής, και επίσης παρέχει δύο σύνθετους δείκτες που αξιολογούν τα εσωτερικευμένα και εξωτερικευμένα προβλήματα. Το *CPRS-R: S* αξιολογεί εναντιωματικά προβλήματα, γνωστικά προβλήματα/διάσπαση προσοχής και υπερκινητικότητα και παρέχει έναν γενικό δείκτη αξιολόγησης των δυσκολιών ΔΕΠ-Υ.

Γενικευμένα αθροιστικά μοντέλα χρησιμοποιήθηκαν για την αξιολόγηση της γραμμικότητας των υπό μελέτη σχέσεων. Στις αναλύσεις για τη διερεύνηση των σχέσεων μεταξύ των εκθέσεων και των εκβάσεων περιλαμβάνονται περιγραφικές μέθοδοι στατιστικής και πολυπαραγοντικά μοντέλα γραμμικής και λογιστικής παλινδρόμησης ανάλογα με το είδος των μεταβλητών στις υπο εξέταση σχέσεις.

Αποτελέσματα

EOP

- Η υψηλή προγεννητική έκθεση σε HCB σχετίστηκε με i) χαμηλότερη επίδοση σε γνωστική, αντιληπτική, επιτελική λειτουργία και λειτουργία μνήμης εργασίας στην ηλικία των 4 ετών, ii) μη λεκτική ικανότητα, γενική ικανότητα, ταχύτητα επεξεργασίας και νοητική ευελιξία και κινητική ταχύτητα σε ηλικία 6 ετών και iii) χαμηλότερες επιδόσεις σε λειτουργίες οπτικοχωρικής ικανότητας, ρέοντος συλλογισμού, μνήμης εργασίας, ποσοτικού συλλογισμού, μη λεκτικής, γενικής ικανότητας και γνωστικής επάρκειας στην ηλικία των 11 ετών.
- Τα υψηλά επίπεδα PCBs στον ορό της μητέρας σχετίστηκαν με μειωμένη επίδοση των παιδιών σε δοκιμασίες μνήμης εργασίας στην προσχολική ηλικία, με αυξημένο χρόνο απόκρισης στο TMT Μέρος Α και χαμηλότερες βαθμολογίες ταχύτητας στο FFT (μη-κυρίαρχο χέρι) στην ηλικία των 6 ετών.
- Τα υψηλά επίπεδα PCBs προγεννητικά σχετίστηκαν με χαμηλότερες βαθμολογίες σε αρκετές βαθμολογίες δεικτών του WISC-V (μνήμη εργασίας, ταχύτητα επεξεργασίας, ποσοτικός συλλογισμός, μη λεκτική ικανότητα, γενική ικανότητα και γνωστική επάρκεια) στην ηλικία των 11 ετών.

- Στην ηλικία των 11 ετών βρήκαμε στατιστικά σημαντικές θετικές σχέσεις μεταξύ των υψηλών επιπέδων HCB και των βαθμολογιών τριών κλιμάκων συμπεριφοράς στα βασικά μοντέλα. Ωστόσο, στα προσαρμοσμένα μοντέλα οι δείκτες εκτίμησης ήταν αποδυναμωμένοι και ενδέχεται να υφίσταται η ύπαρξη πρόσθετων συγχυτικών παραγόντων που να εμπλέκονται στην κατεύθυνση των αποτελεσμάτων.
- Δεν καταδείχθηκε σχέση μεταξύ των υψηλών επιπέδων EOP προγεννητικά με συμπεριφορικές εκβάσεις σε οποιοδήποτε ηλικιακό χρονικό σημείο, με μοναδική εξαίρεση τη σχέση υψηλών προγεννητικών επιπέδων HCB με προβλήματα μεταξύ συνομηλίκων στο SDQ στα 4 έτη.
- Δεν βρέθηκαν σχέσεις μεταξύ των μητρικών συγκεντρώσεων DDE και των νευροαναπτυξιακών και συμπεριφορικών βαθμολογιών στην ηλικία των 4 και 11 ετών. Αναφορικά με την αξιολόγηση στα 6 έτη, βρέθηκε μόνο μία σχέση υψηλών επιπέδων DDE προγεννητικά με τον αυξημένο χρόνο απόκρισης στο TMT Μέρος Β.
- Δεν βρέθηκαν σημαντικές ενδείξεις για τροποποίηση της επίδρασης ανάλογα με το φύλο του παιδιού, τον δείκτη μάζας σώματος (ΔΜΣ) πριν από την εγκυμοσύνη και την TSH της μητέρας κατά τη διάρκεια της εγκυμοσύνης.

Δείκτες Φλεγμονής

- Τα παιδιά προσχολικής ηλικίας με αυξημένες συγκεντρώσεις TNF-α στον ορό σημείωσαν μειωμένες βαθμολογίες στις δοκιμασίες μνήμης, εύρους μνήμης και μνήμης εργασίας και τα παιδιά προσχολικής ηλικίας με υψηλά επίπεδα IFN-γ στον ορό σημείωσαν χαμηλότερες βαθμολογίες στην κλίμακα του εύρους μνήμης. Τα παιδιά ηλικίας 4 ετών με υψηλά επίπεδα IL-8 εμφάνισαν χαμηλότερες βαθμολογίες σε κλίμακα προκοινωνικής συμπεριφοράς.
- Τα παιδιά με αυξημένα επίπεδα IFN-γ στα 4 έτη εμφάνισαν αυξημένες βαθμολογίες στις κλίμακες εναντιωματικών προβλημάτων και υπερκινητικότητας, καθώς και σε βαθμολογίες εσωτερικευμένων και εξωτερικευμένων προβλημάτων του CBCL στην ηλικία των 6 ετών. Τα υψηλά επίπεδα IL-1β στα 4 έτη σχετίστηκαν με περισσότερα εναντιωματικά συμπτώματα και εξωτερικευμένα προβλήματα στην ηλικία των 6 ετών. Βρήκαμε, επίσης, ότι ο αυξημένος λόγος TNF-a/IL10 σχετιζόταν με χαμηλότερες βαθμολογίες διάσπασης προσοχής και ΔΕΠ-Υ στα 6 έτη.
- Τα παιδιά με υψηλά επίπεδα IL-8 στα 4 έτη παρουσίασαν αυξημένες βαθμολογίες στην ταχύτητα επεξεργασίας. Τα παιδιά με υψηλά επίπεδα λόγου IL6/IL-10 στα 4 έτη παρουσίασαν αυξημένες βαθμολογίες στην οπτικοχωρική επίδοση στα 11 έτη.

Τα υψηλά επίπεδα της αντιφλεγμονώδους IL-10 στα 4 έτη σχετίστηκαν με αυξημένες βαθμολογίες γνωστικής επάρκειας. Ο υψηλός λόγος IL6/IL-10 σχετίστηκε με αυξημένες βαθμολογίες γενικής ικανότητας στα 11 έτη. Βρήκαμε, επίσης, ότι τα παιδιά με υψηλά επίπεδα IFN-γ στα 4 έτη παρουσίασαν αυξημένες βαθμολογίες στις κλίμακες υπερκινητικότητας και ΔΕΠ-Υ. Τα υψηλά επίπεδα IL-17α στα 4 έτη σχετίστηκαν με αυξημένες κλίμακες εσωτερικευμένων προβλημάτων στα 11 έτη.

- Τα αποτελέσματά μας κατέδειξαν μεγαλύτερο κίνδυνο για μειωμένες βαθμολογίες λεκτικής επίδοσης για τα αγόρια με υψηλά επίπεδα συγκέντρωσης IL-17α στον ορό, καθώς και χαμηλότερες κινητικές βαθμολογίες στα 4 έτη για τα αγόρια με υψηλές συγκεντρώσεις IL-6. Βρήκαμε επίσης μεγαλύτερο κίνδυνο για χαμηλότερες βαθμολογίες στις κλίμακες μνήμης και εύρους μνήμης στα 4 χρόνια για τα υπέρβαρα/παχύσαρκα παιδιά με υψηλές συγκεντρώσεις TNF-α στον ορό.

Συμπεράσματα

Συμπερασματικά, η παρούσα διατριβή υποστηρίζει και επεκτείνει την προηγούμενη γνώση ότι η προγεννητική έκθεση σε υψηλά επίπεδα EOP μπορεί να σχετιστεί με μειωμένη γνωστική ανάπτυξη παιδιών. Στην πραγματικότητα, αυτή είναι η πρώτη μελέτη που υπογραμμίζει τη σχέση μεταξύ των επιπέδων συγκέντρωσης EOP προγεννητικά και της παιδικής νευροανάπτυξης σε τρία διαφορετικά χρονικά σημεία, στα 4, 6 και 11 έτη, ενισχύοντας τα επιστημονικά στοιχεία για αυτή τη συσχέτιση. Συμπεραίνουμε ότι αυτά τα ευρήματα αυξάνουν την πιθανότητα ότι η έκθεση σε HCB και PCBs μπορεί να διαδραματίσει κρισιμότερο ρόλο στη γνωστική λειτουργία του παιδιού από ό,τι είχε θεωρηθεί προηγουμένως και καταδεικνύουν νέες ερευνητικές κατευθύνσεις στις μελέτες κοόρτης. Η βαθύτερη κατανόηση των περιβαλλοντικών παραγόντων κινδύνου για την μειωμένη γνωστική ανάπτυξη θα μπορούσε να έχει αξιοσημείωτη σημασία για τη δημόσια υγεία λόγω της δυνατότητας τροποποίησης αυτών. Ενώ δεκάδες χιλιάδες βιομηχανικές χημικές ουσίες εξακολουθούν να χρησιμοποιούνται, τα διαθέσιμα στοιχεία για τις πιθανές νευροαναπτυξιακές τους επιπτώσεις παραμένουν ανεπαρκή για τη συντριπτική πλειοψηφία. Μελέτες όπως η τρέχουσα είναι δυνατό να σηματοδοτήσουν ένα πολύτιμο αρχικό βήμα προς τη διερεύνηση περιβαλλοντικών παραγόντων κινδύνου για τις γνωστικές διαταραχές.

Από όσο γνωρίζουμε, πρόκειται για την πρώτη μελέτη που διεξήχθη σε δείγμα γενικού πληθυσμού παιδιών, η οποία υπογραμμίζει τον σημαντικό ρόλο των

αυξημένων επιπέδων φλεγμονής κατά την προσχολική ηλικία στη γνωστική επίδοση του παιδιού σε πολλαπλά χρονικά σημεία. Πράγματι, ενώ ορισμένες μελέτες έχουν εξετάσει την πιθανή σχέση μεταξύ βιοδεικτών φλεγμονής στα παιδιά και της νευροανάπτυξης, οι περισσότερες από αυτές πραγματοποιήθηκαν σε δείγματα εξαιρετικά πρόωρων βρεφών ή σε κλινικά δείγματα παιδιών με διαταραχές του φάσματος του αυτισμού. Επιπλέον, οι περισσότερες από αυτές τις μελέτες έχουν επικεντρωθεί στη σχέση μεταξύ των μητρικών δεικτών φλεγμονής κατά τη διάρκεια της εγκυμοσύνης και των νευροαναπτυξιακών εκβάσεων των παιδιών τους. Καθώς μέχρι σήμερα δεν υπάρχουν μελέτες που να διερευνούν τον τρόπο που οι δείκτες φλεγμονής σχετίζονται με τις μετρήσεις των νευροαναπτυξιακών βαθμολογιών σε ένα δείγμα γενικού πληθυσμού, τα αποτελέσματα αυτά δύνανται να φωτίσουν νέα ερευνητικά μονοπάτια. Τα ευρήματά μας ενισχύουν τα υπάρχοντα στοιχεία ότι η αυξημένη φλεγμονώδης δραστηριότητα μπορεί να εμπλέκεται σε πρώιμες παθοφυσιολογικές διεργασίες, όπως ελλείμματα μνήμης σε συγχρονικό επίπεδο, και σε δυσκολίες συμπεριφοράς σε διαχρονικό επίπεδο. Η καλύτερη κατανόηση των αλληλεπιδράσεων μεταξύ των μοτίβων και των επιπέδων των προ-και-αντιφλεγμονωδών κυτοκινών και των γνωστικών και συμπεριφορικών λειτουργιών θα μπορούσε να μας επιτρέψει να εντοπίσουμε παιδιά σε πρώιμο κίνδυνο για στοχευμένες παρεμβάσεις και να επιτρέψει σε κάθε παιδί να εκπληρώσει το πλήρες αναπτυξιακό του δυναμικό. Οι φλεγμονώδεις βιοδείκτες θα μπορούσαν, επίσης, να χρησιμεύσουν ως προγνωστικοί δείκτες και πιθανώς να οδηγήσουν σε προσεγγίσεις πρόληψης και θεραπείας αναπτυξιακών καθυστερήσεων και διαταραχών συμπεριφοράς για παιδιά σε κίνδυνο.

Abstract in English

Introduction

Persistent organic pollutants (POPs) are considered to be neurotoxic, influencing the synthesis and activity of neurotransmitters and the organization of the developing brain through alterations in basic cellular signaling processes and endocrine function. Human studies on a longitudinal and/or cross-sectional level have associated exposure to POPs in utero and in early childhood with adverse neurodevelopmental outcomes, such as reduced intelligence, ADHD, decreased performance in memory, autism spectrum disorders and other behavioral problems.

Inflammation is a complex natural defense mechanism by body tissues in response to injurious stimuli. This response may stop being protective for the organism and become harmful if it occurs chronically. Inflammatory markers, such as cytokines, are proteins involved in normal aspects of neurodevelopment and there is growing evidence associating them in complex, higher order neurological functions, such as cognition and memory. Imbalanced cytokine production, signaling and regulation may have various neurological consequences. A body of research has evolved around the role of prenatal cytokines as markers of risk for cognitive dysfunction in special and vulnerable populations and extensive research findings report that cytokine levels in plasma and/ or sera are altered in neurodevelopmental disorders, e.g. Autism Spectrum Disorders.

The specific objectives of this thesis are the following:

- To explore the role of high concentration levels of prenatal exposure to HCB, DDE and PCBs in offspring cognitive development at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of prenatal exposure to HCB, DDE and PCBs in offspring behavioral and emotional difficulties at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of several inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) measured in child serum at 4 years of age in child cognitive development at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of several inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) measured in child serum

at 4 years of age in child behavioral and emotional difficulties at 4, 6 and 11 years of age.

Methods

This study uses data from the Rhea Study, a prospective mother-child cohort established in 2007 in Crete, Greece. Pregnant women were recruited in the study at the time of the first ultrasound examination, around the 12th gestational week, from two public and two private prenatal clinics in Heraklion, during a twelve-month period, from 02/2007 until 02/2008. Trained midwives described in detail the study to pregnant women, obtained written informed consent, measured height, weight, and blood pressure, collected urine and blood samples, and completed a thorough questionnaire concerning participants' diet, sociodemographic and lifestyle characteristics, and exposure to various environmental agents. Mother-child pairs were invited to participate in child follow-up assessments when the children were 18 months, 4, 6 and 11 years of age.

POPs: Maternal serum samples were collected at the first prenatal visit around the 3rd and 4th month of pregnancy, in 10 ml Silicone gel separator vacutainer tubes (Becton Dickinson, UK). Tubes were centrifuged within 2 hours from blood collection at 2500rpm for 10min and were then stored in aliquots at -80°C until assayed. The POP analyses were performed in the National Institute for Health and Welfare, Chemicals and Health Unit, Kuopio, Finland with an Agilent 7000B gas chromatograph triple quadrupole mass spectrometer (GC-MS/MS). Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, and DDE were determined. All the results were reported on whole weight and expressed in pg/ml serum, while samples below the limit of quantification (LOQ) were assigned the value 0.5×LOQ. LOQ was 6 pg/ml for PCB118 and PCB156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, PCB180. We chose to use wet-weight levels for the POPs but adjusted for maternal serum triglycerides and cholesterol as continuous variables in all multivariable models to minimize potential biases associated with automatic lipid adjustment. POPs were treated as categorical variables. We calculated total PCB concentrations by summing the concentrations of the 6 individual PCB congeners and studied the associations of interest for the sum of PCBs.

Inflammatory markers: At the 4-year-follow-up assessment, blood samples were collected by venipuncture for each child (10ml) in SST gel separator vacutainer (BD

vacutainers, UK), after written parental consent. For the reduction of pain and discomfort of the children, anesthetic cream 5% EMLA with composition 2.5% lidocaine and 2.5% prilocaine (AstraZeneca, UK) was used. Analyses were performed in the Laboratory of Clinical Nutrition and Epidemiology of Diseases of Medical School, University of Crete. Blood samples were centrifuged (Kubota 4000, Japan) at 2500rpm 10min within 2 hours after collection and stored at -80^{o} C until assayed. The Milliplex Map human high sensitivity T cell magnetic bead panel (Cat. # HSTCMAG-28SK) from Millipore (Billerica, MA) was used for the simultaneous quantification of IFN-γ IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α and TNF-α in the supernatants. The principle of the assay is based on the quantification of multiple bio-molecules concurrently employing fluorescent-coded magnetic beads (MagPlex-C microspheres). The microspheres were incubated with the samples and then were allowed to pass rapidly through laser systems that distinguish the different sets of microspheres and the fluorescent dyes on the reporter bio-molecules. The sensitivity of the assay for every bio-molecule was: 0.3 pg/ml IFN-γ, 0.1 pg/ml IL-1β, 0.1 pg/ml IL-6, 0.1 pg/ml IL-8, 0.6 pg/ml IL-10, 0.3 pg/ml IL-17α, 0.9 pg/ml MIP-1α and 0.2 pg/ml TNF-α. We used a limit of 4 SD based on the statistical convention that observations 4 or more SD from the expected mean can be considered to be "extreme outliers" and thus, excluded from the statistical analyses. The intra-assay precision (%CV) for all biomolecules was <5%. The inter-assay precision (%CV) for IFNγ, IL-6, IL-10 and IL-17α was <20%, for IL-1β, IL-8, MIP-1α and TNF-α was <15%. The above analyses were performed on an automated analyzer Luminex 100 connected with the Luminex xPONENT software.

Cognitive development assessment at 4 years was conducted using the *McCarthy Scales of Children's Abilities* (*MSCA*), which evaluate child development across five domains: verbal, perceptual, quantitative, memory, and motor and offers a composite index of general cognitive development. Cognitive development assessment at 6 years of age was carried out using the *Raven's Colored Progressive Matrices (RCPM)*, which assess non-verbal general intelligence, the *Finger Tapping Test (FTT)*, which assess motor speed, and the *Trail Making Test* part A & part B (*TMT-Part A & TMT-Part B*), which assess visual search, speed of processing, mental flexibility, and executive functions. Cognitive ability at 11 years was carried out using the *Wechsler Intelligence Scale for Children, fifth edition (WISC-V),* assessing intellectual functioning in children across five *Primary Index Scales* [including Verbal Comprehension Index (VCI); Visual

Spatial Index (VSI); Fluid Reasoning Index (FRI); Working Memory Index (WMI); and Processing Speed Index (PSI)] and the four *Ancillary Index Scales* [including Quantitative Reasoning Index (QRI); Nonverbal Index (NI); General Ability Index (GAI) and Cognitive Proficiency Index (CPI)]. Behavioral and emotional development were overall assessed through the parent-report questionnaires. At 4 years of age the *Attention Deficit Hyperactivity Disorder Test (ADHDT)* and the *Strengths and Difficulties Questionnaire (SDQ)* were completed by participants' parents. The *ADHDT* is based on ADHD criteria of DSM-IV and provides 4 indexes, corresponding to 3 subscales (hyperactivity, inattention, impulsivity) and a total ADHD difficulties index. The *SDQ* is a brief screening questionnaire designed to assess behavioral strengths and difficulties in children and evaluates emotional symptoms, conduct problems, hyperactivity and inattention, peer-relationship problems, and prosocial behaviour. SDQ provides two additional composite indexes of internalizing and externalizing problems. Behavioral and emotional problems at 6and 11 years of age were assessed through *Child Behaviour Checklist – Parent Report Form (CBCL)* and the *Conner's Parent Rating Scale, Revised, Short Form (CPRS-R: S)*. The *CBCL* includes 6 scales that correspond to different diagnostic categories of the DSM-IV: affective problems, anxiety problems, somatic problems, attention deficit/hyperactivity problems, oppositional defiant problems, and conduct problems, and two composite indexes of internalizing and externalizing problems. The *CPRS-R: S* assess oppositional problems, cognitive problems/inattention, and hyperactivity, and provides an index of total ADHD symptoms.

Descriptive analyses on the characteristics of the population, and the distribution of the exposures, and the outcomes were conducted. Generalized additive models were used to assess the linearity of the associations of interest. Multiple linear and logistic regression models were used to explore the associations of interest, accordingly.

Results

POPs

- High prenatal HCB exposure was associated with decreased performance in i) cognitive, perceptual, executive and working memory functions at the age of 4, ii) non-verbal general intelligence, processing speed and mental flexibility and motor speed at 6 years of age and iii) visual spatial, fluid

reasoning, working memory, quantitative reasoning, nonverbal, general ability and cognitive proficiency functions at 11 years of age.

- High maternal serum levels of PCBs were associated with offspring decreased performance in working memory tasks at preschool age, increased response time in TMT Part A and lower speed scores in FFT (non-dominant hand) at 6 years of age and lower scores in several WISC-V index scores (working memory, processing speed, quantitative reasoning, nonverbal, general ability and cognitive proficiency) at 11 years of age.
- At 11 years of age we found statistically significant positive associations between high HCB levels and three behavioral scales scores in the basic models. However, in the adjusted models the estimates were weakened and it may be possible there is residual confounding implicated in the direction of the results,
- No association was demonstrated between high prenatal POPs levels with behavioral outcomes at any age-timepoint, with the sole exception of the association of high prenatal HCB levels with child peer problems in SDQ at 4 years.
- No associations were found between maternal DDE concentrations and neurodevelopmental and behavioral scores at 4 and 11 years of age. Regarding 6 years of assessment, we only found one associations of high prenatal DDE levels with increased response time in TMT Part B.
- No indication for effect modification by child sex, maternal pre-pregnancy body mass index (BMI) and maternal TSH during pregnancy was found.

Inflammatory Markers

- Preschoolers with elevated TNF-α concentrations in serum demonstrated decreased scores in memory, memory span and working memory and preschoolers with high IFN-γ serum levels showed lower scores in memory span scale. Children at 4 years with high levels of IL-8 showed lower prosocial behavior scores.
- Children with elevated levels of IFN-γ at 4 years showed increased scores in oppositional and hyperactivity scales, as well as in internalized and externalized CBCL scores at 6 years of age; high IL-1β levels at 4 years were associated with more oppositional symptoms and externalized problems at 6

years of age. We also found that increased TNF-a/IL10 ratio was related with lower inattention and ADHD scores at 6 years.

- Children with high levels of IL-8 at 4 years showed increased scores in processing speed; children with high levels of IL6/IL-10 ratio at 4 years showed increased scores in visual spatial performance at 11 years. High levels of the anti-inflammatory IL-10 at 4 years were associated with increased cognitive proficiency scores; high IL6/IL-10 ratio were related to increased general ability scores at 11 years. We also found that children with high levels of IFN-γ at 4 years demonstrated increased scores in hyperactivity and ADHD scales. High IL-17α levels at 4 years were associated with increased internalized problems scales at 11 years.
- Our results showed greater risk for reduced verbal performance scores for boys with high IL-17α serum concentration levels, as well as lower motor scores at 4 years for boys with high IL-6 serum concentrations. We also found greater risk for lower scores in memory and memory span scales at 4 years for overweight/obese children with high TNF-α serum concentrations.

Conclusions

To conclude, the present thesis supports and extends previous knowledge that prenatal exposure to high levels of POPs can be associated with reduced offspring cognitive development. This is actually the first study highlighting the association between prenatal POPs concentration levels and child neurodevelopment across three different timepoints, at 4, 6 and 11 years of age, strengthening the evidence for this association. We conclude that those findings raise the possibility that exposure to HCB and PCBs may play a more crucial role in child cognition than previously considered and show new directions for research in birth cohort studies. A deeper understanding of environmental risk factors for impaired cognitive development could be of considerable public health importance because of their potential modifiability. While tens of thousands of industrial chemicals are still in use, evidence on their potential neurodevelopmental effects remains inadequate for the vast majority. Studies like the current one may signify a valuable initial step towards exploring environmental risk factors for cognitive disorders.

To our best of knowledge, this the first study conducted in a general population sample of children which highlights the significant role of increased inflammatory levels during preschool years in child cognitive performance across multiple timepoints. In fact, whilst some studies have examined the possible link between child inflammatory biomarkers and neurodevelopment, most of them were carried out with samples of extremely premature infants or with clinical samples of children with autism spectrum disorders. Moreover, most of those studies have focused on the relationship between maternal inflammatory cytokines during pregnancy and their children's neurodevelopmental outcomes. As there are no studies to this date that explored how inflammatory biomarkers relate to measures of neurodevelopmental scores in a general population sample, these results may shed some light in new pathways of investigation. Our findings reinforce the existing evidence that elevated inflammatory activity may be involved in early pathophysiological processes, such as memory deficits at a cross-sectional level, and in behavioral difficulties at a longitudinal level. An increased understanding of the interactions between pro-and-anti-inflammatory cytokine patterns and levels, and cognitive functions could allow us to identify early at-risk children for targeted interventions and allow every child to meet their full developmental potential. Inflammatory biomarkers could also even serve as indicators and possibly lead to prognosis and therapy in order to prevent developmental delays and behavioral problems in at-risk children.

Thesis' publications

This thesis includes 2 published original research papers:

1. **Kyriklaki A**, Vafeiadi M, Kampouri M, Koutra K, Roumeliotaki T, Chalkiadaki G, Anousaki D, Rantakokko P, Kiviranta H, Fthenou E, Bitsios P, Kyrtopoulos S, Kogevinas M, Chatzi L. Prenatal exposure to persistent organic pollutants in association with offspring neuropsychological development at 4 years of age: The Rhea mother-child cohort, Crete, Greece. Environment International. 2016 Dec;97:204–11.

2. **Kyriklaki A,** Margetaki K, Kampouri M, Koutra K, Bitsios P, Chalkiadaki G, Dermitzaki E, Venihaki M, Sarri K, Anousaki D, Kogevinas M, Chatzi L. Association between high levels of inflammatory markers and cognitive outcomes at 4 years of age: The Rhea mother-child cohort study, Crete, Greece. Cytokine. 2019 May;117:1-7.

1. Introduction

1.1. General introduction

Extensive epidemiological research has linked poor early-life conditions with a series of life-long, proinflammatory phenotypes, an increased risk for autoimmune and allergic disorders and physical and mental disorders (1). Several cohort studies have detected associations between the early-life environment and an heightened risk of cardiovascular diseases, autoimmune diseases, type 2 diabetes, obesity, allergies and asthma, migraine and psychiatric disorders (2–6). This century has been marked by the growing evidence of the key role played by epigenetic factors during the intrauterine and in the perinatal period in shaping the development of our organs and tissues (7). Epigenetic mechanisms, triggering changes in the chromosome state without affecting DNA sequences (8), have long been described as the potential underlying mechanism behind development programming in the context of Developmental Origins of Health and Disease (DOHaD) hypothesis (9,10); the field of DOHaD research intends to provide an inclusive perspective on health and disease that reveals how different life experiences influence health and disease risks over the entire life course, from the preconception to advanced age (11).

The influence of intrauterine experiences has been progressively extended from brain development to the susceptibility to neurodegenerative disorders in childhood, as well as in adulthood (10,12). Along with the observable physical maturation, extensive neural formation and network organization take place during the prenatal period; as in postnatal life, the interaction of inherent genetic programs and genetic predispositions with an extensive range of environmental exposures outlines individual differences in neurobehavioral trajectories (13). For instance, there is growing literature suggesting associations between prenatal environmental exposure to household chemicals and child neurodevelopmental outcomes (14), and detrimental effects of maternal stress prenatally in child development and psychopathology (15).

1.2. Early Human Brain Development and Neurodevelopmental Disorders

The Central Nervous System (CNS) is amongst the earliest organ systems of the human body to begin its development prenatally and amongst the last to complete it postnatally (16). Human brain development is a highly complex process that sets the

framework for cognition, behavior and emotions for a person's life (17). It undergoes several critical stages of development from embryonic period to preschool years (18). During these time periods neurons are born, migrate to their final locations, networks are established, and pruning and myelination take place (19). In fact, major progressive and regressive events (e.g. synaptogenesis and synaptic elimination) shape the architecture and development of neural connections, and these have been observed to show distinct regional variations, beginning earlier in primary sensorimotor regions and later in anterior areas such as the prefrontal cortex (16). Those progressive and regressive processes continuously shape the developing brain, suggesting that the measurable development of cognitive skills taking place during typical development might be the result of a process of fine-tuning of brain structure and function (20,21). Some basic behavioral functions are already present at birth, indicating that structural connectivity of the relevant brain circuits have begun, e.g. primary sensory systems, while more complex behaviors such as recognition memory, can even be seen in the term and preterm neonates (22). Despite the lack of their behavioral manifestations, neural circuits related to more complex behaviors, such as working memory, multi-tasking and attention are not present in the neonates, yet they have their neural ontogenies prior to term birth (23).

The developing brain in utero and during the first years of life is highly vulnerable to environmental influences, and gene-environment interactions seem to explain largely the variance in individual differences in behavioral dysfunction, mainly through alterations of the DNA structure and chromatin function that affect gene expression (24). A growing body of evidence suggests that brain development is quite vulnerable in early life compared to later life stages (25). Normal brain development involves multiple complex stages that must be completed consecutively, largely during fetal and early life (26). Impairment of this process could bring about: (a) neurobehavioral disorders (e.g. autism spectrum disorder and attention-deficit hyperactivity disorder, (b) dysfunctional cognitive development, and (c) long-term deleterious effects on well-being and mental health (27).

Neurodevelopmental disorders are have their origins in very early brain development (28,29) and are conditions characterized by impairments of social skills or intelligence with onset in the developmental period (8) and are reported to affect

10-15% of all births (30). Prevalence of neurodevelopmental disorders has increased worldwide, requiring more health and education services (31).

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), enlists intellectual disability, communication disorders, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), specific learning disorders, and motor disorders as neurodevelopmental disorders (32). These conditions can have serious outcomes, such as decline in quality of life and academic achievement, disturbance of behaviour and, in a broader context, greater consequences for the welfare of entire societies (33). Overall, genetic factors seem to account for no more than 30–40% of all cases of those disorders. Hence, non-genetic, environmental exposures are implicated in causation, in some cases probably by interacting with genetically inherited predispositions (25).

1.3. Persistent Organic Pollutants

In the last decades there has been a focus on a mostly of anthropogenic origin subset of harmful organic chemical contaminants, generally referred to as persistent organic pollutants (POPs) (34). POPs are carbon-based organic chemicals that are persistent, bioaccumulative, they are highly resistant to environmental degradation and have long-range transport potential (35) and long half-lives in soils, sediments, air and biota and are able to impose serious health effects in humans, wildlife, and marine biota adjacent to and distant from their origin of emission (34).

These pollutants can bioaccumulate in the fatty tissue of living organisms and become concentrated as they move through the food chain; over 90% of human exposure to POPs is through the consumption of contaminated food, mainly food of animal origin (36–39). Exposure to them, mostly at elevated levels, may cause several health problems such as cardiovascular diseases, cancers, endocrine disruption, diabetes, birth defects, cognitive and behavioral problems and dysfunctional immune and reproductive systems (40–43). Although an international ban on production was enacted at the Stockholm Convention on Persistent Organic Pollutants in 2001 (43), they do remain as a concern to human health; due to their persistence, exposure of the general population still occurs to these compounds banned long time ago, mostly through food chain (44), and due to the chronic exposure and the accumulation in the human body, especially in certain developing countries (35). Sometimes, mixtures of

POPs generate synergistic effects, increasing the toxicities of one another. Consequently, the mixtures of POPs in nature may be more toxic than predicted in the laboratory experiments (45).

1.3.1. Persistent Organic Pollutants and Brain Development

There is robust evidence that industrial chemicals spread in the environment contribute to what is called the global, silent pandemic of neurodevelopmental toxicity (46). The developing human brain, undergoing several major cellular processes (such as neurogenesis, migration, neuronal differentiation, synaptogenesis, myelination, apoptosis, and synaptic plasticity) is exceptionally vulnerable to toxic chemical exposures, and during sensitive stages (e.g. prenatally, in infancy and in early childhood), these chemicals can cause permanent brain injury at low levels of exposure that would have little or no negative effect during adulthood (47). The fetus is poorly protected against industrial chemicals, such as POPs, since the placenta cannot block effectively the passage of several of these toxicants between maternal-fetal circulation and various environmental chemicals can also be transferred postnatally to the infant through breastfeeding (48,49). In addition, during fetal life and early infancy, the blood-brain barrier provides only limited protection against the entry of POPs into the CNS (50), due to its immaturity (51). Lipophilicity and strong chemical attraction for high-lipid tissues seem to be common properties of POPs (52). After having crossed the placenta, most of these pollutants are concentrated to the brain, a largely lipid-rich tissue in the developing fetus (1).

Persistent organic pollutants (POPs) are considered to be neurotoxic, influencing the synthesis and activity of neurotransmitters and the organization of the developing brain through alterations in basic cellular signaling processes and endocrine function (53). The outcomes of developmental neurotoxicity are often potentially permanent and could result in reduced cognitive ability, as expressed in terms of lost IQ points, or behavior problems (25). It has also been suggested that effects of POPs exposure during fetus development occur throughout lifespan (54). In fact, structural brain abnormalities caused by exposure to these chemicals during intrauterine brain development may not be manifested up until the time when normal functions of the corresponding area are expected to occur (47).

1.3.2. Exposure to POPs during pregnancy and offspring's cognitive and behavioral development

As Endocrine Disrupting Chemicals (EDCs), POPs are considered to affect endocrine function through a range of certain mechanisms (55), which are associated with different levels of the hypothalamic-pituitary-gonad, thyroid, and adrenal axes, varying from outcomes on hormone receptors to consequences on hormone synthesis, secretion, or metabolism (56). Human studies on a longitudinal and/or cross-sectional level have linked exposure to EDCs in utero and in early childhood to adverse neurodevelopmental outcomes, such as reduced intelligence, ADHD, decreased performance in memory, autism spectrum disorders and other behavioral problems (57).

Several studies have revealed associations of maternal serum and cord blood levels of PCBs with worse offspring scores in cognitive and psychomotor development in both infancy (46,58–61), and later childhood (62–66), as well as increased risk for ADHD-related behaviors (64,67). However, these adverse effects have not been replicated in other studies (68–75). More specifically, results from different cohort studies that explored the relation of PBCs to cognitive functions in children ranging from 3 to 11 years of age showed associations of these contaminants with at least one domain of cognition, such as lower scores on general intelligence (59,62,63,65,76), processing speed (77), language (69), reading comprehension, (63), motor performance (78) and short-term memory (79). Null effects of PCBs on all cognitive outcomes under examination were also reported (66,72,80,81) and, interestingly, results from two studies that revealed associations between PCBs and intelligence scores at the age of 3 (59,82) demonstrated that these associations did not persist anymore at ages 4 and 6 (66,82). Nonetheless in one study (Oswego study) associations re-appeared when children grew at the age of 9 (65).

Additionally, several studies have outlined associations of prenatal exposure to PCBs and subsequent negative outcomes in behavior, (64,83–87), attention (62,67,69,84,86,88,89) and hyperactivity (64,90), although one study demonstrated that prenatal exposure to PCBs was related to fewer parent-reported ADHD-related behaviors (67). Null effects of PCBs on behavioral outcomes were also reported (79,91–93). Moreover, there are findings suggesting associations of prenatal PCBs with fewer autistic traits (94) and others indicating mixed associations, determined by particular PCB congeners (95). According to a recent review (96), possible mechanisms of PCB

neurotoxicity include: (i) modified dopamine (DA) signaling, (ii) disruption of thyroid hormone signaling, (iii) perturbation of intracellular Ca^{2+} dynamics, and (iv) oxidative stress. These mechanisms have also been implicated in the neurotoxicity of several contemporary POPs.

The vast majority of studies examining organochlorine pesticides (OCPs) in association with neurodevelopment have measured these pollutants in prenatal maternal serum, cord blood, or breast milk and have reported adverse neurobehavioral outcomes, but findings do not seem consistent across outcomes, age groups or specific OCPs (97). There are also studies demonstrating mixed results within the same study populations, with either significant associations at one age group but not at another, or associations with neurodevelopmental outcomes for one specific OCP but not for others (91,98–103).

Regarding prenatal exposure to DDT and DDE, some literature findings suggest adverse effects on cognitive and behavioral outcomes after age 6 months (60,103,104). Darvill and colleagues (105) found no association between intelligence scores at ages 6 or 12 months of age and cord blood DDE concentrations. Moreover, other studies showed higher transplacental exposure to DDE was associated with higher mental scores at 6 months in Bayley Scales of Infant Development but no association persisted at 12 months, (106) and null associations were reported at 24 months (107). Findings concerning older ages are limited; one study provided evidence for null associations between DDE cord serum and memory and learning at 8 years of age (108), while others have reported sex-specific associations between DDT or its metabolites and neurodevelopmental difficulties in early childhood (91,100,109). One study observed no association between p'-DDE and Autism Spectrum Disorder (ASD) diagnosis (99) and another showed that ADHD-related behaviors at 8 years of age were associated with DDT/DDE exposure (64,88).

Concerning prenatal HCB exposure, there are studies reporting lack of association with neuropsychological and behavioral outcomes; specifically, Braun and colleagues (102) suggested that maternal HCB concentrations in maternal serum samples were not associated with occurrence of offspring autistic behaviors at 4 and 5 years old, but also found stronger associations of HCB with autistic behaviors in girls. Another study reported that prenatal exposure to HCB, as measured in cord serum, had no effect on child mental and psychomotor development at 18 months (60).

Similarly, findings from the Flemish mother-newborn cohort reported no significant associations between HCB levels in cord blood and abnormal Strengths and Difficulties Questionnaire (SDQ) scores at the age of 7 to 8 years old (91). On the contrary, results from the Oswego study suggest that placental levels of HCB is a significant predictor of intelligence at the 11-year assessment, as measured by the Wechsler Intelligence Scale for Children (WISC-III) (110). In addition, findings from two Spanish cohorts demonstrate that prenatal exposure to HCB concentrations, as measured in cord serum, is associated with a decrease in the behavioral competence at preschool age, but no association was revealed between HCB and cognitive and psychomotor performance (111).

1.4. Inflammation

Inflammation is a complex and vital process of an organism's response to biological, chemical, and physical stimuli (112). It can be triggered by a wide range of factors, including pathogens, damaged cells, irradiation and toxic compounds (113). At the tissue level, redness, swelling, heat, pain, and loss of tissue function are inflammatory key elements, derived from immune, vascular and inflammatory cell responses to infection or tissue injury (114). After tissue damage, the organism introduces a chemical signaling process that triggers responses intended to heal affected tissues (115). These signals stimulate leukocyte chemotaxis from the general circulation to the damaged areas. These activated leukocytes produce cytokines that, in turn, activate inflammatory responses (116).

Although inflammation is identified as a natural defense mechanism by body tissues in response to injurious stimuli, this response may stop being protective for the organism and become harmful if it occurs chronically (117). Generally, the acute inflammatory response is brought to an end once the triggering insult is annihilated, infection is confronted, and damaged tissue is repaired (113). If the inflammatory process is not terminated by the acute inflammatory response or persists, termination of the acute inflammatory may not take place and a chronic inflammatory state may emerge (118). Chronic inflammation is considered to play a very important part to several diseases, such as arthritis, asthma, atherosclerosis, autoimmune diseases, diabetes and cancer, with the immune system playing a pivotal role (112). In some

chronic inflammatory disorders the inflammatory response can produce more damage to the host than the microbe (119).

