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Maternal thyroid hormones during pregnancy and offspring neuropsychological and physical development, the "Rhea" mother-child cohort study, in Crete, Greece

Kampouri Mariza

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Τομέας Κοινωνικής Ιατρικής

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Ο ρόλος των θυρεοειδικών ορμονών της μητέρας κατά τη διάρκεια της εγκυμοσύνης στη νευροψυχολογική και σωματική ανάπτυξη του παιδιού, Προοπτική Μελέτη μητέρας-παιδιού Κρήτης, Μελέτη“Ρέα”

Καμπούρη Μαρίζα

Ψυχολόγος, BSc

Σχολική Ψυχολογος, MSc

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Principal Supervisor

Leda Chatzi

Professor of Nutritional Epidemiology, Department of Social Medicine, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece

Advising committee

Panayiotis Simos

Professor of Developmental Neuropsychology, Department of Psychiatry and Behavioral Sciences, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece

Maria Venichaki

Assistant Professor of Clinical Chemistry-Biochemistry, Department of Laboratory Medicine, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece

Review Committee

Christos Lionis

Professor of General Practice and Primary Health Care, Department of Social Medicine, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece

Christos Tsatsanis

Professor of Clinical Chemistry and Biochemistry, Department of Laboratory Medicine, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece

Katerina Koutra

Assistant Professor of Clinical Psychology, Department of Psychology, School of Social Sciences, University of Crete, Rethymnon, Crete, Greece

Manolis Kogevinas

Scientific co-director of the Barcelona institute for Global Health ISGlobal, Barcelona, Spain

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Στο μπαμπίνο μου, που μόλις ήρθε!

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Περίληψη / Abstract in Greek

Εισαγωγή: Οι περιβαλλοντικές εκθέσεις κατά τη διάρκεια της πρώιμης ζωής έχουν διαχρονικές επιπτώσεις στην ανάπτυξη και τη μετέπειτα υγεία. Οι θυρεοειδικές ορμόνες αποτελούν σημαντικούς επιγενετικούς παράγοντες, καθώς καθορίζουν τη γενετική έκφραση πολλών γονιδίων κατά την εμβρυϊκή περίοδο, επηρεάζοντας την ανάπτυξη του νέου οργανισμού. Οι μητρικές θυρεοειδικές ορμόνες αποτελούν αποκλειστική πηγή ορμονών για το έμβρυο στην αρχή της κύησης, καθώς ο εμβρυϊκός θυρεοειδής αδένας είναι μεταβολικά ανενεργός μέχρι τη 10^{η} εβδομάδα και ο άξονας υποθαλάμου-υπόφυσης-θυρεοειδούς αδένα ωριμάζει έως και τη 18^{η} εβδομάδα κύησης. Οι φυσιολογικές αλλαγές που συμβαίνουν κατά τη διάρκεια της εγκυμοσύνης οδηγούν σε αυξημένη παραγωγή και έκλυση θυρεοειδικών ορμονών ώστε να καλύψουν την αυξημένη ζήτηση που προκαλεί η κύηση. Ωστόσο, η θυρεοειδική δυσλειτουργία είναι συχνή ορμονική διαταραχή σε γυναίκες αναπαραγωγικής ηλικίας και συνεπώς σύνηθες κλινικό πρόβλημα κατά τη διάρκεια της εγκυμοσύνης, με αρνητικές συνέπειες στη διαθεσιμότητα των θυρεοειδικών ορμονών για το έμβρυο. Η κλινική δυσλειτουργία του θυρεοειδούς κατά τη διάρκεια της εγκυμοσύνης έχει σημαντικές αρνητικές επιπτώσεις στην εμβρυϊκή ανάπτυξη και η μη-κλινική θυρεοειδική δυσλειτουργία της μητέρας πιθανώς συνδέεται με μη επιθυμητά αναπτυξιακά αποτελέσματα για τους απογόνους. Ο στόχος της παρούσας διατριβής είναι η διερεύνηση της σχέσης της μητρικής θυρεοειδικής λειτουργίας κατά τη διάρκεια της εγκυμοσύνης με την ανάπτυξη των απογόνων, με σκοπό τη συμβολή των αποτελεσμάτων στις πολιτικές δημόσιας υγείας για τον εντοπισμό και τη διαχείριση της δυσλειτουργίας του θυρεοειδούς της μητέρας κατά τη διάρκεια της εγκυμοσύνης.

Οι ειδικοί στόχοι της παρούσας διατριβής είναι:

- Να βρεθούν συνιστώσες κοινωνικο-δημογραφικών και περιβαλλοντικών παραγόντων της πρώιμης ζωής και να διερευνηθούν οι σχέσεις των συνιστωσών αυτών με τη γνωστική, κινητική, και συμπεριφορική ανάπτυξη στην προσχολική ηλικία.
- Να διερευνηθεί η σχέση των επιπέδων συγκέντρωσης θυρεοειδικών ορμονών, της μη-κλινικής θυρεοειδικής δυσλειτουργίας (υποθυροξιναιμία, υποκλινικός υποθυρεοειδισμός) και της θυρεοειδικής αυτοανοσίας της μητέρας κατά τη διάρκεια της εγκυμοσύνης με τη γνωστική και κινητική ανάπτυξη των απογόνων από τη βρεφική έως την παιδική ηλικία.
- Να διερευνηθεί η σχέση των επιπέδων συγκέντρωσης θυρεοειδικών ορμονών, της μη-κλινικής θυρεοειδικής δυσλειτουργίας (υποθυροξιναιμία, υποκλινικός υποθυρεοειδισμός) και της θυρεοειδικής αυτοανοσίας της μητέρας κατά τη διάρκεια της εγκυμοσύνης με τη

συναισθηματική και συμπεριφορική ανάπτυξη των απογόνων στη προσχολική και παιδική ηλικία.

- Να διερευνηθεί η σχέση των επιπέδων συγκέντρωσης μητρικών θυρεοειδικών ορμονών, της μη-κλινικής θυρεοειδικής δυσλειτουργίας (υποθυροξιναιμία, υποκλινικός υποθυρεοειδισμός) και της θυρεοειδικής αυτοανοσίας της μητέρας κατά τη διάρκεια της εγκυμοσύνης με τη παχυσαρκία και την καρδιομεταβολική υγεία των απογόνων στη προσχολική και παιδική ηλικία.

Μεθοδολογία: Τα δεδομένα της παρούσας διατριβής προέρχονται από τη προοπτική μελέτη κοόρτης μητέρας-παιδιού «Ρέα», που ξεκίνησε το 2007 στην Κρήτη. Από το Φεβρουάριο του 2007 μέχρι τον Φεβρουάριο του 2008, έγκυες γυναίκες προσεγγίστηκαν κατά την επίσκεψή τους για τον πρώτο υπέρηχο στις 2 δημόσιες και στις 2 ιδιωτικές μαιευτικές κλινικές του Ηρακλείου. Εκπαιδευμένες μαίες περιέγραψαν λεπτομερώς τη μελέτη, πήραν γραπτή συγκατάθεση, μέτρησαν ύψος, βάρος και αρτηριακή πίεση, συνέλλεξαν δείγματα ούρων και αίματος και συμπλήρωσαν ερωτηματολόγια σε σχέση με τη διατροφή, τα κοινωνικο-δημογραφικά στοιχεία, τον τρόπο ζωής, και διάφορους περιβαλλοντικούς παράγοντες. Τα ζεύγη μητέρας-παιδιού προσκλήθηκαν να συμμετάσχουν σε αξιολογήσεις της ανάπτυξης των παιδιών όταν τα παιδιά συμπλήρωσαν τους 18 μήνες, τα 4 έτη, και τα 6 έτη.