Inflammatory markers can be predictive of inflammatory diseases and seem to be related to the causes and outcomes of several diseases (115). After the occurrence of detrimental stimuli, various inflammatory cells, such as macrophages, monocytes and adipocytes, are activated and trigger the production of inflammatory cytokines, which can potentially serve as biomarkers for disease diagnosis, prognosis and designing therapeutic approaches (120–122). Most of those have multiple targets, and multiple functions (123). These intercellular polypeptides act as molecular messengers, are low molecular weight (<200 amino acids) and coordinate and regulate the immune response to infection and inflammatory process by means of a complex, highly coordinated network of cell interactions; nevertheless, disproportionate cytokine production could result to tissue damage, hemodynamic fluctuations, organ failure, and even death (124).

Inflammatory cytokines are categorized as interleukins (ILs; IL-1 to IL-24), colony stimulating factors (CSF), interferons (IFNs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), and chemokines (125). Cytokines that are best known for stimulating inflammatory responses are interleukin 6 (IL-6), interleukin 1 (IL-1), interleukin 2 (IL-2), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and transforming growth factor β (TGF-β) (126). Interleukin 6 (IL-6) mediates the initial stages of the acute inflammatory response, and its levels may remain high in chronic inflammatory conditions (125,126). The IL-1 class of cytokines includes 11 different members that all share pro-inflammatory effects, with IL-1β being one of the more distinguished one, playing an important part in processes such as stimulating the expression of adhesion molecules that upgrades the recruitment of inflammatory cells (127). IL-2 enhances growth and activation of Natural Killer (NK) cells and macrophages, activates the production of cytokines such as IL-1 and IFN-γ and additionally promotes chronic inflammation (112). TNF-α stimulates inflammation by promoting cytokine production, and stimulating cell growth and proliferation. It also contributes in the process of removing dead or dying cells by facilitating apoptosis (126). IFN-γ effectively activates macrophages, promotes the production of other pro-inflammatory cytokines, such as IL-1 and TNF-α, and also promotes the process of migration of inflammatory cells through the vascular system to areas of injured tissues

(112). Interleukin 8 (IL-8) is a chemokine (which is a subclass of small cytokines with chemoattractant properties) involved in moderation of angiogenesis and wound healing (112).

1.4.1. Inflammation and the Brain

Inflammatory markers, such as cytokines, are proteins involved in normal aspects of neurodevelopment, including neuronal development, progenitor cell differentiation, cellular migration within the nervous system and synaptic network formation (128–132) and there is growing evidence associating them in complex, higher order neurological functions, such as cognition and memory (133,134). Imbalanced cytokine production, signaling and regulation may have various neurological consequences (135,136). It has been shown that CNS concentrations of certain cytokines increase, as age increases; individuals without neurological deficits demonstrate a progressive increase in brain expression of IL-1 and microglial activation with advancing age (134). Actually, elevated IL-6 concentrations have been found in the mouse brain with age, possibly as a consequence of increased microglial production (137).

Inflammatory responses are evident in many central nervous system diseases, including autoimmune and neurodegenerative diseases (e.g. Alzheimer's and Parkinson's disease, epilepsy) (115). Activation of the brain's immune cells and microglia that produce pro-inflammatory markers may lead to inflammation-associated CNS diseases (138). Inflammatory brain responses may activate neuronal excitability, injure cells, and increase blood-brain barrier permeability to several molecules (139).

Cytokines also have endocrine effects and it is possible that their production due to fetal injury or maternal infection will have a negative effect in neurodevelopment. All cell types in the developing CNS use cytokines for paracrine and autocrine signalling and normal cytokine-mediated developmental processes are considered to be vulnerable to disruption by possible immune dysregulation (131). It seems therefore quite established that an embryonic cytokine dysregulation is associated with immune imbalance in the developing brain. These consequences on the developing brain have serious effects even in adulthood, e.g. an increased susceptibility to certain mental illnesses (130). For instance, it has been found that

maternal respiratory infection and increased cytokines increase the possibility of schizophrenia occurrence in the offspring (140); such findings tend to be replicated by animal studies, as well, demonstrating that elevated maternal serum cytokines (e.g. IL-6) can play a crucial part in this association (141). Moreover, epidemiological findings imply increased possibilities of autoimmune disorders within the families of patients with Autism Spectrum Disorders, highlighting the important role of the highly stimulated maternal immune response in the risk of neurodevelopmental disorders (142).

The possible important role of cytokines in the pathogenesis of neurodevelopmental disorders has been evident in recent literature, with particular focus to the balance of pro- and anti-inflammatory states that are essential for normal neurodevelopment (143). Cytokine levels are strongly elevated in a number of brain areas and in the cerebral spinal fluid of subjects with autism, at ages varying from 4 to 45 years old (144) and various immune-related genes are also poorly balanced in the brain of autistic and schizophrenic patients (145). Interestingly, studies show that injections of certain cytokines could trigger immediate effects, generating psychiatric symptoms in adults (146–148). However, neurodevelopmental and psychiatric deficits do not necessarily mean neurodegeneration; peripheral cytokines can still affect neurotransmission and be associated with cognitive deficits by means of nutritional consumption and effects on sleep, without directly leading to neuronal death (134). Findings supporting the influence of peripheral cytokine dysregulation on cognitive outcomes has derived from the utilization of cytokines as therapeutic agents; individuals with no psychiatric history receiving frequently cytokines at therapeutic doses, including IFN-α, IL-2 and TNF-α complain about increased somatic symptoms, anorexia, and neuropsychiatric side effects, such as depressed mood, sleep disturbances, difficulties in motivation and thought processing problems (149).

1.4.2. Inflammation and child cognitive and behavioral development

Insight into the link of inflammatory markers role and central nervous system processes has increased, mostly on the basis of animal models. As summarized by Onore and colleagues (132), findings from animal studies indicate that elevated levels of certain cytokines were found to be associated with several behavior and/or cognitive deficits; indicatively, research results show that prolonged expression of IL-

1β hippocampus may weaken spatial memory (150) and be associated with social withdrawal, anxiety, depression and loss of appetite in mice (151). In addition, IL-2 and IL-4 have been suggested to be related to increased repetitive behavioral patterns, which can be typical of ASD (152). Other findings suggest that chronic peripheral IL-2 administration has been related to hippocampal neurodegeneration in rats resulting in impaired memory performance (153) and to deficits in the primary somatosensory cortex, causing poor spatial learning (154).

As Voltas and colleagues (155) point out, few studies have explored the association between inflammatory biomarkers at early age and child neurodevelopmental outcomes, and most of these studies were carried out with special samples of extremely premature infants or with clinical samples of children with ASD. In fact, a body of research has evolved around the role of prenatal cytokines as markers of risk for cognitive dysfunction in special populations, such as children born preterm (156–159), children with low birth weight (160), sickle cell disease (161), cerebral palsy (162) and chronic hepatitis C (163), indicating a potential role for inflammatory processes in neurodevelopmental outcomes for those vulnerable populations.

Extensive research findings report that the levels of immune proteins in plasma and/or sera, such as cytokines and chemokines, are altered in neurodevelopmental disorders, e.g. ASD, where their imbalances seem to be well-documented. In fact, increased plasma levels of pro-inflammatory cytokines in preschool children, such as IL-1β, IL-6, IL-8 and IL-12p40 have been suggested in ASD (164). Case-control studies have found higher circulating IL-6, IL-1β, IL-8 and TNF-α levels in plasma of preschool (164,165) and school-age children with ASD (166–169) compared to typically developing controls. Moreover, plasma levels of IFN-γ and cerebrospinal fluid levels of TNF-α have been reported to be increased in autistic children (170–172) and, likewise, elevated concentrations of IFN-γ have been reported for subjects with ASD compared to controls (166). Conclusively, elevations in cytokines, such as in TNF-α, IL-1β and IL-6 in blood serum, cerebral spinal fluid and brain tissues from children with ASD can be indicative of a disturbed neuro-immune network (132).

Other studies examine the link between inflammation and neurodevelopment within the frames of children living in conditions of poverty and/or adversity. For instance, Jiang and colleagues (173) claimed that inflammatory markers in the first

year of life were associated with neurodevelopmental outcomes in infants living in conditions of poverty, showing that elevated serum levels of pro-inflammatory IL-1β and IL-6 were related to lower scores for motor skills, while elevated serum levels of IL-4 were associated with higher scores for cognitive tasks at 12 and 24 months. Research results by Voltas and colleagues (155) revealed that elevated IL-6 at 12 months predicted elevated scores in internalizing (emotionally reactive, anxious/depressed, withdrawn and attention problems) and externalizing problems (aggressive behavior) at 30 months, high levels of IL-1β at 6 months were related to worse motor skills and no markers were related to mental outcomes, implying that IL-6 and IL-1β could be early markers of later psychological and psychomotor deficits.

1.5. Aim and specific objectives of the thesis

The association between high exposure to POPs during pregnancy with offspring brain development, and especially, offspring performance in cognitive, psychomotor and behavioral domains during infancy, preschool age and later childhood has been of great research interest and has been extensively studied during the last decades. Findings from studies focusing on child cognitive and behavioral development at certain ages remain contradictory or inconclusive. Also, to our knowledge, most of the studies have examined only one, or limited, neurodevelopmental outcomes, making it quite difficult to determine possible risks for cognitive deficits as well as behavioral problems, such as attention-deficit and hyperactivity. Moreover, there are limited research findings regarding examining the same populations at multiple timepoints. The aim of the present study was to evaluate the impact of high levels of prenatal exposure to HCB, DDE and PCBs on offspring neurodevelopment and behavior at three different timepoints: at preschool age (4 years), a critical time period of child development, at mid-childhood (6 years) and at 11 years of age, in a prospective pregnancy cohort (the Rhea Study) in Crete, Greece, after controlling for a wide range of confounders.

Regarding the effect of inflammatory biomarkers during early childhood on child cognitive and behavioral development, there is accumulating research evidence indicating a potential role for inflammatory processes in neurodevelopmental outcomes for vulnerable populations; as it has been thoroughly described in the introduction section, most of these studies were conducted with clinical samples or

with samples of extremely premature infants. To our knowledge, there are no available data discussing the relationship between inflammatory markers levels and neurodevelopment in a general population sample of children. The aim of the present study, within the frames of this pregnancy cohort, is to examine the role of high levels of various inflammatory markers measured in child serum at 4 years of age in neurodevelopmental scores assessed at 4 years of age (cross-sectional design) as well as at 6 years and at 11 years of age, after controlling for a range of confounders.

Overall, with its longitudinal design, the Rhea Study provides the opportunity to investigate the aforementioned associations utilizing repeated reliable and valid measures of various offspring developmental domains.

More specifically, the objectives of this thesis are the following:

- To explore the role of high concentration levels of prenatal exposure to HCB, DDE and PCBs in offspring cognitive development at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of prenatal exposure to HCB, DDE and PCBs in offspring behavioral and emotional difficulties at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of several inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) measured in child serum at 4 years of age in child cognitive development at 4, 6 and 11 years of age.
- To explore the role of high concentration levels of several inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α) measured in child serum at 4 years of age in child behavioral and emotional difficulties at 4, 6 and 11 years of age.

2. Materials and Methods

2.1. Study design

The present thesis utilizes data from the Rhea Study, which is a prospective mother-child cohort established in 2007 in Crete, Greece. The principal aims of the Rhea Study are to identify the various determinants of children's growth and development, to evaluate maternal health during and after pregnancy and to investigate the interaction between environmental stressors and genetic variants in offspring's growth and health; additionally, within its frames, four main outcome

domains are investigated: offspring growth and obesity, neuropsychological and behavioral development, allergies and asthma and genotoxicity (174).

Pregnant women (Greek and immigrants) were recruited in the study at the time of the first ultrasound examination, around week 12 of gestation from two public and two private prenatal clinics in Heraklion city during a 12-month period from February 2007 until February 2008. The inclusion criteria for study participation included residency in the study area, age at pregnancy greater than 16 years, adequate ability of communicating in Greek and first antenatal visit taking place in hospitals or private clinics in Heraklion district.

Initial contact with the study participants took place in the first trimester of pregnancy, at the time of the first major ultrasound test (median 12 weeks of gestation). After that, private meetings were scheduled, where specially trained midwives provided a more detailed description to the women that had already showed their interest in participating. Afterwards, they obtained written informed consent and completed a detailed questionnaire on diet, environmental exposures, sociodemographic and lifestyle characteristics. They also measured height, weight, and blood pressure, and collected urine and blood samples from the pregnant women. The participants were contacted again at the 3rd trimester of pregnancy, when maternal stress questionnaires were completed and medical record information and ultrasound measurements were collected. After that, at child birth admission (median 38 weeks of gestation), infant's weight, length, anogenital distance and head circumference were measured and cord blood samples were collected. In short, mother-child pairs were invited to participate in child follow-up assessments when the children were 9 months, 18 months, 4 years, 6 and 11 years old. The majority of clinical visits were carried out at the University Hospital of Heraklion on Greece; however, for families living in rural areas, visits at four Rural Health Services in Crete were conducted. At each visit, written informed consents were obtained from the mothers. Moreover, all the study protocols were approved by the Ethical Committee of the University Hospital of Heraklion, Crete. Figure 1 presents the clinical visit frequency and timing, as well as the content of Rhea study follow-up visits, summarizing the measures taking place at each timepoint.

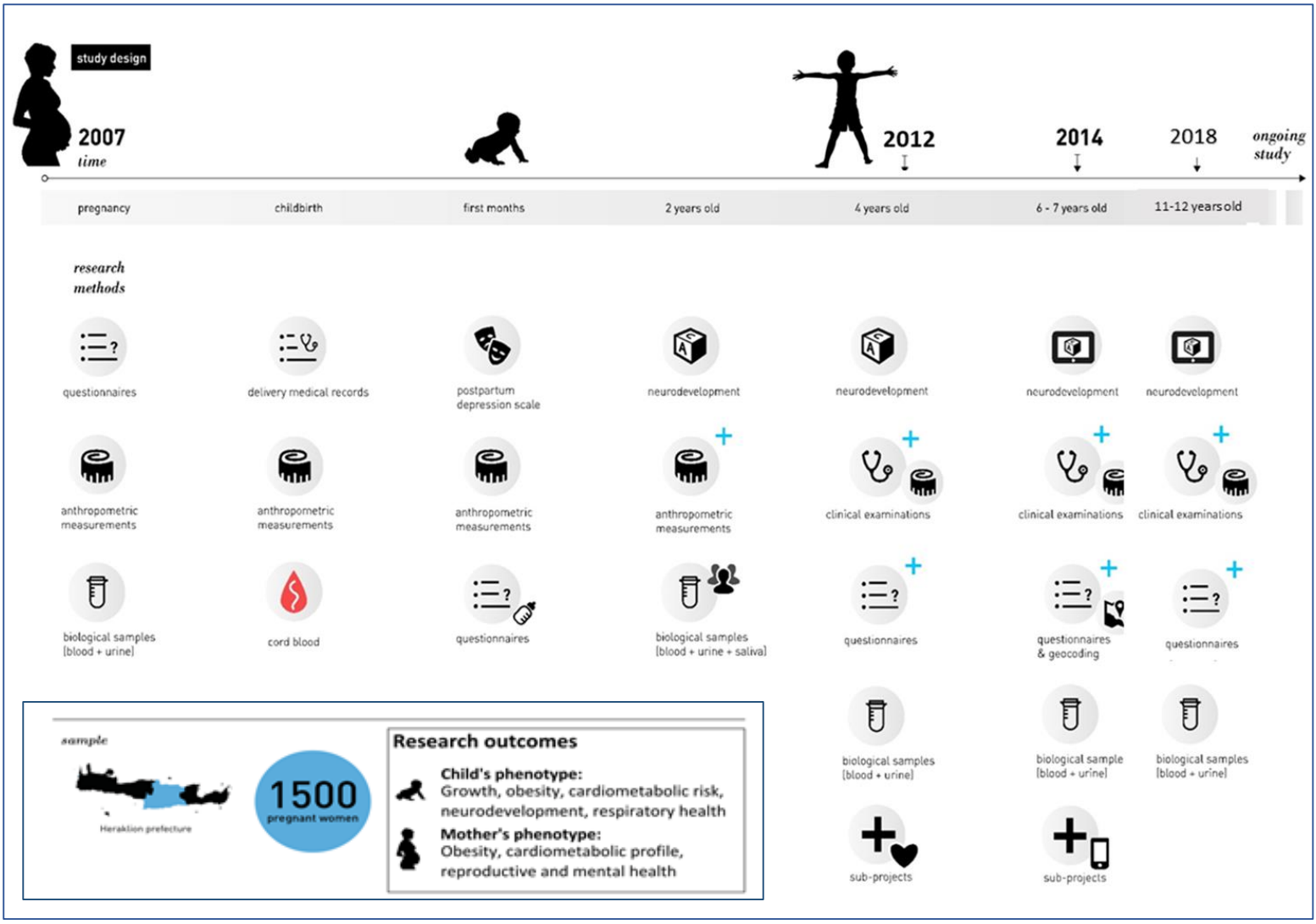

Figure 1. *Follow-up visits and summary of the measures in the Rhea mother-child cohort study*

Considerable strategies have been followed by the study staff in order to keep the participating families engaged in the study and reduce the loss to follow-up. Those efforts included personal invitations sent to all participants by post before each follow-up (e.g. with informative bulletins to keep them updated with the study), as well as personal feedback to the families concerning the child's growth and blood measurements after the clinical examination.

Children who attended the follow-up tended to have older, married parents, of higher education and of Greek origin. Attrition at follow-up was largely due to withdrawal, difficulty in keeping track of changed addresses and contact details, emigration, children's severe health issues and mothers' unwillingness to attend the in-person visits.

2.2. Study population

During the study period 1610 pregnant women agreed to participate and 1363 singleton pregnancies were followed up to delivery. Late recruitment at birth lead in 95 additional mother-child pairs and 1458 live singleton births followed up to birth. At the 18-months follow-up assessment 863 children participated, at the 4-years follow-up assessment 879 children participated, at the 6-years follow-up assessment 607 children participated and at the 11-years follow-up assessment 350 children participated. Figure 2 shows the flow diagram of Rhea Study participation up until the 6-year follow-up assessment. It should be noted here that in each analysis the number of participants differentiates due to the different number of mother-child pairs with available data for the specific analysis.

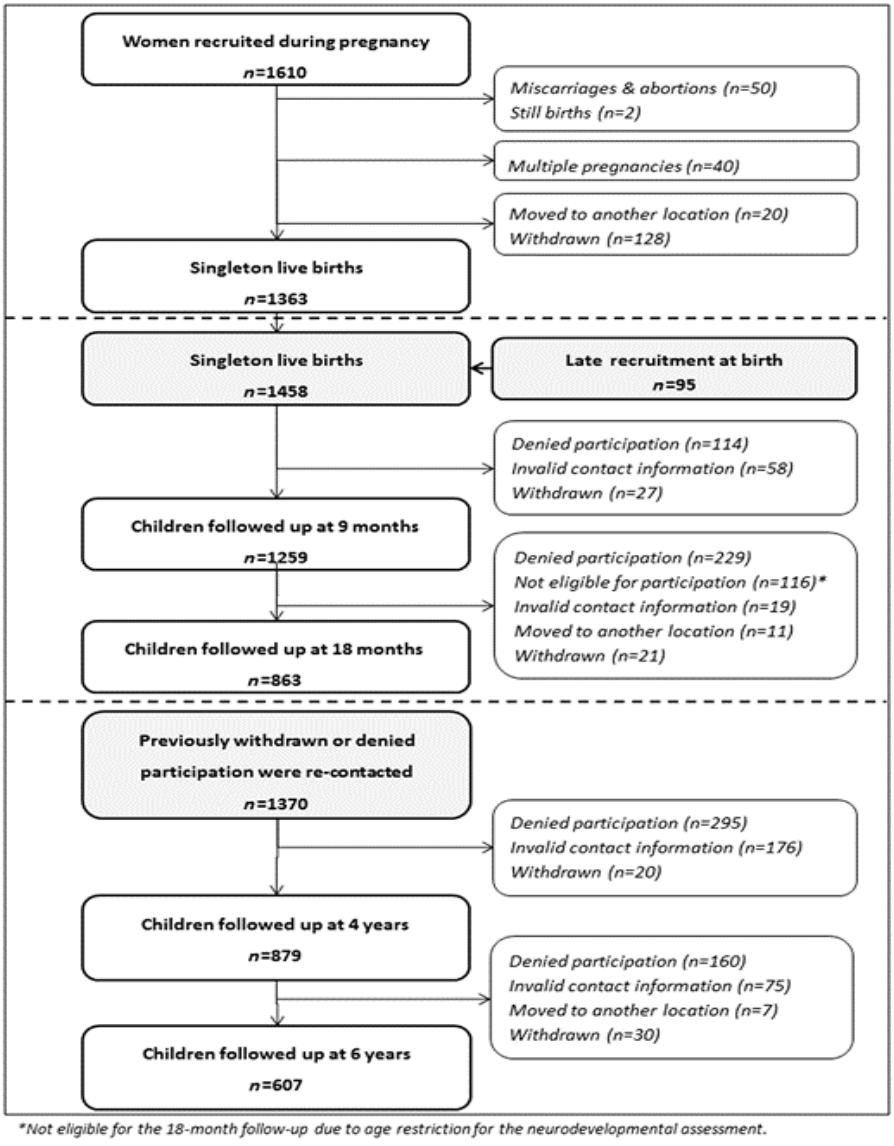

***Figure 2**. Rhea Study flow diagram*

2.2.1. Study population: Prenatal exposure to Persistent Organic Pollutants in association with offspring cognitive development at 4, 6 and 11 years of age

Overall in this study, we had available data on maternal POPs levels from 1110 pregnant women. Specifically, regarding the analyses on the association between exposure to POPs in the first trimester of pregnancy and child cognitive development, complete data for high prenatal POPs exposure, and follow-up neuropsychological assessment, were available for 568 mother–child pairs at 4 years, 383 at 6 years and 254 at 11 years and thus were eligible for analysis. Overall, participating mothers were predominantly Greek, married and had a mean age of 30 years. About half of them had medium educational level and were multiparous. Approximately half of the children were boys. The baseline characteristics of the participants in the analyses are shown in Table 1

Table 1. Baseline characteristics of the study participants at 4, 6 and 11 years of age, Rhea mother-child study, Crete, Greece

	4 years[a] (N=568)	6 years[a] (N=383)	11 years[a] (N=254)
Maternal Characteristics			
Age (years)	30.0 (4.9)	30.3 (4.7)	30.6 (4.4)
Educational level			
Low	88 (15.5)	52 (13.6)	29 (11.4)
Medium	288 (50.7)	193 (50.4)	127 (50.0)
High	192 (33.8)	138 (36.0)	98 (38.6)
Parity			
Primiparous	246 (43.3)	163 (42.6)	112 (44.1)
Multiparous	322 (56.7)	220 (57.4)	142 (55.9)
Ethnic origin			
Greek	534 (94.3)	363 (95.0)	245 (96.5)
Non-Greek	32 (5.7)	19 (5.0)	9 (3.5)
Smoking during pregnancy			
Yes	180 (31.7)	117 (30.5)	77 (30.3)
No	306 (68.3)	266 (69.5)	177 (69.7)
Marital status			

Married	500 (88.2)	343 (89.8)	230 (90.9)
Other	67 (11.8)	39 (10.2)	23 (9.1)
Residence			
Urban	403 (77.1)	279 (78.8)	183 (78.5)
Rural	120 (22.9)	75 (21.2)	50 (21.5)
TSH during pregnancy (μIU/mL)	1.28 (1.0)	1.27 (1.0)	1.31 (1.1)
Pre-pregnancy BMI (kg/m^2)	24.9 (5.0)	24.8 (5.1)	25.1 (5.0)
Child Characteristics			
Sex			
Boy	299 (52.6)	215 (56.1)	145 (57.1)
Girl	269 (47.4)	168 (43.9)	109 (42.9)
Birth weight (g)	3210.9 (453.6)	3193.3 (458.3)	3211.6 (441.1)
Gestational age (weeks)	38.2 (1.6)	38.2 (1.6)	38.3 (1.5)
Breastfeeding (months)	4.3 (4.4)	4.2 (4.2)	4.4 (4.2)
Age at assessment (years)	4.2 (0.2)	6.6 (0.3)	11.0 (0.4)

[a] Data presented as mean (standard deviation) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned

2.2.2. Study population: Prenatal exposure to Persistent Organic Pollutants in association with offspring behavioral and emotional development at 4, 6 and 11 years of age

In the analyses concerning the association between prenatal exposure to POPs and child behavioral and emotional development, we had available complete data on elevated maternal POPs concentrations and information on child behavioral and emotional outcomes for 529 mother-child pairs at 4 years, 399 at 6 years and 255 at 11 years. Overall, participating mothers were mainly Greek, married and had a mean age of approximately 30 years. About half of them had medium educational level and were multiparous. Approximately half of the children were boys. The baseline characteristics of the participants in the analyses are demonstrated in detail in Table 2.

Table 2. Baseline characteristics of the study participants at 4, 6 and 11 years of age, Rhea mother-child study, Crete, Greece

	4 years[a] (N=529)	6 years[a] (N=399)	11 years[a] (N=255)
Maternal Characteristics			
Age (years)	29.9 (4.8)	30.3 (4.7)	30.6 (4.3)
Educational level			
Low	82 (15.5)	54 (13.5)	28 (11.0)
Medium	270 (51.0)	205 (51.4)	127 (49.8)
High	177 (33.5)	140 (35.1)	100 (39.2)
Parity			
Primiparous	231 (43.7)	168 (42.1)	113 (44.3)
Multiparous	298 (56.3)	231 (57.9)	142 (55.7)
Ethnic origin			
Greek	502 (95.1)	382 (96.0)	246 (96.5)
Non-Greek	26 (4.9)	16 (4.0)	9 (3.5)
Smoking during pregnancy			
Yes	159 (30.1)	123 (30.8)	76 (29.8)
No	370 (69.9)	179 (70.2)	152 (70.1)
Marital status			
Married	463 (87.7)	361 (90.7)	231 (90.9)
Other	65 (12.3)	37 (9.3)	23 (9.1)
Residence			
Urban	378 (77.3)	291 (78.4)	183 (78.5)
Rural	111 (22.7)	80 (21.6)	50 (21.5)
TSH during pregnancy (μIU/mL)	1.30 (1.0)	1.30 (1.1)	1.32 (1.1)
Pre-pregnancy BMI (kg/m^2)	24.9 (4.9)	24.9 (5.1)	25.0 (5.1)
Child Characteristics			
Sex			
Boy	281 (53.1)	230 (57.6)	145 (56.9)
Girl	248 (46.9)	169 (42.4)	110 (43.1)
Birth weight (g)	3208.3 (499.9)	3194.9 (446.9)	3212.7 (430.7)
Gestational age (weeks)	38.2 (1.6)	38.2 (1.6)	38.3 (1.5)

Breastfeeding (months)	4.2 (4.3)	4.2 (4.2)	4.4 (4.2)
Age at assessment (years)	4.2 (0.2)	6.6 (0.3)	11.0 (0.3)

[a] Data presented as mean (standard deviation) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned.

2.2.3. Study population: Child inflammatory markers at 4 years in association with child cognitive development at 4, 6 and 11 years of age

Out of 1363 singleton live births, 879 singleton children participated at the 4-years follow-up of the study, during which levels of inflammation markers were measured in 661 children. Analyses on the association between high levels of inflammatory markers at preschool age (4 years) and child cognitive development included full data on inflammation and cognitive assessment for a total of 566 children at 4 years, 366 at 6 years and 244 at 11 years. Regarding this analysis, participating mothers were again mainly Greek, married and had a mean age at pregnancy of approximately 30 years. About half of them had medium educational level and were multiparous. Approximately half of the children were boys. The baseline characteristics of the participants in the analyses are presented in Table 3.

Table 3. Baseline characteristics of the study participants at 4, 6 and 11 years of age, Rhea mother-child study, Crete, Greece

	4 years[a] (N=566)	6 years[a] (N=366)	11 years[a] (N=244)
Maternal Characteristics			
Age (years)	30.0 (4.9)	30.4 (4.8)	30.8 (4.4)
Educational level			
Low	86 (15.2)	44 (12.0)	23 (9.4)
Medium	292 (51.6)	187 (51.1)	121 (49.6)
High	188 (33.2)	135 (36.9)	100 (41.0)
Parity			
Primiparous	262 (46.3)	170 (46.5)	109 (44.7)
Multiparous	304 (53.7)	196 (53.5)	135 (55.3)
Ethnic origin			
Greek	539 (95.4)	351 (95.9)	235 (96.3)

Non-Greek	26 (4.6)	15 (4.1)	9 (3.7)
Marital status			
Married	499 (88.5)	325 (89.3)	221 (91.3)
Other	65 (11.5)	39 (10.7)	21 (8.7)
Residence			
Urban	412 (80.0)	274 (81.8)	186 (82.7)
Rural	103 (20.0)	61 (18.2)	39 (17.3)
Pre-pregnancy BMI (kg/m^2)	24.5 (4.7)	24.3 (4.5)	24.7 (4.8)
Child Characteristics			
Sex			
Boy	305 (53.9)	203 (55.5)	137 (56.2)
Girl	261 (46.1)	163 (44.5)	107 (43.8)
Birth weight (g)	3209.0 (442.7)	3191.5 (459.9)	3209.6 (434.3)
Preterm birth			
Yes	63 (11.1)	45 (12.3)	24 (9.8)
No	503 (88.9)	321 (87.7)	220 (90.2)
Gestational age (weeks)	38.3 (1.5)	38.2 (1.6)	38.3 (1.5)
BMI at age 4 (kg/m^2)	16.4 (1.9)	16.4 (1.9)	16.4 (1.8)
Passive smoking at age 4			
Yes	247 (43.6)	151 (41.3)	105 (43.0)
No	319 (56.4)	215 (58.7)	139 (57.0)
Age at assessment (years)	4.2 (0.2)	6.5 (0.3)	10.9 (0.3)

[a] Data presented as mean (standard deviation) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned.

2.2.4. Study population: Child inflammatory markers at 4 years in association with child behavioral and emotional development at 4, 6 and 11 years of age

Analyses on the association between high levels of inflammatory markers at preschool age (4 years) and child behavioral and emotional outcomes included full data on inflammation and those specific outcomes for a total of 524 children at 4 years, 373 at 6 years and 246 at 11 years. Overall, participating mothers were again mainly of Greek origin, married and had a mean age at pregnancy of approximately 30 years. About half of them had medium educational level and were multiparous.

Approximately half of the children were boys. The baseline characteristics of the participants in the analyses are provided in Table 4.

Table 4. Baseline characteristics of the study participants at 4, 6 and 11 years of age, Rhea mother-child study, Crete, Greece

	4 years[a] (N=524)	6 years[a] (N=373)	11 years[a] (N=246)
Maternal Characteristics			
Age (years)	30.1 (4.9)	30.4 (4.8)	30.7 (4.3)
Educational level			
Low	77 (14.7)	41 (11.0)	22 (8.9)
Medium	275 (52.5)	196 (52.5)	122 (49.6)
High	172 (35.8)	136 (36.5)	102 (41.5)
Parity			
Primiparous	243 (46.4)	172 (46.1)	111 (45.1)
Multiparous	281 (53.6)	201 (53.9)	135 (54.9)
Ethnic origin			
Greek	502 (95.8)	361 (96.8)	237 (96.3)
Non-Greek	22 (4.2)	12 (3.2)	9 (3.7)
Marital status			
Married	460 (88.1)	335 (90.3)	223 (91.4)
Other	62 (11.9)	36 (9.7)	21 (8.6)
Residence			
Urban	381 (72.2)	280 (81.6)	187 (82.7)
Rural	100 (20.8)	63 (18.4)	39 (17.3)
Pre-pregnancy BMI (kg/m^2)	24.5 (4.6)	24.4 (4.6)	24.6 (4.8)
Child Characteristics			
Sex			
Boy	282 (53.8)	212 (56.8)	137 (55.7)
Girl	242 (46.2)	161 (43.2)	109 (44.3)
Birth weight (g)	3209.7 (437.6)	3189.6 (452.4)	3210.9 (422.4)
Preterm birth			

Yes	59 (11.3)	46 (12.3)	23 (9.4)
No	465 (88.7)	327 (87.7)	223 (90.6)
Gestational age (weeks)	38.2 (1.5)	38.2 (1.6)	38.4 (1.4)
BMI at age 4 (kg/m^2)	16.4 (1.9)	16.4 (1.9)	16.4 (1.8)
Passive smoking at age 4			
Yes	222 (42.4)	159 (42.6)	104 (42.3)
No	302 (57.6)	214 (57.4)	142 (57.7)
Age at assessment (years)	4.2 (0.2)	6.5 (0.3)	10.9 (0.3)

[a] Data presented as mean (standard deviation) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned.

2.3. Biological sample collection and exposure assessment

2.3.1. Persistent Organic Pollutants concentration levels

Maternal serum samples were collected at the first prenatal visit around the 3rd and 4h month of pregnancy, in 10 mlτ Silicone gel separator vacutainer tubes (Becton Dickinson, UK). Tubes were centrifuged within 2 hours from blood collection at 2500rpm for 10min and were then stored in aliquots at -80°C until assayed (175). The POP analyses were performed in the National Institute for Health and Welfare, Chemicals and Health Unit, Kuopio, Finland with an Agilent 7000B gas chromatograph triple quadrupole mass spectrometer (GC-MS/MS). Pretreatment of serum samples for GC-MS/MS analysis has been described elsewhere (176). Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, and DDE were determined. All the results were reported on whole weight and expressed in pg/ml serum, while samples below the limit of quantification (LOQ) were assigned the value 0.5×LOQ. LOQ was 6 pg/ml for PCB118 and PCB156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, PCB180. We chose to use wet-weight levels for the POPs but adjusted for maternal serum triglycerides and cholesterol as continuous variables in all multivariable models to minimize potential biases associated with automatic lipid adjustment (177). POPs were treated as categorical variables. We calculated total PCB concentrations by summing the concentrations of the 6 individual PCB congeners and studied the associations of interest for the sum of PCBs.

Regarding our analyses, maternal POPs concentrations are presented in Table 5. The highest concentrations were found for DDE, followed by the sum of the six

measured PCBs, PCB153 and HCB. Spearman correlation coefficients (p-value) were 0.48 (<0.001) for DDE-PCBs, 0.48 (<0.001) for DDE-HCB, and 0.65 (<0.001) for PCBs-HCB.

Table 5. First trimester maternal serum POP levels (pg/ml, n=689)

				Percentile		
Contaminants	**Mean ± SD**	**min**	**max**	**25th**	**50th**	**75th**
HCB	109.2±102.4	19.5	1330.5	62.2	82.4	116.6
DDE	2947.9±3071.1	181.6	23175.4	1190.9	1955.9	3535.3
PCB118	20.3±12.5	3.0	143.6	12.0	17.8	25.3
PCB153	149.7±106.6	13.0	1348.9	85.5	125.7	188.3
PCB138	80.0±54.8	5.0	742.8	45.3	68.8	102.0
PCB156	8.2±7.6	3	81.5	3	6.5	10.7
PCB180	85.6±77.6	5	979.6	44.5	67.0	104.9
PCB170	43.3±40.6	5	542.3	21.9	33.8	53.5
Total PCBs	387.1±288.4	34.0	3758.3	217.3	320.8	484.8

Mothers that were older, multiparous, and had higher pre-pregnancy BMI, were more likely to have higher POP levels in early pregnancy, as shown in Table 6.

Table 6. Study participants characteristics by concentrations of first trimester maternal serum POPs levels (pg/ml)

		HCB	DDE	PCBs
Characteristic	N	r[a] or Mean ± SD	r[a] or Mean ± SD	r[a] or Mean ± SD
Maternal characteristics				
Maternal age (years)	688	0.53*	0.41*	0.66*
<20	17	52.2±21.5	1022.8±679.8	122.9±82.9
≥20-30	280	82.5±81.3	2178.2±2232.3	267.5±171.7
≥30-40	372	126.3±96.2	3617.2±3490.0	483.2±315.8
≥40	19	277.8±274.9	5870.4±3611.9	681.5±412.4
Ethnic origin				
Greek	653	109.7±103.5	2969.0±3030.1	391.5±295.7
Other	37	123.1±120.8	3652.8±4038.4	366.9±257.7
Education				
Low	104	94.4±70.9	2580.6± 2598.7	293.6± 219.6

Medium	351	102.8±94.2	2884.0 ± 3079.0	382.8± 326.1
High	228	129.2±128.6	3303.7±3118.1	439.8±231.2
Parity		0.12*	0.12*	0.11*
Primiparous	282	99.3±83.5	2520.1±2340.0	379.9±354.3
Multiparous	386	115.2±101.7	3354.6±3544.0	395.5±238.0
Pre-pregnancy BMI (kg/m²)	681	0.14*	0.08*	-0.09*
Underweight (<18.5)	21	77.5±90.7	2272.0±2139.5	327.5±239.9
Normal (≥18.5-25)	439	106.5±103.6	2923.6±2899.5	411.1±313.7
Overweight (≥25-30)	139	112.7±86.2	3312.4±3615.1	366.4±238.3
Obese (≥30)	82	136.7±136.4	2868.9±2842.9	309.3±166.7
Weight gain during pregnancy (kg)	572	-0.08*	-0.11*	-0.04
Smoking during pregnancy				
Never	562	107.1±100.6	2928.4±2942.5	387.3±297.4
Ever	111	125.9±125.7	3079.5±3319.6	387.2±222.4
Marital Status				
Married	678	109.4±102.8	2961.9±3072.8	387.9±289.7
Other	12	104.4±102.0	1515.2±1039.5	352.7±266.8
Never	81	128.3±135.3	3030.2±2913.9	390.7±240.5
Ever	588	108.5±100.6	3051.6±3256.5	391.6±295.3
Residence				
Urban	479	117.0±117.4	3006.7±3026.0	394.265.0
Rural	144	93.4±68.3	2859.4±3043.2	338.7±309.3
TSH during pregnancy (µIU/mL)	643			
Infant characteristics				
Sex				
Boy	359	105.7±92.8	3027.5±3057.7	384.2±314.7
Girl	336	115.0±115.4	3018.5±3295.1	400.1±280.1
Birth weight (g)	692	-0.09*	-0.08*	-0.05
Breastfeeding (months)	669	0.05	0.05	0.12*

2.3.2. Inflammatory Markers concentration levels

Following the completion of the 4-year-follow–up assessments, blood samples were collected by venipuncture for each child (10ml) in SST gel separator vacutainer

(BD vacutainers, UK), after written parental consent. For the reduction of pain and discomfort of the children, anesthetic cream 5% EMLA with composition 2.5% lidocaine and 2.5% prilocaine (AstraZeneca, UK) was used. Analyses were performed in the Laboratory of Clinical Nutrition and Epidemiology of Diseases of Medical School, University of Crete. Blood samples were centrifuged (Kubota 4000, Japan) at 2500rpm 10min within 2 hours after collection and stored at -80^{o} C until assayed. The Milliplex Map human high sensitivity T cell magnetic bead panel (Cat. # HSTCMAG-28SK) from Millipore (Billerica, MA) was used for the simultaneous quantification of IFN-γ IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α and TNF-α in the supernatants. The principle of the assay is based on the quantification of multiple bio-molecules concurrently employing fluorescent-coded magnetic beads (MagPlex-C microspheres). The microspheres were incubated with the samples and then were allowed to pass rapidly through laser systems that distinguish the different sets of microspheres and the fluorescent dyes on the reporter bio-molecules. The sensitivity of the assay for every bio-molecule was: 0.3 pg/ml IFN-γ, 0.1 pg/ml IL-1β, 0.1 pg/ml IL-6, 0.1 pg/ml IL-8, 0.6 pg/ml IL-10, 0.3 pg/ml IL-17α, 0.9 pg/ml MIP-1α and 0.2 pg/ml TNF-α. We used a limit of 4 SD based on the statistical convention that observations 4 or more SD from the expected mean can be considered to be "extreme outliers" and thus, excluded from the statistical analyses. The intra-assay precision (%CV) for all biomolecules was <5%. The inter-assay precision (%CV) for IFNγ, IL-6, IL-10 and IL-17α was <20%, for IL-1β, IL-8, MIP-1α and TNF-α was <15%. The above analyses were performed on an automated analyzer Luminex 100 connected with the Luminex xPONENT software.

Child inflammatory levels in serum at 4 years are presented in Table 7 and Table 8 illustrates correlation coefficients calculated for all those markers.

Table 7. Child inflammatory levels at 4 years of age (pg/ml)

Inflammatory Marker	N	Median (IQR)	Geometric Mean (GSD)	Percentile	
				10th	90th
IFN-γ pg/ml	636	26.1 (22.7)	21.8 (2.3)	6.5	50.3
IL-1β pg/ml	637	1.3 (1.0)	1.1 (2.1)	0.5	2.3
IL-6 pg/ml	635	1.1 (0.8)	1.1 (1.8)	0.5	2.3
IL-8 pg/ml	635	3.5 (1.9)	3.6 (1.5)	2.2	5.7
TNF-α pg/ml	639	6.0 (3.2)	5.8 (1.5)	3.4	9.3
IL-17α pg/ml	634	11.0 (12.0)	10.7 (2.2)	4.1	26.8
MIP-1α pg/ml	641	13.4 (7.6)	12.8 (1.5)	6.9	21.1
IL-10 pg/ml	641	5.3 (5.0)	5.4 (2.1)	2.1	13.3

GSD: Geometric Standard Deviation

Table 8. Spearman's rho correlation coefficients calculated for all inflammatory markers at 4 years of age

IFN-γ	1.000							
IL-1β	0.597*	1.000						
IL-6	0.340*	0.410*	1.000					
IL-8	0.203*	0.288*	0.255*	1.000				
IL-10	0.386*	0.451*	0.488*	0.342*	1.000			
IL-17α	0.675*	0.488*	0.271*	0.178*	0.363*	1.000		
TNF-a	0.305 *	0.236*	0.283*	0.304*	0.377*	0.253*	1.000	
MIP-1α	0.637 *	0.468*	0.245*	0.121*	0.354*	0.549*	0.238*	1.000
	IFN-γ	**IL-1β**	**IL-6**	**IL-8**	**IL-10**	**IL-17α**	**TNF-α**	**MIP-1α**

** $p < 0.05$*

2.4. Assessment of offspring development

2.4.1. Cognitive development

Preschoolers' cognitive development *at 4 years of age* was assessed through the *McCarthy Scales of Children's Abilities (MSCA)* (178). The *MSCA* are developed for children of ages 2½–8½ years, and are designed to assess children's cognitive and motor development in order to identify possible developmental delay. Overall, the

MSCA include 18 tasks, which are summarized in five scales: (i) verbal (verbal expression and comprehension), (ii) perceptual performance (reasoning), (iii)

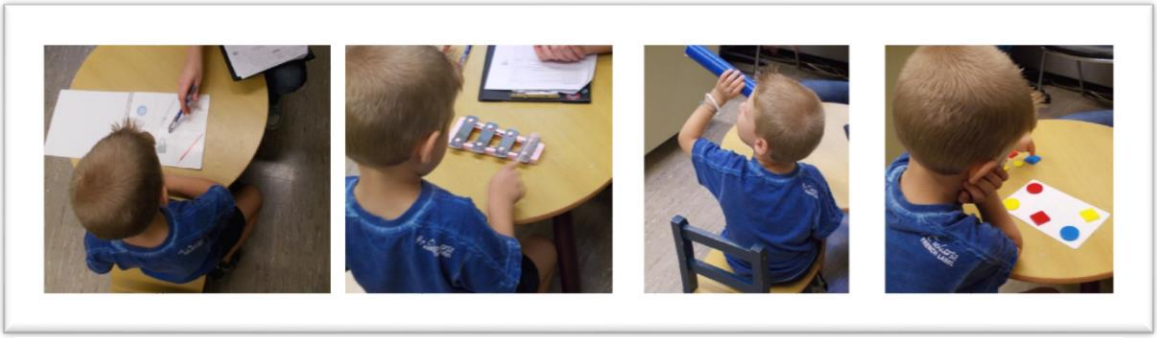

***Figure 3**. MSCA assessment during the 4-year-follow-up*

quantitative scale (numerical aptitude and interest), (iv) memory (verbal and non-verbal short-term memory), and (v) motor (gross and fine motor ability) and a general cognitive scale (overall cognitive ability), which is a composite scale of verbal, perceptual performance and quantitative scales. Executive function, working memory, memory span and cognitive functions of posterior cortex are four additional scales derived from the MSCA test in accordance with their association with specific neurocognitive function areas (179).

The translation and cross-cultural adaptation of the *MSCA* were performed according to the internationally recommended methodology. Internal consistency (Cronbach's alpha) varied between r_a = .76 and r_a = .89, showing adequate reliability for all the scales. Confirmatory factor analysis was performed using AMOS software (v.22, IBM SPSS Statistics, Armonk, NY) and maximum likelihood estimation method. A model with five correlated latent variables was confirmed, and the confirmatory factor analysis indices supported good fit of the model (v2 /df = 2, comparative fit index = .83, goodness-of-fit index = .97, root mean square error of approximation = .034). Raw scores of the *MSCA* were standardized for child's age at test administration using a parametric method for the estimation of age-specific reference intervals (180). The parameters of the distribution were modeled as fractional polynomial functions of age and estimated by maximum likelihood. Standardized residuals were then typified having a mean of 100 points with an SD of 15 to homogenize the scales (parameters conventionally used in psychometrics for IQ assessment). Scores were treated as continuous variables with higher scores representing better performance.

The *MSCA* were administered individually to the participants by two trained psychologists at random. The inter-observer variability was <1%. Right after each

neuropsychological assessment the psychologists completed a brief, standard form regarding the assessment conditions and difficulties encountered during administration, such as child's behavior (bad moods, nervousness) and physical condition (tiredness, colds). This report was used for creating the "quality of assessment index" for the MSCA, which was flagged as "excellent", "bad" or "very bad". Families received detailed feedback on their children's performance.

Cognitive development assessment *at 6 years of age* [mean (SD): 6.6 (0.3) years], was computerized and included the *Raven's Colored Progressive Matrices (RCPM)* (181), the Trail Making Test part A & part B (*TMT-A Part* & *TMT-B Part*) (182), and the *Finger Tapping Test (FTT)* (182). The *RCPM* consists of 36 items that are presented in 3 sets of 12, with an increasing level of difficulty within each set. *RCPM* is a non-verbal assessment of general intelligence for individuals over 5 years of age. The proposed task is to apply logical reasoning to identify the pattern in the presented material; by pinpointing the relationship between the elements of the system, the respondent successfully solves each of the problems. The outcome we utilized was the standardized score of the total sum of correct responses. The *TMT* assesses visual search, speed of processing, mental flexibility, and executive functions; it consists of two parts: in *TMT-A part* the participant has to draw lines sequentially connecting 25 framed numbers distributed on the screen, in *TMT-B part* the participant has to connect numbers sequentially, while alternating between framed and encircled symbols. The outcome we used is the time spent to finish the task in seconds, in each part. The *FTT* is designed to assess motor speed. The participant in instructed to repeatedly press a button at a stable joystick, as fast as they can for 15 seconds and 4 trials (2 trials with the dominant and 2 trials with the non-dominant). The outcome we used in the present analyses was the sum of hits of each hand.

Cognitive ability at 11 years was assessed through the greek version of the *Wechsler Intelligence Scale for Children, fifth edition (WISC-V)* (183). *WISC-V* is a standardized, widely used and well-validated individually administered psychometric instrument assessing intellectual functioning in children in ages 6–16 years of age. Seven core subtests (i.e., Similarities, Vocabulary, Block Design, Matrix Reasoning, Figure Weights, Digit Span, and Coding) contribute to the Full Scale IQ (FSIQ), whereas 10 subtests are required to derive the five *Primary Index Scales*, derived from factor analysis and designed to measure the five common specific intelligence

domains: Verbal Comprehension Index (VCI); Visual Spatial Index (VSI); Fluid Reasoning Index (FRI); Working Memory Index (WMI); and Processing Speed Index (PSI). Briefly stated, the VCI (Similarities and Vocabulary subtests) measures a child's verbal reasoning abilities, the VSI (Block Design and Visual Puzzles subtests) measures a child's visuospatial reasoning abilities. The FRI (Matrix Reasoning and Figure Weights subtests) provides a measure of inductive and quantitative reasoning, while the WMI (Digit Span and Picture Span subtests) assesses working memory and the PSI (Coding and Symbol Search) assesses speed of thinking and motor speed. At the *Ancillary Index Scales* level, there are scores that reflect various theoretical combinations of primary and secondary subtests, such as the Quantitative Reasoning Index (QRI) (including Figure Weights subtest), Nonverbal Index (NI) (including Block Design, Matrix Reasoning, Coding, Figure Weights, Visual Puzzles and Picture Span subtests), General Ability Index (GAI) (including Block Design, Similarities, Matrix Reasoning, Vocabulary and Figure Weights subtests) and Cognitive Proficiency Index (CPI) (including Digit Span, Coding, Picture Span, Symbol Search and Information subscales). The primary index scores and the ancillary index scores are on a standard score metric with a mean of 100 and an SD of 15, parameters conventionally used in psychometrics for IQ assessment. Scores were treated as continuous variables with higher scores representing better performance. As documented in its technical manual, the WISC-V boasts strong psychometric properties including test-retest reliability. All participants were administered the standard paper and pencil version of the WISC-V. Two trained school psychologists administered the WISC-V and verified its scoring. The inter-observer variability was <1%. Right after each neuropsychological assessment the psychologists completed a brief, standard form regarding the assessment conditions and difficulties encountered during administration, such as child's behavior (bad moods, nervousness) and physical condition (tiredness, colds). This report was used for creating the "quality of assessment index" for WISC-V, which was flagged as "excellent", "bad" or "very bad".

2.4.2. Behavioral and emotional development

Behavioral and emotional *symptoms* at *4 years of age* were assessed through the parent-report standardized questionnaires *Attention Deficit Hyperactivity Disorder Test (ADHDT)* (184) and the *Strengths and Difficulties Questionnaire (SDQ)* (185). The *ADHDT* is based on ADHD criteria of DSM-IV (186) and it is composed of 36 items

which assess ADHD related symptoms in ages 3-23 years. The instrument provides 4 indexes, corresponding to 3 subscales (13 items on the hyperactivity subscale, 13 items on the inattention subscale, and 10 items on the impulsivity subscale) and there is also a total ADHD difficulties score. Raters are instructed to mark the appropriate quantifier beside each behavior/characteristic. Ratings range from 0 (not a problem) to 1 (mild problem) and 2 (severe problem). All 36 items are summed, equally contributing to generate an index for total ADHD difficulties (possible range, 0-72). Higher scores indicate higher and more severe ADHD related symptomatology. The ADHDT has been translated and adapted for the Greek population (187). The *SDQ* is a brief behavioral screening questionnaire designed to assess strengths and difficulties in behavior from 3 to 16 years of age. The *SDQ* includes 25 items that assess emotional symptoms, conduct problems, hyperactivity and inattention, peer-relationship problems, and prosocial behaviour. The questionnaire provides also indexes of two broad-band scales assessing internalizing problems (emotional symptoms + peer-relationship problems) and externalizing problems (conduct problems + hyperactivity/inattention). . Items are rated on a 3-point Likert scale as either not true, somewhat true, or certainly true. Responses are scored 0-2 for negatively-worded items and prosocial items. Positively-worded items from the difficulties subscales are reverse-coded. Subscales range from 0 to 10, and the total difficulties score (which is a sum of the difficulties subscales), ranges from zero to 40.The *SDQ* was translated and adapted for the Greek population (188).

Behavioral and emotional problems at *6 and 11 years of age* were assessed through the parent-report questionnaires *Child Behaviour Checklist – Parent Report Form (CBCL)* (189) and the *Conners' Parent Rating Scale, Revised, Short Form (CPRS-R: S)* (190). The *CBCL* is a widely used parent-report questionnaire composed of 113 items and designed to assess behavioral and emotional difficulties in children between 6 and 18 years of age. The *CBCL* offers two alternative ways to summarize its items, the empirically-based syndrome scales and the DSM-oriented scales. The DSM-oriented scales, which were used in the research papers included in this thesis, include 6 scales that correspond to different diagnostic categories of the DSM-IV (186) (Affective problems, Anxiety problems, Somatic problems, Attention Deficit/Hyperactivity problems, Oppositional Defiant problems, and Conduct problems). The two broad-band scales of Internalizing problems & Externalizing problems were also used in the included research papers. The *CBCL* is translated, adapted, and standardized for the

Greek population (191). The *CPRS-R: S* is designed to assess ADHD symptoms and includes 27 items and assess oppositional problems, cognitive problems/inattention, and hyperactivity, as well as an index for total ADHD symptoms. The translation and cross-cultural adaptation of the *CPRS-R: S* was performed according to the recommended methodology (forward translation by two independent translators and synthesis of one translation, back-translation by bilingual expert in psychology, review of the original and the translated version of the manuscript by expert-panel, and pre-testing) (192).

Scores in each scale of *ADHDT*, *SDQ*, *CBCL*, and *CPRS-R* were treated as continuous variables. We should also note that scores indicate perceived symptoms and the severity of those symptoms and do not represent clinical disorders.

2.5. Covariates' selection

We selected the potential confounders based upon previously described covariates of our exposure and outcome variables of interest. In our analyses, the change in estimate, as well as the Directed Acyclic Graphs (DAGs) methodological approaches have been applied to determine the potential confounders that were taken into account for the adjusted multivariate models. When the change in estimate method was adopted, the cut-off point that was applied for the covariates inclusion in the models was a change greater than the 10% of the initial values of the estimates.

DAGs were also applied, since they represent causal relationships among variables; they have been extensively used in epidemiologic research to identify variables that must be measured and controlled in order to obtain unconfounded effect estimates (193). More specifically, a DAG is composed of variables and arrows between the variables (directed edges); the graph is acyclic, thus it is not possible to start at a variable, follow the directed edges of the arrows and end up back at the same variable. A causal DAG illustrates the causal inferences behind each research hypothesis and facilitates in determining the confounders of each relationship, avoiding the risk for selection bias that can be caused when other traditional methods are used (194).

Covariates' selection: Prenatal exposure to Persistent Organic Pollutants in association with offspring cognitive and behavioral development at 4, 6 and 11 years of age

We applied the change-in-estimate criterion to determine the confounders of the association between exposure to POPs in the first trimester of pregnancy and child cognitive and behavioral development during the three time points. The potential confounders were selected on the basis of literature concerning our study's hypotheses and were searched within a wide variety of factors, such as, maternal age during pregnancy, maternal educational level, maternal marital status at pregnancy, maternal ethnic origin, maternal residency, maternal tobacco smoking during pregnancy, maternal BMI, maternal IQ, parity, child's gestational age and weight at birth, breastfeeding duration, child's sex, and child's age at the time of assessment.

Child sex (male, female), child's age at the time of the assessment (years), quality of neuropsychological assessment (excellent, bad, very bad) (at 4 and 11-year assessments) and examiner (psychologist 1, psychologist 2) (at 4 and 11-year assessments), maternal serum triglycerides and cholesterol levels during pregnancy were a priori considered as potential confounding factors and included in all multivariate models. Additionally, we assessed whether maternal age (years), maternal educational level [low level: ≤ 9 years of mandatory schooling, medium level: > 9 years of schooling up to attending post-secondary school education and high level: attending university or having a university/ technical college degree], mother's tobacco smoking habits during pregnancy (never, ever), parity (primiparous, multiparous), and breastfeeding duration (months) had further influence on the effect estimates. Potential confounding by these factors was examined by each at a time in a basic model already containing serum triglycerides and cholesterol and the a priori defined cofounders. If inclusion of a variable altered the contaminant coefficient by 10% or more for a neurodevelopmental scale, we retained the variable in the final set of covariates. Following these methods our first model (basic model) was adjusted for maternal serum levels of triglycerides and total cholesterol, child sex, quality of assessment and examiner; the second model (fully-adjusted model) was additionally adjusted for maternal age, maternal educational level, mother's tobacco smoking habits during pregnancy, breastfeeding duration and parity.

Covariates' selection: Inflammatory markers at 4 years in association with offspring cognitive and behavioral development at 4, 6 and 11 years of age

In these analyses, we followed the DAGs approach to determine the potential confounders of the association between child inflammatory markers at 4 years and cognitive and behavioral development at 4, 6 and 11 years of age. Figure 4 illustrates the causal inferences concerning the association of child inflammation during preschool age and neurodevelopment for the fully adjusted model; the minimal sufficient adjustment set for estimating the total effect included the following factors: parity (primiparous/multiparous), maternal education [low level: ≤ 9 years of mandatory schooling, medium level: > 9 years of schooling up to attending post-secondary school education and high level: attending university or having a university/technical college degree], maternal age at pregnancy (years), maternal pre-pregnancy Body Mass Index (BMI) (kg/m^2), child BMI at 4 years (kg/m^2), preterm birth (yes, no), weight of birth (g) and passive smoking at 4 years (yes, no). Quality of assessment (good, bad, very bad), (at 4 and 11-year assessments), examiner (psychologist 1, psychologist 2) (at 4 and 11-year assessments), child sex (male, female), child age (years) were included a priori in all our models.

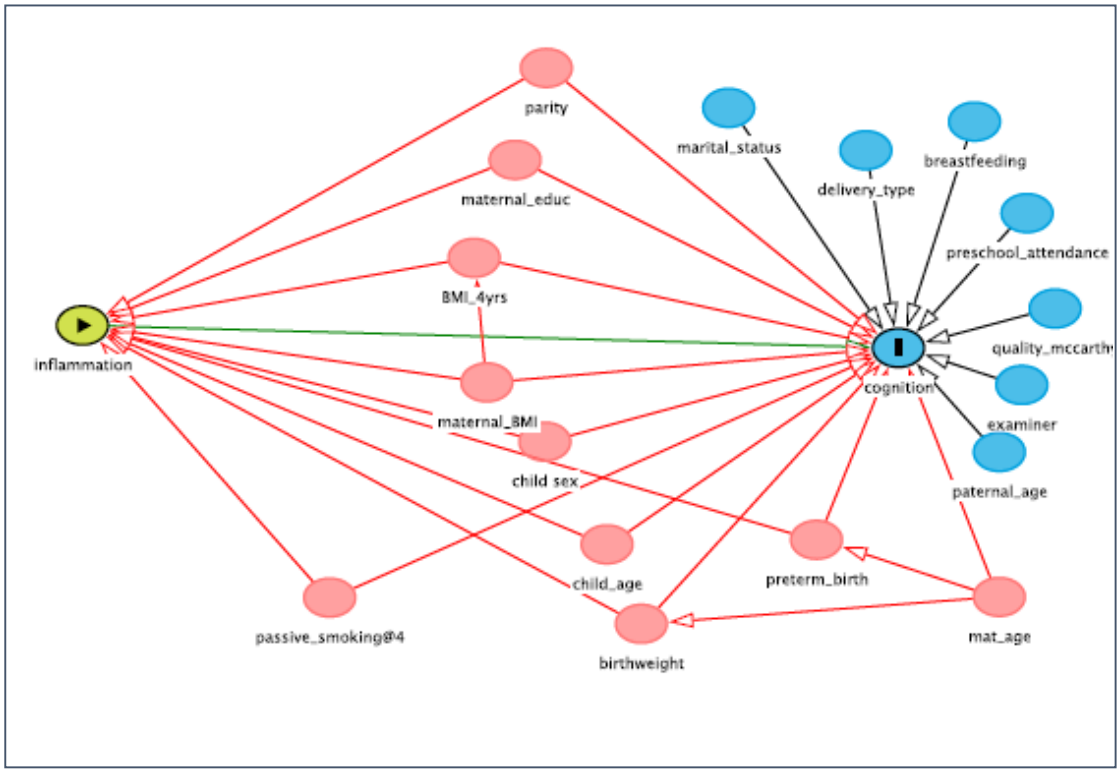

Figure 4*. Directed Acyclic Graph (DAG) for the selection of confounders on the inflammation-neurodevelopment analysis*

2.6. Statistical analysis

Descriptive analyses of the study population characteristics, exposures, and outcomes were conducted for each analysis of the present thesis. Overall, bivariate associations between normally distributed continuous variables and categorical variables were studied using either Student's t-test or ANOVA. Bivariate associations between non-normally continuous variables were studied using non-parametric statistical methods (Mann-Whitney, Kruskal-Wallis), and associations of categorical variables were tested using Pearson's Chi-square test.

Generalized additive models (GAMs) were applied in order to explore the shape of the associations between exposures of interest (POPs and inflammatory markers) and outcomes under study (cognitive and behavioral scores). These models did not indicate clear linear relationships (p-gain defined as the difference in normalized deviance between the generalized additive model and the linear model for the same exposure and outcome >0.1). Thus, since non-linearity was evident we used both maternal serum concentrations of child inflammatory biomarkers as categorical variables. Multivariate linear regression models were used to estimate crude and

adjusted beta coefficients and the corresponding 95% confidence intervals (95% CI's) for the exposure-outcome associations.

In addition, in the analyses of the association of POPs and inflammatory levels with child behavioral and emotional symptoms at 4 and 6 years (*ADHDT*, *SDQ*, and *CBCL* questionnaires), we had to deal with missing data (the missing values reached 24%). Therefore, we generated 20 complete data sets using multiple imputations with chained equations (MICE) (195). In the imputation model, all the questionnaire items (raw data) were regressed on all the other items (196). For the imputation of *ADHDT* and *SDQ* items, ordinal regression models were applied. This method was not feasible for the imputation of *CBCL* due to empty cells. Thus, Predictive Mean Matching (PMM) was applied. Although PMM is widely used for continuous variables, it has been shown that it can yield plausible inference for ordered categorical data as well (197). After obtaining the full datasets, all sub-scales were calculated separately for each imputed data set. No such problem occurred with the *CPRS-R: S* and with covariate information, where missing data did not exceed 2.1%. Estimations of the imputed data sets were combined using Rubin's rules (198). To explore potential differences between imputed and observed values, complete-case analysis was conducted. No meaningful change of the estimates was observed, thus we present effect estimates based on the multiply imputed data.

All hypothesis testing was conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. The standardization of the MSCA and all other statistical analyses were performed using Stata Software, version 13 (Stata Corp LP, College Station, TX, USA).

Statistical analysis: Prenatal exposure to Persistent Organic Pollutants in association with offspring cognitive and behavioral development at 4, 6 and 11 years of age

Since we found evidence of non-linearity between maternal serum POPS levels and the cognitive and behavioral outcomes (GAMs), in the analyses of the association of POPs with those outcomes, exposure variables were used as categorical. Figure 5 shows the GAMs for some main outcomes at 4 years of age. GAMs were also repeated in order to explore the shape of the associations between POPs and neurodevelopmental outcomes at 6 and 11 years of age, indicating similar results (non-linear relationships) (Figure 6 and 7).

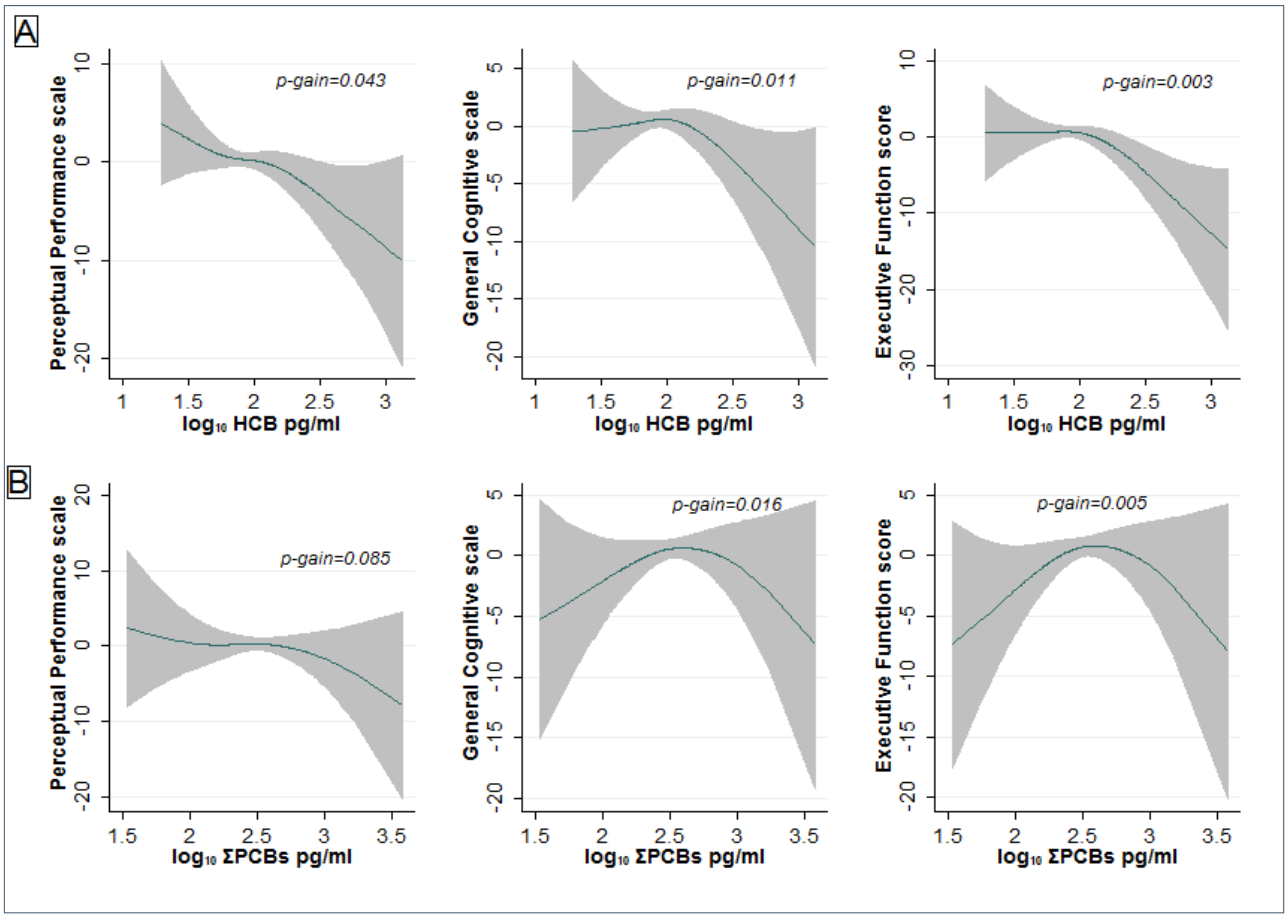

***Figure 5**. Adjusted associations (95% CIs) of (A) HCB and (B) sum of PCBs with offspring MSCA perceptual performance, general cognitive and executive function score at 4 years of age. All models were adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination, maternal age at birth, maternal educational level, smoking during pregnancy, parity and breastfeeding duration.*

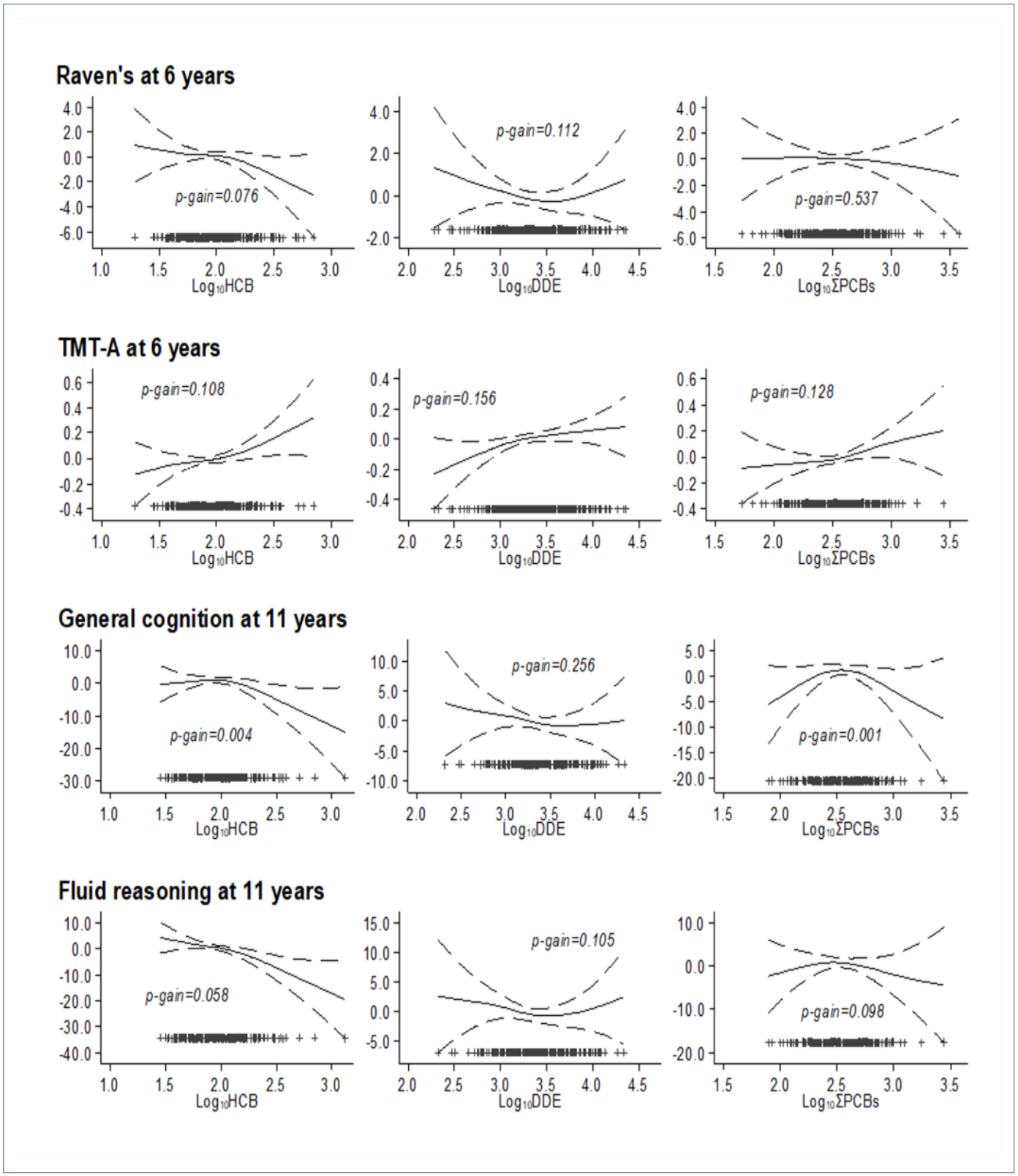

Figure 6. *Adjusted associations (95% CIs) of HCB, DDE and sum of PCBs levels with offspring cognitive scales at 6 and 11 years of age. Models regarding 6 years were adjusted for maternal serum triglycerides and cholesterol, child sex, child age, maternal age at birth, maternal educational level, smoking during pregnancy, parity and breastfeeding duration. Models regarding 11 years were adjusted for maternal serum triglycerides and cholesterol, child sex, child age, quality of assessment, examiner at 4 year assessment, maternal age at birth,*

maternal educational level, smoking during pregnancy, parity and breastfeeding duration. Plus symbols (+) represent observations.

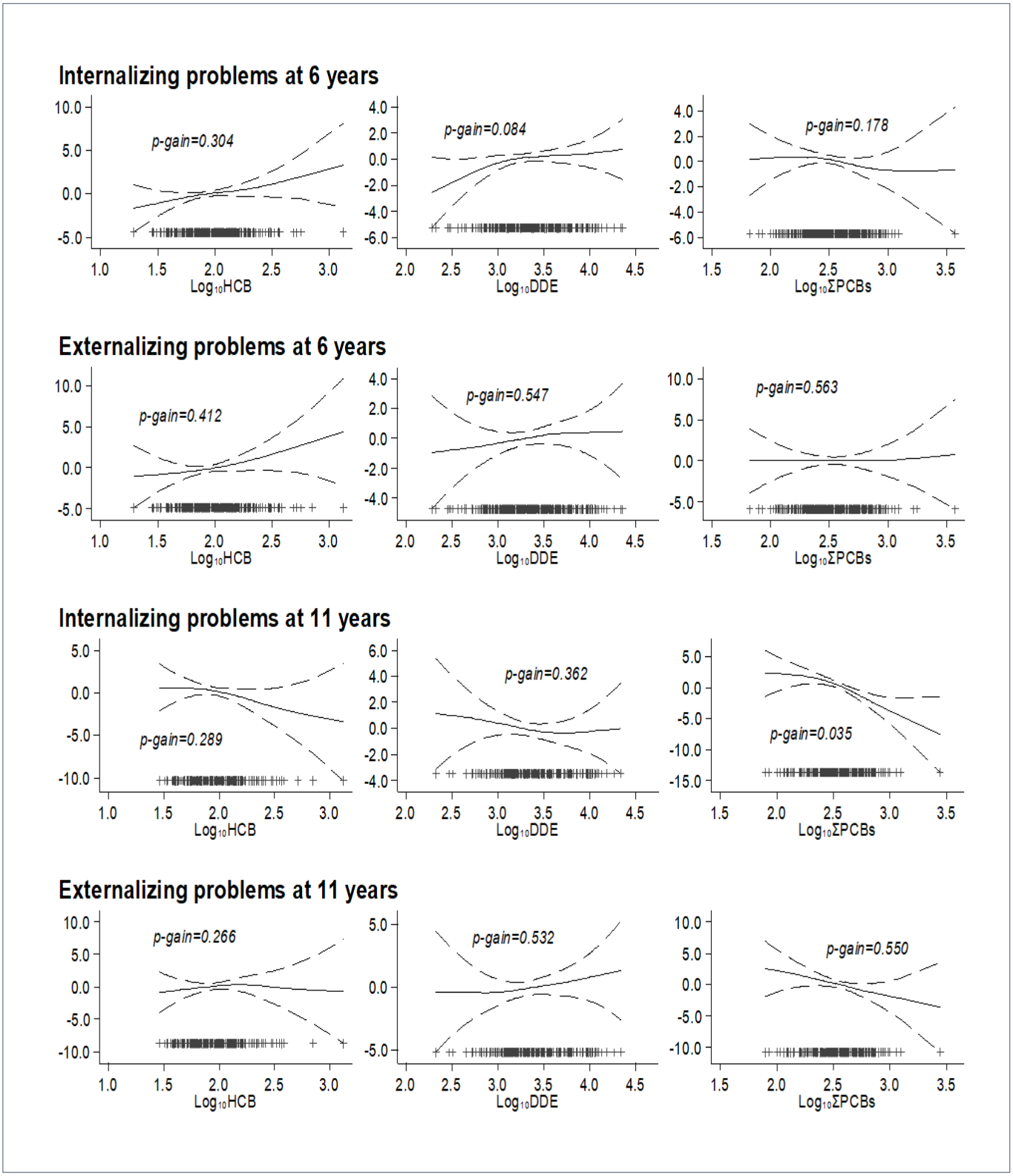

Figure 7. *Adjusted associations (95% CIs) of HCB, DDE and sum of PCBs levels with offspring behavioral and emotional scales at 6 and 11 years of age. Models regarding 6 and 11 years were adjusted for maternal serum triglycerides and cholesterol, child sex, child age,*

maternal age at birth, maternal educational level, smoking during pregnancy, parity and breastfeeding duration. Plus symbols (+) represent observations.

The non-linear association between maternal HCB levels and child's neurodevelopmental scores at 4 years of age was found slightly positive up to concentrations of the 90th percentile (which corresponds to concentrations of ~2.70 pg/ml for HCB levels) and after that point a more negative change in the slope was evident (Figure 8, scatterplot with a moving average Lowess curve). Similar patterns were observed for the rest of the contaminants and timepoints (6 and 11 years of age). Thus, POPs levels in maternal serum were treated as categorical variables; the categories were defined as the "high exposure group" (≥90th percentile) and the reference group (<90th percentile). Multivariate regression models were used to examine the association between prenatal POPs exposure and children's neurodevelopmental and behavioral outcomes at the three ages of interest.

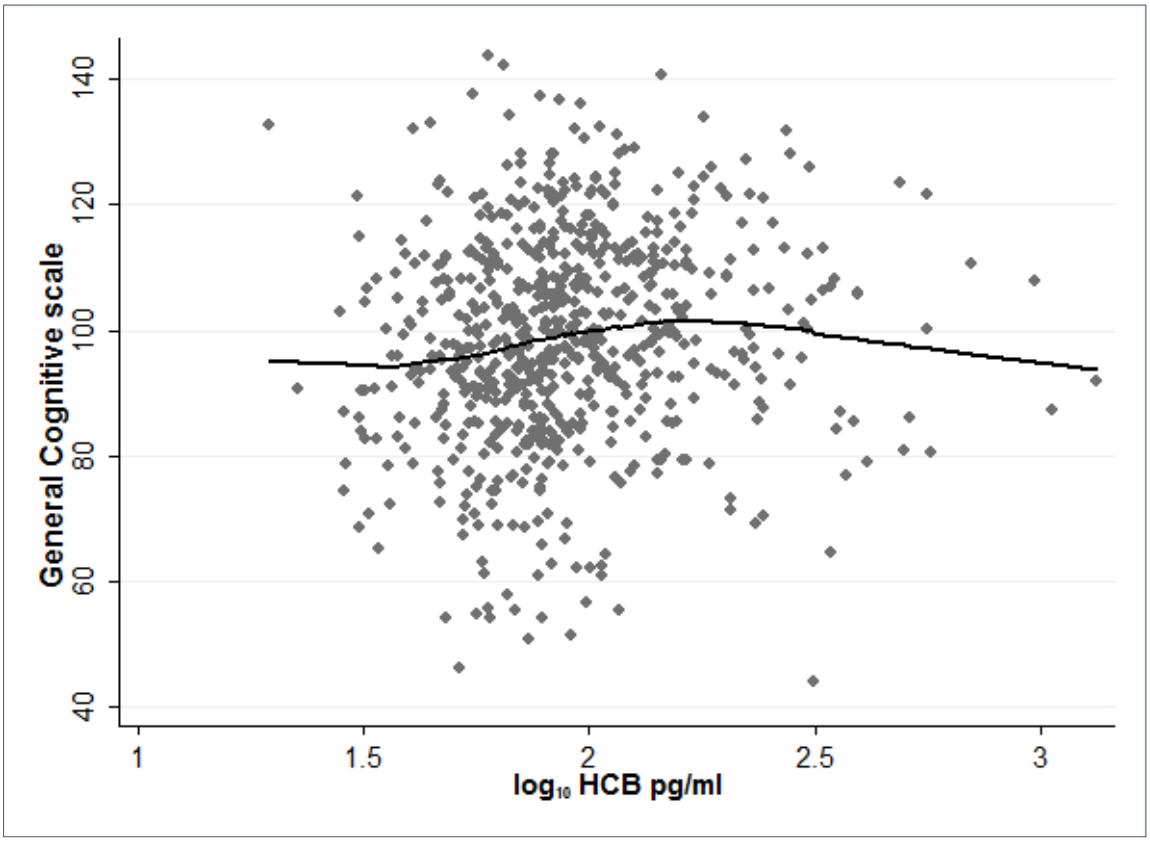

***Figure 8**. Association between maternal HCB levels during pregnancy (log10 transformed) and child general cognitive scale at 4 years of age. The line represents the mean change in the MSCA general cognitive score for every 10-fold increase in maternal HCB concentration.*

We also looked for heterogeneity in associations related to child sex (male, female), and maternal pre-pregnancy BMI status (<25 kg/m^2, ≥25 kg/m^2) and maternal TSH

during pregnancy by including interaction terms in the models. We repeated some of the analyses excluding children who had been born preterm (< 37 gestational weeks) in order to explore remaining confounding by prematurity. Finally, we performed further adjustment for maternal TSH during pregnancy (n=554) and for maternal intelligence for a subsample of the study population (n=266) with available information on maternal cognition. Maternal IQ was measured using the Raven's Standard Progressive Matrices (Raven 1998). Moreover, we studied the associations of interest in a multi-pollutant model including HCB, DDE and PCBs.