Δείγματα αίματος των εγκύων γυναικών συνελέχθησαν στη 14η εβδομάδα κύησης (Τ.Α. = 3.6 εβδομάδες κύησης) σε σωλήνες κενού των 10 ml και αποθηκεύτηκαν στους -80^{O} C μέχρι την ανάλυσή τους, αφού φυγοκεντρήθηκαν. Η θυρεοειδική λειτουργία των εγκύων γυναικών αξιολογήθηκε με ποσοτική ανάλυση των επιπέδων της θυρεοειδοτρόπου ορμόνης (TSH), της ελεύθερης θυροξίνης (fT4), των αντισωμάτων έναντι της θυρεοειδικής υπεροξειδάσης (TPO-Abs) και των αντισωμάτων έναντι της θυρεοσφαιρίνης (Tg-Abs) με τη μέθοδο της ξηρής φάσης χημειοφωταυγούς ανοσομετρικής εξέτασης από το σύστημα Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, CA). Στις αναλύσεις της παρούσας μελέτης χρησιμοποιήθηκαν όρια φυσιολογικού εύρους τιμών βασισμένα στον πληθυσμό και ειδικά ως προς το τρίμηνο κύησης, σύμφωνα με τις σχετικές οδηγίες για τον εντοπισμό και τη διαχείριση της θυρεοειδικής δυσλειτουργίας κατά τη διάρκεια της εγκυμοσύνης. Τα φυσιολογικά όρια για το πρώτο τρίμηνο εγκυμοσύνης ήταν για την TSH: 0.05 - 2.53 μIU/mL και για την fT4: 0.95 - 1.53 ng/dL, και για το δεύτερο τρίμηνο για την TSH από 0.18 έως 2.73 μIU/mL και για την fT4 από 0.87 έως 1.45 ng/dL. Στην παρούσα ανάλυση χρησιμοποιήθηκαν επίσης κατηγορικές μεταβλητές ήπιας θυρεοειδικής δυσλειτουργίας: ο υποκλινικός υποθυρεοειδισμός ορίστηκε ως υψηλότερη συγκέντρωση της TSH από το ανώτερο φυσιολογικό όριο αλλά χαμηλότερη από 10 mIU/mL και συγκέντρωση της fT4 εντός των φυσιολογικών ορίων και η υποθυροξιναιμία ορίστηκε ως εντός των φυσιολογικών ορίων συγκέντρωση της TSH και

συγκέντρωση της fT4 χαμηλότερη από το 5ο εκατοστημόριο. Τα αντιθυρεοειδικά αντισώματα θεωρήθηκαν αυξημένα αν η συγκέντρωσή τους ήταν ίση ή υψηλότερη από 35 IU/mL για τα αντισώματα έναντι της θυρεοειδικής υπεροξειδάσης και υψηλότερη από 40 IU/mL για τα αντισώματα έναντι της θυρεοσφαιρίνης.

Η αξιολόγηση της γνωστικής και κινητικής ανάπτυξης στους 18 μήνες πραγματοποιήθηκε με τις κλίμακες Bayley Scales of Infant and Toddler Development (Bayley-III, τρίτη έκδοση), οι οποίες αξιολογούν τη βρεφική και νηπιακή ανάπτυξη σε 5 τομείς: τον γνωστικό τομέα, τη λεκτική κατανόηση, τη λεκτική έκφραση, την αδρή κινητικότητα, και τη λεπτή κινητικότητα. Η αξιολόγηση της γνωστικής και κινητικής ικανότητας στα 4 έτη έγινε με τις κλίμακες *McCarthy Scales of Children's Abilities (MSCA)*, οι οποίες αξιολογούν την ανάπτυξη σε 5 τομείς: τον λεκτικό τομέα, την αντίληψη, την αριθμητική ικανότητα, τη μνήμη, και τον κινητικό τομέα. Στα 6 έτη η αξιολόγηση της γνωστικής και κινητικής ανάπτυξης έγινε με την κλίμακα *Raven's Colored Progressive Matrices (RCPM)*, που αξιολογεί τη μη-λεκτική γενική γνωστική ικανότητα, το *Finger Tapping Test (FTT)*, που αξιολογεί την ψυχοκινητική 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 χορήγηση έγινε με τη χρήση ηλεκτρονικού υπολογιστή. Η συμπεριφορική και συναισθηματική ανάπτυξη αξιολογήθηκε 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 παρέχει δείκτες που αξιολογούν τα συναισθηματικά συμπτώματα, τα προβλήματα διαγωγής, την υπερκινητικότητα και διάσπαση προσοχής, τα προβλήματα με τους συνομηλίκους και την προκοινωνική συμπεριφορά, και παρέχει επίσης δύο σύνθετους δείκτες που αξιολογούν προβλήματα εσωτερίκευσης και εξωτερίκευσης. Τα συμπεριφορικά και συναισθηματικά προβλήματα στα 6 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, έλλειψη προσοχής/υπερκινητικότητα, εναντιωματικά/προκλητικά προβλήματα, και προβλήματα διαγωγής, και επίσης παρέχει δύο σύνθετους δείκτες που αξιολογούν προβλήματα εσωτερίκευσης και εξωτερίκευσης. Το *CPRS-R: S* αξιολογεί εναντιωματικά προβλήματα, γνωστικά προβλήματα/διάσπαση προσοχής και

υπερκινητικότητα και παρέχει έναν γενικό δείκτη αξιολόγησης των δυσκολιών ΔΕΠ-Υ. Οι πληροφορίες για το βάρος και το ύψος γέννησης προέρχονται από τους ιατρικούς φακέλους. Οι σωματομετρήσεις στα 4 και 6 έτη έγιναν από εκπαιδευμένο ερευνητικό προσωπικό και με βάση συγκεκριμένα ερευνητικά πρωτόκολλα. Οι σωματομετρήσεις συμπεριέλαβαν βάρος, ύψος, περίμετρο μέσης, δερματικές πτυχές και ανάλυση βιοηλεκτρικής εμπέδυσης. Επίσης, μετρήθηκε η συστολική και διαστολική αρτηριακή πίεση και στο τέλος της κλινικής αξιολόγησης συνελέχθησαν δείγματα αίματος για την ανάλυση των λιπιδίων.

Για την αξιολόγηση της γραμμικότητας των υπό μελέτη σχέσεων χρησιμοποιήθηκαν γενικευμένα αθροιστικά μοντέλα. Για τη διερεύνηση των συνιστωσών των πρώιμων κοινωνικοδημογραφικών και περιβαλλοντικών παραγόντων χρησιμοποιήθηκε ανάλυση κύριων συνιστωσών. Για την ανάλυση των καμπυλών διαχρονικής μη-λεκτικής γνωστικής ανάπτυξης από τη βρεφική έως τη παιδική ηλικία χρησιμοποιήθηκαν μοντέλα καμπυλών ανάπτυξης βασισμένα σε ομάδες. Στις αναλύσεις που χρησιμοποιήθηκαν για τη διερεύνηση των σχέσεων μεταξύ των εκθέσεων και των αποτελεσμάτων περιλαμβάνονται περιγραφικές μέθοδοι στατιστικής και πολυπαραγοντικά μοντέλα γραμμικής και λογιστικής παλινδρόμησης ανάλογα με το είδος των μεταβλητών στις υπο εξέταση σχέσεις.