Statistical analysis: Child inflammatory markers at 4 years in association with child cognitive and behavioral development at 4, 6 and 11 years of age

Evidence of non-linearity between child inflammatory marker levels at 4 years and the cognitive and behavioral outcomes at 4, 6 and 11 years was revealed, as found in the GAMs that were performed. Figure 9 illustrates the GAMs for two main outcomes at 4 years of age; GAMs were also applied in order to explore the shape of the associations between inflammation at 4 years and neurodevelopmental outcomes at 6 and 11 years of age, indicating similar results (non-linear relationships) (Figure 10 and 11).

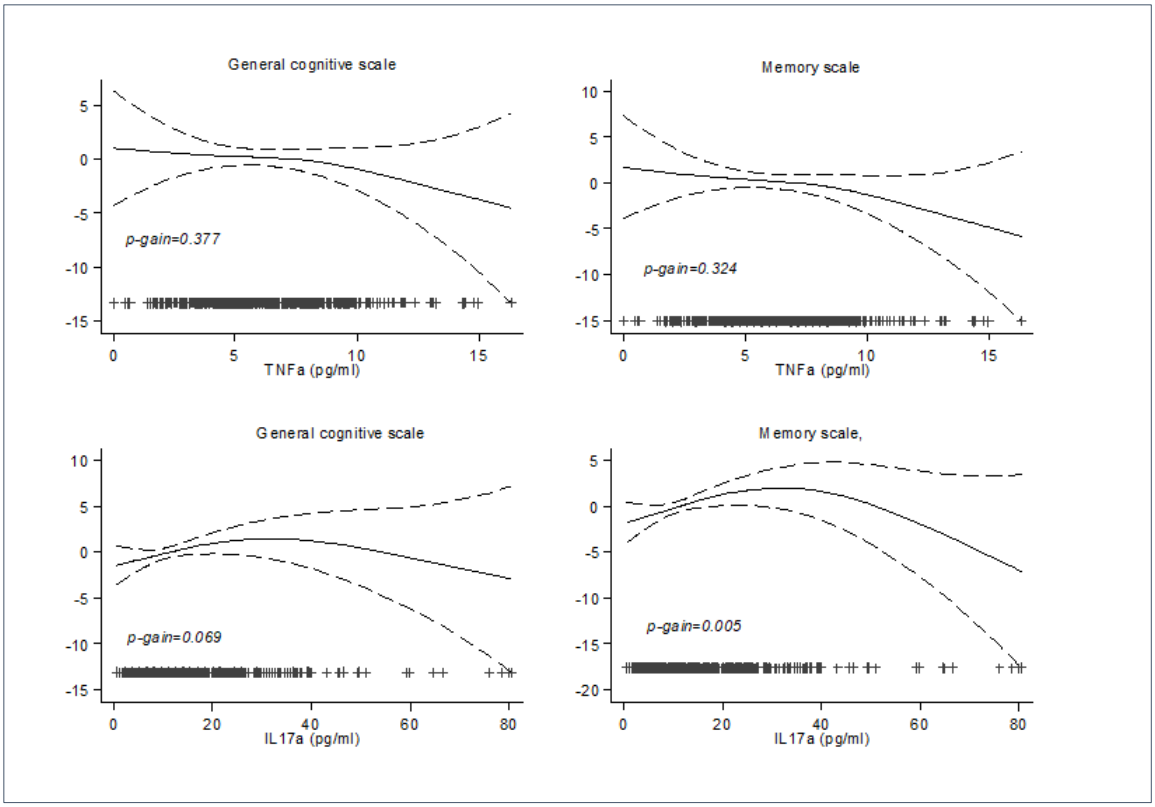

***Figure 9**. GAMS for adjusted associations (95% CIs) of TNF-a and IL-17a with child MSCA general cognitive and memory scale at 4 years of age. All models were adjusted for examiner, quality of assessment, child sex, maternal age in pregnancy, maternal educational level, BMI pre-pregnancy, parity, passive smoking at 4 years, weight of birth, preterm birth and BMI at 4 years. Plus symbols (+) represent observations.*

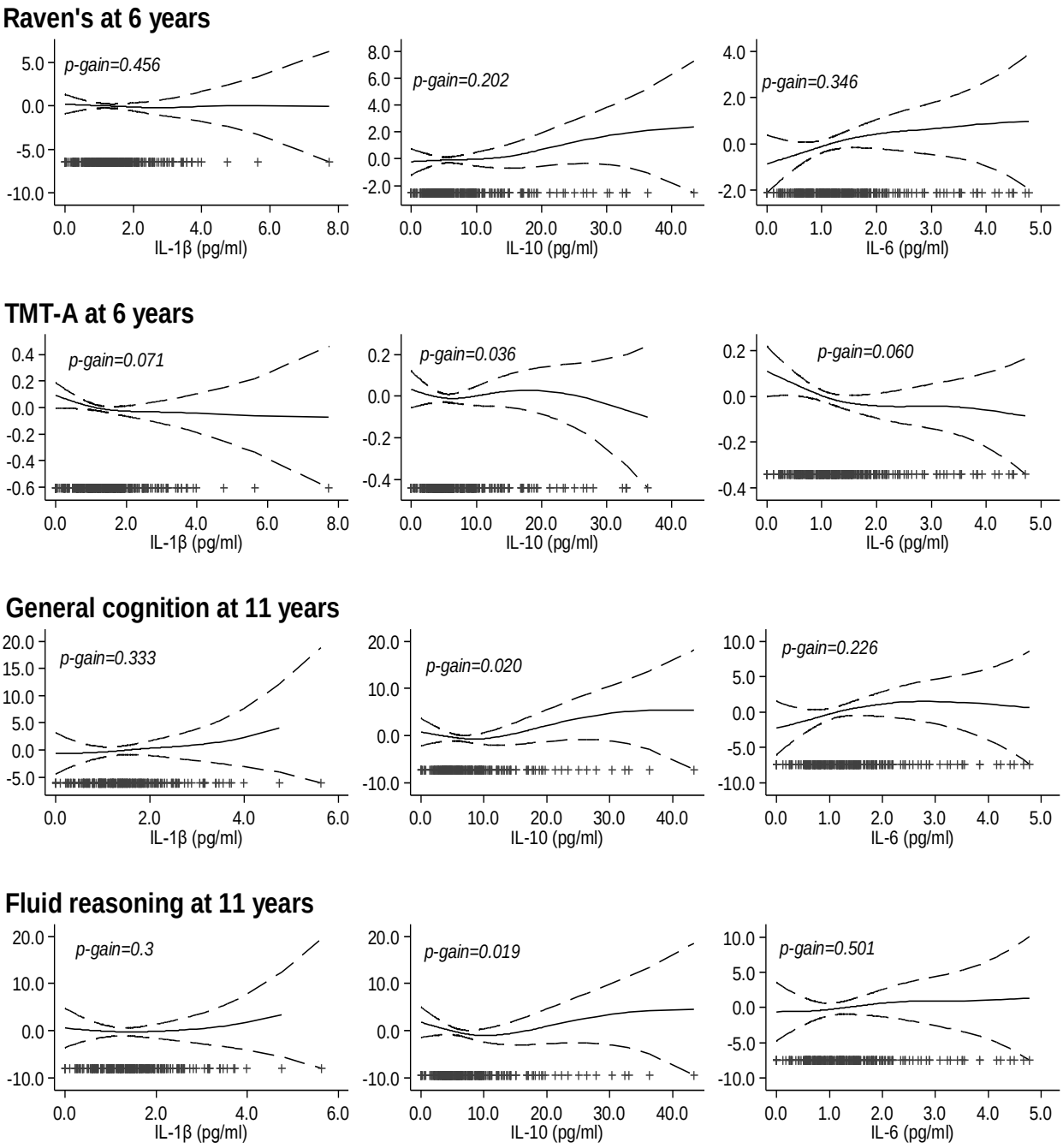

Figure 10. *Adjusted associations (95% CIs) of child IL-1β, IL-10 and IL-6 levels with cognitive outcomes at 6 and 11 years of age. Models regarding 6 years were adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, weight of birth, maternal BMI pre-pregnancy, parity, passive smoking at 4 years, preterm birth and BMI at 4 years. Models regarding 11 years were adjusted for child sex, child age, quality of assessment, examiner at 4 year assessment, maternal age at birth, maternal educational level, parity, weight of birth, maternal BMI pre-pregnancy, parity, passive smoking at 4 years, preterm birth and BMI at 4 years. Plus symbols (+) represent observations.*

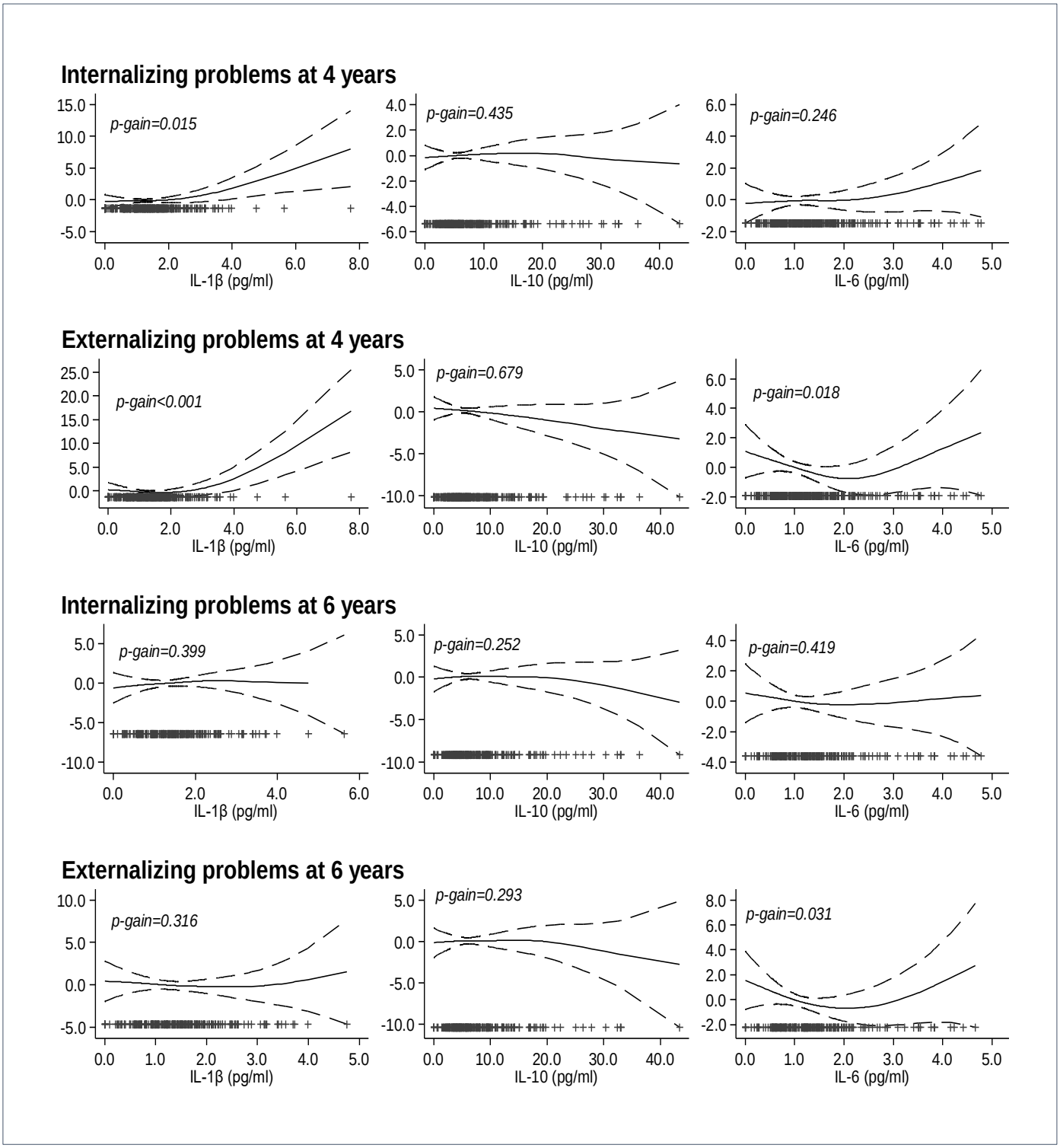

Figure 11. *Adjusted associations (95% CIs) of child IL-1β, IL-10 and IL-6 levels with behavioral and emotional outcomes at 4 and 6 years of age. All models were adjusted for for child sex, child age, maternal age at birth, maternal educational level, parity, weight of birth, maternal BMI pre-pregnancy, parity, passive smoking at 4 years, preterm birth and BMI at 4 years. Plus symbols (+) represent observations.*

In these analyses we used serum concentrations of inflammatory biomarkers as categorical variables; the categories were defined as the "high exposure group" (≥90th percentile) and the reference group (<90th percentile). This categorization was also decided upon graphical inspection of the relationship between outcomes and exposures after the application of a spline knot (Figure 12).

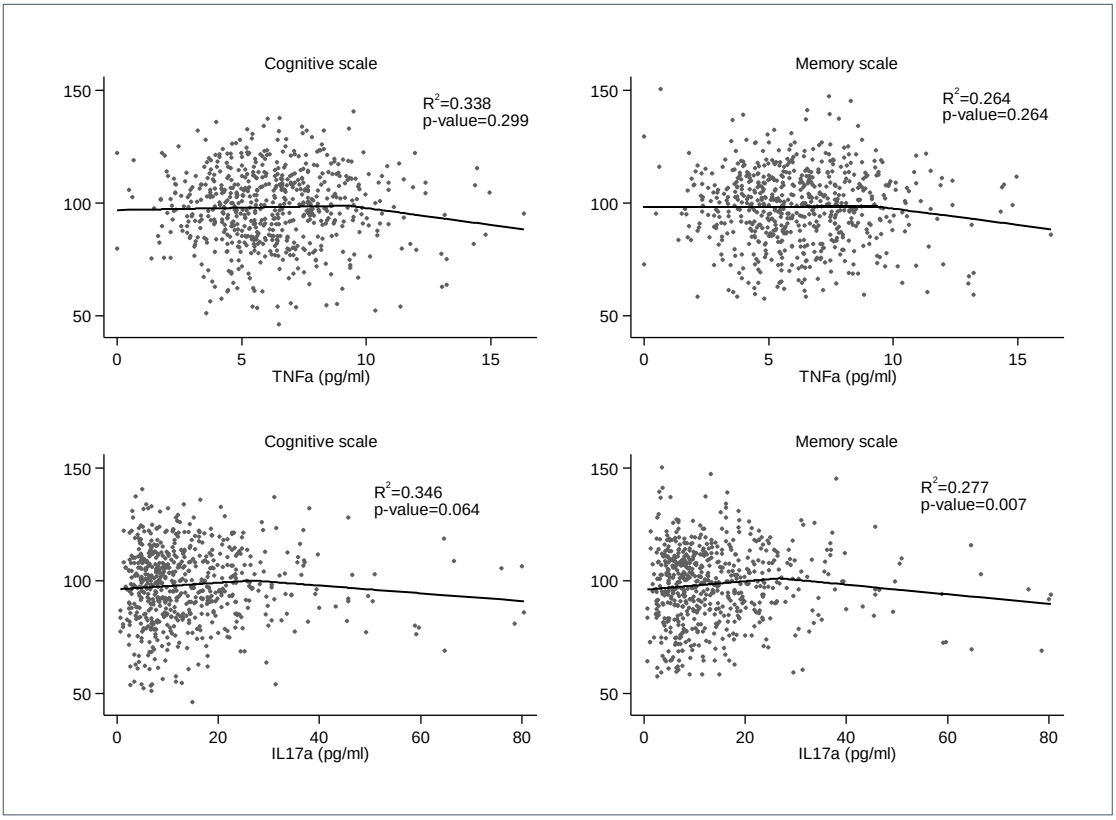

***Figure 12**. Association between child inflammatory marker levels at 4 years (pg/ml) and child neurodevelopmental scores at 4 years of age. The line represents the mean change in scores for every unit increase in child inflammatory marker levels. R-squared and p-values were derived from the fully adjusted model. The p-value assesses the significance of the change of the slope.*

We performed various sensitivity analyses to assess the robustness of our results. First, in order to assess if our studied associations were modified by child sex, child BMI at 4 years (normal weight vs. overweight or obese), maternal pre-pregnancy BMI (normal weight vs. overweight or obese), passive smoking exposure, appropriate interaction terms were included in the regression models. We stratified the sample in the cases that significant interactions were detected. Second, we repeated all analyses excluding preterm (<37 gestational weeks) neonates. Third, because relations of inflammatory markers with cognitive outcomes could be confounded by chronic child health diseases and infections, we repeated the analysis after further adjusting for

asthma occurrence at 4 years (yes/no), allergic rhinitis symptoms in the last 12 months at 4 years of age (yes/no), and helicobacter pylori seropositivity at 4 years of age (yes/no).

3. Results

3.1. Prenatal exposure to Persistent Organic Pollutants in association with offspring cognitive and behavioral development at 4, 6 and 11 years of age

Four years of age

Table 9 shows the association between high maternal POPs serum levels and MSCA scores attained at age four. Children with high levels of prenatal HCB exposure (≥ 90th percentile) demonstrated decreased scores in perceptual performance (adjusted β = -6.07; 95% CI: -10.17, -1.97), general cognition (adjusted β = -4.97; 95% CI: -8.99, -0.96) (Table 9), executive function (adjusted β = -6.24; 95% CI: -10.36, -2.11), as well as in working memory subscale (adjusted β = -4.71; 95% CI: -9.05, -0.36); high prenatal exposure to several PCBs (≥ 90th percentile) was associated with a 4.62 points reduction in working memory score (95% CI: -9.10, -0.14) (Table 10). High DDE levels were not associated with child MSCA scores at 4 years.

No association was demonstrated between prenatal POPs levels neither with SDQ nor with ADHDT scores at 4 years of age, with the sole exception of the association of high prenatal HCB levels with child peer problems in SDQ (see Table 11 and Table 12). To assess whether specific PCB congeners had an influence on the outcomes of our study, we performed separate analyses for the 6 PCB congeners (118, 153, 138, 156, 180 and 170). Individual PCB congeners had similar associations with the outcomes to those for total PCBs (data not shown).

Multi-pollutant model including elevated levels of DDE, HCB, and PCBs showed that associations with most of the outcomes (although not statistically significant) were mainly driven by HCB. However, our results indicate that the association with working memory subscale was equally driven by HCB and PCBs (Table 13). Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results (data not shown). No indication for effect modification by child sex, maternal pre-pregnancy body mass index (BMI) and maternal TSH during pregnancy was found (p-interaction > 0.10) (data not shown). Repeating the analysis after adjustment for maternal IQ in a subsample of 266 mother-

child pairs with available IQ data showed substantially the same inverse trend between prenatal POPs levels and offspring neurodevelopmental scores, though confidence intervals were wider, probably due to small sample size (Table 14).

Table 9. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 4 years

	McCarthy Scales of Children's Abilities											
	Verbal		Perceptual Performance		Quantitative		General Cognitive		Memory		Motor Scale	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants												
HCB pg/ml ≥90th perc												
Basic Model[1]	0.79	(-3.05, 4.63)	-2.12	(-6.04, 1.80)	-0.14	(-4.15, 3.86)	-0.39	(-4.29, 3.51)	0.75	(-3.11, 4.61)	-0.09	(-4.22, 4.05)
Adjusted Model[2]	-2.98	(-7.00, 1.05)	**-6.07**	**(-10.17, -1.97)**	-4.10	(-8.36, 0.15)	**-4.97**	**(-8.99, -0.96)**	-3.47	(-7.59, 0.65)	-2.97	(-7.49, 1.55)
DDE pg/ml ≥90th perc												
Basic Model[1]	-0.09	(-4.01, 3.84)	1.93	(-2.08, 5.94)	0.92	(-3.17, 5.01)	0.76	(-3.23, 4.74)	0.83	(-3.12, 4.78)	1.51	(-2.71, 5.74)
Adjusted Model[2]	-3.49	(-7.53, 0.55)	-1.29	(-5.43, 2.86)	-2.11	(-6.40, 2.17)	-3.01	(-7.05, 1.03)	-2.49	(-6.63, 1.66)	0.01	(-4.53, 4.55)
ΣPCBs[3] pg/ml ≥90th perc												
Basic Model[1]	1.96	(-2.03, 5.95)	0.19	(-3.90, 4.27)	0.07	(-4.10, 4.24)	1.26	(-2.80, 5.32)	2.57	(-1.44, 5.58)	1.38	(-2.93, 5.68)
Adjusted Model[2]	-1.41	(-5.57, 2.75)	-2.37	(-6.62, 1.88)	-3.44	(-7.83, 0.95)	-2.36	(-6.52, 1.79)	-0.43	(-4.69, 3.83)	0.22	(-4.44, 4.89)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination (N=609)
[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=586)
[3] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 10. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 4 years

	Subscales derived from McCarthy Scales of Children's Abilities							
	Executive Function		Working Memory		Memory Span		Functions of Posterior Cortex	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants								
HCB pg/ml ≥90th perc								
Basic Model[1]	-2.02	(-6.00, 1.96)	-1.18	(-5.17, 2.81)	1.13	(-3.87, 4.14)	1.09	(-2.74, 4.92)
Adjusted Model[2]	**-6.24**	**(-10.36, -2.11)**	**-4.71**	**(-9.05, -0.36)**	-3.71	(-8.02, 0.59)	-3.09	(-7.09, 0.90)
DDE pg/ml ≥90th perc								
Basic Model[1]	-0.14	(-4.21, 3.93)	-0.86	(-4.94, 3.22)	1.47	(-2.62, 5.56)	1.83	(-2.09, 5.74)
Adjusted Model[2]	-3.84	(-7.99, 0.32)	-3.66	(-8.03, 0.71)	-1.49	(-5.82, 2.83)	-1.50	(-5.52, 2.52)
ΣPCBs[3] pg/ml ≥90th perc								
Basic Model[1]	0.01	(-4.13, 4.16)	-1.59	(-5.74, 2.56)	2.95	(-1.21, 7.11)	2.08	(-1.91, 6.06)
Adjusted Model[2]	-3.58	(-7.85, 0.69)	**-4.62**	**(-9.10, -0.14)**	0.34	(-4.10, 4.78)	-1.09	(-5.22, 3.04)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination (N=608)
[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=585)
[3] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 11. Associations between prenatal exposure to POPs and child behavioral outcomes at 4 years of age [Attention Deficit Hyperactivity Disorder Test (ADHDT)]

	Attention Deficit Hyperactivity Disorder Test							
	Total score		Hyperactivity		Impulsivity		Inattention	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants								
HCB pg/ml ≥90th perc								
Basic Model[1]	-1.87	(-5.44, 1.70)	-1.14	(-2.62, 0.35)	0.41	(-1.58, 0.76)	-0.34	(-1.59, 0.92)
Adjusted Model[2]	0.85	(-3.12, 4.83)	0.19	(-1.45, 1.83)	0.42	(-0.86, 1.71)	0.50	(-0.90, 1.90)
DDE pg/ml ≥90th perc								
Basic Model[1]	-1.67	(-5.15, 1.81)	-1.13	(-2.62, 0.35)	-0.58	(-1.75, 0.59)	-0.54	(-1.81, 0.73)
Adjusted Model[2]	1.06	(-2.75, 4.88)	0.37	(-1.21, 1.95)	0.33	(-0.93, 1.61)	0.21	(-1.17, 1.60)
ΣPCBs[4] pg/ml ≥90th perc								
Basic Model[1]	-1.81	(-5.46, 1.84)	-0.46	(-1.96, 1.05)	-0.87	(-2.07, 0.33)	-0.43	(-1.74, 0.88)
Adjusted Model[2]	0.78	(-3.22, 4.77)	0.81	(-0.82, 2.45)	0.07	(-1.24, 1.39)	0.37	(-1.07, 1.81)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex and child age at assessment (N=524)
[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=441)
[4] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 12. Associations between prenatal exposure to POPs and child behavioral outcomes at age 4 years [Strengths and Difficulties Questionnaire (SDQ)]

	Strengths and Difficulties Questionnaire											
	Total score		Hyperactivity/ Inattention		Emotional problems		Conduct problems		Peer problems		Prosocial behavior	
	β	95% CI	β	95% CI	β	95% CI	B	95% CI	β	95% CI	β	95% CI
Contaminants												
HCB pg/ml ≥90th perc												
Basic Model[1]	-0.74	(-4.91, 3.42)	-2.12	(-6.23, 1.99)	-0.89	(-4.87, 3.09)	-1.92	(-6.03, 2.19)	2.32	(-1.60, 6.24)	-1.40	(-5.37, 2.57)
Adjusted Model[2]	3.83	(-0.73, 8.38)	1.69	(-2.16, 6.16)	0.19	(-4.23, 4.62)	0.38	(-4.21, 4.97)	**6.67**	**(2.41, 10.91)**	-0.03	(4.78, 4.07)
DDE pg/ml ≥90th perc												
Basic Model[1]	-2.65	(-6.86, 1.57)	-2.12	(-6.23, 1.99)	-1.62	(-5.66, 2.42)	-0.55	(-4.72, 3.61)	-0.94	(-4.92, 3.03)	0.82	(-3.20, 4.84)
Adjusted Model[2]	1.44	(-3.08, 5.97)	-2.05	(-6.59, 2.49)	-0.65	(-5.07, 3.77)	2.86	(-1.69, 7.40)	3.08	(-1.19, 7.36)	0.69	(-3.71, 5.09)
ΣPCBs[4] pg/ml ≥90th perc												
Basic Model[1]	-2.37	(-6.83, 2.06)	-1.50	(-5.84, 2.85)	-1.25	(-5.42, 2.93)	-1.54	(-5.77, 2.70)	-2.82	(-6.93, 1.28)	-3.31	(-7.42, 0.80)
Adjusted Model[2]	1.38	(-3.40, 6.15)	1.97	(-2.69, 6.64)	-0.01	(-4.62, 4.60)	1.23	(-3.43, 5.90)	-0.32	(-4.78, 4.14)	-3.15	(-7.70, 1.41)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex and child age at assessment (N=540)

[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=485)

[4] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 13. Association between POPs and outcomes in multipollutant models including HCB, DDE and PCBs (≥ 90th perc) at age 4 years

Outcome	**Exposure (≥ 90th perc)**	**Adjusted Model[1]**
Verbal scale[2]	HCB	-2.54 (-6.69, 1.61)
	DDE	-3.19 (-7.26, 0.89)
	ΣPCBs[6]	-0.19 (-4.80, 3.74)
Perceptual scale[2]	HCB	**-5.79 (-10.03, -1.56)**
	DDE	-0.62 (-4.77, 3.54)
	ΣPCBs[6]	-0.95 (-5.30, 3.41)
Quantitative scale[2]	HCB	-3.39 (-7.78, 1.00)
	DDE	-1.56 (-5.86, 2.75)
	ΣPCBs[6]	-2.50 (-7.02, 2.01)
General cognitive scale[2]	HCB	**-4.48 (-8.62, -0.34)**
	DDE	-2.46 (-6.52, 1.60)
	ΣPCBs[6]	-1.09 (-5.35, 3.17)
Memory scale[2]	HCB	-3.38 (-7.63, 0.88)
	DDE	-2.19 (-6.36, 1.98)
	ΣPCBs[6]	0.56 (-3.81, 4.94)
Motor scale[2]	HCB	-3.21 (-7.87, 1.46)
	DDE	0.26 (-4.32, 4.84)
	ΣPCBs[6]	0.96 (-3.84, 5.76)
Executive function[3]	HCB	**-5.47 (-9.72, -1.23)**
	DDE	-3.11 (-7.27, 1.05)
	ΣPCBs[6]	-2.01 (-6.38, 2.35)
Working memory[3]	HCB	-3.63 (-8.10, 0.85)
	DDE	-3.00 (-7.39, 1.39)

	ΣPCBs[6]	-3.50 (-8.10, 1.10)
Memory span[3]	HCB	-3.90 (-8.34, 0.54)
	DDE	-1.21 (-5.57, 3.14)
	ΣPCBs[6]	1.37 (-3.20, 5.94)
Functions of posterior cortex[3]	HCB	-2.91 (-7.04, 1.22)
	DDE	-1.18 (-5.23, 2.87)
	ΣPCBs[6]	-0.30 (-4.55, 3.95)
Total score ADHDT[4]	HCB	0.62 (-3.49, 4.73)
	DDE	0.96 (-2.90, 4.81)
	ΣPCBs[6]	0.55 (-3.57, 4.67)
Total score SDQ[5]	HCB	3.64 (-1.03, 8.31)
	DDE	1.16 (-3.38, 5.70)
	ΣPCBs[6]	0.53 (-4.35, 5.41)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination, maternal age at birth, maternal educational level, smoking during pregnancy and parity

[2] N=586

[3] N=585

[4] Adjusted for maternal serum triglycerides and cholesterol, child sex, child age at assessment, maternal age at birth, maternal educational level, smoking during pregnancy and parity (N=441)

[5] Adjusted for maternal serum triglycerides and cholesterol, child sex, child age at assessment, maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=485)

[6] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 14. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 4 years – mediator model also adjustment for maternal Intelligence Quotient (IQ) (N = 266)

	Contaminants					
	HCB pg/ml ≥ 90th perc		DDE pg/ml ≥ 90th perc		ΣPCBs[1] pg/ml ≥ 90th perc	
	B	95% CI	β	95% CI	β	95% CI
Neurodevelopmental scores						
Verbal	-0.42	(-6.32, 5.49)	-5.31	(-11.81, 1.21)	-0.86	(-6.96, 5.25)
Perceptual Performance	-5.14	(-10.82, 0.54)	-3.41	(-9.73, 2.91)	-3.87	(-9.75, 2.02)
Quantitative	-0.85	(-6.61, 4.91)	0.89	(-5.49, 7.27)	-6.54	(-12.43, -0.64)
General Cognitive	-2.55	(-8.27, 3.15)	-2.83	(-6.83, 1.17)	-3.41	(-9.30, 2.48)
Memory	-1.77	(-7.58, 4.04)	-2.41	(-6.53, 1.71)	-1.86	(-7.87, 4.14)
Motor Scale	-3.57	(-10.04, 2.91)	-0.03	(-4.60, 4.54)	-2.05	(-8.75, 4.65)
Executive function	-3.06	(-8.99, 2.87)	-4.20	(-10.76, 2.35)	-3.73	(-9.85, 2.39)
Working Memory	-0.93	(-6.81, 4.93)	-1.51	(-8.01, 4.99)	**-6.26**	**(-12.27, -0.25)**
Memory Span	-3.01	(-9.00, 2.99)	-3.47	(-10.11, 3.17)	-3.29	(-9.48, 2.91)
Posterior Cortex	-1.83	(-7.46, 3.81)	-3.26	(-9.49, 2.97)	-2.38	(-8.19. 3.44)

[1] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Six years of age

Table 15 shows the association between high maternal POPs serum levels and neuropsychological tasks attained at six years of age. Children with high levels of prenatal HCB exposure (≥ 90th percentile) showed decreased scores in Raven's Coloured Progressive Matrices (adjusted β = -1.90; 95% CI: -3.61, -0.18), increased response time in TMT Part A (adjusted β = 0.16; 95% CI: 0.01, 0.31) and Part B (adjusted β = 0.24; 95% CI: 0.07, 0.40), as well as decreased scores in FFT (dominant hand) (adjusted β = -6.58; 95% CI: -13.13, -0.03); high DDE levels in maternal serum during the first trimester were associated with increased response time in TMT Part B (adjusted β = 0.15; 95% CI: 0.01, 0.30) in offsprings. Additionally, high prenatal exposure to several PCBs (≥ 90th percentile) was associated with a 0.14 points increase in response time in TMT Part A (95% CI: 0.01, 0.27) and with a -7.15 points decrease in FFT (non-dominant hand) scores (95% CI: -13.13, 1.17). No association was demonstrated between high prenatal POPs levels neither with CPRS-R: S nor with CBCL scores at 6 years of age (Table 16).

Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results, although associations were found non-significant

due to limited power (N=45) (data not shown). No indication for effect modification by child sex was found, other than one interaction between child sex and HCB levels in response to one behavioral scale (p for interaction < 0.05); stratified analysis revealed increased CPRS-R: S ADHD index scores for boys with high concentrations of HCB (adjusted β = 2.47; 95% CI: -0.27, 5.21), whereas these associations in girls were in the opposite direction (adjusted β = -1.58; 95% CI: -4.23, 1.08). Finally, no indication for effect modification by maternal pre-pregnancy body mass index (BMI), child BMI and maternal TSH during pregnancy was found (p-interaction > 0.10) (data not shown).

Table 15. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 6 years

	Neuropsychological developmental outcomes at 6 years									
	RCPM: Total score[1]		TMT: Part A (log-transformed)[1]		TMT: Part B (log-transformed)[1]		FTT: Dominant hand[1]		FTT: Non-dominant hand[1]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants										
HCB pg/ml ≥90th perc										
Basic Model[2]	-0.88	(-2.44, 0.68)	0.13	(-0.01, 0.26)	**0.17**	**(0.02, 0.32)**	-4.94	(-10.81, 0.93)	-2.66	(-8.65, 3.33)
Adjusted Model[3]	**-1.90**	**(-3.61, -0.18)**	**0.16**	**(0.01, 0.31)**	**0.24**	**(0.07, 0.40)**	**-6.58**	**(-13.13, -0.03)**	-3.87	(-10.74, 2.99)
DDE pg/ml ≥90th perc										
Basic Model[2]	1.15	(-0.32, 2.61)	**0.12**	**(0.01, 0.24)**	0.08	(-0.06, 0.22)	-4.11	(-9.46, 1.24)	-0.12	(-5.64, 5.39)
Adjusted Model[3]	0.89	(-0.68, 2.46)	0.07	(-0.07, 0.20)	**0.15**	**(0.01, 0.30)**	-3.82	(-9.60. 1.97)	-0.16	(-6.21, 5.90)
ΣPCBs[4] pg/ml ≥90th perc										
Basic Model[2]	-0.13	(-1.58, 1.32)	**0.17**	**(0.02, 0.32)**	0.09	(-0.05, 0.22)	-4.61	(-9.88, 0.65)	**-6.75**	**(-12.01, -1.47)**
Adjusted Model[3]	-0.26	(-1.84, 1.33)	**0.14**	**(0.01, 0.27)**	0.11	(-0.03, 0.26)	-4.66	(-10.50, 1.18)	**-7.15**	**(-13.13, -1.17)**

[1]Abbreviations: TMT: Trail Making Test, FTT: Finger Tapping Test, RCPM: Raven's Coloured Progressive Matrices

[2] Adjusted for maternal serum triglycerides and cholesterol, child sex, child age (N=409)

[3]As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=383)

[4] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 16. Associations between prenatal exposure to POPs and child behavioral outcomes at age 6 years

	Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S) (N=388)								Child Behaviour Checklist – Parent Report Form (CBCL): broad-band scales (N=399)			
	Oppositional		Inattention		Hyperactivity		ADHD index		Externalized problems		Internalized problems	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants												
HCB pg/ml ≥90th perc												
Basic Model[2]	-0.31	(-1.36, 0.74)	-0.01	(-1.08, 1.08)	-0.70	(-1.67, 0.26)	0.18	(-1.59, 1.94)	-0.68	(-2.66, 1.30)	-0.54	(-1.95, 0.87)
Adjusted Model[3]	-0.22	(-1.4, 0.96)	0.01	(-1.24, 1.26)	-0.47	(-1.59, 0.64)	0.37	(-1.65, 2.39)	0.57	(-1.72, 2.86)	-0.08	(-1.70, 1.54)
DDE pg/ml ≥90th perc												
Basic Model[2]	-0.59	(-1.55, 0.38)	-0.63	(-1.64, 0.38)	-0.29	(-1.18, 0.60)	-0.67	(-2.31, 0.98)	-1.25	(-3.13, 0.63)	-0.36	(-1.70, 0.99)
Adjusted Model[3]	-0.53	(-1.57, 0.52)	-0.55	(-1.66, 0.57)	-0.02	(-1.02, 0.97)	-0.46	(-2.26, 1.33)	-0.41	(-2.46, 1.64)	0.17	(-1.28, 1.62)
ΣPCBs[4] pg/ml ≥90th perc												
Basic Model[2]	-0.34	(-1.33, 0.65)	-0.79	(-1.81, 0.22)	-0.34	(-1.25, 0.57)	-0.03	(-1.70, 1.64)	-0.91	(-2.77, 0.94)	-1.28	(-2.60, 0.03)
Adjusted Model[3]	0.07	(-1.02, 1.15)	-0.75	(-1.90, 0.40)	0.12	(-0.92, 1.15)	0.46	(-1.39, 2.31)	0.68	(-1.41, 2.78)	-0.59	(-2.07, 0.89)

[2] Adjusted for maternal serum triglycerides and cholesterol, child sex and child age (N=419)
[3] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=392)
[4] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Eleven years of age

Associations between high maternal POPs serum levels and neuropsychological tasks at eleven years of age are displayed in Table 17 and 18. Children with high levels of prenatal HCB exposure (≥ 90th percentile) showed decreased scores in various primary and ancillary WISC-V index scales at 11 years, such as Visual Spatial Index (adjusted β = -6.26; 95% CI: -12.23, -0.30), Fluid Reasoning Index (adjusted β = -8.89; 95% CI: -14.38, -3.39), Working Memory Index (adjusted β = -7.73; 95% CI: -13.22, -2.24), Processing Speed Index (adjusted β = -7.81; 95% CI: -13.78, -1.84), Quantitative Reasoning Index (adjusted β = -8.68; 95% CI: -14.49, -2.87), Nonverbal Index (adjusted β = -9.40; 95% CI: 15.02, -3.79), General Ability Index (adjusted β = -7.55; 95% CI: 12.77, -2.34) and Cognitive Proficiency Index (adjusted β = -8.85; 95% CI: 13.95, -3.75). High PCBs levels (≥ 90th percentile) in maternal serum during the first trimester were associated with lower scores in two primary index scales [Working Memory Index (adjusted β = -6.68; 95% CI: -11.71, -1.65), Processing Speed Index (adjusted β = -8.01; 95% CI: -13.44, -2.58)] and all the ancillary WISC-V index scales [Quantitative Reasoning Index (adjusted β = -5.62; 95% CI: -10.95, -0.27), Nonverbal Index (adjusted β = -7.35; 95% CI: 12.51, -2.19), General Ability Index (adjusted β = -5.58; 95% CI: -10.37, -0.78) and Cognitive Proficiency Index (adjusted β = -7.07; 95% CI: 11.76, -2.39)].