Αποτελέσματα:

1. Το υψηλό γονεϊκό κοινωνικό επίπεδο, η παρακολούθηση παιδικού σταθμού και οι λιγότερες ώρες παρακολούθησης τηλεόρασης, το «μη κάπνισμα» κατά τη διάρκεια της εγκυμοσύνης και ο θηλασμός για μεγαλύτερο χρονικό διάστημα, και η εμπλοκή και των δύο γονέων στην ανατροφή του παιδιού αποτελούν ισχυρά προστατευτικούς παράγοντες για τη γνωστική και συμπεριφορική ανάπτυξη των παιδιών στα 4 έτη. Η αυξημένη σειρά γέννησης συνδέεται με μειωμένη λεκτική ικανότητα και μειωμένα συμπτώματα ΔΕΠ-Υ στα 4 έτη. Η συνιστώσα των σωματομετρικών χαρακτηριστικών των βρεφών στη γέννηση δεν βρέθηκε να σχετίζεται με τη γνωστική και συμπεριφορική ανάπτυξη των παιδιών στα 4 έτη.
2. Η υποθυροξιναιμία της μητέρας κατά τη διάρκεια της κύησης σχετίζεται με μειωμένες βαθμολογίες των απογόνων σε κλίμακες λεκτικής και κινητικής ανάπτυξης στην προσχολική και την παιδική ηλικία. Η έκθεση σε θυρεοειδική αυτοανοσία σχετίζεται με μειωμένη αντιληπτική επίδοση, χαμηλότερη κινητική ικανότητα και αυξημένο ρίσκο για δυσμενή μη-λεκτική νοητική ανάπτυξη διαχρονικά, από τη βρεφική έως τη παιδική ηλικία.
3. Ο υποκλινικός υποθυρεοειδισμός της μητέρας κατά τη διάρκεια της εγκυμοσύνης σχετίζεται με συμπεριφορικές και συναισθηματικές δυσκολίες στην προσχολική και παιδική ηλικία. Η έκθεση σε θυρεοειδική αυτοανοσία της μητέρας κατά τη διάρκεια της κύησης καθιστά πιο ισχυρές τις

σχέσεις του μητρικού υποκλινικού υποθυρεοειδισμού και των συμπεριφορικών και συναισθηματικών προβλημάτων των απογόνων. Τα αυξημένα αντιθυρεοειδικά αντισώματα σε ευθυρεοειδικες μητέρες κατά την κύηση συνδέονται με αυξημένα συμπεριφορικά προβλήματα των απογόνων. Δεν παρατηρήθηκε σχέση μεταξύ της μητρικής υποθυροξιναιμίας και της συμπεριφορικής και συναισθηματικής ανάπτυξης των απογόνων. Δεν βρέθηκε επίδραση του φύλου στη σχέση μεταξύ της μητρικής θυρεοειδικής λειτουργίας κατά τη διάρκεια της κύησης και της συμπεριφορικής και συναισθηματικής ανάπτυξης των απογόνων.

4. Ο μητρικός υποκλινικός υποθυρεοειδισμός σχετίζεται με αυξημένο δείκτη μάζας σώματος (ΔΜΣ) και αυξημένη ολική λιπώδη μάζα στους απογόνους στα 4 έτη. Η μητρική υποθυροξιναιμία συνδέεται με αυξημένη περίμετρο μέσης των απογόνων στα 4 έτη. Δεν παρατηρήθηκε κάποια σχέση μεταξύ της μητρικής μη κλινικής θυρεοειδικής δυσλειτουργίας και των λοιπών υπό μελέτη δεικτών καρδιομεταβολικής υγείας στα 4 και στα 6 έτη (λιπιδικό προφίλ, αρτηριακή πίεση).

Συμπεράσματα: Η παρούσα διατριβή υποστηρίζει έναν καινοτόμο τρόπο επιλογής ομάδων και στόχων κατά τον σχεδιασμό προγραμμάτων πρόληψης, καθώς αναδεικνύει την ύπαρξη ισχυρών σχέσεων μεταξύ συνιστωσών πρώιμων κοινωνικοδημογραφικών και περιβαλλοντικών παραγόντων και της νευροψυχολογικής ανάπτυξης των παιδιών, και παρέχει ερευνητικές αποδείξεις για τον ρόλο της μητρικής θυρεοειδικής λειτουργίας κατά τη διάρκεια της κύησης με τη μετέπειτα ανάπτυξη των απογόνων. Ειδικότερα, τα παρουσιαζόμενα ευρήματα υποστηρίζουν ότι η μητρική υποθυροξιναιμία σχετίζεται με μειωμένη γνωστική και κινητική ανάπτυξη των απογόνων και ο υποκλινικός υποθυρεοειδισμός με αυξημένα συμπεριφορικά και συναισθηματικά συμπτώματα στους απογόνους στην προσχολική και παιδική ηλικία. Επίσης, τα ευρήματα υποστηρίζουν ότι η θυρεοειδική αυτοανοσία συνδέεται με την ανάπτυξη των απογόνων, καθώς αυξημένα αντισώματα σε ευθυρεοειδικές γυναίκες σχετίζονται με αρνητικές επιπτώσεις στη γνωστική, συμπεριφορική και συναισθηματική ανάπτυξη και σε συνδυασμό με υποκλινικό υποθυρεοειδισμό καθιστούν ισχυρότερες τις σχέσεις που παρατηρήθηκαν με την αύξηση των συμπεριφορικών και συναισθηματικών προβλημάτων. Οι επαναλαμβανόμενες μετρήσεις της γνωστικής ανάπτυξης από τη βρεφική έως τη παιδική ηλικία χρησιμοποιήθηκαν για τη δημιουργία καμπύλων διαχρονικής, μη-λεκτικής γνωστικής ανάπτυξης από τη βρεφική έως την παιδική ηλικία, και τα σχετικά αποτελέσματα έδειξαν αυξημένο ρίσκο της θυρεοειδικής αυτοανοσίας για τη διαχρονική μη-λεκτική γνωστική ανάπτυξη των απογόνων. Μελλοντικές μελέτες με πολλαπλές μετρήσεις του επιπέδου των θυρεοειδικών ορμονών της μητέρας είναι απαραίτητες προκειμένου να αποσαφηνιστεί εάν τα αυξημένα αντισώματα αποτελούν ένδειξη ενός ανεξάρτητου ρίσκου για την ανάπτυξη των απογόνων ή εάν η σχέση που παρατηρήθηκε διαμεσολαβείται από την επίδραση των

αυξημένων αντισωμάτων στα επίπεδα θυρεοειδικών ορμονών της μητέρας κατά την εγκυμοσύνη. Τα παρόντα αποτελέσματα επίσης υποστηρίζουν πιθανή εμπλοκή των μητρικών θυρεοειδικών ορμονών στη σωματική ανάπτυξη των απογόνων. Η σχέση του επιπέδου των θυρεοειδικών ορμονών της μητέρας με τη σωματική ανάπτυξη των απογόνων θα πρέπει να επιβεβαιωθεί από άλλες μελέτες και να διαπιστωθεί αν συνεχίζει να υφίσταται η συσχέτιση μετά την προσχολική ηλικία, καθώς τα παρόντα αποτελέσματα στα 6 έτη δεν επιβεβαίωσαν τα ευρήματα των 4 ετών.