Findings regarding high prenatal POPs levels and behavioral outcomes showed that, although in the basic model there were statistically significant associations between high HCB levels (≥ 90th percentile) and oppositional (adjusted β= -1.45; 95% CI: -2.74, -0.17), and hyperactivity CPRS-R: S scales (adjusted β = -1.44; 95% CI: -2.47, -0.40), and CBCL externalized (adjusted β = -3.41; 95% CI: -5.91, -0.92) and internalized problems scales (adjusted β = -2.28; 95% CI: -4.51, -0.05), in the adjusted model the estimates were weakened and non-significant (see Table 19). No other association was found between high prenatal POPs levels and CPRS-R: S and CBCL scores at 11 years of age (Table 19).

Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results, although associations were found non-significant due to limited power (N=27) (data not shown). No indication for effect modification by child sex, maternal pre-pregnancy body mass index (BMI) and maternal TSH during pregnancy was found (p-interaction > 0.10) (data not shown).

Table 17. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 11 years

	Wechsler Intelligence Scale for Children-V (WISC-V) *Primary Index Scales*									
	Verbal Comprehension Index		Visual Spatial Index		Fluid Reasoning Index		Working Memory Index		Processing Speed Index	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants										
HCB pg/ml ≥90th perc										
Basic Model[1]	1.36	(-3.39, 6.12)	-0.57	(-5.96, 4.81)	-4.30	(-9.36, 0.74)	-2.58	(-7.55, 2.39)	-2.58	(-7.84, 2.69)
Adjusted Model[2]	-3.41	(-8,53, 1.70)	**-6.26**	**(-12.23, -0.30)**	**-8.89**	**(-14.38, -3.39)**	**-7.73**	**(-13.22, -2.24)**	**-7.81**	**(-13.78, -1.84)**
DDE pg/ml ≥90th perc										
Basic Model[1]	3.07	(-1.77, 7.91)	4.50	(-0.96, 9.96)	4.29	(-0.86, 9.45)	2.93	(-2.13, 8.00)	3.03	(-2.32, 8.41)
Adjusted Model[2]	-0.18	(-5.03, 4.66)	1.08	(-4.60, 6.76)	1.95	(-3.34, 7.24)	0.28	(-4.98, 5.55)	-0.33	(-6.04, 5.38)
ΣPCBs[3] pg/ml ≥90th perc										
Basic Model[1]	0.99	(-3.55, 5.23)	-0.97	(-6,10, 4.16)	-1.41	(-6.25, 3.42)	-2.80	(-7.54, 1.93)	-3.45	(-8.46, 1.56)
Adjusted Model[2]	-2.90	(-7.58, 1.78)	-4.89	(-10.35, 0.58)	-4.60	(-9.69, 0.5)	**-6.68**	**(-11.71, -1.65)**	**-8.01**	**(-13.44, -2.58)**

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex, child age, quality of assessment, examiner at 11-year assessment (N=268)
[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=254)
[3] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 18. Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 11 years

	Wechsler Intelligence Scale for Children-V (WISC-V) *Ancillary Index Scales*							
	Quantitative Reasoning Index		Nonverbal Index		General Ability Index		Cognitive Proficiency Index	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants								
HCB pg/ml ≥90th perc								
Basic Model[1]	-4.38	(-9.69, 0.92)	-3.33	(-8.60, 1.93)	-1.57	(-6.57, 3.42)	-2.85	(-7.75, 2.06)
Adjusted Model[2]	**-8.68**	**(-14.49, -2.87)**	**-9.40**	**(-15.02, -3.79)**	**-7.55**	**(-12.77, -2.34)**	**-8.85**	**(-13.95, -3.75)**
DDE pg/ml ≥90th perc								
Basic Model[1]	4.19	(-1.22, 9.61)	4.90	(-0.45, 10.26)	4.58	(-0.49, 9.64)	3.96	(-1.03, 8.95)
Adjusted Model[2]	1.76	(-3.81, 7.34)	1.40	(-4.01, 6.28)	1.07	(-3.93, 6.07)	0.49	(-4.43, 5.42)
ΣPCBs[3] pg/ml ≥90th perc								
Basic Model[1]	-2.49	(-7.57, 2.58)	-2.93	(-7.95, 2.10)	-1.03	(-5.80, 3.73)	-2.34	(-7.02, 2.35)
Adjusted Model[2]	**-5.62**	**(-10.95, -0.27)**	**-7.35**	**(-12.51, -2.19)**	**-5.58**	**(-10.37, -0.78)**	**-7.07**	**(-11.76, -2.39)**

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex, child age at 11-year assessment (N=268)
[2] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=254)
[3] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

Table 19. Associations between prenatal exposure to POPs and child behavioral outcomes at age 11 years

	Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S) (N=254)								**Child Behaviour Checklist – Parent Report Form (CBCL): broad-band scales (N=246)**			
	Oppositional		Inattention		Hyperactivity		ADHD index		Externalized problems		Internalized problems	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Contaminants												
HCB pg/ml ≥90th perc												
Basic Model[2]	**-1.45**	**(-2.74, -0.17)**	-0.03	(-1.28, 1.21)	**-1.44**	**(-2.47, -0.40)**	-1.80	(-3.97, 0.36)	**-3.41**	**(-5.91, -0.92)**	**-2.28**	**(-4.51, -0.05)**
Adjusted Model[3]	-1.19	(-2.65, 0.28)	0.44	(-0.99, 1.88)	-0.95	(-2.13, 0.23)	-1.22	(-3,78, 1.33)	-2.02	(-4.89, 0.86)	-2.25	(-4.81, 0.32)
DDE pg/ml ≥90th perc												
Basic Model[2]	-0.79	(-2.07, 0.49)	-0.36	(-1.61, 0.89)	-0.62	(-1.64, 0.41)	-0.41	(-2.60, 1.78)	-0.64	(-3.10, 1.81)	-0.51	(-2.71, 1.68)
Adjusted Model[3]	-0.33	(-1.70, 1.04)	0.24	(-1.10, 1.59)	-0.05	(-1.14, 1.04)	0.53	(-1.85, 2.29)	0.95	(-1.70, 3.60)	0.12	(-2.26, 2.49)
ΣPCBs[4] pg/ml ≥90th perc												
Basic Model[2]	-0.62	(-1.85, 0.60)	0.01	(-1.18, 1.21)	-0.84	(-1.83, 0.15)	-1.27	(-3.36, 0.82)	-1.83	(-4.28, 0.63)	-2.76	(-4.88, -0.63)
Adjusted Model[3]	-0.03	(-1.37, 1.30)	0.58	(-0.73, 1.89)	-0.28	(-1.36, 0.8)	-0.27	(-2.60, 2.06)	-0.13	(-2.76, 2.51)	-2.10	(-4.44, 0.24)

[1] Adjusted for maternal serum triglycerides and cholesterol, child sex and child age (N=267)
[2]As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy, breastfeeding duration and parity (N=253)
[3] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180

3.2. Child inflammatory markers at 4 years in association with child cognitive and behavioral development at 4, 6 and 11 years of age

Four years of age

Table 20 shows regression results for high inflammatory marker levels in child serum in relation to neurodevelopmental outcomes (MSCA scores) at 4 years of age. Children with high IFN-γ serum levels (≥ 90th percentile) showed lower scores in memory span scale (adjusted β = -3.4; 95% CI: -7.3, -0.4). Children with high TNF-α serum levels (≥ 90th percentile) demonstrated decreased scores in memory (adjusted β = -4.0; 95% CI: -7.7, -0.2), working memory (adjusted β = -4.0; 95% CI: -8.0, -0.1) as well as in memory span scale (adjusted β = -4.0; 95% CI: -7.9, -0.1). High TNF-α/IL-10 ratio was associated with decreased quantitative (adjusted β = -4.3; 95% CI: -8.2, -0.4), motor (adjusted β = -3.5; 95% CI: -7.5, -0.5), executive function (adjusted β = -4.8; 95% CI: -8.5, -1.1), general cognitive (adjusted β = -3.6; 95% CI: -7.3, -0.1), memory (adjusted β = -3.8; 95% CI: -7.6, -0.1), working memory (adjusted β = -3.5; 95% CI: -7.5, -0.5) and memory span scores (adjusted β = -5.3; 95% CI: -9.1, -1.4). No other association was detected between high inflammatory levels and other cognitive and behavioral scores at 4 years of age, with the sole exception of the association of high IL-8 levels with low prosocial behavior in SDQ (adjusted β = -0.50; 95% CI: -0.99, -0.01) (see Table 23 and Table 24).

Further analyses showed evidence for an interaction between child sex and IL-17α levels in response to neurodevelopmental scores (p for interaction < 0.05). Stratified analysis revealed reduced verbal scale scores for boys with high concentrations of IL-17α (adjusted β = -4.2; 95% CI: -10.2, 1.7), whereas these associations in girls were in the opposite direction. Moreover, boys with high concentrations of IL-6 had lower motor (adjusted β = -0.2; 95% CI: -6.2, 5.8) scale scores (Table 21). Further stratified analysis according to child BMI status, showed reduced scores in memory (adjusted β = -11.4; 95% CI: -20.6, -2.2) and memory span (adjusted β = -11.3; 95% CI: -20.2, -2.4) scores for overweight/obese children with high concentrations of TNF-α in serum compared to children with normal weight (Table 22). We found no evidence of significant interactions between maternal pre-pregnancy BMI, child BMI, exposure to passive smoking and child inflammatory biomarker levels at 4 years of age (p for interaction > 0.10). Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results. Finally,

after further adjustment for asthma, allergic rhinitis and helicobacter pylori seropositivity at 4 years, our results did not differ substantially from those derived from the main analysis (data not shown).

Table 20. Adjusted associations between child inflammatory markers levels at age 4 years and child neurodevelopmental outcomes at age 4 years

McCarthy Scales of Children's Abilities

	Verbal	Perceptual	Quantitative	Motor	Exec. Function	General cognitive	Memory	Working memory	Memory span
	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)
Markers with pro-inflammatory activity (≥90th percentile)									
IFN-γ	-2.3 (-5.9, 1.3)	1.7 (-1.9, 5.4)	-1.0 (-4.8, 2.8)	2.5 (-1.5, 6.4)	-1.7 (-5.3, 2.0)	-1.0 (-4.6, 2.6)	-2.8 (-6.5, 1.0)	-2.1 (-6.1, 1.8)	**-3.4 (-7.3, -0.4)**
IL-1β	-2.2 (-5.8, 1.4)	-2.1 (-5.7, 1.6)	1.2 (-2.7, 5.0)	-2.2 (-6.2, 1.7)	-0.8 (-4.5, 2.8)	-1.9 (-5.5, 1.7)	-2.6 (-6.3, 1.2)	1.3 (-2.6, 5.3)	-2.7 (-6.5, 1.2)
IL-6	2.1 (-1.6, 5.7)	1.9 (-1.8, 5.5)	0.8 (-3, 4.7)	3.7 (-.3, 7.6)	1.0 (-2.7, 4.6)	2.1 (-1.5, 5.8)	3.0 (-0.8, 6.8)	0.1 (-3.9, 4.1)	2.9 (-1.0, 6.8)
IL-8	-1.0 (-4.6, 2.7)	-1.0 (-4.6, 2.7)	-0.9 (-4.8, 2.9)	0.8 (-3.2, 4.7)	-1.7 (-5.3, 2.0)	-1.2 (-4.8, 2.5)	-1.2 (-4.9, 2.6)	-2.0 (-5.9, 2.0)	-0.8 (-4.7, 3.1)
TNF-α	-3.2 (-6.9, .4.0)	-1.7 (-5.3, 2.0)	-2.6 (-6.5, 1.2)	-2.4 (-6.3, 1.6)	-2.8 (-6.4, 0.9)	-3.0 (-6.6, 0.7)	**-4.0 (-7.7, -0.2)**	**-4.0 (-8.0, -0.1)**	**-4.0 (-7.9, -0.1)**
IL-17α	1.4 (-2.3, 5.0)	-2.3 (-6.0, 1.4)	1.6 (-2.3, 5.5)	-1.5 (-5.5, 2.5)	0.3 (-3.4, 4.1)	0.1 (-3.5, 3.8)	0.9 (-2.9, 4.7)	1.9 (-2.1, 5.9)	0.3 (-3.6, 4.2)
MIP-1α	-0.2 (-3.8, 3.4)	-0.5 (-4.2, 3.1)	0.7 (-3.1, 4.5)	0.8 (-3.1, 4.7)	1.3 (-2.3, 4.9)	-0.3 (-3.9, 3.3)	-1.5 (-5.2, 2.2)	1.1 (-2.8, 5.0)	-1.4 (-5.2, 2.4)
Markers with anti-inflammatory activity (≥90th percentile)									
IL-10	1.2 (-2.5, 4.8)	2.7 (-1.0, 6.3)	0.7 (-3.1, 4.6)	2.8 (-1.2, 6.7)	2.0 (-1.6, 5.7)	1.8 (-1.9, 5.4)	0.1 (-3.6, 3.9)	-0.4 (-4.4, 3.5)	1.0 (-2.8, 4.9)
Ratios (≥90th percentile)									
IL-6/IL10	-0.2 (-3.9, 3.5)	-1.5 (-5.2, 2.2)	-1.3 (-5.2, 2.6)	-2.6 (-6.6, 1.3)	-1.8 (-5.5, 1.9)	-0.9 (-4.6, 2.8)	0.8 (-3.0, 4.6)	-0.7 (-4.7, 3.3)	0.6 (-3.3, 4.5)
TNF-α/IL-10	-3.0 (-6.7, 0.7)	-2.9 (-6.6, 0.8)	**-4.3 (-8.2, -0.4)**	**-3.5 (-7.5, -0.5)**	**-4.8 (-8.5, -1.1)**	**-3.6 (-7.3, -0.1)**	**-3.8 (-7.6,- 0.1)**	**-3.5 (-7.5, -0.5)**	**-5.3 (-9.1, -1.4)**

Adjusted for examiner, quality of assessment, child sex, maternal age in pregnancy, maternal education, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years (N = 634)

Table 21. Adjusted associations between child inflammatory markers levels at age 4 years and child neurodevelopmental outcomes at age 4 years stratified by child sex

McCarthy Scales of Children's Abilities

		Verbal	Perceptual	Quantitative	Motor	Exec. Function	General cognitive	Memory	Working memory	Memory span
		beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)
	Markers with pro-inflammatory activity (≥90th percentile)									
IFN-γ	Boys	-2.1 (-7.5, 3.3)	3.5 (-2.1, 9.1)	1.4 (-4.4, 7.2)	2.3 (-3.8, 8.4)	-1.0 (-6.8, 4.7)	0.2 (-5.2, 5.7)	-2.5 (-8.0, 3.0)	0.9 (-5.0, 6.7)	-3.2 (-8.8, 2.4)
	Girls	-2.4 (-7.2, 2.4)	0.5 (-4.2, 5.2)	-3.1 (-8.1, 2.0)	3.0 (-2.0, 8.0)	-2.0 (-6.6, 2.5)	-1.8 (-6.6, 2.9)	-2.7 (-7.9, 2.4)	-5.0 (-10.4, 0.4)	-3.8 (-9.1, 1.4)
IL-1β	Boys	-0.3 (-5.8, 5.1)	-1.2 (-6.9, 4.4)	5.1 (-0.8, 11.0)	-0.3 (-6.6, 5.9)	1.3 (-4.6, 7.1)	0.3 (-5.3, 5.8)	-0.7 (-6.3, 4.8)	6.1 (0.1, 12.0)	-1.3 (-7.0, 4.4)
	Girls	-3.5 (-8.3, 1.3)	-2.5 (-7.2, 2.2)	-2.0 (-7.1, 3.1)	-3.4 (-8.4, 1.5)	-2.2 (-6.8, 2.3)	-3.5 (-8.2, 1.2)	-3.9 (-9.0, 1.3)	-2.1 (-7.5, 3.3)	-3.9 (-9.1, 1.4)
IL-6	Boys	1.7 (-3.6, 7.0)	-1.0 (-6.5, 4.5)	0.8 (-4.9, 6.5)	**-0.2 (-6.2, 5.8) ‡**	1.2 (-4.5, 6.8)	0.8 (-4.5, 6.2)	3.1 (-2.3, 8.5)	1.6 (-4.2, 7.4)	2.2 (-3.4, 7.7)
	Girls	2.2 (-2.9, 7.3)	2.9 (-2.1, 7.8)	1.4 (-4.0, 6.7)	**6.7 (1.4, 11.9) ‡**	0.1 (-4.7, 4.9)	2.8 (-2.1, 7.8)	2.7 (-2.7, 8.2)	-0.7 (-6.4, 5.0)	3.8 (-1.8, 9.3)
IL-8	Boys	-3.5 (-8.9, 1.9)	-3.2 (-8.8, 2.4)	-3.2 (-9.0, 2.7)	-2.4 (-8.5, 3.7)	-4.6 (-10.3, 1.2)	-4.0 (-9.4, 1.5)	-3.3 (-8.7, 2.2)	-5.5 (-11.3, 0.4)	-3.0 (-8.6, 2.6)
	Girls	1.5 (-3.3, 6.4)	1.0 (-3.7, 5.8)	1.8 (-3.4, 6.9)	3.8 (-1.2, 8.9)	1.2 (-3.4, 5.8)	1.5 (-3.2, 6.3)	1.0 (-4.2, 6.2)	1.8 (-3.6, 7.3)	1.6 (-3.8, 6.9)
TNF-α	Boys	-3.4 (-8.2, 1.5)	-1.4 (-6.5, 3.7)	-1.4 (-6.6, 3.9)	-1.4 (-6.9, 4.1)	-2.6 (-7.8, 2.6)	-2.7 (-7.6, 2.2)	-4.2 (-9.1, 0.8)	-3.9 (-9.2, 1.4)	-4.6 (-9.7, 0.4)
	Girls	-3.3 (-8.7, 2.2)	-1.3 (-6.7, 4.1)	-3.8 (-9.5, 2.0)	-2.8 (-8.4, 2.9)	-3.0 (-8.2, 2.2)	-3.1 (-8.5, 2.3)	-3.1 (-8.9, 2.8)	-3.7 (-9.8, 2.4)	-2.2 (-8.2, 3.8)
IL-17α	Boys	**-4.2 (-10.2, 1.7) ‡**	-4.6 (-10.8, 1.6)	-1.8 (-8.2, 4.7)	-3.7 (-10.5, 3.0)	-4.1 (-10.4, 2.3)	-4.7 (-10.7, 1.3)	-2.8 (-8.8, 3.3)	-1.3 (-7.8, 5.2)	-3.9 (-10, 2.3)
	Girls	**4.8 (0.2, 9.4) ‡**	-1.1 (-5.7, 3.5)	3.6 (-1.4, 8.6)	0.6 (-4.3, 5.5)	2.6 (-1.8, 7.1)	2.9 (-1.7, 7.5)	3.6 (-1.4, 8.6)	4.0 (-1.3, 9.2)	2.8 (-2.4, 7.9)
MIP-1α	Boys	1.0 (-3.9, 5.9)	-2.1 (-7.2, 3.0)	3.8 (-1.4, 9.1)	-1.0 (-6.6, 4.5)	2.0 (-3.2, 7.2)	0.4 (-4.6, 5.4)	-0.6 (-5.6, 4.4)	5.2 (-0.2, 10.5)	0.6 (-4.5, 5.7)
	Girls	-1.3 (-6.6, 4.0)	1.5 (-3.7, 6.7)	-2.7 (-8.2, 2.9)	3.4 (-2.1, 8.9)	0.6 (-4.4, 5.7)	-0.8 (-6.0, 4.4)	-2.2 (-7.9, 3.4)	-3.7 (-9.6, 2.2)	-3.8 (-9.6, 2.0)
	Markers with anti-inflammatory activity (≥90th percentile)									

IL-10	Boys	1.5 (-3.5, 6.5)	3.7 (-1.5, 8.9)	1.3 (-4.1, 6.7)	1.1 (-4.6, 6.8)	2.3 (-3.1, 7.6)	2.4 (-2.7, 7.5)	0.4 (-4.7, 5.5)	-0.5 (-6.0, 5.0)	2.9 (-2.4, 8.1)
	Girls	0.5 (-4.9, 5.9)	1.7 (-3.6, 7.0)	1.0 (-4.7, 6.7)	5.3 (-0.3, 10.9)	1.5 (-3.6, 6.6)	1.1 (-4.2, 6.4)	0.9 (-4.9, 6.7)	0.60 (-5.4, 6.7)	0.4 (-5.5, 6.3)
	Ratios (≥90th percentile)									
IL-6/ IL10	Boys	0.9 (-4.2, 6.1)	-1.1 (-6.3, 4.2)	-2.2 (-7.6, 3.3)	-3.1 (-8.8, 2.7)	-0.7 (-6.1, 4.8)	-0.3 (-5.5, 4.9)	1.0 (-4.2, 6.2)	-1.3 (-6.9, 4.2)	0.1 (-5.3, 5.4)
	Girls	-2.1 (-7.5, 3.4)	-4.8 (-10.1, 0.5)	-0.6 (-6.3, 5.1)	-4.7 (-10.4, 0.9)	-3.7 (-8.9, 1.5)	-3.0 (-8.3, 2.4)	-0.6 (-6.4, 5.2)	0.9 (-5.2, 7.0)	-0.3 (-6.3, 5.7)
TNF-α/ IL-10	Boys	-1.6 (-6.6, 3.5)	-1.5 (-6.7, 3.7)	-1.7 (-7.1, 3.7)	-4.5 (-10.2, 1.1)	-2.4 (-7.8, 3.0)	-1.7 (-6.8, 3.4)	-2.5 (-7.6, 2.6)	-1.6 (-7.1, 3.9)	-4.7 (-10, 0.5)
	Girls	-4.2 (-9.6, 1.2)	-3.7 (-8.9, 1.5)	-7.2 (-12.8, -1.6)	-1.9 (-7.4, 3.7)	-7.2 (-12.3, -2.2)	-5.3 (-10.6, -0.1)	-4.9 (-10.6, 0.8)	-5.2 (-11.1, 0.8)	-5.7 (-11.5, 0.2)

Adjusted for examiner, quality of assessment, maternal age in pregnancy, maternal education, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years. ‡ p for interaction with sex < 0.05

Table 22. Adjusted associations between child inflammatory markers levels at age 4 years among normal weight and obese/overweight children

McCarthy Scales of Children's Abilities

		Verbal beta (95% CI)	Perceptual beta (95% CI)	Quantitative beta (95% CI)	Motor beta (95% CI)	Exec. Function beta (95% CI)	General cognitive beta (95% CI)	Memory beta (95% CI)	Working memory beta (95% CI)	Memory span beta (95% CI)
	Markers with pro-inflammatory activity (≥90th percentile)									
IFN-γ	Normal weight	-2.1 (-6.1, 2.0)	2.1 (-1.9, 6.1)	-0.9 (-5.1, 3.3)	2.6 (-1.6, 6.9)	-1.6 (-5.6, 2.4)	-0.7 (-4.7, 3.3)	-2.8 (-6.8, 1.3)	-2.4 (-6.6, 1.8)	-3.4 (-7.6, 0.8)
	Obese/overweight	-4.7 (-13.4, 4.0)	-3.2 (-12.8, 6.5)	-4.2 (-14.1, 5.7)	-1.4 (-11.7, 8.9)	-5.7 (-14.5, 3.2)	-4.8 (-14, 4.3)	-4.6 (-14.9, 5.6)	-3.6 (-14.4, 7.3)	-7.2 (-17, 2.6)
IL-1β	Normal weight	-2.1 (-6.2, 2.1)	-1.5 (-5.6, 2.6)	2.0 (-2.3, 6.3)	-0.9 (-5.2, 3.5)	-0.9 (-5.1, 3.2)	-1.4 (-5.5, 2.7)	-1.3 (-5.5, 2.9)	1.8 (-2.6, 6.1)	-1.4 (-5.8, 2.9)
	Obese/overweight	-3.9 (-11.8, 4.0)	-4.9 (-13.5, 3.7)	-3.5 (-12.5, 5.5)	-7.1 (-16.3, 2.0)	-1.6 (-9.7, 6.5)	-5.1 (-13.4, 3.1)	-8.9 (-18, 0.2)	-1.6 (-11.5, 8.3)	-8.6 (-17.4, 0.2)
IL-6	Normal weight	1.3 (-2.6, 5.2)	1.6 (-2.2, 5.5)	1.3 (-2.8, 5.3)	3.7 (-0.4, 7.8)	0.5 (-3.4, 4.4)	1.8 (-2.1, 5.6)	2.2 (-1.7, 6.1)	0.4 (-3.7, 4.5)	2.2 (-1.8, 6.3)
	Obese/overweight	9.2 (-2.0, 20.3)	2.8 (-9.4, 15.1)	-1.7 (-14.6, 11.3)	1.4 (-11.8, 14.5)	4.2 (-7.3, 15.8)	5.4 (-6.4, 17.2)	10.6 (-2.5, 23.7)	-0.6 (-14.7, 13.4)	8.8 (-3.8, 21.5)
IL-8	Normal weight	-0.9 (-4.9, 3.0)	-2.2 (-6.1, 1.7)	-0.3 (-4.4, 3.8)	0.3 (-3.9, 4.5)	-1.6 (-5.6, 2.3)	-1.5 (-5.4, 2.4)	-1.3 (-5.3, 2.6)	-1.4 (-5.5, 2.8)	-0.8 (-4.9, 3.4)
	Obese/overweight	-0.9 (-11.4, 9.6)	8.8 (-2.6, 20.3)	-1.8 (-13.9, 10.2)	4.6 (-7.7, 16.8)	-1.6 (-12.5, 9.2)	2.9 (-8.1, 13.9)	2.9 (-9.4, 15.2)	-3.1 (-16.2, 10.1)	1.8 (-10, 13.7)
TNF-α	Normal weight	-1.6 (-5.8, 2.6)	-1.1 (-5.3, 3.0)	-0.8 (-5.1, 3.6)	-1.0 (-5.5, 3.4)	-1.9 (-6.1, 2.3)	-1.5 (-5.6, 2.6)	**-1.9 (-6.1, 2.4) ‡**	-2.5 (-6.8, 1.9)	**-1.6 (-6, 2.7) ‡**
	Obese/overweight	-9.7 (-17.5, -1.8)	-2.3 (-11.0, 6.4)	-8.5 (-17.6, 0.6)	-7.4 (-16.7, 1.8)	-5.0 (-13.1, 3.2)	-8.1 (-16.4, 0.3)	**-11.4 (-20.6, -2.2) ‡**	-9.2 (-19.1, 0.6)	**-11.3 (-20.2, -2.4) ‡**
IL-17α	Normal weight	2.0 (-2.3, 6.3)	-0.9 (-5.2, 3.4)	2.6 (-1.9, 7.1)	-0.1 (-4.7, 4.5)	1.3 (-3.0, 5.6)	1.3 (-3.0, 5.5)	2.3 (-2.0, 6.6)	2.7 (-1.8, 7.2)	1.8 (-2.7, 6.3)
	Obese/overweight	-1.2 (-8.8, 6.3)	-8.2 (-16.2, -0.2)	-1.3 (-10, 7.3)	-7.7 (-16.1, 0.7)	-4.1 (-11.9, 3.6)	-4.6 (-12.4, 3.2)	-3.7 (-12.5, 5.0)	-0.7 (-10.1, 8.8)	-4.2 (-12.6, 4.2)

MIP-1α	Normal weight	0.8 (-3.3, 4.9)	0.4 (-3.7, 4.5)	0.9 (-3.3, 5.2)	2.1 (-2.3, 6.4)	2.3 (-1.8, 6.4)	0.6 (-3.4, 4.7)	-0.9 (-5.1, 3.2)	1.9 (-2.3, 6.2)	-1.1 (-5.4, 3.2)
	Obese/overweight	-4.5 (-12.3, 3.3)	-6.2 (-14.6, 2.2)	-1.2 (-10.1, 7.7)	-6.8 (-15.8, 2.1)	-4.3 (-12.2, 3.6)	-5.2 (-13.3, 2.9)	-4.1 (-13.2, 5.0)	-1.9 (-11.6, 7.7)	-4.1 (-12.8, 4.7)
	Markers with anti-inflammatory activity (≥90th percentile)									
IL-10	Normal weight	-4.5 (-12.3, 3.3)	-6.2 (-14.6, 2.2)	-1.2 (-10.1, 7.7)	-6.8 (-15.8, 2.1)	-4.3 (-12.2, 3.6)	-5.2 (-13.3, 2.9)	-4.1 (-13.2, 5.0)	-1.9 (-11.6, 7.7)	-4.1 (-12.8, 4.7)
	Obese/overweight	1.6 (-2.5, 5.7)	3.0 (-1.0, 7.1)	2.3 (-1.9, 6.6)	3.9 (-0.5, 8.2)	2.0 (-2.1, 6.1)	2.5 (-1.5, 6.5)	1.3 (-2.8, 5.5)	0.5 (-3.7, 4.8)	2.5 (-1.8, 6.8)
	Ratios (≥90th percentile)									
IL-6/ IL10	Normal weight	-0.1 (-4.2, 4.1)	-0.4 (-4.5, 3.6)	-0.1 (-4.4, 4.1)	-0.9 (-5.2, 3.5)	-0.8 (-4.9, 3.4)	-0.1 (-4.2, 3.9)	0.5 (-3.6, 4.7)	0.4 (-3.9, 4.7)	-0.1 (-4.4, 4.2)
	Obese/overweight	-0.6 (-9.3, 8.1)	-5.9 (-15.3, 3.4)	-5.7 (-15.6, 4.1)	-8.0 (-18, 2.0)	-6.0 (-14.8, 2.7)	-4.0 (-13.1, 5.0)	3.0 (-7.2, 13.2)	-6.5 (-17.2, 4.2)	4.1 (-5.7, 13.9)
TNF-α/ IL-10	Normal weight	-1.6 (-5.8, 2.7)	-2.3 (-6.5, 1.8)	-3.3 (-7.6, 1.0)	-2.8 (-7.3, 1.6)	-3.8 (-8.0, 0.4)	-2.5 (-6.7, 1.6)	-2.6 (-6.8, 1.6)	-2.8 (-7.2, 1.6)	-4.7 (-9.0, -0.3)
	Obese/overweight	-8.8 (-17.1, -0.5)	-6.3 (-15.4, 2.7)	-10.1 (-19.5, -0.7)	-3.7 (-13.5, 6.1)	-10.3 (-18.7, -1.9)	-9.0 (-17.7, -0.3)	-9.3 (-19.1, 0.6)	-10.4 (-20.6, -0.2)	-8.3 (-17.8, 1.2)

Adjusted for examiner, quality of assessment, child sex, maternal age in pregnancy, maternal education, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight and preterm birth. ‡ p for interaction with child BMI status < 0.05

Table 23. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 4 years [Attention Deficit Hyperactivity Disorder Test (ADHDT)]

	Attention Deficit Hyperactivity Disorder Test							
	Total score		Hyperactivity		Impulsivity		Inattention	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)								
IFN-γ	1.36	(-1.90, 4.62)	0.39	(-1.04, 1.81)	-0.03	(-1.17, 1.12)	1.00	(-0.18, 2.17)
IL-1β	1.95	(-1.20, 5.09)	0.87	(-0.51, 2.25)	0.24	(-0.87, 1.35)	0.84	(-0.30, 1.97)
IL-6	0.54	(-2.79, 3.87)	0.25	(-1.20, 1.70)	0.31	(-0.86. 1.47)	-0.01	(-1.21, 1.19)
IL-8	-2.42	(-5.73, 0.90)	-0.49	(-1.93, 0.96)	-0.99	(-2.15, 0.17)	-0.94	(-2.13, 0.25)
TNF-α	-0.26	(-0.69, 0.17)	-0.08	(-0.27, 0.10)	-0.11	(-0.26, 0.04)	-0.06	(-0.22, 0.09)
IL-17α	-1.42	(-4.76, 1.92)	-0.22	(-1.68, 1.23)	-0.19	(-1.36, 0.99)	-0.05	(-0.12, 0.01)
MIP-1α	-0.18	(-0.36, 0.01)	-0.05	(-0.13, 0.02)	-0.07	(-0.14, 0.01)	-0.05	(-0.12, 0.01)
Markers with anti-inflammatory activity (≥90th percentile)								
IL-10	-0.04	(-0.21, 0.13)	-0.01	(-0.09, 0.06)	-0.01	(-0.07, 0.05)	-0.02	(-0.08, 0.04)
Ratios (≥90th percentile)								
IL-6/IL10	-1.04	(-4.62, 2.55)	-0.62	(-2.20, 0.95)	-0.44	(-1.70, 0.82)	0.03	(-1.26, 1.32)
TNF-α/IL-10	-1.64	(-5.15, 1.88)	-0,73	(-2.28, 0.82)	-0.72	(-1.96, 0.51)	-0.19	(-1.45, 1.09)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years (N=524)

Table 24. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 4 years [Strengths and Difficulties Questionnaire (SDQ)]

	Strengths and Difficulties Questionnaire											
	Total score		Hyperactivity/ Inattention		Emotional problems		Conduct problems		Peer problems		Prosocial behavior	
	β	95% CI	β	95% CI	β	95% CI	B	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)												
IFN-γ	0.79	(-0.50, 2.07)	0.42	(-0.10, 1.03)	0.21	(-0.25, 0.67)	0.17	(-0.27, 0.60)	-0.06	(-0.44, 0.33)	-0.23	(-0.74, 0.28)
IL-1β	1.03	(-0.21, 2.26)	0.31	(-0.24, 0.86)	0.29	(-0.16, 0.73)	0.23	(-0.19, 0.64)	0.21	(-0.16, 0.57)	-0.23	(-0.74, 0.29)
IL-6	0.08	(-1.23, 1.38)	-0.45	(-1.03, 0.12)	0.28	(-0.19, 0.74)	0.19	(-0.25, 0.63)	0.06	(-0.32, 0.45)	-0.01	(-0.53, 0.51)
IL-8	-0.30	(-1.60, 1.01)	-0.21	(-0.79, 0.37)	-0.06	(-0.52, 0.41)	-0.31	(-0.47, 0.41)	0.01	(-0.38, 0.38)	**-0.50**	**(-0.99, -0.01)**
TNF-α	-0.03	(-0.19, 0.14)	-0.02	(-0.09, 0.05)	-0.02	(-0.04, 0.09)	-0.02	(-0.08, 0.04)	-0.01	(-0.06, 0.04)	0.02	(-0.05, 0.09)
IL-17α	0.05	(-1.25, 1.36)	-0.23	(-0.08, 0.35)	0.39	(-0.08, 0.86)	0.09	(-0.35, 0.54)	-0.20	(-0.58, 0.17)	0.10	(-0.43, 0.63)
MIP-1α	-0.02	(-0.09, 0.05)	-0.18	(-0.05, 0.01)	0.01	(-0.01, 0.04)	-0.01	(-0.03, 0.02)	-0.01	(-0.04, 0.01)	0.02	(-0.01, 0.05)
Markers with anti-inflammatory activity (≥90th percentile)												
IL-10	-0.01	(-0.08, 0.06)	0.01	(-0.04, 0.02)	-0.01	(-0.03, 0.02)	-0.01	(-0.03, 0.02)	0.01	(-0.02, 0.02)	-0.01	(-0.04, 0.02)
Ratios (≥90th percentile)												
IL-6/IL10	-0.47	(-1.89, 0.96)	-0.36	(-0.99, 0.27)	0.05	(-0.46, 0.56)	0.15	(-0.33, 0.62)	-0.30	(-0.73, 0.12)	-0.27	(-0.84, 0.30)
TNF-α/IL-10	-1.11	(-2.50, 0.29)	-0.34	(-0.96, 0.27)	-0.09	(-0.58, 0.41)	-0.32	(-0.79, 0.15)	-0.35	(-0.77, 0.06)	-0.38	(-0.18, 0.94)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years (N=524)

Six years of age

We found no association between child inflammatory markers levels at 4 years and child neurodevelopmental outcomes attained at six years of age (Table 25). Regarding behavioral outcomes, children with high levels of IFN-γ at 4 years (≥ 90th percentile), showed increased scores in CPRS-R: S oppositional (adjusted β = 1.19; 95% CI: 0.12, 2.26) and hyperactivity (adjusted β = 1.15; 95% CI: 0.13, 2.18) scales, as well as with CBCL externalized (adjusted β = 2.47; 95% CI: 0.28, 4.66) and internalized (adjusted β = 1.51; 95% CI: 0.02, 3.00) problems scale. Additionally, high IL-1β levels at 4 years (≥ 90th percentile) were associated with increased CPRS-R: S oppositional scale (adjusted β = 1.11; 95% CI: 0.04, 2.17) and CBCL internalized problems scales (adjusted β = 1.54; 95% CI: 0.09, 3.00). However, high TNF-α/IL-10 ratio was associated with decreased CPRS-R: S inattention (adjusted β = -1.51; 95% CI: -2.84, 0.18) and ADHD index scales (adjusted β = -2.51; 95% CI: (-4.71, -0.31) at 6 years (Table 26).

Further analyses revealed evidence for an interaction between child sex and certain inflammatory markers levels in response to cognitive and behavioral scores (p for interaction < 0.05); stratified analysis indicated increased response time in TMT Part B (adjusted β = 0.09; 95% CI: -0.10, 0.28) at 6 years for boys with high concentrations of MIP-1α at 4 years, whereas these associations in girls were in the opposite direction (adjusted β = -0.25; 95% CI: -0.48, -0.01). Moreover, boys with elevated concentrations of IL-6 attained higher CBCL internalized problems scale scores (adjusted β = 2.01; 95% CI: -0.03, 4.06) at 6 years, compared to girls (adjusted β = -1.00; 95% CI: -3.16, 1.16). It is also noteworthy that boys with increased IL-17α levels at 4 years demonstrated higher scores in all CPRS-R: S scales at 6 years related to girls' performance, such as in Oppositional (adjusted β = 1.94; 95% CI: -0.07, 3.80), Inattention (adjusted β = 1.72; 95% CI: -0.29, 3.73), Hyperactivity (adjusted β = 1.26; 95% CI: -0.53, 3.06) and ADHD index scales (adjusted β = 3.34; 95% CI: 0.16, 6.52) (Table 27).

We found no evidence of significant interactions between maternal pre-pregnancy BMI, child BMI, exposure to passive smoking and child inflammatory biomarker levels at 4 years (p for interaction > 0.10). Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results. Finally, after further adjustment for asthma, allergic rhinitis and helicobacter pylori seropositivity at 4 years, our results did not differ substantially from those derived from the main analysis (data not shown).