Abstract

Introduction: Early-life environmental events have long-term impact on human health and development. Thyroid hormones are well-established epigenetic factors that regulate the genetic expression of multiple genes and drive fetal growth and development. The fetal thyroid gland becomes metabolically active after the 10^{th} gestational week and the fetal hypothalamus-pituitary-thyroid axis matures after the 18^{th} gestational week, consequently the fetus is exclusively dependent on maternal thyroid hormones' supply until mid-gestation and remains partially dependent until birth. In most pregnant women multiple physiological changes occur and facilitate the increased demand of thyroid hormones' production, however, thyroid dysfunction is a common hormonal abnormality among women of the reproductive age and consequently it consists of a usual clinical problem in pregnancy that affects the availability of thyroid hormones for the fetus. There is robust evidence that maternal clinical thyroid dysfunction during pregnancy has detrimental effects on offspring development and in recent decades observational studies have suggested that even mild thyroid dysfunction may lead to suboptimal offspring developmental outcomes. In this thesis, we aim to explore the link between maternal thyroid functioning in early pregnancy and offspring development and provide evidence that will contribute to informed public health policies regarding the unresolved relevant dispute on the necessity of universal maternal thyroid screening and the appropriate management of mild maternal thyroid dysfunction in pregnancy.

The specific objectives of this thesis are:

- To identify patterns of early-life sociodemographic and environmental factors and explore their association with child cognitive, motor, and behavioral development in preschool age.
- To explore the association of the concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity with offspring cognitive and motor development from infancy to early childhood.
- To explore the association of the concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity with offspring behavioral and emotional development in preschool age and childhood.
- To explore the association of the concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity with offspring obesity and cardiometabolic health in preschool age and childhood.

Methods: This thesis uses data from the "Rhea" birth cohort, which is a prospective mother-child cohort that was 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 years, and 6 years of age.

Maternal blood samples were collected at the first prenatal visit (mean gestational age 14.1 weeks, SD 3.6 weeks). Serum samples were collected in 10 ml vacutainer tubes, were centrifuged and stored in aliquots at -80° C until assayed. Maternal thyroid functioning was assessed by quantitative analysis of serum thyroid stimulating hormone (TSH), free thyroxine (fT4), antibodies to thyroid peroxidase (TPO-Abs), and antibodies to thyroglobulin (Tg-Abs), by Immulite 2000 immunoassay system (Siemens Healthcare Diagnostics, Los Angeles, CA). For TSH, the inter- and intra-assay variability were < 5.3 and < 6.4 respectively, for levels of 0.32 - 39 mIU/mL. For fT4, the inter- and intra-assay variability were < 7.8% and < 7.1% respectively, for levels of 0.51 - 4.82 ng/dL or 6.56 - 62.03 pmol/L. For antibodies to thyroglobulin (Tg-Abs), the inter- and intra-assay variability were < 4.9% and < 5.8% and for antibodies to thyroid peroxidase (TPO-Abs) < 7.4% and 7.2%, respectively. Population-based, and trimester-specific reference intervals, were used in the analyses, according to the relevant guidelines for thyroid dysfunction screening and management in pregnancy. The normal reference ranges for the 1st trimester were for TSH: 0.05 - 2.53 μIU/mL and for fT4: 0.95 - 1.53 ng/dL, and for the 2nd trimester for TSH: 0.18 - 2.73 μIU/mL and for fT4: 0.87 - 1.45 ng/dL. Categorical entities of mild thyroid dysfunction were also used in the analyses of present thesis. Subclinical hypothyroidism was defined as TSH above the upper limit of the trimester-specific reference interval but below 10 mIU/mL and fT4 within the normal range and hypothyroxinemia was defined as TSH within the normal trimester-specific reference range and fT4 below the 5th percentile. Maternal thyroid antibodies were considered elevated if TPO-Abs were equal or greater than 35 IU/mL, and if Tg-Abs were greater than 40 IU/mL, according to the limits proposed by the manufacturer.

The assessment of offspring's cognitive and motor development was conducted at 18 months using the 3rd edition of Bayley Scales of Infant and Toddler Development (Bayley-III), which assess infant and toddler development across five domains: cognitive, receptive communication, expressive

communication, gross motor ability, and fine motor ability. Cognitive and motor development assessment at 4 years was conducted using the *McCarthy Scales of Children's Abilities* (*MSCA*), which evaluate children's development across five domains: verbal, perceptual, quantitative, memory, and motor and offers a composite index of general cognitive development. Cognitive and motor development assessment at 6 years of age was conducted 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. Behavioral and emotional development were 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 6 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. Information on birthweight and birth-length/height was obtained from medical records. Anthropometry at the follow up examinations at 4 and 6 years of age was conducted by trained research assistants according to standard protocols. The anthropometric measures included weight, height, waist circumference, skinfold thickness, and bioelectric impedance analysis. Systolic and diastolic blood pressure were also measured. At the end of the clinical assessment blood samples were collected to assess the children's lipid profile.

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. Principal component analysis was used to identify patterns of early-life sociodemographic and environmental factors. Group-based trajectory modelling was used to construct

trajectories of longitudinal non-verbal cognitive development from infancy to early childhood. Multiple linear and logistic regression models were used to explore the associations of interest, accordingly.

Results: The results of the current thesis support that:

1. Higher parental social status, preschool attendance and less TV watching, nonsmoking during pregnancy and breastfeeding, and parental involvement in child life are protective factors of child cognitive and behavioral development at 4 years of age. Increased child birth order is associated with decreased verbal ability at 4 years of age. Increased child birth order is associated with decreased ADHD-related symptoms at 4 years of age. Offspring's size at birth is not associated with any measure of child cognitive and behavioral development at 4 years of age.
2. Maternal hypothyroxinemia during gestation is related with decreased offspring verbal and motor ability scores in preschool age and childhood. Exposure to maternal thyroid autoimmunity is associated with decreased child perceptual performance and motor ability scores. Maternal thyroid autoimmunity in pregnancy increases the risk for adverse child non-verbal cognitive development from infancy to childhood.
3. Maternal subclinical hypothyroidism in early pregnancy is associated with behavioral and emotional difficulties in early childhood. Maternal thyroid autoimmunity further reinforces the association of subclinical hypothyroidism with child behavioral and emotional difficulties in early childhood. Elevated maternal thyroid antibodies in euthyroid pregnant women are associated with adverse behavioral outcomes. No association between maternal hypothyroxinemia and child behavioral development is observed. No sex-related differences are identified in the association of mild maternal thyroid dysfunction with child behavioral and emotional development.
4. Maternal subclinical hypothyroidism is associated with increased offspring BMI and total fat mass at 4 years of age. Maternal hypothyroxinemia is associated with increased waist circumference at 4 years of age. Maternal thyroid mild dysfunction is not associated with any other offspring measure of cardiometabolic health at 4 and 6 years of age (lipid profile, blood pressure).