Table 25. Adjusted associations between child inflammatory markers levels at age 4 years and child neurodevelopmental outcomes at age 6 years

	Neuropsychological developmental outcomes at 6 years									
	RCPM: Total score[1]		TMT: Part A (log-transformed)[1]		TMT: Part B (log-transformed)[1]		FTT: Dominant hand[1]		FTT: Non-dominant hand[1]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)										
IFN-γ	0.91	(-0.62, 2.44)	-0.05	(-0.18, 0.09)	-0.01	(-0.15, 0.14)	-2.51	(-8.12, 3.11)	-3.31	(-9.15, 2.53)
IL-1β	-0.50	(-2.02, 1.03)	0	(-0.13, 0.13)	0.01	(-0.13, 0.15)	-0.91	(-6.64, 4.83)	-0.08	(-5.98, 5.82)
IL-6	0.83	(-0.75, 2.42)	-0.04	(-0.18, 0.10)	0.06	(-0.09, 0.21)	0.38	(-5.55, 6.30)	1.50	(-4.59, 7.59)
IL-8	1.50	(-0.07, 3.07)	-0.13	(-0.27, 0.01)	-0.02	(-0.17, 0.13)	-1.69	(-7.36, 3.99)	2.99	(-2.85, 8.84)
TNF-α	1.36	(-0.19, 2.90)	-0.02	(-0.15, 0.12)	-0.04	(-0.18, 0.11)	4.59	(-0.94, 10.12)	-4.10	(-9.83, 1.62)
IL-17α	0.65	(-0.98, 2.29)	-0.09	(-0.23, 0.05)	-0.08	(-0.23, 0.07)	-3.91	(-9.81, 2.00)	-3.68	(-9.90, 2.55)
MIP-1α	0.05	(-1.53, 1.62)	-0.01	(-0.14, 0.13)	-0.04	(-0.19, 0.10)	0.30	(-5.48, 6.09)	-3.10	(-9.12, 2.92)
Markers with anti-inflammatory activity (≥90th percentile)										
IL-10	0.62	(-0.91, 2.14)	0.07	(-0.07, 0.20)	0.04	(-0.11, 0.19)	-1.65	(-7.32, 4.01)	-2.89	(-8.76, 2.98)
Ratios (≥90th percentile)										
IL-6/IL10	0.67	(-1.12, 2.46)	0.06	(-0.09, 0.22)	0.07	(-0.10, 0.23)	-0.05	(-6.66, 6.56)	-1.82	(-8.79, 5.15)
TNF-α/IL-10	-0.45	(-2.39, 1.48)	0.15	(-0.01, 0.32)	0.11	(-0.07, -0.29)	0.92	(-5.99, 7.83)	3.27	(-3.98, 10.53)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years (N=340)

[1]Abbreviations: TMT: Trail Making Test, FTT: Finger Tapping Test, RCPM: Raven's Coloured Progressive Matrices

Table 26. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 6 years

	Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S) (N=360)								**Child Behaviour Checklist – Parent Report Form (CBCL): broad-band scales (N=375)**			
	Oppositional		Inattention		Hyperactivity		ADHD index		Externalized problems		Internalized problems	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)												
IFN-γ	**1.19**	**(0.12, 2.26)**	0.96	(-0.12, 2.04)	**1.15**	**(0.13, 2.18)**	0.96	(-0.86, 2.79)	**2.47**	**(0.28, 4.66)**	**1.51**	**(0.02, 3.00)**
IL-1β	**1.11**	**(0.04, 2.17)**	0.75	(-0.36, 1.86)	0.81	(-0.21, 1.83)	0.96	(-0.89, 2.80)	2.00	(-0.13, 4.13)	**1.54**	**(0.09, 3.00)**
IL-6	-0.28	(-1.38, 0.82)	-0.14	(-1.28, 0.99)	-0.91	(-1.96, 0.14)	-0.40	(-2.30, 1.50)	0.23	(-1.99, 2.44)	0.59	(-0.91, 2.08)
IL-8	0.33	(-0.75, 1.41)	0.06	(-1.05, 1.18)	0.46	(-0.58, 1.49)	-0.73	(-2.58, 1.11)	0.42	(-1.73, 2.57)	0.98	(-0.49, 2.45)
TNF-α	-0.45	(-1.51, 0.61)	-0.14	(-1.25, 0.98)	0.01	(-1.01, 1.03)	-0.36	(-2.18, 1.45)	-0.94	(-3.09, 1.21)	-0.21	(-1.67, 1.25)
IL-17α	0.08	(-1.05, 1.21)	-0.08	(-1.26, 1.09)	-0.11	(-1.19, 0.97)	0.51	(-1.41, 2.43)	-1.72	(-3.99, 0.56)	-0.11	(-1.67, 1.46)
MIP-1α	-0.20	(-1.29, 0.88)	-0.38	(-1.50, 0.73)	0.25	(-0.79, 1.28)	-0.65	(-2.50, 1.19)	-1.44	(-3.62, 0.74)	-0.21	(-1.29, 1.71)
Markers with anti-inflammatory activity (≥90th percentile)												
IL-10	0.16	(-0.88, 1.20)	-0.55	(-1.63, 0.53)	-0.15	(-1.15, 0.85)	-1.48	(-3.29, 0.32)	-0.07	(-2.20, 2.07)	0.30	(-1.16, 1.75)
Ratios (≥90th percentile)												
IL-6/IL10	-0.36	(-1.61, 0.89)	0.61	(-0.67, 1.89)	-0.23	(-1.43, 0.96)	-0.28	(-2.40, 1.84)	0.70	(-1.85, 3.25)	0.21	(-1.53, 1.95)
TNF-α/IL-10	-0.64	(-1.94, 0.67)	**-1.51**	**(-2.84, -0.18)**	-0.95	(-2.20, 0.30)	**-2.51**	**(-4.71, -0.31)**	-1.09	(-1.09, -3.76)	-0.56	(-2.39, 1.26)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years (N=333)

Table 27. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 6 years stratified by child sex

		Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S)				**Child Behaviour Checklist – Parent Report Form (CBCL): broad-band scales**	
		Oppositional	Inattention	Hyperactivity	ADHD index	Externalized problems	Internalized problems
		beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)
Markers with pro-inflammatory activity (≥90th percentile)							
IFN-γ	Boys	1.60 (0.06, 3.15)	2.01 (0.46, 3.57)	1.96 (0.49, 3.42)	2.28 (-3.34, 4.90)	3.88 (0.72, 7.03)	2.08 (-0.07, 4.23)
	Girls	0.79 (-0.71, 2.29)	-0.03 (-1.53, 1.48)	0.40 (-1.02, 1.82)	0.27 (-2.81, 2.27)	1.15 (-1.91, 4.21)	0.97 (-1.11, 3.05)
IL-1β	Boys	1.18 (-0.29, 2.65)	0.36 (-1.19, 1.92)	1.11 (-0.30, 2.51)	1.04 (-1.53, 3.62)	1.45 (-1.49, 4.38)	0.88 (-1.12, 2.88)
	Girls	0.79 (-0.71, 2.29)	1.15 (-0.45, 2.75)	0.48 (-1.01, 1.96)	0.86 (-1.78, 3.51)	2.61 (-0.48, 5.71)	2.28 (0.17 4.39)
IL-6	Boys	0.77 (-0.75, 2.30)	0.20 (-1.40, 1.81)	-0.65 (-2.11, 0.82	0.84 (-1.84, 3.52)	1.93 (-1.11, 4.98)	**2.01 (-0.03, 4.06)‡**
	Girls	-1.40 (-2.97, 0.18)	-0.50 (-2.11, 1.12)	-1.19 (-2.70, 0.32)	-1.64 (-4.33, 1.05)	-1.67 (-4.88, 1.54)	**-1.00 (-3.16, 1.16)‡**
IL-8	Boys	1.15 (-0.44, 2.74)	0.42 (-1.23, 2.06)	0.61 (-0.92, 2.14)	-0.08 (-2.88, 2.64)	1.04 (-2.14, 4.22)	1.32 (0.85, 3.49)
	Girls	-0.37 (-1.84, 1.10)	-0.24 (-1.75, 1.28)	0.33 (-1.08, 1.74)	-1.29 (-3.80, 1.22)	-0.11 (-3.05, 2.83)	-0.69 (-1.32, 2.70)
TNF-α	Boys	-0.37 (-1.71, 0.96)	-0.02 (-1.42, 1.39)	0.28 (-1.00, 1.56)	-0.41 (-2.75, 1.94)	-0.25 (-2.98, 2.48)	0.75 (-1.11, 2.60)
	Girls	-0.59 (-2.36, 1.18)	-0.34 (-2.17, 1.49)	-0.46 (-2.16, 1.24)	-1.06 (-4.09, 1.97)	-2.06 (-5.57, 1.44)	1.78 (-4.15, 0.60)
IL-17α	Boys	**1.94 (0.07, 3.80) ‡**	**1.72 (-0.29, 3.73)‡**	**1.26 (-0.53, 3.06)‡**	**3.34 (0.16, 6.52)‡**	-0.55 (-4.39, 3.29)	1.81 (-0.82, 4.43)
	Girls	**-0.97 (-2.37, 0.43) ‡**	**-1.01 (-2.46, 0.43)‡**	**-0.88 (-2.24, 0.47)‡**	**-1.08 (-3.47, 1.30)‡**	2.36 (-5.19, 0.48)	-1.15 (0.04, 3.09)
MIP-1α	Boys	-0.39 (-1.77, 0.98)	0.01 (-1.40, 1.43)	0.47 (-0.85, 1.79)	0.25 (-2.03, 2.52)	-1.80 (-4.60, 1.00)	0.04 (1.89, 1.96)
	Girls	0.12 (-1.66, 1.90)	-1.04 (-2.87, 0.79)	-0.13 (-1.84, 1.58)	-1.43 (-4.45, 1.58)	-0.88 (-4.39, 2.26)	0.47 (-1.94, 2.88)

	Markers with anti-inflammatory activity (≥90th percentile)						
IL-10	Boys	0.47 (-0.83, 1.77)	-0.88 (-2.24, 0.47)	-0.29 (-1.53, 0.96)	-1.77 (-4.05, 0.51)	-0.68 (-3.33, 1.97)	0.09 (-1.72, 1.91)
	Girls	-0.39 (2.13, 1.35)	0.02 (-1.77, 1,80)	0.10 (-1.57, 1.77)	-1.01 (-3.96, 1.94)	1.04 (-2.51, 4.59)	0.66 (1.77, 3.09)
	Ratios (≥90th percentile)						
IL-6/ IL10	Boys	-0.58 (-2.19, 1.03)	0.61 (-1.04, 2.26)	0.04 (-1.50, 1.58)	0.23 (-2.51, 2.96)	1.22 (-2.07, 4.51)	-0.23 (-2.48, 2.02)
	Girls	-0.03 (-2.03, 1.97)	0.62 (-1.43, 2.66)	-0.64 (-2.56, 1.27)	-1.07 (-4.45, 2.32)	-0.10 (-4.18, 3.98)	0.88 (-1.91, 3.66)
TNF-α/							
IL-10	Boys	-1.18 (-2.75, 0.40)	-1.39 (-3.00, 0.21)	-1.49 (-2.99, 0.02)	-3.22 (-5.87, -0.56)	-1.85 (-5.08, 1.38)	-1.42 (-3.62, 0.78)
	Girls	0.53 (-1.78, 2.83)	-1.75 (-4.10, 0.60)	0.21 (-1.99, 2.41)	-0.98 (-4.87, 2.91)	0.54 (-4.17, 5.26)	1.29 (-1.93, 4.50)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years. ‡ p for interaction with sex < 0.05

Eleven years of age

We found that children with high levels of IL-8 at 4 years (≥ 90th percentile), showed increased scores in Processing Speed Index WISC-V scale (adjusted β = 5.66; 95% CI: 0.72, 10.59) and children with high levels of IL6/IL-10 ratio at 4 years showed increased scores in Visual Spatial Index WISC-V scale (adjusted β = 8.41; 95% CI: 1.43, 15.38) at 11 years (Table 28). Regarding WISC-V Ancillary Index Scales, high IL-10 levels at 4 years (≥ 90th percentile) were associated with increased Cognitive Proficiency Index scores (adjusted β = 5.06; 95% CI: 0.90, 9.22), and high IL6/IL-10 ratio at 4 years (≥ 90th percentile) were related to increased General Ability Index scores at 11 years (adjusted β = 6.62; 95% CI: 0.42, 12.82) (Table 29).

Concerning behavioral outcomes at 11 years of age, we found that children with high levels of IFN-γ at 4 years (≥ 90th percentile), showed increased scores in CPRS-R: S Hyperactivity (adjusted β = 1.10; 95% CI: 0.12, 2.09) and ADHD index (adjusted β = 2.19; 95% CI: 0.02, 4.37) scales (Table 30). Moreover, high IL-17α levels at 4 years was associated with increased CBCL internalized problems scales (adjusted β = 3.35; 95% CI: 1.16, 5.55) (Table 30).

Further analyses revealed evidence for an interaction between child sex and certain inflammatory markers levels in response to one behavioral score (p for interaction < 0.05); stratified analysis showed increased CPRS-R: S Inattention scale scores at 11 years for boys with high levels of IL-8 at 4 years (adjusted β = 1.69; 95% CI: 0.12, 3.49), whereas these associations in girls were in the opposite direction (adjusted β = -0.75; 95% CI: -2.29, 0.79). However, we found no evidence of significant interaction between maternal pre-pregnancy BMI, child BMI, exposure to passive smoking and child inflammatory biomarker levels at 4 years of age (p for interaction > 0.10). Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results. Finally, after further adjustment for asthma, allergic rhinitis and helicobacter pylori seropositivity at 4 years, our results did not differ substantially from those derived from the main analysis (data not shown).

Table 28. Adjusted associations between child inflammatory markers levels at age 4 years and child neurodevelopmental outcomes at age 11 years

	Wechsler Intelligence Scale for Children-V (WISC-V) *Primary Index Scales*									
	Verbal Comprehension Index		Visual Spatial Index		Fluid Reasoning Index		Working Memory Index		Processing Speed Index	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)										
IFN-γ	1.88	(-2.39, 6.15)	2.35	(-2.51, 7.21)	-0.55	(-5.29, 4.19)	2.94	(-1.83, 7.72)	0.28	(-4.66, 5.22)
IL-1β	0.52	(-3.66, 4.70)	0.91	(-3.84, 5.67)	0.71	(-3.93, 5.35)	2.53	(-2.14, 7.21)	-0.90	(-5.74, 3.94)
IL-6	1.65	(-2.83, 6.13)	1.83	(-3.28, 6.95)	0.68	(-4.28, 5.63)	0.42	(-4.57, 5.4)	-0.94	(-6.09, 4.20)
IL-8	-0.43	(-4.74, 3.87)	-0.92	(-5.80, 3.97)	0.25	(-4.54, 5.03)	2.30	(-2.51, 7.11)	**5.66**	**(0.72, 10.59)**
TNF-α	1.16	(-3.02, 5.34)	1.02	(-3.75, 5.79)	3.41	(-1.20, 8.02)	-1.68	(-6.36, 2.99)	-1.89	(-6.73, 2.94)
IL-17α	2.00	(-2.52, 6.51)	0.53	(-4.61, 5.67)	-1.37	(-6.38, 3.64)	-1.21	(-6.25, 3.84)	-3.91	(-9.12, 1.29)
MIP-1α	2.23	(-2.23, 6.69)	2.79	(-2.31, 7.89)	0.74	(-4.17, 5.64)	4.59	(-0.41, 9.30)	0.27	(-4.92, 5.46)
Markers with anti-inflammatory activity (≥90th percentile)										
IL-10	3.62	(-0.53, 7.77)	3.22	(-1.51, 7.96)	2.49	(-2.13, 7.12)	3.69	-(0.97, 8.34)	2.58	(-2.24, 7.41)
Ratios (≥90th percentile)										
IL-6/IL10	5.28	(-0.86, 11.43)	**8.41**	**(1.43, 15.38)**	6.43	(-0.43, 13,29)	-2.36	(-9.36, 4.63)	-2.46	(-9.57, 4.65)
TNF-α/IL-10	1.09	(-4.91, 7.09)	3.04	(-3.81, 9.88)	2.52	(-4.19, 9.22)	0.97	(-5.83, 7.76)	-4.37	(-11.25, 2.52)

Adjusted for child sex, child age, quality of assessment, examiner, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years (N=244)

Table 29. Adjusted associations between child inflammatory markers levels at age 4 years and child neurodevelopmental outcomes at age 11 years

	Wechsler Intelligence Scale for Children-V (WISC-V) *Ancillary Index Scales*							
	Quantitative Reasoning Index		Nonverbal Index		General Ability Index		Cognitive Proficiency Index	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)								
IFN-γ	2.39	(-2.64, 7.43)	1.04	(-3.59, 5.68)	0.88	(-3.41, 4.99)	2.39	(-1.91, 6.68)
IL-1β	1.16	(-3.77, 6.09)	2.19	(-2.33, 6.72)	0.79	(-3.42, 4.99)	1.00	(-3.22, 5.21)
IL-6	1.12	(-4.09, 6.34)	0.38	(-4.48, 5.23)	1.42	(-3.08, 5.91)	2.38	(-2.11, 6.87)
IL-8	0.02	(-5.06, 5.10)	1.72	(-2.94, 6.38)	0.27	(-4.06, 4.60)	1.79	(-2.54, 6.11)
TNF-α	2.93	(-1.98, 7.83)	0.46	(-4.09, 5.00)	2.13	(-2.07, 6.32)	1.44	(-2.77, 5.66)
IL-17α	-1.66	(-6.99, 3.67)	-2.52	(-7.41, 2.36)	0.67	(-3.87, 5.21)	0.03	(-4.52, 4.58)
MIP-1α	3.24	(-1.98, 7.83)	2.05	(-2.77, 6.87)	1.60	(-2.86, 6.05)	3.13	(-1.31, 7.58)
Markers with anti-inflammatory activity (≥90th percentile)								
IL-10	3.04	(-1.87, 7.95)	3.45	(-1.06, 7.96)	4.04	(-0.13, 8.21)	**5.06**	**(0.90, 9.22)**
Ratios (≥90th percentile)								
IL-6/IL10	6.93	(-0.34, 14.20)	3.54	(-3.14, 10.22)	**6.62**	**(0.42, 12.82)**	5.19	(-1.03, 11.42)
TNF-α/IL-10	6.13	(-0.94, 13.20)	1.27	(-5.23, 7.77)	1.18	(-4.89, 7.25)	0.65	(-5.43, 6.73)

Adjusted for child sex, child age, quality of assessment, examiner, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years (N=244)

Table 30. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 11 years

	Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S) (N=243)								**Child Behaviour Checklist – Parent Report Form (CBCL): broad-band scales (N=230)**			
	Oppositional		Inattention		Hyperactivity		ADHD index		Externalized problems		Internalized problems	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Markers with pro-inflammatory activity (≥90th percentile)												
IFN-γ	0.16	(-1.17, 1.48)	0.64	(-0.60, 1.89)	**1.10**	**(0.12, 2.09)**	**2.19**	**(0.02, 4.37)**	0.96	(-1.62, 3.53)	-0.60	(-2.83, 1.63)
IL-1β	-0.79	(-2,07, 0.50)	-0.01	(-1.21, 1.18)	-0.13	(-1.13, 0.86)	-1.26	(-3.37, 0.85)	-1.07	(-3.59, 1.45)	0.15	(-2.01, 2.30)
IL-6	0.65	(-0.69, 2.00)	-0.07	(-1.35, 1.22)	-0.56	(-1.60, 0.47)	-0.18	(-2.41, 2.05)	0.42	(-2.28, 3.12)	-0.07	(-2.33, 2.18)
IL-8	0.73	(-0,54, 2.01)	0.28	(-0.91, 1.46)	-0.36	(-1.33, 0.30)	-0.30	(-2.39, 1.73)	0.16	(-2.27, 2.59)	-0.48	(-2.59, 1.62)
TNF-α	-0.18	(-1.50, 1.15)	0.09	(-1.16, 1.33)	-0.02	(-1.02, 0.99)	1.90	(-0.26, 4.06)	-0.36	(-2.85, 2.13)	-0.06	(-2.25, 2.13)
IL-17α	0.66	(-0.72, 2.04)	0.54	(-0.73, 1.82)	0.62	(-0.42, 1.67)	2.19	(-0.05, 4.42)	1.35	(-1.30, 4.00)	**3.35**	**(1.16, 5.55)**
MIP-1α	-1.61	(-3.00, 0.23)	-0.30	(-1.60, 1.01)	0.17	(-0.84, 1.19)	0.68	(-1.63, 2.98)	-1.58	(-4.3, 1.14)	-0.62	(-2.92, 1.68)
Markers with anti-inflammatory activity (≥90th percentile)												
IL-10	0.37	(-0.90, 1.65)	0.28	(-0.91, 1.47)	0.54	(-0.43, 1.50)	1.56	(-0.52, 3.63)	1.21	(-1.22, 3.65)	-0.12	(-2.21, 1.98)
Ratios (≥90th percentile)												
IL-6/IL10	-0.62	(-2.56, 1.32)	0.41	(-1.38, 2.20)	-0.71	(-2.18, 0.77)	-0.76	(-2.53, -4.05)	-2.36	(-6.27, 1.55)	0.50	(-2.92, 3.92)
TNF-α/IL-10	-0.52	(-2.34, 1.31)	-0.51	(-2.19, 1.17)	-0.41	(-0.41, -1.79)	-1.70	(-4.68, 1.27)	-2.73	(-6.36, 0.90)	-1.97	(-5.01, 1.07)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years

Table 31. Adjusted associations between child inflammatory markers levels at age 4 years and child behavioral outcomes at age 11 years stratified by child sex

		Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R: S)			
		Oppositional	Inattention	Hyperactivity	ADHD index
		beta (95% CI)	beta (95% CI)	beta (95% CI)	beta (95% CI)
Markers with pro-inflammatory activity (≥90th percentile)					
IFN-γ	Boys	0.29 (-1.61, 2.18)	0.21 (-1.60, 2.02)	0.93 (0.49, 2.34)	2.67 (-0.52, 5.86)
	Girls	0.03 (-1.82, 1.88)	1,02 (-0.69, 2.74)	1.28 (-0.11, 2.66)	1.77 (-1.23, 4.78)
IL-1β	Boys	-0.80 (-2.57, 0.97)	-0.47 (-2.11, 1.18)	-0.55 (-1.90, 0.79)	-2.04 (-4.93, 0.85)
	Girls	-0.77 (-2.63, 1.09)	0.49 (-1.24, 2.21)	0.36 (-1.10, 1.82)	0.40 (-3.43, 2.63)
IL-6	Boys	1.29 (-0.65, 3.22)	-1.47 (-3.34, 0.40)	-0.17 (-1.67, 1.32	-0.91 (-4.12, 2.31)
	Girls	0.06 (-1.81, 1.94)	1.17 (-0.58, 2.29)	-0.93 (-2.37, 0.52)	0.50 (-2.61, 3.61)
IL-8	Boys	1.06 (-0.89, 3.01)	**1.69 (0.12, 3.49)‡**	-0.31 (-1.79, 1.17)	0.91 (-2.29, 4.11)
	Girls	0.49 (-1.18, 2.17)	**-0.75 (-2.29, 0.79)‡**	-0.40 (-1.68, 0.87)	-1.19 (-3.93, 1.55)
TNF-α	Boys	-0.20 (-1.78, 1.38)	0.24 (-1.25, 1.73)	0.03 (-1.16, 1.23)	1.99 (-0.58, 4.56)
	Girls	-0.13 (-2.57, 2.31)	-0.25 (-2.52, 2.01)	-0.14 (-1.99, 1.71)	1.68 (-2.28, 5.65)
IL-17α	Boys	0.97 (-1.12, 3.06)	0.96 (-0.98, 2.90)	1.47 (-0.11, 3.04)	3.81 (0.42, 7.19)
	Girls	0.41 (-1.43, 2.26)	0.22 (-1.49, 1.93)	-0.04 (-1.43, 1.35)	0.92 (-2.06, 3.91)
MIP-1α	Boys	-1.52 (-3.40, 0.37)	-0.67 (-2.39, 1.05)	0.14 (-1.22, 1.49)	0.95 (-2.08, 3.98)
	Girls	-1.73 (-3.80, 0.34)	0.22 (-1.80, 2.24)	0.22 (-1.31, 1.75)	0.30 (-3.26, 3.86)
Markers with anti-inflammatory activity (≥90th percentile)					
IL-10	Boys	0.28 (-1.27, 1.84)	-0.13 (-1.60, 1.34)	0.43 (-0.75, 1.61)	2.22 (-0.32, 4.75)

	Girls	0.55 (-1.58, 2.68)	1.02 (-0.95, 2,98)	0.74 (-0.88, 2.36)	0.31 (-3.14, 3.76)
	Ratios (≥90th percentile)				
IL-6/ IL10	Boys	-0.74 (-3.20, 1.72)	-0.64 (-2.90, 1.62)	-0.77 (-2.64, 1.10)	-3.69 (-7.95, 0.56)
	Girls	-0.42 (-3.56, 2.72)	2.13 (-0.76, 5.01)	-0.60 (-2.99, 1.78)	3.41 (-1.67, 8.48)
TNF-α/					
IL-10	Boys	-0.98 (-3.25, 1.29)	-0.06 (-2.90, 1.62)	-0.45 (-2.18, 1.28)	-1.42 (-5.13, 2.29)
	Girls	0.34 (-2.74, 3.41)	2.13 (-0.76, 4.01)	-0.33 (-2.67, 2.01)	-2.21 (-7.21, 2.79)

Adjusted for child sex, child age, maternal age at birth, maternal educational level, parity, BMI pre-pregnancy, birth weight, preterm birth, passive smoking at 4 years, BMI at 4 years. ‡ p for interaction with sex < 0.05

4. Discussion

4.1. Prenatal exposure to Persistent Organic Pollutants in association with offspring cognitive and behavioral development at 4, 6 and 11 years of age

In this population-based pregnancy cohort study we found for the first time that prenatal exposure to high HCB levels was associated with reduced child cognitive performance at various age timepoints. We also found that high concentrations of maternal serum PCBs levels were associated with decreased neuropsychological performance at 4, 6 and 11 years of age. These results persisted after adjustment for several maternal and child characteristics. Prenatal exposure to organochlorine pesticides and PCBs was not associated with child behavioral and ADHD-related problems, with the sole exception of the association of high prenatal HCB levels with child peer problems in SDQ at 4 years of age.

In fact, this is the first epidemiological study that provides three distinct timepoints of assessing the outcomes under study, revealing robust negative associations of high prenatal HCB exposure with i) cognitive, perceptual, executive and working memory functions at the age of 4 years (MSCA), ii) non-verbal general intelligence (via *RCPM*), processing speed and mental flexibility (via *TMT-Part A & TMT-Part B*), and motor speed (via FTT) at 6 years of age, and iii) visual spatial, fluid reasoning, working memory, quantitative reasoning, nonverbal, general ability and cognitive proficiency functions (via WISC-V) at 11 years of age. The above findings observed at various timepoints suggest consistency over time.

No association was demonstrated between high prenatal POPs levels with behavioral outcomes, with the exception of the association of high prenatal HCB levels with child peer problems in SDQ at 4 years. Moreover, at 11 years of age we found statistically significant positive associations between high HCB levels and three behavioral scales scores in the basic models. However, it is noteworthy that in the adjusted models the estimates were weakened and it may be possible there is residual confounding implicated in the direction of the results; nevertheless, the explanation for the direction of the associations remains unclear.

Our findings on the negative association between HCB exposure and child cognitive performance are in line with the Oswego study in the US, which revealed that placental levels of HCB were a significant predictor of lower WISC-III IQ scores

in 11-year-old children (110). Findings from the New Bedford Cohort study also observed weak positive associations between HCB in cord serum and working memory tasks among adolescents (199). To our knowledge, only one other study has examined the association between cord blood HCB levels with cognitive and psychomotor performance at preschool age, which, in contrast with our findings, did not find an association in children from the INMA cohort in Spain (111). Other studies examining the effects of organochlorine pesticides on cognitive outcomes in infancy also reported null associations (60,200). Similarly, a recent cohort study revealed that prenatal HCB levels were not associated with any of the Bayley-III subscales at 18-month toddlers (201). Studies examining the effect of prenatal HCB exposure in older children are sparse; one study found no association between cord serum HCB concentrations and inhibition outcomes among adolescents (202).

It is noteworthy that adverse health effects of endocrine-disrupting chemicals, such as POPs, could follow non-monotonic dose responses with increased risks at lower concentrations and null or inverse risks at higher concentrations (203), thus the substantial differences in the exposure levels may explain the disparity in findings across studies.

The mechanisms by which HCB may lead to developmental impairments are not clear; an animal study assessing the possible developmental neurotoxicity following premating maternal exposure to hexachlorobenzene in rats suggested that this compound possibly interferes with myelination during development, an important process for the normal functioning of all ascending and descending neural (204). Overall, impairment related to HCB exposure has been described in animal models, but little information is available in humans (205). A possible suggested mechanism is that exposure to HCB at background levels may affect thyroid function during pregnancy and these findings are of particular significance, since thyroid hormones of maternal origin may play an essential role in fetal neurodevelopment (206). The first trimester of pregnancy is considered a crucial time period when brain development is more dependent on maternal thyroid hormone levels and more susceptible to endocrine disruptor effects (207,208). However, in our study maternal TSH did not seem to have a substantial influence on the association between HCB and neurodevelopment Weak or no associations between HCB and neurodevelopmental outcomes found in other studies could possibly be explained by the fact that exposure

assessment has been performed at late pregnancy or birth and not at the early stages of pregnancy.

In this study we found that high maternal serum levels of PCBs were associated with offspring decreased performance in working memory tasks at preschool age, increased response time in TMT Part A and lower speed scores in FFT (non-dominant hand) at 6 years of age. Our findings additionally showed that high prenatal PCBs levels were associated with lower scores in several WISC-V index scores (working memory, processing speed, quantitative reasoning, nonverbal, general ability and cognitive proficiency) at 11 years of age. A review examining the effects of prenatal PCBs exposure on cognitive performance (75) highlights a vulnerability of executive functions, such as working memory, planning and mental flexibility. Boucher and colleagues (75) assumed that cognitive deficits linked to attention, verbal performance and working memory functions might be more affected by PCB exposure prenatally, in contrast to auditory and visual motor performance which seems to be less affected. Likewise, a literature review on PCB developmental neurotoxicity assumes that executive function is possibly one of the most affected behavioral domains related to PCB exposure, with working memory and inhibitory control appearing to be the most impacted (209). Our findings are also consistent with other studies suggesting that prenatal exposure to PCBs predicted poorer working memory in early childhood (77,210) and in school age (63,84). Again, these studies have assessed the outcomes under study during one specific timepoint, while our analysis reveals various statistically significant results at three different age groups.

Since the prefrontal structures of the brain are considered to be of particular importance in higher-order functions, like executive functions and working memory (211), one plausible underlying mechanism could be the effect of prenatal exposure to PCBs on prefrontal cortex (75). This hypothesis is in line with animal studies that showed dysregulation in dopamine levels of the prefrontal cortex in rats exposed to PCB congeners in utero (53). The most widely suggested mechanisms of PCB neurotoxicity include: disruption of the hypothalamic-pituitary-thyroid axis, altered neurotransmitter signaling -particularly decreased dopamine levels and increased GABA signaling- modulation of neuronal Ca^{2+} signals and increased intracellular levels of reactive oxygen species (212).

Apart from the aforementioned results on cognitive performance, we did not find any other significant associations between maternal PCBs levels and behavioral scores at the three timepoints. Similarly, a recent Greenlandic cohort study found no consistent evidence of associations between prenatal PCBs levels and problematic behavior assessed with SDQ at 3-5 years of age (213). No associations were also found between cord PCB levels and behavioral problems reported by teachers for Inuit children living in Arctic Quebec at the age of 8–14 years (83). Several studies of PCB exposure during pregnancy have reported no associations with neuropsychological and behavioral domains, as well (68–74,82). However, there is much variation in human studies exploring these relations (51,64–66,68–72,205).

In our analysis, no associations were found between maternal DDE concentrations and neurodevelopmental and behavioral scores at 4 and 11 years of age. Regarding 6 years of assessment, we only found one associations of high prenatal DDE levels with increased response time in TMT Part B. Within the frames of the Spanish INMA cohort, no associations were observed between maternal levels of DDE and Bayley Scales of Infant Development (BSID) at 14 months (200). In addition, the North Carolina Infant Feeding Study detected no association between cord DDE levels and scores on MSCA at ages 3, 4 or 5 years (73). Likewise, no associations were reported between prenatal levels of DDE and BSID scores at 8 months and WISC scores at 7 years in the US Collaborative Perinatal Project (103,214). On the contrary, there are cohorts that have detected negative associations between prenatal DDE exposure and social, mental and psychomotor development, mainly in infancy (60,103,104) and to a lesser extent in preschool (215) and school years (64,199), but it should be noted that most of those population samples were largely exposed to high levels of DDE. Overall, epidemiological findings on prenatal DDE levels and cognitive development vary greatly; potential explanations for this heterogeneity in results are not quite apparent, although they may include different study designs, study sample sizes and different neurodevelopmental/behavioral outcomes examined by each study.

As mentioned above, our findings suggest that prenatal exposure to organochlorine pesticides and PCBs was not associated with child behavioral and ADHD-related outcomes, with the sole exception of the association of high prenatal HCB levels with child peer problems in SDQ at 4 years of age. A small number of studies have reported positive associations between POPs and behavioral or ADHD-related symptoms (64,216–219), while other studies have reported null or inconsistent

results (220). In fact, a pooled analysis of seven European birth cohort studies of 4437 children, did not demonstrate any association between either pre- or post-natal exposure (up to 24 months) to PCB-153, *p-p´*-DDE and HCB and the risk of ADHD before the age of 10 years (221).

Exposure levels to HCB, DDE and ΣPCBs in our cohort study were close to but generally lower than the median exposure in other pregnant populations (78,110,111,214). Possible variations on the observed median POP levels may be due to the fact that in other studies the exposure measurements were conducted at different time points or in different sample types (e.g. samples from cord or placental blood).

Strengths of the present study include the prospective population-based design, the long follow-up period and the reliable, valid, and comprehensive psychometric instruments that were used to assess child cognition and behavior. In the present analysis we also used standardized neurodevelopmental scales (mean of 100 points with a 15 SD). There is extensive literature on the public health impact of a 1-point loss of a neuropsychological scale, most are based on effects of lead exposure on IQ (46). Although a seemingly small change of a 1-point decrease in IQ score might not be relevant at the individual level, at the population level this is possible to swift the distribution of IQ to the left and increase the number of persons below the normal range (222). The exclusion of women who gave birth to twins as well as adjustment for several socio-demographic variables reduced the likelihood of confounding. Imputation of the missing values of questionnaires were applied in order to avoid any bias due to selective response to specific items of the questionnaires. This study is not free of limitations; we cannot rule out the possibility that prenatal and/or postnatal exposure to other unmeasured chemicals correlated to POPs may have confounded the associations under study. We could not separate the potential effects of postnatal exposures because we have not measured POPs at other time points, but we tried to control for these possible effects by including breastfeeding duration in our model. Children included in the present analysis who had complete data were more socially advantaged than the remainder of the cohort and this could lead to underestimation of the observed associations. Due to the young age of the children, we relied on their parents as source of information about behavioral symptoms and problems. Finally, although we incorporated a number of confounding variables regarding potential social and environmental aspects that are associated with child neurodevelopment, we

acknowledge that residual confounding may still occur, due to possible unmeasured factors such as social economic status, and quality of home environment.

4.2. Child inflammatory markers at 4 years in association with child cognitive and behavioral development at 4, 6 and 11 years of age

In the present analysis we examined for the first time the relationship between inflammatory marker levels and neurodevelopment at three different time points in a general population sample of children and our main findings are summarized as follows:

i) Preschoolers with elevated TNF-α concentrations in serum demonstrated reduced scores in memory, memory span and working memory and preschoolers with high IFN-γ serum levels showed lower scores in memory span scale. Children at 4 years with high levels of IL-8 showed lower prosocial behavior scores.

ii) Children with elevated levels of IFN-γ at 4 years showed increased scores in oppositional and hyperactivity scales, as well as in internalized and externalized CBCL scores at 6 years of age; high IL-1β levels at 4 years were associated with more oppositional symptoms and externalized problems at 6 years of age. However, we also found that increased TNF-a/IL10 ratio was related with lower inattention and ADHD scores at 6 years.

iii) Children with high levels of IL-8 at 4 years showed increased scores in processing speed at 11 years and children with high levels of IL6/IL-10 ratio at 4 years showed increased scores in visual spatial performance at 11 years. High IL-10 levels at 4 years were associated with increased cognitive proficiency scores and high IL6/IL-10 ratio were related to increased general ability scores at 11 years. We also found that children with high levels of IFN-γ at 4 years demonstrated increased scores in hyperactivity and ADHD scales. Moreover, high IL-17α levels at 4 years were associated with increased internalized problems scales at 11 years.

As mentioned above, in the cross-sectional analysis, we found that elevated TNF-α plasma levels at 4 years were associated with lower performance in various memory scales at that age. However, in the longitudinal associations between those TNF-α and memory scales at 6 and 11 years, we observed that these associations were not attenuated. Comparison with other studies is rather complex mainly because of

different methodological approaches study design, type and size of study samples, age of the participants and outcomes examined. Overall, studies that have established well the association between TNF-α levels with cognitive deficits mainly include samples of elderly populations; elevated TNF-α serum concentrations have been detected in patients with cognitive decline, such as Alzheimer's disease (223–226), suggesting that TNF-α-driven processes may contribute to cognitive and memory deficits of the disease and that inhibition of TNF-α can be effective for treating it (227–229). In addition, a study conducted with adult patients with depressive disorder demonstrated that elevated expression of TNF-α, TNFRSF1A and TNFRSF1B genes correlates negatively, among others, with working memory, direct and delayed auditory-verbal memory and effectiveness of learning processes and verbal fluency (230). Available data on child inflammation and neurodevelopmental outcomes are largely based on ASD samples; a recent cross-sectional study investigating the association between peripheral cytokine levels (including TNF-α) and cognitive profiles in children with ASD found negative correlations of IL-6 and IFN-γ serum levels with WISC verbal comprehension index and working memory index respectively, suggesting that cytokines may play an important role in the neural development in ASD (231).

In general, inflammatory signaling is considered to be a critical contributor to the short-and-long term regulation of mood and cognition, but the exact mechanisms by which cytokines may modulate memory remain unknown (232). TNF-α concentrations are found elevated in various neuropathological states that are related to learning and memory deficits, highlighting a possible role in plasticity (233). For this purpose, much work has been carried out in the hippocampus; in fact, animal studies provide evidence that mice over-expressing TNF-α demonstrate memory impairments and disrupted learning capabilities (234,235), supporting the notion that TNF-α activity at the hippocampus and the synaptic level may influence brain function and behavior (236–238). Consistently, a negative effect of TNF-α was found following intra-hippocampal administration to rats, which lead to impaired hippocampal-dependent working memory, as shown by an increased number of errors and longer latencies regarding the runway task (239). Moreover, increased TNF-α in rats following peripheral nerve injury may not only contribute to chronic pain, but also to memory deficits by dysfunction of hippocampus (240). A study conducted in adults showed that higher concentrations of TNF-α are associated with smaller

hippocampal volumes suggesting that the balance between the hypothalamic-pituitary adrenal axis and inflammation processes might explain hippocampal volume reductions (241). In the cross-sectional analysis we also found that elevated IFN-γ levels at 4 years were associated with memory span scales at the age of 4; regarding high IFN-γ levels, animal models have shown that they can act as a negative regulator of neuroplastic changes such as hippocampus structure, cell density, neuronal morphology and synaptic plasticity and, therefore may be associated with poorer performance in learning and memory tasks (242). We also found that high TNF-α/IL-10 ratio was associated with decreased quantitative, motor, executive function, general cognitive, memory, working memory and memory span scores. Since this is the first study illustrating the associations of these ratios with child cognitive domains, further data is required to validate these results and clarify the underlying mechanisms of these findings.