Conclusions: In conclusion, the present thesis showed that patterns of early-life sociodemographic and environmental factors are strongly associated with child neuropsychological development, proposing an innovative method to select target groups and aims of preventive programmes and extended previous knowledge regarding the role of mild maternal thyroid dysfunction on offspring development, providing evidence for public health policies regarding thyroid management in pregnancy. More specifically, the results support that maternal hypothyroxinemia is linked with offspring suboptimal cognitive and motor

development, maternal subclinical hypothyroidism is associated with increased offspring behavioral and emotional problems, and thyroid autoimmunity is associated with adverse offspring cognitive, motor and behavioral outcomes. In addition, the repeated assessment of child cognitive development from infancy to childhood has been used to construct trajectories of non-verbal cognitive development; the consequent analyses support that maternal thyroid autoimmunity in euthyroid women increases the risk for adverse non-verbal cognitive development longitudinally, from infancy to early childhood. In addition, maternal thyroid autoimmunity is found to reinforce the observed relationships between maternal subclinical hypothyroidism and offspring behavioral and emotional problems, indicating that maternal thyroid autoimmunity may pose an additional risk for subclinically hypothyroid pregnant women. Future studies with multiple measurements of maternal thyroid hormones during pregnancy are essential to clarify whether elevated thyroid antibodies impact independently on offspring development or through their effect on thyroid hormones' concentration levels. Our results also indicate that maternal thyroid hormones may be implicated in offspring physical development and body composition, however further studies are needed to replicate the specific results, since the relevant associations are evident in 4 years but are not observed in 6 years of age.

Thesis' publications

This thesis includes 3 published original research papers:

1. **Kampouri M**, Kyriklaki A, Roumeliotaki T, Koutra K, Anousaki D, Sarri K, et al. Patterns of early-life social and environmental exposures and child cognitive development, Rhea Birth Cohort, Crete, Greece. Child development. 2018;89(4):1063-73.

2. **Kampouri M**, Margetaki K, Koutra K, Kyriklaki A, Karakosta P, Sarri K, Anousaki D, Chalkiadaki G, Vafeiadi M, Kogevinas M, Chatzi L. Maternal mild thyroid dysfunction and child behavioral and emotional difficulties at 4 and 6 years of age: The Rhea mother-child cohort study, Crete, Greece. Hormones and behavior. 2019; 116:104585.

3. **Kampouri M**, Margetaki K, Koutra K, Kyriklaki A, Karakosta P, Anousaki D, Chalkiadaki G, Vafeiadi M, Kogevinas M, Chatzi L. Maternal mild thyroid dysfunction and child ognitive and motor development from infancy to childhood. Journal of Epidemiology & Community Health; 2020.

1. Introduction

1.1. General introduction

Early-life environmental events have long-term impact on health and susceptibility to disease in later life (1). This link was initially proposed by the developmental origins of health and disease hypothesis (DOHaD), which suggested that environmental deprivations during the fetal period force the organism to trade-off normal development in order to survive, and as a result the fetus suffers damaging consequences in later life ("thrifty phenotype hypothesis") (2). The original DOHaD hypothesis provided a novel perspective to abnormal development and disease, but it focused solely on severe deprivations and extreme phenotypes; updating the DOHaD hypothesis with the biological mechanism of developmental plasticity provided an enriched and broad framework, which describes both normal and abnormal human development, health and disease (3).

Developmental plasticity refers to the capacity of an organism to adapt to the specific, either regular or irregular environmental conditions it develops in; and offers an evolutionary advantage by matching the developing organism to the particular environment it inhabits (4). Therefore, developmental plasticity explains how severe environmental insults can result in gross developmental abnormalities and how mild stressors can cause subtle phenotypic responses, posing a risk for common, non-communicable diseases in adult life (5). Evidence from epidemiological and clinical studies confirms this theoretical model demonstrating that early life environmental conditions are associated with various non-communicable diseases in later life, such as cardiovascular and metabolic diseases, difficulties in cognition and mental health, asthma and lung diseases, osteoporosis, and certain cancers (6-12).

The associations between early life environment and later health/disease are mediated by epigenetic modifications. Epigenetics refer to changes in genetic expression without any alteration of the DNA sequence and they are accountable for the diversity of phenotypes derived from a sole genome (5, 13). Hormones in general and thyroid hormones specifically are well-established epigenetic factors which are heavily involved in the expression of a wide range of genes that drive fetal growth, neurodevelopment, and metabolic regulation in mammals (14).

1.2. Thyroid hormones during pregnancy

1.2.1. Thyroid hormones' synthesis and metabolism

The thyroid gland is located below the larynx and anterior to the upper part of the trachea. It is consisted of two lobes connected by a narrow band of thyroid tissue, the isthmus. The functional units of the thyroid gland are hollow spherical structures named follicles. Follicles are filled with colloid, a viscous material which is the storage of thyroglobulin. Thyroglobulin is a large glycoprotein and the precursor molecule of thyroid hormones (15). The synthesis of thyroid hormones also requires iodine, a trace element which is acquired from diet. The daily intake requirement of iodine is 90 μg for children below 6 years of age, 120μg for children above 6 years of age, 150 μg for adults, and 250 μg for pregnant and lactating women (16, 17). The metabolism of iodine starts when iodide, an iodine compound, gets trapped from the capillaries into the follicular cells by an active transport system, and continues with the oxidation of iodide to iodine by the enzyme thyroid peroxidase (TPO) in the colloid (18). Iodine gets attached on a number of the approximately 120 tyrosine residues of the thyroglobulin protein to form two different kinds of iodotyrosines, the monoiodotyrosine (MIT) and the diiodotyrosine (DIT). Then, 20% of MIT and DIT are coupled and form thyroid hormones, when two DITs are coupled they form a thyroxine (T4), and when one MIT is coupled with one DIT they form a triiodothyronine (T3) molecule. The whole chain of reactions is catalysed by thyroid peroxidase (19). Thyroglobulin, at this point, consists of a thyroid hormones' storage in the thyroid gland until the time of their release to the vascular system.

T4 is the main hormone released by the thyroid gland, corresponding to 90% of the total secretion, however the majority of T4 is locally converted to T3. T3 is the biologically potent hormone that interacts with the target-cells. The vast majority of circulating thyroid hormones (≈ 99%) is bound to proteins, mainly to thyroxine-binding globulin (TBG) and a smaller percent to albumin, and transthyretin (20). The binding of the hydrophobic thyroid hormones to proteins enables their transportation and slows their clearance prolonging their half-life and supporting a stable T4 concentration in the vascular system (21). A proportion smaller than 1% of the total thyroid hormones is free and immediately biologically active (0.01% - 0.02% of T4 and 0.2 of T3) (22).

Thyroid hormone synthesis and secretion are controlled by a complex mechanism of positive and negative regulation loops. Thyroid stimulating hormone (TSH) is a glycoprotein secreted by the anterior pituitary that stimulates both the production and the secretion of thyroid hormones. TSH consists of two subunits, a chorionic gonadotropin subunit, which is similar to human chorionic gonadotropin (hCG), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), and a TSHB subunit, which is the TSH-specific part of the hormone (23). TSH secretion is initiated by thyroid releasing hormone (TRH), which is

synthesized by the hypothalamus, and is inhibited by other hypothalamic factors (dopamine and somatostatin) and by circulating thyroid hormones (T3 and T4) (24-26).

1.2.2. Fetal and maternal thyroid function during gestation

The fetal thyrocytes are derived from the embryonic endoderm in the floor of the primitive pharynx. They form a structure, which is visible in the pharynx from embryonic day 16, then they proliferate and migrate reaching their final position in embryonic day 40 to 50 (27). Even though thyroid hormones can be detected in embryonic fluids at 4 weeks after conception, and nuclear thyroid hormones receptors have been identified from the 9th gestational week, the fetal thyroid gland is metabolically inactive until the 10th gestational week, the hypothalamus-pituitary-thyroid axis reaches maturity at the 18th gestational week and even at the end of gestation the fetal thyroid gland does not produce sufficient levels of hormones (28). Consequently, the fetus is dependent exclusively on maternal thyroid hormone supply until the second trimester, and remains partly dependent on maternal supply until birth (27-29).

Multiple physiological changes in pregnant women facilitate the increased need of thyroid hormones and result in a 50% increase in thyroid hormones' production in pregnancy. More specifically, the maternal thyroid gland increases the secretion of T4 to maintain a normal free T4 (fT4) concentration, since fT4 levels are vulnerable due to the high levels of oestrogens during pregnancy that result in increased concentration of TBG, thus in increased binding of T4 to TBG (30, 31). Another important physiological change resulting in thyroid hormones' increase in early pregnancy occurs due to the thyrotropic effect of maternal hCG on the thyroid gland. In more detail, hCG, which is structurally similar to TSH, is raised gradually in the first trimester, peaks at the 10th gestational week, and then decreases again, affecting accordingly the levels of thyroid hormones (32, 33). High hCG levels also influence TSH concentration; more specifically TSH is suppressed in the first trimester and progressively increases in the second trimester to pre-pregnancy values, remaining unchanged after this point until birth (34). Moreover, the maternal thyroid gland increases in size by 10% during pregnancy in iodine sufficient countries and by 20% - 40% in iodine deficient countries, to boost the production of thyroid hormones (35-37). The above adaptations cannot be effective if dietary iodine is not sufficient, thus an increase by 50% in iodine intake has been recommended during pregnancy (16). The increase of iodide clearance from the renal system by 50% in pregnancy (from 30ml/min to 45-60ml/min) and the transference of a fraction of iodine to the fetal-placental complex, makes the rise of iodine intake crucial for maintaining euthyroidism in gestation (38).

As a result of the aforementioned changes, in normal pregnancy and iodine sufficient populations, total T4 levels elevate in the first trimester and remain high until term; fT4 levels increase in the first trimester, decrease by 20% - 30% at the second and the third trimester and are slightly lower at term than in non-pregnancy; total T3 and free T3 (fT3) levels increase slightly during the whole gestation period (39-41); and TSH is suppressed in the first trimester and increases progressively during the second trimester (34). Studies regarding the fetal concentration of thyroid hormones have demonstrated that T3 levels increase progressively during gestation, but do not reach adult levels even at term (42-44); fetal T4 concentration increases progressively throughout gestation and reaches adult levels at 36th gestational week (42, 44); and fetal TSH concentration increases gradually from the first trimester until the end of the second trimester and then remains stable to term (45, 46).

1.2.3. Maternal thyroid hormone dysfunction in pregnancy

The aforementioned adaptations happen immaculately in the majority of pregnant women, however thyroid disease is a relatively common hormonal abnormality among women of the reproductive age and consequently a usual clinical problem in pregnant women.

Clinical hypothyroidism, is defined as decreased concentration levels of thyroid hormones with elevated TSH. The common symptoms of clinical hypothyroidism are excessive tiredness, weight gain, cold sensitivity, weakness and pain in muscles and joints, itchy and dry skin, constipation, puffy eyes, hoarse voice, depressed mood, difficulties in memory and attention, irregular and heavy menses (47). In most cases, hypothyroidism is the result of autoimmune thyroiditis or moderate to severe iodine deficiency, but it can also be caused by medical interventions (thyroid removal, radioactive thyroid treatment, drugs) and genetic factors (congenital thyroid agenesis) (48-50). The prevalence of clinical hypothyroidism in pregnant women varies from 0.5% to 6.6% (51-55). Untreated hypothyroidism in pregnancy increases the risk for gestational hypertension, preterm birth, low birth weight, and miscarriage and has detrimental effects on offspring neurodevelopment (56-61). Thus, the medical treatment of pregnant women with clinical hypothyroidism with levothyroxine is mandatory (37, 62). The treatment goal is TSH in the lower half of the trimester-specific normal range or at least TSH below 2.5 mU/L (37).

Subclinical hypothyroidism is defined as elevated TSH with normal concentration levels of thyroid hormones. Subclinical hypothyroidism is usually asymptomatic or causes subtle symptoms, and it is prevalent in 2% to 13.7% in pregnant women (55, 63-69). It has been associated with increased risk for preeclampsia and eclampsia, placental abnormalities, miscarriage, preterm birth, and low birth weight

(56-59, 70, 71), even though there are findings from other studies that have not identified any association between subclinical hypothyroidism and several of these adverse pregnancy outcomes (72, 73). The variance in the findings may be the result of the different cut-off points which were used regarding the upper limit of normal TSH in the definition of the subclinical hypothyroidism. It is noteworthy that, even though the impact of hypothyroidism on child neurodevelopment is destructive, the effect of maternal subclinical hypothyroidism on child neurocognitive development is not clear (64). Furthermore, the evidence regarding the effectiveness of thyroid medication is limited. Findings of two randomized controlled trials (RCTs) did not identify any difference in the intellectual development between children of mildly hypothyroid mothers who were treated with levothyroxine during pregnancy and the untreated control group (74) or between children of subclinically hypothyroid or hypothyroxinemic mothers who were treated with levothyroxine and the respective control groups (75). Results from another RCT on the effectiveness of medication for mild hypothyroidism accompanied with thyroid peroxidase antibodies (TPO-Abs) positivity, showed that medication with levothyroxine reduced a composite index of adverse pregnancy outcomes (76). However, the interpretation of these results is complex, since the control group of this study included women with subclinical hypothyroidism and low concentration of thyroid antibodies. Due to the limited evidence on the effectiveness of levothyroxine treatment for subclinical hypothyroidism in pregnancy and considering the evidence from the aforementioned recent RCT (76), current recommendation suggests evaluation of TPO-Abs status in women with TSH > 2.5 mU/L, and levothyroxine treatment if the antibodies are also elevated (37).

Thyroid autoimmunity is characterized by elevated TPO-Abs or elevated thyroglobulin antibodies (Tg-Abs). Epidemiological studies have demonstrated that 2% - 18% of pregnant women are either positive to TPO-Abs or/and to Tg-Abs (prevalence varies mainly due to different ethnicity of the samples) (65, 77-84). Thyroid autoimmunity is a silent disease, since the thyroid gland initially increases in size to keep up with the need of thyroid hormones which is compromised due to the antibodies presence. However, eventually the thyroid gland decreases in size, thyroid hormones' production is reduced, and the patient develops hypothyroidism. Elevated thyroid antibodies are present in approximately 70% - 90% of pregnant women with clinical hypothyroidism, 30% - 60% of pregnant women with subclinical hypothyroidism, and 10% of pregnant women with hypothyroxinemia (85). Furthermore, thyroid autoimmunity is the leading cause of hypothyroidism in iodine-sufficient populations and amplifies the risk for hypothyroidism in pregnant women, due to its combination with the stress imposed on the thyroid gland to carry out the pregnancy-related adjustments that have been previously described (59, 76, 86).