We also found that children at 4 years with high levels of IL-8 attained lower SDQ prosocial behavior scores. A low prosocial score is generally considered a good predictor of ASD diagnosis in children (243). To our knowledge, there is one study exploring the relation of pro-inflammatory cytokines with prosocial behavior, reporting that IL-6 is associated with increased odds of abnormal prosocial behavior in 5-year-old children (244)

In the present study, we also found that children with elevated levels of IFN-γ at 4 years demonstrated increased scores in oppositional and hyperactivity scales, as well as in externalized CBCL scores at 6 years of age and also showed increased scores in hyperactivity and ADHD scales at 11 years of age. Studies suggest that variations in cytokine concentration levels might regulate the basal ganglia and play an important part in the dopamine synthesis in the brain, which is implicated in behavioral problems (245). In fact, several cytokines have been researched as possible neurochemical markers of behavioral problems, such as ADHD-related symptoms, and there seems to be high heterogeneity among biomarkers, making the interpretations of study findings quite challenging (246). Moreover, while both preclinical and clinical studies often report that certain cytokines may regulate neurodevelopment, whether any of these markers is related to child behavioral outcomes remains unknown and less-studied (244). It is noteworthy that there is only one recent cross-sectional study (data derived from the French national mother-child

cohort EDEN) reporting associations between serum levels of individual cytokines and specific behavioral dimensions in 5-year-old children assessed by the SDQ, supporting the notion that certain cytokines under study (IL-6, IL-10, IL-15, IL-17A, and TNFα; IFN-γ was not included) were associated with behavioral outcomes in children (244). A case-control study reported that children with ADHD showed increased IL-6 and IL-10 serum levels than normal children, but no significant differences were found for IFN-γ (and other cytokines) between patients and controls (247). Similar finding were also reported by Oades and colleagues (248). Another study examining the relation between IL-6, IL-1β, IL-4 at 6 and 12 months of age and neurodevelopment and psychological and 30 months showed that IL-6 at 12 months predicted higher scores in internalizing and externalizing problems at 30 months (155). However, to our knowledge, there are no other available study findings on longitudinal associations regarding IFN-γ or other inflammatory markers at preschool age and externalized and/or ADHD-related outcomes at later timepoints.

Our findings also suggest that children with elevated levels of IFN-γ at 4 years demonstrated increased scores in internalized CBCL scores at 6 years of age. Many studies have suggested increased circulation levels of IFN-γ among depressed individuals (249,250). One cross-sectional study also reported that IFN-γ plasma concentrations were significantly higher in the depression group than in the control group among adolescents (251). Comparable results were found in a study of depressive female adolescents, where plasma levels of IL-6 and IFN-γ were associated with depression severity and anxiety symptoms (252). It is also noteworthy that many commonly used antidepressants are supposed to antagonize IFN-γ signaling (253,254).

We also found that high IL-1β levels at 4 years were associated with more oppositional symptoms and externalized problems at 6 years of age. These results are in line with a cross-sectional study reporting that IL-1β was significantly associated with lower cognitive, language, and social emotional development in a cohort of children growing up in adversity (255). Other studies have found significant increases in plasma levels of some cytokines such as IL-1β in a group of children with ASD compared to a control group, indicating greater impairment in communication, social interaction and repetitive behaviors (164,245). In animal models, central administration of IL-1β seems to stimulate the hypothalamic–pituitary–adrenal axis,

decrease hippocampal brain-derived neurotrophic factor, and weaken hippocampal-dependent learning (256). Ψιλοασχετο;

Our findings also include that high IL-17α levels at 4 years were associated with increased internalized problems scales at 11 years. To our best knowledge, there are no study findings in line with this association, although studies in animal models have overall demonstrated that elevated concentrations of IL-17A are associated with depression (257). The role of IL-17a is more extensively studied and well-established in the pathophysiology of ASD (258,259).

Finally, in this study we came across the following findings regarding inflammatory markers at 4 years and cognitive outcomes at 11 years of age: High IL-10 levels were also associated with increased cognitive proficiency score and children with elevated levels of IL6/IL-10 ratio showed increased scores in visual spatial performance and general ability scores. While the role of pro-inflammatory cytokines in various disorders has been extensively studied in recent years, the same is not true for anti-inflammatory ones, such as IL-10 (260). A study conducted in 70 healthy subjects found that elevated IL-10 levels have been associated with enhanced scores of vocabulary and arithmetic intelligence subtests, highlighting that that anti-inflammatory cytokine such as IL-10 may have positive influence on cognitive intelligence (261). In fact, evidence from animal models report that anti-inflammatory cytokines, such as IL-10, can suppress neuroinflammation and have significant therapeutic potentials in ameliorating neurodegenerative disorders such as Alzheimer's disease, suggesting that anti-inflammatory cytokines can potentially function as neuromodulators or neurohormones and even signify a novel approach to treating neurodegenerative disorders through anti-inflammatory signaling patterns (262). An unexpected finding in our study was that fact that children with high levels of IL-8 demonstrated increased scores in processing speed at 11 years of age. IL-8 is overall a promising biomarker of ASD, linked to the social and cognitive functions of children with ASD and can be even associated with the pathogenesis of ASD (263). Thus, it is possible that residual confounding has affected the above reported finding.

Our results showed greater risk for reduced verbal performance scores for boys with high IL-17α serum concentrations, as well as lower motor scores for boys with high IL-6 serum concentrations at 4 years. Additionally, at both timepoints of 6 and 11 years of age, our results highlight greater risk for increased behavioral problems scores

for boys with high inflammatory markers. To our knowledge, this is the first study revealing sex-related differences in inflammation-specific cognitive and behavioral domain associations in a cross-sectional and longitudinal level. Although further study is required to validate and clarify the underlying mechanisms and the clinical utility of these findings, increased male prevalence has been frequently reported in various neurodevelopmental disorders, highlighting the concept of a male vulnerability model (264). It is hypothesized that this male susceptibility occurs partly because microglia and inflammatory molecules are involved in the normal developmental process of sexual differentiation (265) and also because males have more activated innate immune cells in the developing brain under normal conditions (266). We also found greater risk for lower scores in memory and memory span scales for overweight/obese children at 4 years with high TNF-α serum concentrations. This finding is in line with evidence from human clinical studies showing that obesity may increase the risk of mild cognitive impairment, in the form of short-term memory and executive function deficits (267). Obesity is considered to be a low-grade pro-inflammatory state, and studies have reported low-grade elevation of TNF-α in obese individuals (268–270). Research in rodent models show that obesity-induced inflammation may directly interfere with synaptic communication in the hippocampus (271).

The results of this study should be definitely interpreted in light of its limitations; although we incorporated extensive information on potential child and social factors that are associated with child neurodevelopment, we acknowledge that residual confounding because of other unmeasured confounders may still occur. In addition, children included in the present analysis who had complete data were more socially advantaged than the remainder of the cohort and this could lead to underestimation of the observed associations. Due to the young age of the children, we relied on their parents as source of information about behavioral symptoms and problems. On the other hand, the strengths of the present study include the heterogeneous and relatively large sample size, the long follow-up period, the multiple-timepoint outcome assessment and use of various reliable, robust and valid psychometric instruments in order to assess child cognition and behavior. In this analysis we also used standardized neurodevelopmental scales. Furthermore, we used multiple imputations with chained equations in order to increase precision and reduce bias.

4.3. General discussion

This thesis includes sections that aim to explore the role inflammation and early exposure to POPs in various domains of offspring neurodevelopment (cognitive, emotional and behavioral development) at three different timepoints, from preschool years to 11 years of age. It is well-established that inflammatory markers are related to complex, higher order neurological functions. There is also growing evidence that in utero exposure to POPs might disrupt child neurodevelopment. The present analyses aim to broaden our understanding of the impact of those exposures and provide evidence that can assist proper public health policies in ensuring optimum early life conditions which can bring about advantageous developmental outcomes in offsprings. The following section provides a brief summary of the study findings, (discussed already in detail in the results section).

4.3.1. What this study adds & future research

- Although POPs are well-known for their deleterious effect on child neurodevelopment, this is the first study indicating that prenatal exposure to high HCB levels is associated with decreased child cognitive performance at multiple-age timepoints. We also provided evidence that high concentrations of maternal serum PCBs levels were associated with impaired neuropsychological performance at 4, 6 and 11 years of age. Overall, this is the first epidemiological study suggesting consistency over time for these observed associations at various timepoints. Further research is needed to elucidate the link of prenatal POPs exposure and child neuropsychological development at a longitudinal level, so as to evaluate more thoroughly which factors impact on POPs and which factors modify their relation with child cognitive development, as well as to discover the underlying mechanisms of the associations under study. Future research combining extensive neuropsychological assessment and possible other functional cognitive measures, such as school performance and educational achievement, as well as neuroimaging techniques is necessary to explore the role of POPs in child neurodevelopment, and maybe discover whether the effect of those contaminants is part-specific within the brain.
- We have provided evidence of the link of high inflammatory markers levels at preschool age with impaired offspring's neuropsychological development (mainly with memory scales) cross-sectionally. High inflammatory

concentrations at preschool age were also found to be associated with more impaired behavioral development at 6 years, as well as at 11 years of age. This is the first time that a study reveals this kind of pattern regarding timepoints. Further studies are needed in order to replicate the above mentioned findings and explore how offspring cognitive and behavioral profiles are established in later childhood and adulthood. Our results are in line with growing evidence supporting that systemic inflammation during early development is linked to changes to the developing brain with possibly persistent effects. Further studies are needed to pinpoint how to best measure inflammation and evaluate the specific neurocognitive changes that mediate the relationship between inflammation and neurodevelopmental outcomes.

4.3.2. General conclusion

To conclude, the present thesis supports and extends previous knowledge that prenatal exposure to high levels of POPs is associated with reduced offspring cognitive development. This is actually the first study highlighting the association between prenatal POPs concentration levels and child neurodevelopment across three different timepoints, at 4, 6 and 11 years of age, strengthening the evidence for this association. Using Rhea Study's longitudinal design and its valid, multi-domain, and comprehensive developmental assessment, we conclude that these findings raise the possibility that exposure to HCB and PCBs may play a more crucial role in child cognition than previously considered and show new directions for research in birth cohort studies. A deep understanding of environmental risk factors for impaired cognitive development could be of considerable public health importance because of their potential modifiability. While tens of thousands of industrial chemicals remain in use, evidence on their potential neurodevelopmental effects is inadequate for the vast majority. Studies like the current one may signify an valuable initial step towards exploring environmental risk factors for cognitive disorders.

To our best of knowledge, this the first study conducted in a general population sample of children which highlights the significant role of increased inflammatory levels during preschool years in child cognitive performance across multiple timepoints. In fact, a few studies have examined the possible link between child inflammatory biomarkers and neurodevelopment, and most of them were carried out with samples of extremely premature infants or with clinical samples of children with autism spectrum disorders. Moreover, most of those studies have focused on the

relationship between maternal inflammatory cytokines during pregnancy and their children's neurodevelopmental outcomes. As there are no studies to this date that explored how inflammatory biomarkers relate to measures of neurodevelopmental scores in a general population sample, these results may shed some light in new pathways of investigation. Our findings reinforce the existing evidence that elevated inflammatory activity may be involved in early pathophysiological processes, such as memory deficits at a cross-sectional level, and in behavioral difficulties at a longitudinal level. An increased understanding of the interactions between pro-and-anti-inflammatory cytokine levels and patterns, and cognitive functions could allow us to identify early at-risk children for targeted interventions and allow every child to meet their full developmental potential. Inflammatory biomarkers could also even serve as prognostic indicators and possibly lead to prognosis and therapy in order to prevent developmental delays and behavioral problems in at-risk children.

5. Thesis' Publications

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Prenatal exposure to persistent organic pollutants in association with offspring neuropsychological development at 4 years of age: The Rhea mother-child cohort, Crete, Greece

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ABSTRACT

Background: Persistent Organic Pollutants (POPs) are highly-resistant compounds to environmental degradation and due to fat solubility they bioaccumulate through the food chain. As they cross the placenta, in utero exposure to POPs could disrupt child neurodevelopment as they are considered to be neurotoxic.
Aims: We examined whether in utero exposure to levels of different POPs is associated with offspring cognitive and behavioral outcomes at 4 years of age in a mother-child cohort in Crete, Greece (Rhea study).
Methods: We included 689 mother-child pairs. Concentrations of several polychlorinated biphenyls (PCBs) and other organochlorine compounds (dichlorodiphenyl dichloroethene [DDE], hexachlorobenzene [HCB]) were determined in maternal serum collected in the first trimester of pregnancy by triple quadrupole mass spectrometry. Neurodevelopment at 4 years was assessed by means of the McCarthy Scales of Children's Abilities. Behavioral difficulties were assessed by Strengths and Difficulties Questionnaire and Attention Deficit Hyperactivity Disorder Test. Linear regression analyses were used to estimate the associations between the exposures and outcomes of interest after adjustment for potential confounders.
Results: Children with "high" HCB concentrations (≥90th percentile) in maternal serum, demonstrated decreased scores in perceptual performance (adjusted $\beta = -6.07$; 95% CI: −10.17, −1.97), general cognitive (adjusted $\beta = -4.97$; 95% CI: −8.99, −0.96), executive function (adjusted $\beta = -6.24$; 95% CI: −10.36, −2.11) and working memory (adjusted $\beta = -4.71$; 95% CI: −9.05, −0.36) scales at 4 years of age. High exposure to PCBs (≥90th percentile) during pregnancy was associated with a 4.62 points reduction in working memory score at 4 years of age (95% CI: −9.10, −0.14). Prenatal exposure to DDE, HCB and PCBs was not associated with child behavioral difficulties.
Conclusions: The findings suggest that prenatal exposure to HCB and PCBs may contribute to reduced cognitive development at preschool age. Our results raise the possibility that exposure to HCB may play a more important role in child cognition than previously considered.

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List of abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
ADHDT	Attention Deficit Hyperactivity Disorder Test
BMI	body mass index
DDE	dichlorodiphenyl dichloroethene
DDT	dichlorodiphenyl trichloroethane
GAMs	generalized additive models
HCB	hexachlorobenzene

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(continued)

IQ	Intelligence Quotient
MSCA	McCarthy Scales of Children's Abilities
PCBs	polychlorinated biphenyls
PBDEs	polybrominateddiphenyl ethers
SD	standard deviation
SDQ	Strengths and Difficulties Questionnaire
95% CI	95% Confidence Interval

1. Introduction

Prevalence of neurodevelopmental disorders has increased worldwide, requiring more health and education services (Boyle et al., 2011). These disorders have their origins in very early brain development (de Graaf-Peters and Hadders-Algra, 2006; Sonnander and Claesson, 1999). According to the developmental origins of health and disease (DOHaD) hypothesis environmental exposures experienced early in life have the potential to "program" development, predisposing to various cognitive and behavioral phenotypes (Meaney, 2010; Wadhwa et al., 2009).

Persistent organic pollutants (POPs) are considered to be neurotoxic, influencing the synthesis and activity of neurotransmitters and the organization of the developing brain through alterations in basic cellular signaling processes and endocrine function (Seegal, 1996). These compounds are highly resistant to environmental degradation and as lipid soluble they bioaccumulate in human and animal tissue (World Health Organization, 2010). Organochlorine compounds, such as polychlorinated biphenyls (PCBs), dichlorodiphenyl trichloroethane (DDT), its metabolite dichlorodiphenyl dichloroethene (DDE), polybrominateddiphenyl ethers (PBDEs), and hexachlorobenzene (HCB) are POPs whose production and use has been banned (PCBs, HCB, some PBDEs) or restricted (DDT) by the Stockholm agreement (Stockholm Convention on Persistent Organic Pollutants, 2004). However, due to their persistence, exposure of the general population continues to these compounds banned long time ago, mostly through food chains (Porta et al., 2008). Maternal concentrations of POPs are transmitted to the developing fetus prenatally through the placenta and postnatally via breast milk (Needham et al., 2010; Shen et al., 2007).

Maternal serum and cord blood levels of PCBs have been associated in several studies with worse scores in cognitive and psychomotor development in both infancy (Grandjean and Landrigan, 2006; Park et al., 2010; Patandin et al., 1999; Ribas-Fitó et al., 2003; Walkowiak et al., 2001) and later childhood (Forns et al., 2012b; Jacobson and Jacobson, 1996; Sagiv et al., 2010; Stewart et al., 2008; Vreugdenhil et al., 2002), as well as with increased risk for ADHD-related behaviors (Neugebauer et al., 2015; Sagiv et al., 2010). However, these adverse effects have not been replicated in other studies (Boucher et al., 2009; Daniels et al., 2003; Gladen and Rogan, 1991; Grandjean et al., 2001; Gray et al., 2005; Nakajima et al., 2006; Newman et al., 2014; Wilhelm et al., 2008).

Cohort studies examining the effects of prenatal organochlorine exposure on child neurodevelopment and behavior have detected a negative association between cognition and behavior in infancy or preschool age and DDE (Eskenazi et al., 2006; Ribas-Fitó et al., 2003; Torres-Sánchez et al., 2007) and HCB exposure (Ribas-Fitó et al., 2007; Stewart et al., 2012). Other studies found null associations regarding DDE (Darvill et al., 2000; Gladen et al., 1988; Rogan and Gladen, 1991; Sagiv et al., 2010), and HCB exposure (Darvill et al., 2000; Forns et al., 2012b; Ribas-Fitó et al., 2003; Ribas-Fitó et al., 2006).

Findings from studies focusing on child cognitive and behavioral development at preschool age remain contradictory or inconclusive. To our knowledge, most of the studies have examined only one neurodevelopmental outcome, making it hard to determine possible risks for cognitive deficits and behavioral problems, such as attention-deficit and hyperactivity. The aim of the present study was to evaluate the impact of prenatal exposure to HCB, DDE and PCBs on child neurodevelopment and behavior at preschool age, a critical time period of child development, in a prospective pregnancy cohort in Crete, Greece, after controlling for a wide range of confounders and mediators.

2. Materials and methods

2.1. Study population

The Rhea study prospectively examines a population-based sample of pregnant women and their children at the prefecture of Heraklion, Crete, Greece. Methods are described in detail elsewhere (Chatzi et al., 2009). Briefly, female residents (Greek and immigrants) who became pregnant during a period of one year starting in February 2007 were contacted and asked to participate in the study. The first contact was made at the time of the first major ultrasound examination (mean ± SD 11.96 ± 1.49 weeks) and several contacts followed (6th month of pregnancy, at birth, 6 months, 1st year and 4 years after birth). To be eligible for inclusion in the study, women had to have a good understanding of the Greek language and be older than 16 years of age. Face-to-face structured questionnaires along with self-administered questionnaires and medical records were used to obtain information on several psychosocial, dietary, and environmental exposures during pregnancy and early childhood. The study was approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece, and all participants provided written, informed consent after complete description of the study.

Out of 1363 singleton live births, 879 children participated at the 4 years follow-up of the study, during which neuropsychological development was assessed in 785 children. From those, complete data for prenatal POPs exposure, and follow-up interviews were available for 689 mother–child pairs and thus were eligible for analysis.

2.2. Biological sample collection and exposure assessment

Maternal serum samples were collected at the first prenatal visit around the 3rd and 4th month of pregnancy, in 10 ml Silicone gel separator vacutainer tubes (Becton Dickinson, UK). Tubes were centrifuged within 2 h from blood collection at 2500 rpm for 10 min and were then stored in aliquots at −80 °C until assayed (Vafeiadi et al., 2015). The POP analyses were performed in the National Institute for Health and Welfare, Chemicals and Health Unit, Kuopio, Finland with an Agilent 7000B gas chromatograph triple quadrupole mass spectrometer (GC–MS/MS). Pretreatment of serum samples for GC–MS/MS analysis has been described elsewhere (Koponen et al., 2013). Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, and DDE were determined. All the results were reported on whole weight and expressed in pg/ml serum, while samples below the limit of quantification (LOQ) were assigned the value 0.5 × LOQ. LOQ was 6 pg/ml for PCB118 and PCB156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, PCB180. We chose to use wet-weight levels for the POPs but adjusted for maternal serum triglycerides and cholesterol as continuous variables in all multivariable models to minimize potential biases associated with automatic lipid adjustment (Schisterman et al., 2005). POPs were treated as categorical variables. We calculated total PCB concentrations by summing the concentrations of the 6 individual PCB congeners and studied the associations of interest for the sum of PCBs.

2.3. Outcome assessment

Children's cognitive and motor development was assessed by two trained psychologists, with the McCarthy Scales of Children's Abilities (MSCA), at the 4 year clinical visit at the University Hospital of Heraklion, Greece. In brief, MSCA test aims to identify possible developmental delay in different skills with the use of six scales: the Verbal, the Perceptual-Performance, the Quantitative, the General Cognitive, the Memory and the Motor scale (McCarthy, 1972). Executive function, working memory, memory span and cognitive functions of posterior cortex are four

additional scales derived from the MSCA test in accordance with their association with specific neurocognitive function areas (Julvez et al., 2007). The translation and cross-cultural adaptation of the MSCA was performed according to internationally recommended methodology. Raw scores of the neurodevelopmental assessment scales were standardized for child's age at test administration using a method for the estimation of age-specific reference intervals based on fractional polynomials (Royston and Wright, 1998). Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales (parameters conventionally used in psychometrics for IQ assessment). Scores were treated as continuous variables with higher scores representing better performance. Children were assigned to the two psychologists at random. The inter-observer variability was <1%. Right after each MSCA assessment psychologists completed a brief report regarding difficulties encountered during administration, such as child's behavior (bad moods, nervousness) and physical condition (tiredness, colds). This report was used for creating the quality of assessment index for the MSCA, which was flagged as "excellent", "bad" or "very bad". 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; (Gilliam, 1995)] is designed to identify and evaluate ADHD in ages 3–23 years. It is composed of 36 items for three subscales; 13 items on the hyperactivity subscale, 13 items on the inattention subscale, and 10 items on the impulsivity subscale. Raters are instructed to mark the appropriate quantifier beside each behavior/characteristic. Ratings range from 0 (not a problem) to 1 (mild problem) and 2 (severe problem). All 36 items are summed, equally contributing to generate an index for total ADHD difficulties (possible range, 0–72). Higher scores indicate higher and more severe symptomatology. The ADHDT has been translated and adapted for the Greek population (Maniadaki and Kakouros, 2002).

The parent version of the Strengths and Difficulties Questionnaire [SDQ; (Goodman, 1997)] is a behavioral screening instrument designed to assess strengths and difficulties of children aged 3–16 year old. It consists of five subscales each measured by five items, which cover emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems and prosocial behavior. Items are rated on a 3-point Likert scale as either not true, somewhat true, or certainly true. Responses are scored 0–2 for negatively-worded items and prosocial items. Positively-worded items from the difficulties subscales are reverse-coded. Subscales range from 0 to 10, and the total difficulties score (which is a sum of the difficulties subscales), ranges from zero to 40. The SDQ has been translated and adapted for the Greek population (Mpipou-Nakou et al., 2001). Scores in each scale of ADHDT and SDQ test were treated as continuous variables. These scores delineate symptoms and their perceived severity and not a clinical disorder.

2.4. Data analysis

Descriptive analyses of the study population characteristics, exposures and outcomes were conducted. Generalized additive models (GAMs) were applied to explore the shape of the relationships between POPs in maternal serum and outcomes under study. These models did not indicate clear linear relationships (p-gain defined as the difference in normalized deviance between the GAM model and the linear model for the same predictor <0.05). Fig. 1 shows the GAMs for some main outcomes.

The non-linear association between maternal HCB levels and child's neurodevelopmental scores was slightly positive up to concentrations of the 90th percentile (which corresponds to concentrations of ~2.70 pg/ml for HCB levels) and after that point a more negative change in the slope was evident (Fig. 2, scatterplot with a moving average Lowess curve). Similar patterns were observed for the rest of the contaminants. Thus, POPs levels in maternal serum were treated as categorical variables; the categories were defined as the "high exposure group" (≥90th percentile) and the reference group (<90th percentile). Multivariate regression models were used to examine the association between prenatal POPs exposure and children's neurodevelopmental and behavioral outcomes. Estimated associations were described in terms of β-coefficients and their 95% confidence intervals (CI). Child sex (male, female), quality of assessment (excellent, bad, very bad) and examiner (psychologist 1, psychologist 2) were a priori considered as potential confounding factors and included in all multivariate models. Additionally, we assessed whether maternal age (years), maternal educational level [low level: ≤9 years of mandatory schooling, medium level: >9 years of schooling up to attending post-secondary school education and high level: attending university or having a university/technical college degree], mother's tobacco smoking habits during pregnancy (never, ever), parity (primiparous, multiparous), birth weight (g) and breastfeeding duration (months) had further influence on the effect estimates. Potential confounding by these factors was examined by each at a time in a basic model already containing serum triglycerides and cholesterol and the a priori defined cofounders. If inclusion of a variable altered the contaminant coefficient by 10% or more for a neurodevelopmental scale, we retained the variable in the final set of covariates. Following these methods our first model (basic model) was adjusted for maternal serum levels of triglycerides and total cholesterol, child sex, quality of assessment and examiner; the second model (confounder model) was additionally adjusted for maternal age, maternal educational level, mother's tobacco smoking habits during pregnancy and parity. In the third model (mediator model), we additionally adjusted for birth weight and breastfeeding duration.

We also looked for heterogeneity in associations related to child sex (male, female), and maternal pre-pregnancy BMI status (<25 kg/m^2, ≥25 kg/m^2) and maternal TSH during pregnancy by including interaction terms in the models. We repeated the analyses excluding children who had been born preterm (<37 gestational weeks) in order to explore remaining confounding by prematurity. Finally, we performed further adjustment for maternal TSH during pregnancy (n = 554) and for maternal intelligence for a subsample of the study population (n = 266) with available information on maternal cognition. Maternal IQ was measured using the Raven's Standard Progressive Matrices (Raven, 1998). Moreover, we studied the associations of interest in a multi-pollutant model including HCB, DDE and PCBs.

All hypothesis testing was conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. The standardization of the MSCA and all other statistical analyses were performed using Stata Software, version 13 (Stata Corp LP, College Station, TX, USA).

3. Results

Table 1 is describing the study population characteristics. Participating mothers were predominantly Greek (94.7%), married (98.3%) and had a mean (±SD) age of 29.8 ± 5.0 years. About half of them had medium educational level (51.4%) and were multiparous (57.8%). Before pregnancy, 32.4% of mothers were overweight/obese, almost all mothers (87.9%) initiated breastfeeding and the mean length of breastfeeding was 4.1 ± 4.3 months. About half of the children (51.6%) were boys, the mean (±SD) birth weight of the study population was 3212.2 (±454.2) g and the mean age at assessment was 4.2 (±0.21) years.

Maternal POPs concentrations are presented in Table 2. The highest concentrations were found for DDE, followed by the sum of the six measured PCBs, PCB153 and HCB. Spearman correlation coefficients (p-value) were 0.48 (<0.001) for DDE-PCBs, 0.48 (<0.001) for DDE-HCB, and 0.65 (<0.001) for PCBs-HCB. Mothers that were older, multiparous, and had higher pre-pregnancy BMI, were more likely to have higher POP levels in early pregnancy (see Supplemental material, Table 1).

Table 3 shows regression results for maternal POPs levels in relation to neurodevelopmental outcomes at 4 years of age. Children with high levels of prenatal HCB exposure (≥90th percentile) demonstrated decreased scores in perceptual performance (adjusted β = −6.07; 95% CI: −10.17, −1.97), general cognition (adjusted β = −4.97; 95% CI: −8.99, −0.96) (Table 3), executive function (adjusted β = −6.24;

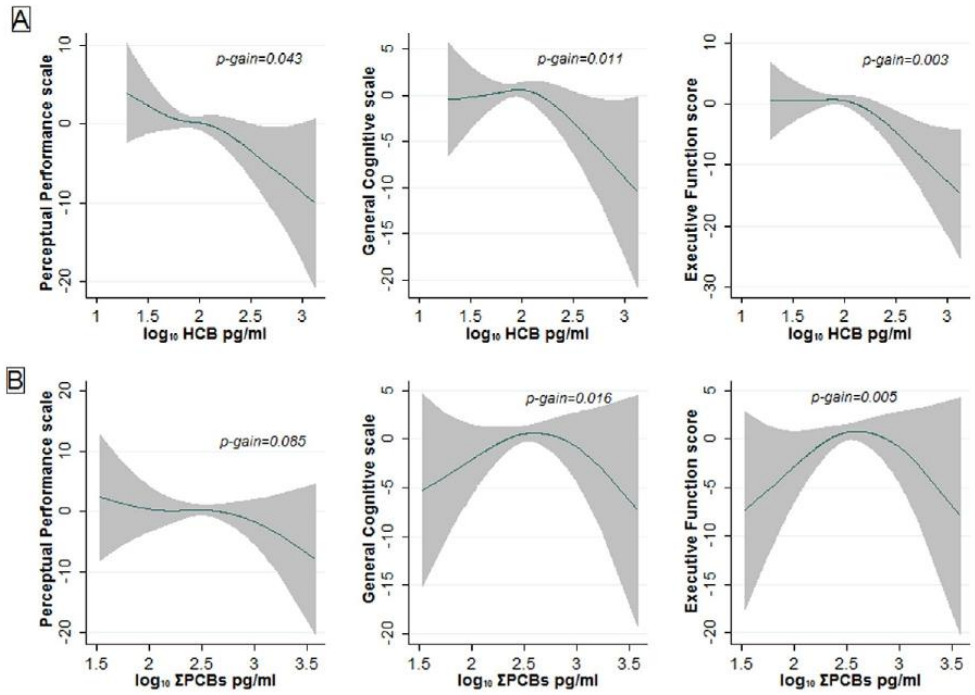

Fig. 1. Adjusted associations (95% CIs) of (A) HCB and (B) sum of PCBs with offspring perceptual performance, general cognitive and executive function score at 4 years of age. All models were adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination, maternal age at birth, maternal educational level, smoking during pregnancy, parity, infant weight and breastfeeding duration.

95% CI: −10.36, −2.11), as well as in working memory subscale (adjusted β = −4.71; 95% CI: −9.05, −0.36) at 4 years of age (Table 4). Further adjustment for child characteristics (mediator model) did not modify these associations (see Supplemental material, Table 2 and Table 3). High prenatal exposure to several PCBs (≥ 90th percentile) was associated with a 4.62 points reduction in working memory score at 4 years of age (95% CI: −9.10, −0.14). Further adjustment for child characteristics (mediator model) did not change these results (see Supplemental material, Table 3). DDE levels were not associated with child neurodevelopmental scores at 4 years. No association was demonstrated between prenatal POPs levels neither with SDQ nor with ADHDT scores at 4 years of age (see Supplemental material, Table 4 and Table 5). To assess whether specific PCB congeners had an influence on the outcomes of our study, we performed separate analyses for the 6 PCB congeners (118, 153, 138, 156, 180 and 170). Individual PCB congeners had similar associations with the outcomes to those for total PCBs (data not shown).

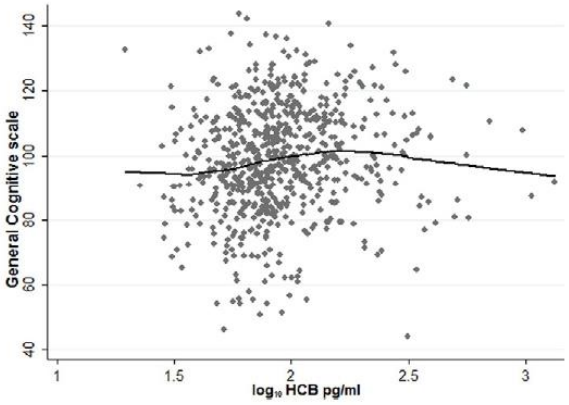

Fig. 2. Association between maternal HCB levels during pregnancy (log_{10} transformed) and child general cognitive scale at 4 years f age. The line represents the mean change in the general cognitive score for every 10-fold increase in maternal HCB concentration.

Multi-pollutant model including elevated levels of DDE, HCB, and PCBs showed that associations with most of the outcomes (although not statistically significant) were mainly driven by HCB. However, our results indicate that the association with working memory subscale was equally driven by HCB and PCBs (see Supplemental material, Table 6). Sensitivity analyses excluding preterm newborns (<37 gestational weeks) did not meaningfully change our results (data not shown). No indication for effect modification by child sex, maternal pre-pregnancy body mass index (BMI) and maternal TSH during pregnancy was found (p-interaction > 0.10) (data not shown). Additionally adjusted model for maternal TSH during pregnancy (Confounder model plus TSH) did not change our results substantially (Table 3 and Table 4). Repeating the analysis after adjustment for maternal IQ in a subsample of 266 mother-child pairs with available IQ data showed substantially the same inverse trend between prenatal POPs levels and offspring neurodevelopmental scores, though confidence intervals were wider, probably due to small sample size (see Supplemental material, Table 7).

4. Discussion

In this population-based pregnancy cohort study we found for the first time that prenatal exposure to HCB was associated with reduced child cognitive performance at preschool age. We also found that high concentrations of maternal serum PCBs levels were associated with

Table 1
Study participants characteristics.

Characteristic	Total	
	N	% or Mean ± SD
Maternal characteristics		
Maternal age (years)	688	29.8 ± 5.0
<20	17	2.4
≥20–30	280	40.7
≥30–40	372	54.1
≥40	19	2.8
Ethnic origin		
Greek	653	94.7
Other	37	5.3
Education		
Low	104	15.2
Medium	351	51.4
High	228	33.4
Parity		
Primiparous	282	42.2
Multiparous	386	57.8
Pre-pregnancy BMI (kg/m^2)	681	24.4 ± 4.7
Underweight (<18.5)	21	3.1
Normal (≥18.5–25)	439	64.5
Overweight (≥25–30)	139	20.4
Obese (≥30)	82	12.0
Weight gain during pregnancy (kg)	572	13.9 ± 5.6
Smoking during pregnancy		
Never	562	83.5
Ever	111	16.5
Marital status		
Married	678	98.3
Other	12	1.7
Never	81	12.1
Ever	588	87.9
Residence		
Urban	479	76.9
Rural	144	23.1
TSH during pregnancy (μIU/mL)	643	1.29 ± 1.0
Infant characteristics		
Sex		
Boy	359	51.6
Girl	336	48.4
Birth weight (g)	692	3212.2 ± 454.2
Breastfeeding (months)	669	4.1 ± 4.3

decreased child working memory scores. These results persisted after adjustment for several maternal and child characteristics. Prenatal exposure to organochlorine pesticides and PCBs was not associated with child behavioral and ADHD-related problems.

This is the first epidemiological study that reveals significant association of prenatal HCB exposure with cognitive, perceptual, executive and working memory functions at the age of 4 years. Our findings on the negative association between HCB exposure and child cognitive performance are in consistence with the Oswego study in the US, which revealed that placental levels of HCB were a significant predictor of lower WISC-III IQ scores in 11-year-old children (Stewart et al., 2012). To our knowledge, only one other study has examined the association between cord blood HCB levels with cognitive and psychomotor performance at preschool age, which, in contrast with our findings, did not find an association (Ribas-Fitó et al., 2007) in children from the INMA cohort in Spain. Other studies examining the effects of organochlorine pesticides on cognitive outcomes in infancy also reported null associations (Forns et al., 2012a; Ribas-Fitó et al., 2003). Adverse health effects of endocrine-disrupting chemicals, such as POPs, could follow non-monotonic dose responses with increased risks at lower concentrations and null or inverse risks at higher concentrations (Vandenberg et al., 2012), thus the substantial differences in the exposure levels may explain the disparity in findings across these studies.

Table 2
First trimester maternal serum POP levels (pg/ml, n = 689).

Contaminants	Mean ± SD	min	max	25th	Percentile	
					50th	75th
HCB	109.2 ± 102.4	19.5	1330.5	62.2	82.4	116.6
DDE	2947.9 ± 3071.1	181.6	23,175.4	1190.9	1955.9	3535.3
PCB118	20.3 ± 12.5	3.0	143.6	12.0	17.8	25.3
PCB153	149.7 ± 106.6	13.0	1348.9	85.5	125.7	188.3
PCB138	80.0 ± 54.8	5.0	742.8	45.3	68.8	102.0
PCB156	8.2 ± 7.6	3	81.5	3	6.5	10.7
PCB180	85.6 ± 77.6	5	979.6	44.5	67.0	104.9
PCB170	43.3 ± 40.6	5	542.3	21.9	33.8	53.5
Total PCBs	387.1 ± 288.4	34.0	3758.3	217.3	320.8	484.8

The mechanisms by which HCB may lead to developmental impairments are not clear; an animal study assessing the possible developmental neurotoxicity following premating maternal exposure to hexachlorobenzene in rats suggested that this compound possibly interferes with myelination during development, an important process for the normal functioning of all ascending and descending neural pathways (Goldey and Taylor, 1992). Overall, impairment related to HCB exposure has been described in animal models, but little information is available in humans (Korrick and Sagiv, 2008). A possible suggested mechanism is that exposure to HCB at background levels may affect thyroid function during pregnancy and these findings are of particular significance, since thyroid hormones of maternal origin may play an essential role in fetal neurodevelopment (Chevrier et al., 2008). However, in our study maternal TSH did not seem to have a substantial influence on the association between HCB and neurodevelopment. The first trimester of pregnancy is considered a crucial time period when brain development is more dependent on maternal thyroid hormone levels and more susceptible to endocrine disruptor effects (Colborn, 2004; de Escobar et al., 2004). Weak or no associations between HCB and neurodevelopmental outcomes found in other studies could possibly be explained by the fact that exposure assessment has been performed at late pregnancy or birth and not at the early stages of pregnancy.

In this study we found that high maternal serum levels of PCBs were associated with decreased performance in working memory tasks at preschool age. This is in line with other studies which suggested that prenatal exposure to PCBs predicted poorer working memory in early childhood (Jacobson et al., 1990; Jacobson et al., 1992) and in school age (Jacobson and Jacobson, 1996; Jacobson and Jacobson, 2003). Since the prefrontal structures of the brain are considered to be of particular importance in higher-order functions, like working memory (Jurado and Rosselli, 2007), one plausible underlying mechanism could be the effect of prenatal exposure to PCBs on prefrontal cortex (Boucher et al., 2009). This hypothesis is in line with animal studies that showed dysregulation in dopamine levels of the prefrontal cortex in rats exposed to PCB congeners in utero (Seegal et al., 2005). Apart from the aforementioned results, no other significant associations were demonstrated between maternal PCBs levels and neurodevelopmental and behavioral scores at 4 years of age. Several studies of PCB exposure during pregnancy have reported no associations with neuropsychological and behavioral domains, as well (Boucher et al., 2009; Daniels et al., 2003; Gladen and Rogan, 1991; Grandjean et al., 2001; Gray et al., 2005; Nakajima et al., 2006; Newman et al., 2014; Stewart et al., 2003; Wilhelm et al., 2008). However, there is much variation in human studies exploring these relations (Forns et al., 2012b; Grandjean and Landrigan, 2006; Jacobson and Jacobson, 1996; Neugebauer et al., 2015; Patandin et al., 1999; Ribas-Fitó et al., 2003; Sagiv et al., 2010; Stewart et al., 2008; Vreugdenhil et al., 2002; Walkowiak et al., 2001).

In our analysis, no associations were found between maternal DDE concentration and neurodevelopmental and behavioral scores at 4 years of age. Similarly, in the Spanish INMA cohort no associations were observed between maternal levels of DDE and Bayley Scales of Infant Development (BSID) at 14 months (Forns et al., 2012a). In addition,

Table 3
Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 4 years.