Therefore, the current recommendation for thyroid autoimmunity in pregnancy is TSH assessment in early pregnancy and reassessment every 4 weeks for the rest of the pregnancy (37).

Thyroid autoimmunity has been associated with increased risk for miscarriage, adverse perinatal outcomes and perinatal mortality (72, 87), while maternal thyroid autoimmunity accompanied with increased TSH has been linked with increased risk for miscarriage, preterm delivery and fetal adverse neuropsychological outcomes (61, 88-91). Previous studies have not identify any association of maternal thyroid autoimmunity with maternal hypertensive disorders (92). It has to be noted that the majority of the aforementioned studies has been focused on TPO-antibodies exclusively.

Hypothyroxinemia, is defined as decreased fT4 concentration levels (the most frequently cut-off points are the 2.5th, the 5th percentile, and the 10th percentile), with normal TSH concentration (37, 93). The prevalence of hypothyroxinemia in pregnancy varies from 1.3% to 26.5% (17, 70, 72, 83, 94-96). The variance in the prevalence may be the result of the different cut-off points that are applied for the definition of hypothyroxinemia, of the different levels of iodine-sufficiency, and of different trimesters in which the hormones have been assessed. Even though the findings regarding maternal hypothyroxinemia and several pregnancy outcomes are mixed (37, 88, 97-99), there is overall relatively consistent evidence for its negative effect on offspring neuropsychological development. Nevertheless, the evidence regarding the effectiveness of hypothyroxinemia treatment with levothyroxine has not confirm any benefit of treatment for offspring neuropsychological development who were born to mothers with hypothyroxinemia (74, 75). In addition, recent findings have supported that overtreatment with levothyroxine in pregnancy may increase the risk for suboptimal offspring neurodevelopment (100). Thus, current guidelines recommend no treatment of hypothyroxinemia in pregnancy (37).

Hyperthyroidism is defined as suppressed TSH concentration accompanied with increased levels of thyroid hormones (thyrotoxicosis). Hyperthyroidism occurs in 1% to 2% of the general population (54, 101) and in 0.1% - 0.4% in pregnant women (102-104). The common symptoms of hyperthyroidism are nervousness, sweating, increased heart rate, resistance to insulin, heat intolerance, hyperactivity, tremor, weakness, diarrhea, hyperdynamic precordium, and weight loss (105). The primary causes of hyperthyroidism in pregnancy are Graves' disease, molar pregnancy and gestational transient thyrotoxicosis; less common causes include toxic adenoma, subacute thyroiditis, multinodular toxic goiter, iodine-induced thyrotoxicosis, and thyrotoxicosis factitia (104). Subclinical hyperthyroidism is defined as suppressed TSH concentration with normal ranges of thyroid hormones and it has to be differentiated from the normal suppression of TSH due to increased hCG in early pregnancy (low but

detectable TSH is possibly not of clinical significance). Subclinical hyperthyroidism has not been related with adverse outcomes for the mother and the fetus; however, moderate to severe hyperthyroidism has been associated with increased risk for miscarriage, pregnancy induced hypertension, prematurity, low birth weight, intrauterine growth restriction, stillbirth, and maternal congestive heart failure and increased risk for neurodevelopmental difficulties and neural problems in offspring (seizures) (37). Anti-thyroid medication has a potential teratogenic effect on the fetus, thus, relevant guidelines suggest for the physicians to reconsider anti-thyroid medication if a pregnancy is initiated (37).

1.3. Maternal thyroid functioning and offspring's development

1.3.1. Thyroid hormones and the development of the central nervous system

Thyroid hormones are crucial for the development of the central nervous system (CNS), influencing neurogenesis, neuronal migration, neuronal and glial cell differentiation, and myelination (106). The action of thyroid hormones on the fetal brain is mainly genomic and the predominant model of genomic thyroid action is the genetic regulation through the interaction of T3 with nuclear receptors in the genes, for which temporal- and regional-specific regulating mechanisms are crucial (107).

More specifically, the nuclear pathway of thyroid hormones' action is mediated by thyroid hormones' receptors (TRs), which are widely expressed in neurons and glial cells (oligodendrocytes and astrocytes) (108-110). TRs bind to specific DNA sequences in the regulatory region of target genes [thyroid hormone response elements (TREs)], and repress or activate the corresponding gene expression depending on the absence or the presence of T3 (111). In the absence of T3, TRs recruit proteins, which are called corepressors, in order to form large protein-complexes that have histone deacetylase activity; due to this process chromatin position is repressed and genetic transcription is inhibited (112). Conversely, in the presence of T3 at the TRs, corepressors are released, proteins called coactivators are recruited and histone acetylation modifies the structure of chromatin, initiating genetic transcription (113). This mechanism enables temporal specificity of the developmental change in the CNS, through the repression of the expression of specific genes until T3 is present and their genetic expression is initiated (114). The temporal and regional specificity of the action of thyroid hormones also involves the local transformation of T4 to T3; more specifically, T4 is the predominant maternal thyroid hormone that is transferred across the placenta and can enter in the CNS more readily compared to T3, where it is transformed locally to the biologically active hormone T3 (106).

Neural proliferation is affected by thyroid hormones via their interaction with the extracellular matrix protein integrin $a_v\beta_3$ (115), which affects the size of the basal and intermediate progenitor pool; thus, thyroid hormones deficiency results in selective loss of upper layer neurons and reduced cortical thickness (116). Conversely, evidence from a stem-cells' study has shown that thyroid hormones activate the switch from cell proliferation to differentiation (115), thus the presence of thyroid hormones repress the proliferation and initiates differentiation. This heterogeneity of outcomes of thyroid hormone action may be dependent on the different neurodevelopmental timepoints of thyroid hormones' action in the aforementioned studies (117).

Thyroid hormones also influence cell migration in the cortex, hippocampus, and the cerebellum via multiple pathways. A possible pathway involves the regulation of the maturation of the radial glia cells by thyroid hormones. Radial glia cells are precursors of the oligodendrocytes, the astrocytes, and the ependymal cells and serve as a scaffold for cell migration (118, 119), therefore thyroid hormones may be involved in both neurogenesis and migration via this pathway. In addition, thyroid hormones directly affect the expression of *the gene Reln,* which encodes reelin, a protein which stops migrating neurons and facilitates the establishment of neocortical layers and the inside-out development of the brain (120, 121).

Oligodendrocytes are responsible for myelin production and the differentiation of their precursors is dependent on thyroid hormones' action (122). In addition, thyroid hormones regulate the expression of multiple genes encoding myelin proteins (123). Consequently, thyroid hormones deprivation is associated with decreased myelin content and decreased axon myelination, while hyperthyroidism with accelerated myelination (124-126). It is noteworthy that the reduction of total white matter volume due to deficient thyroid hormones status persists even after the organism returns to a euthyroid status (127).