Contaminants	McCarthy Scales of Children's Abilities											
	Verbal		Perceptual performance		Quantitative		General cognitive		Memory		Motor scale	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
HCB pg/ml ≥90th perc												
Basic model[a]	0.79	(−3.05, 4.63)	-2.12	(−6.04, 1.80)	−0.14	(−4.15, 3.86)	−0.39	(−4.29, 3.51)	0.75	(−3.11, 4.61)	−0.09	(−4.22, 4.05)
Confounder model[b]	−2.98	(−7.00, 1.05)	−6.07	(−10.17, −1.97)	−4.10	(−8.36, 0.15)	−4.97	(−8.99, −0.96)	−3.47	(−7.59, 0.65)	−2.97	(−7.49, 1.55)
Confounder model (plus TSH)[c]	−3.06	(−7.23, 1.11)	−6.08	(−10.32, −1.83)	−3.65	(−8.08, 0.78)	−4.96	(−9.16, −0.77)	−3.31	(−7.57, 0.95)	−2.54	(−7.19, 2.10)
DDE pg/ml ≥90th perc												
Basic model[a]	−0.09	(−4.01, 3.84)	1.93	(−2.08, 5.94)	0.92	(−3.17, 5.01)	0.76	(−3.23, 4.74)	0.83	(−3.12, 4.78)	1.51	(−2.71, 5.74)
Confounder model[b]	−3.49	(−7.53, 0.55)	−1.29	(−5.43, 2.86)	−2.11	(−6.40, 2.17)	−3.01	(−7.05, 1.03)	−2.49	(−6.63, 1.66)	0.01	(−4.53, 4.55)
Confounder model (plus TSH)[c]	−3.63	(−7.86, 0.59)	−1.45	(−5.79, 2.89)	−1.95	(−6.46, 2.55)	−3.14	(−7.40, 1.13)	−2.91	(−7.24, 1.42)	−0.27	(−4.99, 4.45)
ΣPCBs[d] pg/ml ≥90th perc												
Basic model[a]	1.96	(−2.03, 5.95)	0.19	(−3.90, 4.27)	0.07	(−4.10, 4.24)	1.26	(−2.80, 5.32)	2.57	(−1.44, 5.58)	1.38	(−2.93, 5.68)
Confounder model[b]	−1.41	(−5.57, 2.75)	−2.37	(−6.62, 1.88)	−3.44	(−7.83, 0.95)	−2.36	(−6.52, 1.79)	−0.43	(−4.69, 3.83)	0.22	(−4.44, 4.89)
Confounder model (plus TSH)[c]	−1.42	(−5.70, 2.86)	−1.61	(−5.99, 2.78)	−3.06	(−7.60, 1.49)	−1.96	(−6.28, 2.35)	−0.60	(−4.98, 3.78)	1.16	(−3.60, 5.93)

[a] Adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination (N = 609).
[b] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy and parity (N = 586).
[c] N = 554.
[d] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180.

the North Carolina Infant Feeding Study revealed no association between cord DDE levels and scores on MSCA at ages 3, 4 or 5 years (Gladen and Rogan, 1991). Likewise, no associations were reported between prenatal levels of DDE and BSID scores at 8 months and WISC scores at 7 years in the US Collaborative Perinatal Project (Jusko et al., 2012). On the contrary, there are cohorts that have detected negative associations between prenatal DDE exposure and social, mental and psychomotor development, mainly in infancy (Eskenazi et al., 2006; Ribas-Fitó et al., 2003; Torres-Sánchez et al., 2007) and to a lesser extent in preschool (Ribas-Fitó et al., 2006) and school years (Sagiv et al., 2010), but it should be noted that most of those population samples were overall exposed to high levels of DDE. Overall, epidemiological findings on prenatal DDE levels and cognitive development vary greatly; potential explanations for this heterogeneity in results are not apparent, although they may also include different study designs, study sample sizes and different neurodevelopmental/behavioral outcomes examined by each study.

Exposure levels to HCB, DDE and ΣPCBs in our cohort study were close to but generally lower than the median exposure in other pregnant populations (Jusko et al., 2012; Ribas-Fitó et al., 2007; Roze et al., 2009; Stewart et al., 2012). Possible variations on the observed median POP levels may be due to the fact that in other studies the exposure measurements were conducted at different time points or in different sample types (e.g. samples from cord or placental blood).

Strengths of the present study include the prospective population-based design. Neurodevelopment assessment at preschool age was performed with the use of MCSA (McCarthy, 1972), a valid, standardized psychometric test which provides both a general level of child's intellectual functioning and an assessment of separate neurodevelopmental domains. In the present analysis we used standardized neurodevelopmental scales (mean of 100 points with a 15 SD). There is extensive literature on the public health impact of a 1-point loss of a neuropsychological scale, most are based on effects of lead exposure on IQ (Grandjean and Landrigan, 2006). Although a seemingly small change of a 1-point

Table 4
Associations between prenatal exposure to POPs and child neurodevelopmental outcomes at age 4 years.

Contaminants	Subscales derived from McCarthy Scales of Children's Abilities						
	Executive function		Working memory		Memory span		Functions of posterior cortex	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
HCB pg/ml ≥90th perc								
Basic model[a]	−2.02	(−6.00, 1.96)	−1.18	(−5.17, 2.81)	1.13	(−3.87, 4.14)	1.09	(−2.74, 4.92)
Confounder model[b]	−6.24	(−10.36, −2.11)	−4.71	(−9.05, −0.36)	−3.71	(−8.02, 0.59)	−3.09	(−7.09, 0.90)
Confounder model (plus TSH)[c]	−6.00	(−10.30, −1.71)	−4.43	(−8.91, 0.05)	−3.74	(−8.18, 0.70)	−3.23	(−7.41, 0.95)
DDE pg/ml ≥90th perc								
Basic model[a]	−0.14	(−4.21, 3.93)	−0.86	(−4.94, 3.22)	1.47	(−2.62, 5.56)	1.83	(−2.09, 5.74)
Confounder model[b]	−3.84	(−7.99, 0.32)	−3.66	(−8.03, 0.71)	−1.49	(−5.82, 2.83)	−1.50	(−5.52, 2.52)
Confounder model (plus TSH)[c]	−3.72	(−8.10, 0.66)	−4.05	(−8.59, 0.49)	−1.80	(−6.31, 2.71)	−1.85	(−6.10, 2.40)
ΣPCBs[d] pg/ml ≥90th perc								
Basic model[a]	0.01	(−4.13, 4.16)	−1.59	(−5.74, 2.56)	2.95	(−1.21, 7.11)	2.08	(−1.91, 6.06)
Confounder model[b]	−3.58	(−7.85, 0.69)	−4.62	(−9.10, −0.14)	0.34	(−4.10, 4.78)	−1.09	(−5.22, 3.04)
Confounder model (plus TSH)[c]	−2.96	(−7.39, 1.47)	−3.95	(−8.54, 0.64)	0.54	(−4.02, 5.10)	−0.91	(−5.21, 3.38)

[a] Adjusted for maternal serum triglycerides and cholesterol, child sex, quality of assessment, examiner at 4 year examination (N = 608).
[b] As in basic model plus adjustment for maternal age at birth, maternal educational level, smoking during pregnancy and parity (N = 585).
[c] N = 553.
[d] Individual PCB congeners included: 118, 138, 153, 156, 170 and 180.

decrease in IQ score might not be relevant at the individual level, at the population level this is possible to swift the distribution of IQ to the left and increase the number of persons below the normal range (Bellinger, 2012). The exclusion of women who gave birth to twins as well as adjustment for several socio-demographic variables reduced the likelihood of confounding. The inclusion of maternal intelligence, although available for a subsample of the total population, should be considered as an additional strength of the present study. This study is not free of limitations; we cannot rule out the possibility that prenatal and/or postnatal exposure to other unmeasured chemicals correlated to POPs may have confounded the associations under study. We could not separate the potential effects of postnatal exposures because we have not measured POPs at other time points, but we tried to control for these possible effects by including breastfeeding duration in our models. Children included in the present analysis who had complete data were more socially advantaged than the remainder of the cohort and this could lead to underestimation of the observed associations. Due to the young age of the children, we relied on their parents as source of information about behavioral symptoms and problems (SDQ and ADHDT scores). Finally, although we incorporated a number of confounding factors regarding potential social and environmental aspects that are associated with child neurodevelopment, we acknowledge that residual confounding may still occur, due to possible unmeasured factors such as social economic status, and quality of home environment.

5. Conclusions

Overall, in the present study we found that prenatal exposure to HCB and PCBs was associated with reduced offspring cognitive development at preschool age. This is actually the first study highlighting the association between prenatal HCB with perceptual, cognitive and executive domains at 4 years of age. These findings raise the possibility that exposure to HCB may play a more important role in child cognition than previously considered and shows new directions for research in birth cohort studies. Further research is needed to replicate these results and to explore potential underlying biological mechanisms.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2016.09.012.

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Association between high levels of inflammatory markers and cognitive outcomes at 4 years of age: The Rhea mother-child cohort study, Crete, Greece

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ABSTRACT

There is growing evidence associating inflammatory markers in complex, higher order neurological functions, such as cognition and memory. We examined whether high levels of various inflammatory markers are associated with cognitive outcomes at 4 years of age in a mother-child cohort in Crete, Greece (Rhea study). We included 642 children in this cross-sectional study. Levels of several inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-17α, IL-10, MIP-1α, TNF-α and the ratios of IL-6 to IL-10 and TNF-α to IL-10) were determined in child serum via immunoassay. Neurodevelopment at 4 years was assessed by means of the McCarthy Scales of Children's Abilities. Multivariate linear regression analyses were used to estimate the associations between the exposures and outcomes of interest after adjustment for various confounders. Our results indicate that children with high TNF-α concentrations ($\geq 90^{th}$ percentile) in serum demonstrated decreased scores in memory (adjusted $\beta = -4.0$; 95% CI: −7.7, −0.2), working memory (adjusted $\beta = -4.0$; 95% CI: −8.0, −0.1) as well as in memory span scale (adjusted $\beta = -4.0$; 95% CI: −7.9, −0.1). We also found that children with high IFN-γ serum levels showed lower scores in memory span scale (adjusted $\beta = -3.4$; 95% CI: −7.3, −0.4). Children with elevated TNF-α/IL-10 ratio demonstrated decreased quantitative (adjusted $\beta = -4.3$; 95% CI: −8.2, −0.4), motor (adjusted $\beta = -3.5$; 95% CI: −7.5, −0.5), executive function (adjusted $\beta = -4.8$; 95% CI: −8.5, −1.1), general cognitive (adjusted $\beta = -3.6$; 95% CI: −7.3, −0.1), memory (adjusted $\beta = -3.8$; 95% CI: −7.6, −0), working memory (adjusted $\beta = -3.5$; 95% CI: −7.5, −0.5) and memory span scores (adjusted $\beta = -5.3$; 95% CI: −9.1, −1.4) The findings suggest that high levels of TNF-α may contribute to reduced memory performance at preschool age.

1. Introduction

Inflammation is identified as a natural defense mechanism by body tissues in response to injury, but this process may stop being protective for the organism and become harmful when it occurs chronically [1]. Inflammatory markers, such as cytokines, are proteins involved in normal aspects of neurodevelopment, including progenitor cell differentiation, cellular migration within the nervous system and synaptic

Abbreviations: IFN-γ, Interferon γ; IL-1β, Interleukin 1β; IL-6, Interleukin 6; IL-8, Interleukin 8; IL-17α, Interleukin 17α; IL-10, Interleukin 10; MIP-1α, Macrophage Inflammatory Protein 1α; TNF-α, Tumor Necrosis Factor α; ASD, autism spectrum disorders; DAGs, directed acyclic graphs; BMI, Body Mass Index; IQ, Intelligence Quotient; MSCA, McCarthy Scales of Children's Abilities; SD, Standard Deviation; 95% CI, 95% confidence interval; PCBs, polychlorinated biphenyls; GAMs, generalized additive models

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network formation [2–6] and there is growing evidence associating them in complex, higher order neurological functions, such as cognition and memory [7,8]. Imbalanced cytokine production, signaling and regulation may have various neurological consequences [9,10].

Insight into the link of inflammatory markers function and central nervous system processes has increased, mostly on the basis of animal models. As Voltas et al. [11] recently pointed out, few studies have examined the potential association between child inflammatory biomarkers and neurodevelopment, and most of these studies were carried out with samples of extremely premature infants or with clinical samples of children with autism spectrum disorders (ASD). In fact, a body of research has evolved around the role of prenatal cytokines as markers of risk for cognitive dysfunction in special populations, such as children born preterm [12–14], children with low birth weight [15], sickle cell disease [16] and chronic hepatitis C [17], indicating a potential role for inflammatory processes in neurodevelopmental outcomes for those vulnerable populations. Some clinical studies have linked cytokine imbalances during development and throughout life to ASD; case-control studies have found higher circulating IL-6, IL-1β, IL-8 and TNF-α levels in plasma of preschool [18,19] and school-age children with ASD [20–22] compared to typically developing controls. Moreover, plasma levels of IFN-γ and cerebrospinal fluid levels of TNF-α have been reported to be increased in autistic children [23–25] and, likewise, elevated concentrations of IFN-γ have been reported for subjects with ASD compared to controls [20].

However, to our knowledge, there are no available data discussing the relationship between inflammatory markers levels and neurodevelopment in a general population sample of children. The aim of the present study is to examine the role of various inflammatory markers (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α, TNF-α and two pro- to anti-inflammatory cytokine ratios, IL-6 to IL-10 and TNF-α to IL-10) measured in child serum at 4 years of age in neurodevelopmental scores assessed at 4 years of age in a cross-sectional study nested in the pregnancy cohort in Crete, Greece, after controlling for a range of confounders. It is hypothesized that increased levels of inflammation will be associated with elevated risk for inferior neurodevelopmental scores at 4 years of age.

2. Materials and methods

2.1. Study population

The Rhea study prospectively examines a population-based sample of pregnant women and their children at the prefecture of Heraklion, Crete, Greece. Methods are described in detail elsewhere [26]. Briefly, female residents (Greek and immigrants) who became pregnant during a period of one year starting in February 2007 were contacted and asked to participate in the study. The first contact was made at the time of the first major ultrasound examination (mean ± SD 12.0 ± 1.5 weeks) and several contacts followed (6th month of pregnancy, at birth, 6 months, 1st year and 4 years after birth). To be eligible for inclusion in the study, women had to have a good understanding of the Greek language and be older than 16 years of age. Face-to-face structured questionnaires along with self-administered questionnaires and medical records were used to obtain information on several psychosocial, dietary, and environmental exposures during pregnancy and early childhood. The study was conducted according to the guidelines of the Declaration of Helsinki and all procedures were approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece. Written informed consent was obtained from all the participants after complete description of the study.

The present analysis is a cross-sectional study, nested within the Rhea cohort. Out of 1363 singleton live births, 879 singleton children participated at the 4 years follow-up of the study, during which inflammation markers were measured in 661 children. From those, complete data for neurodevelopment was available for 642 children. Of those, 59 children had incomplete information regarding pre-pregnancy BMI, smoking early in pregnancy, parity, birth weight, preterm birth, BMI at the age of 4 and passive smoking of the child at 4 years of age. Thus, full data was available for a total of 583 children (90.8% of the children with exposure data and outcome assessment). We observed differences in some of the exposures and outcome data ($p < 0.05$) between the children that had full data available (n = 583) and those that had incomplete covariate information (n = 59). Due to those differences, the incomplete covariate information was imputed.

2.2. Biological sample collection and exposure assessment

Following the completion of the 4-year-follow–up assessments, blood samples were collected by venipuncture for each child (10 ml) in SST gel separator vacutainer (BD vacutainers, UK), after written parental consent. For the reduction of pain and discomfort of the children, anesthetic cream 5% EMLA with composition 2.5% lidocaine and 2.5% prilocaine (AsraZeneca, UK) was used. Analyses were performed in the Laboratory of Clinical Nutrition and Epidemiology of Diseases of Medical School, University of Crete. Blood samples were centrifuged (Kubota4000, Japan) at 2500 rpm 10 min within 2 hrs after collection and stored at −80° C until assayed. The Milliplex Map human high sensitivity T cell magnetic bead panel (Cat. # HSTCMAG-28SK) from Millipore (Billerica, MA) was used for the simultaneous quantification of IFN-γ IL-1β, IL-6, IL-8, IL-10, IL-17α, MIP-1α and TNF-α in the supernatants. The principle of the assay is based on the quantification of multiple bio-molecules concurrently employing fluorescent-coded magnetic beads (MagPlex-C microspheres). The microspheres were incubated with the samples and then were allowed to pass rapidly through laser systems that distinguish the different sets of microspheres and the fluorescent dyes on the reporter bio-molecules. The sensitivity of the assay for every bio-molecule was: 0.3 pg/ml IFN-γ, 0.1 pg/ml IL-1β, 0.1 pg/ml IL-6, 0.1 pg/ml IL-8, 0.6 pg/ml IL-10, 0.3 pg/ml IL-17α, 0.9 pg/ml MIP-1α and 0.2 pg/ml TNF-α. We used a limit of 4 SD based on the statistical convention that observations 4 or more SD from the expected mean can be considered to be "extreme outliers" and thus, excluded from the statistical analyses. The intra-assay precision (%CV) for all biomolecules was < 5%. The inter-assay precision (%CV) for IFNγ, IL-6, IL-10 and IL-17α was < 20%, for IL-1β, IL-8, MIP-1α and TNF-α was < 15%. The above analyses were performed on an automated analyzer Luminex 100 connected with the Luminex xPONENT software.

2.3. Outcome assessment

2.3.1. McCarthy scales of Children's Abilities (MSCA)

Children's cognitive and motor development was assessed by two trained psychologists, with the McCarthy Scales of Children's Abilities (MSCA), at the 4 year clinical visit at the University Hospital of Heraklion, Greece. In brief, MSCA test aims to identify possible developmental delay in different skills with the use of six scales: the Verbal, the Perceptual-Performance, the Quantitative, the General Cognitive, the Memory and the Motor scale [27]. Executive function, working memory and memory span are three additional scales derived from the MSCA test in accordance with their association with specific neurocognitive function areas [28].

The translation and cross-cultural adaptation of the MSCA was performed according to the internationally recommended methodology. Children were assigned to the two psychologists at random. The inter-observer variability was < 1%. Right after each MSCA assessment psychologists completed a brief report regarding difficulties encountered during administration, such as child's behavior (bad moods, nervousness) and physical condition (tiredness, colds). This report was used for creating the quality of assessment index for the MSCA, which was flagged as "good", "bad" or "very bad". Additional information on children's behavior was obtained via maternal report on standardized

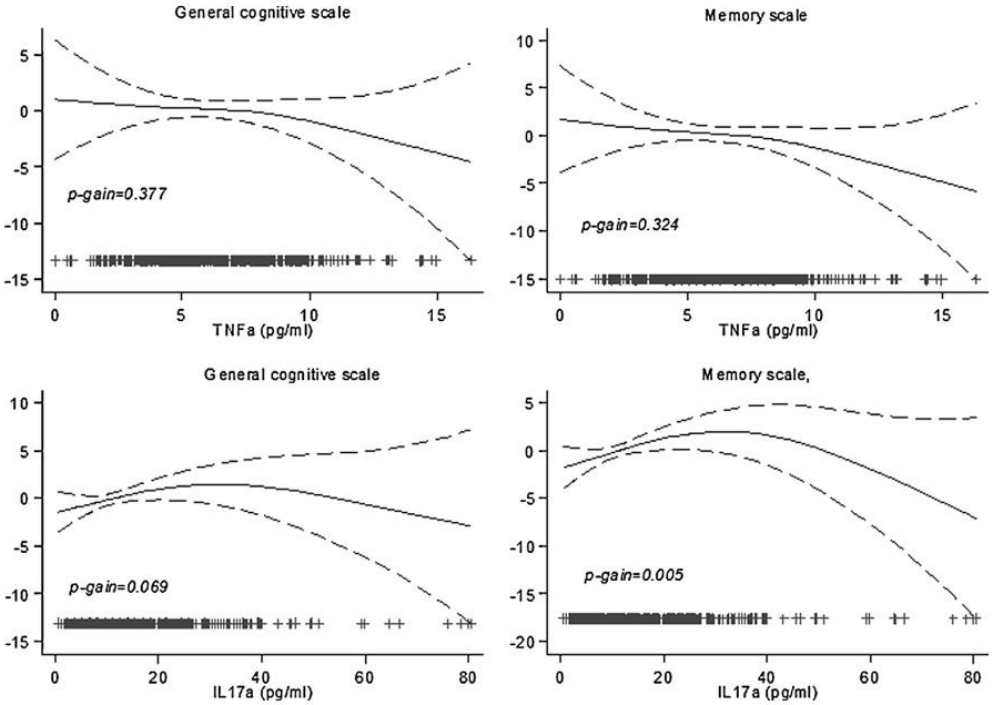

Fig. 1. GAMS for adjusted associations (95% CIs) of TNF-α and IL-17α with child general cognitive and memory scale at 4 years of age. All models were adjusted for examiner, quality of assessment, child sex, maternal age in pregnancy, maternal education, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years. Plus symbols (+) represent observations.

child behavior scales which were administered at the 4 years of age follow-up.

Raw scores of the neurodevelopmental assessment scales were standardized for child's age at test administration using a method for the estimation of age-specific reference intervals based on fractional polynomials [29]. Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales (parameters conventionally used in psychometrics for IQ assessment). Scores were treated as continuous variables with higher scores representing better performance.

2.4. Statistical analysis

Descriptive analysis of the study population was conducted. We performed generalized additive models (GAMs) to assess the linear relationship between inflammatory markers and outcomes. Fig. 1 shows the GAMs for two main outcomes. Because GAMs did not show linearity (p-gain, defined as the difference in normalized deviance between the GAM model and the linear model for the same exposure and outcome > 0.1), we used serum concentrations of inflammatory biomarkers as categorical variables; the categories were defined as the "high exposure group" ($\geq 90^{th}$ percentile) and the reference group (< 90th percentile). This categorization was also decided upon graphical inspection of the relationship between outcomes and exposures after the application of a spline knot (Supplementary Fig. 1).

We estimated associations between serum concentrations of inflammatory marker levels and continuous scores in cognitive and motor development by performing multivariate linear models. Estimated associations were described in terms of β-coefficients and their 95% confidence intervals (CI). The significance of the β-coefficients was evaluated by the Wald's test. We assessed the association between serum concentrations of inflammatory markers and each neurodevelopmental outcome in an adjusted model for quality of MSCA assessment (good, bad, very bad), examiner (psychologist 1, psychologist 2), child sex (male, female), and maternal characteristics, such as maternal age in pregnancy (years), maternal education [low level: ≤ 9 years of mandatory schooling, medium level: > 9 years of schooling up to attending post-secondary school education and high level: attending university or having a university/technical college degree] parity (primiparous, multiparous) and maternal pre-pregnancy Body Mass Index (BMI) and child characteristics, such as passive smoking at 4 years (yes, no), birth weight (g), preterm birth (yes, no) and child BMI at 4 years (kg/m^2). For the fully adjusted model, we selected the covariates using directed acyclic graphs (DAGs) (Supplementary Fig. 2).

We performed various sensitivity analyses to assess the robustness of our results. First, in order to assess if our studied associations were modified by child sex, child BMI at 4 years (normal weight vs. overweight or obese), maternal pre-pregnancy BMI (normal weight vs. overweight or obese), Child nursery attendance (yes/no) or passive smoking exposure, appropriate interaction terms were included in the regression models. We stratified the sample in the cases that significant interactions were detected. Second, we repeated all analyses excluding preterm (< 37 gestational weeks) and low birth weight (< 2500 g) neonates. Third, because relations of inflammatory markers with cognitive outcomes could be confounded by chronic child health diseases and infections, we repeated the analysis after further adjusting for asthma occurrence at 4 years (yes/no), allergic rhinitis symptoms in the last 12 months at 4 years of age (yes/no), and helicobacter pylori seropositivity at 4 years of age (yes/no). We also included in the models exposures during pregnancy that have been previously shown to affect child neurodevelopment such as maternal serum concentrations of polychlorinated biphenyls (PCBs, pg/ml) [30] and maternal serum levels of Vitamin D (nmol/l) [31].

Due to the relatively high percentage of missing covariates (9.2%)

Table 1
Study participants characteristics.

	Total	
	N	% or Mean ± SD
Maternal characteristics		
Maternal age (years)	634	29.8 ± 5.0
Ethnic origin		
Greek	602	94.7
Other	34	5.3
Education		
Low	95	15.3
Medium	320	51.7
High	204	33.0
Parity		
Primiparous	298	46.5
Multiparous	343	53.5
BMI before pregnancy (kg/m^2)	608	24.5 ± 4.7
Child characteristics		
Sex		
Boy	336	52.3
Girl	306	47.7
Age at 4 years follow-up	642	4.2 ± 0.2
Birth weight (g)	618	3195.4 ± 449.2
Preterm birth	669	4.1 ± 4.3
Yes	74	11.8
No	551	88.2
BMI at age 4 (kg/m^2)	640	16.4 ± 1.8
Passive smoking at age 4		
Yes	280	43.8
No	359	56.2

we used multiple imputations with chained equations (MICE) in order to increase precision and reduce bias. The imputation model included exposures, outcomes, and covariates under study, as well as additional auxiliary variables [32]. In analytic models, we combined estimates from the 20 imputed data sets generated with the use of Rubin's rules [33]. Results were similar between multiple imputation and complete case analysis, and hence, we present effect estimates based on imputed data.

All hypothesis testing was conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. The standardization of the MSCA and all other statistical analyses were performed using Stata Software, version 13 (Stata Corp LP, College Station, TX, USA).

3. Results

Table 1 describes the study population characteristics. Participating mothers were predominantly Greek (94.7%) and had a mean (± SD) age of 29.8 (± 5.0) years. About half of them had medium educational level (51.7%) and were multiparous (53.5%). Before pregnancy, the mean maternal BMI was 24.5 (± 4.7) kg/m^2. About half of the children (52.3%) were boys, the mean (± SD) birth weight of the study population was 3195.4 (± 449.2) g and the mean age at assessment was 4.2 (± 0.2) years. A total of 280 (43.8%) were exposed to passive smoking and the mean (± SD) BMI at age 4 was 16.4 (± 1.8) kg/m^2.

Child inflammatory levels in serum at 4 years are presented in Table 2 and Supplementary Table 1 illustrates correlation coefficients calculated for all those markers.

Table 3 shows regression results for high inflammatory marker levels in child serum in relation to neurodevelopmental outcomes (MSCA scores) at 4 years of age. Children with high IFN-γ serum levels (≥90th percentile) showed lower scores in memory span scale (adjusted β = −3.4; 95% CI: −7.3, −0.4). Children with high TNF-α serum levels (≥90th percentile) demonstrated decreased scores in memory (adjusted β = −4.0; 95% CI: −7.7, −0.2), working memory (adjusted β = −4.0; 95% CI: −8.0, −0.1) as well as in memory span scale (adjusted β = −4.0; 95% CI: −7.9, −0.1). High TNF-α/IL-10 ratio was associated with decreased quantitative (adjusted β = −4.3; 95% CI: −8.2, −0.4), motor (adjusted β = −3.5; 95% CI: −7.5, −0.5), executive function (adjusted β = −4.8; 95% CI: −8.5, −1.1), general cognitive (adjusted β = −3.6; 95% CI: −7.3, −0.1), memory (adjusted β = −3.8; 95% CI: −7.6, −0), working memory (adjusted β = −3.5; 95% CI: −7.5, −0.5) and memory span scores (adjusted β = −5.3; 95% CI: −9.1, −1.4). No other association was detected between high inflammatory levels and other neurodevelopmental scores at 4 years of age.

Table 2
Child inflammatory levels at 4 years of age (pg/ml).

Inflammatory Marker	N	Median (IQR)	Geometric Mean (GSD)	Percentile	
				10th	90th
IFN-γ pg/ml	636	26.1 (22.7)	21.8 (2.3)	6.5	50.3
IL-1β pg/ml	637	1.3 (1.0)	1.1 (2.1)	0.5	2.3
IL-6 pg/ml	635	1.1 (0.8)	1.1 (1.8)	0.5	2.3
IL-8 pg/ml	635	3.5 (1.9)	3.6 (1.5)	2.2	5.7
TNF-α pg/ml	639	6.0 (3.2)	5.8 (1.5)	3.4	9.3
IL-17α pg/ml	634	11.0 (12.0)	10.7 (2.2)	4.1	26.8
MIP-1α pg/ml	641	13.4 (7.6)	12.8 (1.5)	6.9	21.1
IL-10 pg/ml	641	5.3 (5.0)	5.4 (2.1)	2.1	13.3

GSD: Geometric Standard Deviation.

Further analyses showed evidence for an interaction between child sex and IL-17α levels in response to neurodevelopmental scores (p for interaction < 0.05). Stratified analysis revealed reduced verbal (adjusted β = −4.2; 95% CI: −10.2, 1.7) scale scores for boys with high concentrations of IL-17a, whereas these associations in girls were in the opposite direction. Moreover, boys with high concentrations of IL-6 had lower motor (adjusted β = −0.2; 95% CI: −6.2, 5.8) scale scores (Supplementary Table 2). Further stratified analysis according to child BMI status, showed reduced scores in memory (adjusted β = −11.4; 95% CI: −20.6, −2.2) and memory span (adjusted β = −11.3; 95% CI: −20.2, −2.4) scores for overweight/obese children with high concentrations of TNF-α in serum compared to children with normal weight (Supplementary Table 3). We found no evidence for any significant interaction between maternal pre-pregnancy overweight/obesity, exposure to passive smoking or child nursery attendance and child high inflammatory biomarker levels at 4 years of age (p for interaction > 0.05). Sensitivity analyses excluding preterm newborns (< 37 gestational weeks) and low birth weight neonates (< 2500 g) did not meaningfully change our results (data not shown). After further adjustment for asthma, allergic rhinitis and helicobacter pylori seropositivity at 4 years, as well as exposure to environmental pollutants and pregnancy serum levels of Vitamin D, our results did not differ substantially from those derived from the main analysis (Supplementary Table 4).

4. Discussion

In the present analysis we examined for the first time the relationship between inflammatory marker levels and neurodevelopment in a general population sample of children and found that preschoolers with elevated TNF-α concentrations in serum demonstrated decreased scores in memory, memory span and working memory tasks. These associations persisted after the sequential adjustment for several maternal and child factors. We also found that children with high IFN-γ serum levels showed lower scores in memory span scale. Elevated levels of the rest inflammatory markers under examination (IL-1β, IL-6, IL-8, IL-17α, IL-10 and MIP-1α) were not associated with any other child neurodevelopmental scores.

Comparison with other studies is rather complex mainly because of different methodological approaches study design, type and size of study samples, age of the participants and outcomes examined. Studies with elderly populations have well-established the association between TNF-α levels with cognitive deficits. Elevated TNF-α serum

Table 3
Adjusted associations (β coefficients & 95% CIs) of child inflammatory markers levels with MSCA scales at 4 years of age (n = 634).

	Verbal beta (95% CI)	Perceptual beta (95% CI)	Quantitative beta (95% CI)	Motor beta (95% CI)	Exec. Function beta (95% CI)	General cognitive beta (95% CI)	Memory beta (95% CI)	Working memory beta (95% CI)	Memory span beta (95% CI)
Markers with pro-inflammatory activity									
IFN-γ[a]	−2.3 (−5.9, 1.3)	1.7 (−1.9, 5.4)	−1.0 (−4.8, 2.8)	2.5 (−1.5, 6.4)	−1.7 (−5.3, 2.0)	−1.0 (−4.6, 2.6)	−2.8 (−6.5, 1.0)	−2.1 (−6.1, 1.8)	**−3.4 (−7.3, 0.4.0)**
IL-1β[a]	−2.2 (−5.8, 1.4)	−2.1 (−5.7, 1.6)	1.2 (−2.7, 5.0)	−2.2 (−6.2, 1.7)	−0.8 (−4.5, 2.8)	−1.9 (−5.5, 1.7)	−2.6 (−6.3, 1.2)	1.3 (−2.6, 5.3)	−2.7 (−6.5, 1.2)
IL-6[a]	2.1 (−1.6, 5.7)	1.9 (−1.8, 5.5)	0.8 (−3, 4.7)	3.7 (−0.3, 7.6)	1.0 (−2.7, 4.6)	2.1 (−1.5, 5.8)	3.0 (−0.8, 6.8)	0.1 (−3.9, 4.1)	2.9 (−1.0, 6.8)
IL-8[a]	−1.0 (−4.6, 2.7)	−1.0 (−4.6, 2.7)	−0.9 (−4.8, 2.9)	0.8 (−3.2, 4.7)	−1.7 (−5.3, 2.0)	−1.2 (−4.8, 2.5)	−1.2 (−4.9, 2.6)	−2.0 (−5.9, 2.0)	−0.8 (−4.7, 3.1)
TNF-α[a]	−3.2 (−6.9, 0.4.0)	−1.7 (−5.3, 2.0)	−2.6 (−6.5, 1.2)	−2.4 (−6.3, 1.6)	−2.8 (−6.4, 0.9)	−3.0 (−6.6, 0.7)	**−4.0 (−7.7, −0.2)**	**−4.0 (−8.0, −0.1)**	**−4.0 (−7.9, −0.1)**
IL-17α[a]	1.4 (−2.3, 5.0)	−2.3 (−6.0, 1.4)	1.6 (−2.3, 5.5)	−1.5 (−5.5, 2.5)	0.3 (−3.4, 4.1)	0.1 (−3.5, 3.8)	0.9 (−2.9, 4.7)	1.9 (−2.1, 5.9)	0.3 (−3.6, 4.2)
MIP-1α[a]	−0.2 (−3.8, 3.4)	−0.5 (−4.2, 3.1)	0.7 (−3.1, 4.5)	0.8 (−3.1, 4.7)	1.3 (−2.3, 4.9)	−0.3 (−3.9, 3.3)	−1.5 (−5.2, 2.2)	1.1 (−2.8, 5.0)	−1.4 (−5.2, 2.4)
Markers with anti inflammatory activity									
IL-10[a]	1.2 (−2.5, 4.8)	2.7 (−1.0, 6.3)	0.7 (−3.1, 4.6)	2.8 (−1.2, 6.7)	2.0 (−1.6, 5.7)	1.8 (−1.9, 5.4)	0.1 (−3.6, 3.9)	−0.4 (−4.4, 3.5)	1.0 (−2.8, 4.9)
Ratios									
IL-6/IL10[a]	−0.2 (−3.9, 3.5)	−1.5 (−5.2, 2.2)	−1.3 (−5.2, 2.6)	−2.6 (−6.6, 1.3)	−1.8 (−5.5, 1.9)	−0.9 (−4.6, 2.8)	0.8 (−3.0, 4.6)	−0.7 (−4.7, 3.3)	0.6 (−3.3, 4.5)
TNF-α/IL-10[a]	−3.0 (−6.7, 0.7)	−2.9 (−6.6, 0.8)	**−4.3 (−8.2, −0.4)**	**−3.5 (−7.5, 0.5)**	**−4.8 (−8.5, −1.1)**	**−3.6 (−7.3, 0.1)**	**−3.8 (−7.6, 0)**	**−3.5 (−7.5, 0.5)**	**−5.3 (−9.1, −1.4)**

All models are adjusted for examiner, quality of assessment, child sex, maternal age in pregnancy, maternal education, BMI pre-pregnancy, parity, passive smoking at 4 years, birth weight, preterm birth and BMI at 4 years.
Bold text indicates statistically significant associations at $p < 0.05$.
[a] ≥ 90th percentile.

concentrations have been detected in patients with cognitive decline, such as Alzheimer's disease [34–36] suggesting that TNF-α-driven processes may contribute to cognitive and memory deficits of the disease and that inhibition of TNF-α can be effective for treating it [37–40]. In addition, a study conducted with adult patients with depressive disorder demonstrated that elevated expression of TNF-α, TNFRSF1A and TNFRSF1B genes correlates negatively, among others, with working memory, direct and delayed auditory-verbal memory and effectiveness of learning processes and verbal fluency [41].

Available data on child inflammation and neurodevelopmental outcomes are mainly based on ASD samples; a recent cross-sectional study investigating the association between peripheral cytokine levels (including TNF-α) and cognitive profiles in children with ASD found negative correlations of IL-6 and IFN-γ serum levels with WISC verbal comprehension index and working memory index respectively, suggesting that cytokines may play a role in the neural development in ASD [42].

In general, inflammatory signaling is considered to be a critical contributor to the short-and long term regulation of mood and cognition, but the exact mechanisms by which cytokines may modulate memory remain unknown [43]. TNF-α concentrations are found elevated in various neuropathological states that are related to learning and memory deficits, highlighting a possible role in plasticity [44]. For this purpose, much work has been carried out in the hippocampus; in fact, animal studies provide evidence that mice over-expressing TNF-α demonstrate memory impairments and disrupted learning capabilities [45,46], supporting the notion that TNF-α activity at the hippocampus and the synaptic level may influence brain function and behavior [47,48]. Consistently, a negative effect of TNF-α was found following intra-hippocampal administration to rats, which lead to impaired hippocampal-dependent working memory, as shown by an increased number of errors and longer latencies regarding the runway task [49]. Moreover, increased TNF-α in rats following peripheral nerve injury may not only contribute to chronic pain, but also to memory deficits by dysfunction of hippocampus [50]. A study conducted in adults showed that higher concentrations of TNF-α are associated with smaller hippocampal volumes suggesting that the balance between the hypothalamic-pituitary adrenal axis and inflammation processes might explain hippocampal volume reductions [51]. Regarding high IFN-γ levels, animal models have shown that they can act as a negative regulator of neuroplastic changes such as hippocampus structure, cell density, neuronal morphology and synaptic plasticity and, therefore may be associated with poorer performance in learning and memory tasks [52]. We also found that high TNF-α/IL-10 ratio was associated with decreased quantitative, motor, executive function, general cognitive, memory, working memory and memory span scores. Since this is the first study illustrating the associations of these ratios with child cognitive domains, further data is required to validate these results and clarify the underlying mechanisms of these findings.

Our results showed greater risk for reduced verbal performance scores for boys with high IL-17α serum concentrations, as well as lower motor scores for boys with high IL-6 serum concentrations. To our knowledge, this is the first study revealing sex-related differences in inflammation-specific cognitive domain associations in children. Although further study is required to validate and clarify the underlying mechanisms and the clinical utility of these findings, increased male prevalence has been frequently reported in various neurodevelopmental disorders, highlighting the concept of a male vulnerability model [53]. It is hypothesized that this male susceptibility occurs partly because microglia and inflammatory molecules are involved in the normal developmental process of sexual differentiation [54] and also because males have more activated innate immune cells in the developing brain under normal conditions [55]. We also found greater risk for lower scores in memory and memory span scales for overweight/obese children with high TNF-α serum concentrations. This finding is in line with evidence from human clinical studies showing that obesity

may increase the risk of mild cognitive impairment, in the form of short-term memory and executive function deficits [56]. Obesity is considered to be a low-grade pro-inflammatory state, and studies have reported low-grade elevation of TNF-α in obese individuals [57–59]. Research in rodent models show that obesity-induced inflammation may directly interfere with synaptic communication in the hippocampus [60].

The results of this study should be interpreted in light of its limitations; the cross-sectional design of the study does not permit inferences on causality. Although we incorporated extensive information on potential child and social factors that are associated with child neurodevelopment, we acknowledge that residual confounding because of other unmeasured confounders may still occur. On the other hand, the strengths of the present study include the heterogeneous and relatively large sample size. In addition, we carefully assessed neurodevelopmental data using a robust instrument such as MSCA [27], a valid, standardized psychometric test which provides both a general level of child's intellectual functioning and an assessment of separate neurodevelopmental domains. Moreover, we used multiple imputations with chained equations in order to increase precision and reduce bias.

To our knowledge, this the first study conducted in a general population sample of children which highlights the significant role of increased TNF-α levels during preschool years in child memory performance. As there are no studies to this date that analyzed how inflammatory biomarkers relate to measures of neurodevelopmental scores in a general population sample, these results may shed some light in new pathways of investigation. Our findings reinforce the existing evidence that elevated inflammatory activity may be involved in early pathophysiological processes, such as memory deficits and further investigation on the meaning of these associations can provide new insights. The follow-up of this cohort could provide additional data about the potential predictive role of those biomarkers and elucidate some of the questions raised by the results.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.01.010.

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