1.3.2. Maternal thyroid functioning and offspring's cognitive and motor development

The structural development and the formation of synapses and neural pathways provide the basis for smooth flow of neural impulses, which are manifested behaviorally in cognitive and motor functioning, while myelination allows the acceleration of the neural impulses' speed (128). Similarly to neurodevelopment, cognitive and motor development have sequential character; each one of cognitive and motor development stages is the result of the previous stages, leads to next stages and consists of an essential part of the general outcome of the maturational process (129, 130). During its development the brain is more susceptible to environmental determinants (131) while the outcomes of such effects may

be manifested behaviorally in cognitive and motor functions in later developmental stages, due to the sequential character of human cognitive and motor development. Thus, thyroid hormones mild dysfunction in early life and during critical windows of brain development can impact brain development and these impacts may be manifested behaviorally in cognitive and motor functioning in later developmental stages.

Evidence from observational studies in humans has suggested that even mild maternal thyroid dysfunction during gestation may result in adverse offspring outcomes in neuropsychological development. However, there is considerable heterogeneity among the findings as a result of significant differences in the methodological approach of the relevant studies. More specifically, there are differences regarding the indicators of maternal thyroid dysfunction (i.e. subclinical hypothyroidism, hypothyroxinemia, clinical and subclinical hypothyroidism combined, thyroid autoimmunity), the trimester during which thyroid function was assessed, the cut-off points for the definition of the exposure, the neuropsychological outcome which was assessed (e.g. intelligence, cognitive development, reaction speed) and the psychometric tools that were used (e.g. parent report questionnaires, direct neuropsychological assessment, computerized testing), as well as differences in the characteristics of the populations in each study (e.g. iodine sufficiency, iron status).

The role of maternal hypothyroxinemia on offspring neuropsychological development has been the predominant focus of studies regarding the link between maternal thyroid dysfunction and child development. Originally a small study reported higher risk of poor motor development at 12 months and 24 months of age in offspring of mothers with fT4 concentration levels below the 10^{th} percentile during the first trimester of pregnancy (132). Other observational studies during the last two decades have supported that low maternal fT4 concentration levels are related with decreased mental performance (133) and intelligence (100, 132, 134, 135), reduced quantitative ability and general cognitive development (136), reduced cognitive ability, perceptual performance, and memory (137), slower response speed (138), and increased risk for delay in expressive language and non-verbal cognition (96).

Conversely, other observational studies have identified no association between low circulation levels of T4 at early pregnancy and offspring's verbal, visuospatial, and motor skills, at 6 months and 3 years of age (139), neither between low T4 levels in late pregnancy and offspring's cognitive abilities at 5.5 years of age (136). Furthermore, no association was found between maternal low fT4 levels at the second trimester and child cognitive and motor development at 24 months of age (140), and between maternal low fT4 levels at the third trimester and offspring's cognitive and motor development at 6, 12,

24 and 60 months of age (141). It has been previously supported that the timing of the thyroid deficiency is an important factor for the effect of maternal hypothyroxinemia on child cognition and motor development; since a thyroid hormones deficiency may exert greater impact in early than in late pregnancy (142). Another factor that contributes in the heterogeneity of the results is the definition of low thyroxine levels; it seems that the fT4 constitutes a better indicator of maternal thyroid hormones supply to the fetus than total thyroxine circulating levels (143).

Studies regarding maternal subclinical hypothyroidism have been scarce and the relevant findings have been inconclusive. There are previous results that have supported a relation of subclinical hypothyroidism with neurodevelopmental delays in infancy (144), decreased intelligence in toddlerhood (34), and reduced verbal, memory and cognitive scores in prematurely born infants (136). While, results from two previous large observational studies did not identify any association of maternal subclinical hypothyridism or decreased levels of TSH with offspring cognitive development (96, 133).

Considerable variance of evidence is also observed regarding the role of maternal thyroid autoimmunity on child neuropsychological development (34, 90, 91, 145, 146). Therefore, it has been emphasised that future studies are necessary to clarify if the effect of maternal thyroid autoimmunity on child cognition is permanent or transient (146) or whether the differences are depended on specific relevant characteristics of the populations under study, as is the iodine sufficiency (145).

Recent findings using brain imaging techniques have been used to identify possible differentiations between children of mothers with thyroid dysfunction in pregnancy and children of euthyroid mothers. The results have revealed that offspring of hypothyroid mothers who did not receive any thyroid medication during pregnancy, had morphological aterations in corpus callosum at 9-12 years of age (147) and smaller hippocampal volume (148). Evidence from a large population based study has supported that both increased and decreased maternal fT4 is associated with reduced grey matter volume and total brain matter volume (100).

To summarize, the evidence from observational studies points to a link of maternal hypothyroxinemia during early pregnancy with child cognitive and motor development despite considerable methodological differences across studies. Conversely, the existing evidence is not sufficient to support that maternal subclinical hypothyroidism and maternal thyroid autoimmunity are implicated in offspring cognitive and motor development. Evidence from brain imaging studies has supported that offspring of mothers with thyroid clinical dysfunction have anatomical alterations in brain regions

important for cognitive development and that both increased and decreased fT4 may be associated with decreased brain matter development.

1.3.3. Maternal thyroid functioning and offspring's behavioral and emotional development

The most common mental health disorders in childhood fall into three categories; (i) emotional disorders, which include symptoms of anxiety, depression, phobias, or obsessions, (ii) behavioral disorders, which are characterized by oppositional, aggressive, or antisocial behaviours, and (iii) hyperactivity disorder (ADHD), which involves inattention and overactivity. The joint prevalence of emotional and behavioral disorders varries between 9% and 22% and the worldwide pooled prevalence of ADHD reaches the 5.29% of the general polulation (149-155).

Magnetic Resonance Imaging (MRI) and functional MRI (fMRI) studies on clinical populations with the aforementioned disorders have pinpointed structural and functional alterations in several brain regions including the cerebral cortex, the hippocampus, the corpus callosum and the cerebellum (156-162) that have also been found altered in offspring of mothers with overt hypothyroidism (147, 148, 163) as well as in rats offspring of mothers with hypothyroxinemia during pregnancy (164).

Furthermore, the association between maternal thyroid and child cognitive development has been explored and it is evident that difficulties in cognitive development during childhood underpin behavioral and emotional difficulties. For instance, increased externalizing symptoms (i.e. aggression, hyperactivity, attention problems) have been associated with reduced executive functioning (165, 166), impaired general cognitive ability, and difficulties in learning and memory (167). Increased internalizing symptoms (i.e. depression, anxiety, and withdrawal) have been linked with impairments in verbal fluency and memory (168), problems in verbal processing (169), and poor executive functioning (167). However, the studies regarding the role of maternal throid function and offspring internalizing and externalizing sympotms are scarce and primarely focused on ADHD-related difficulties.

Findings derived from the Generation R project have supported that maternal TSH concentration levels are positively associated with child externalizing symptoms and that maternal thyroid autoimmunity increases the risk for offspring ADHD symptoms at 3 years of age (91, 170), while at the same population at 8 years of age maternal hypothyroxinemia was related with child ADHD symptoms and maternal TSH was not associated with any child behavioral outcome (171). Findings from other observational studies have shown an association of maternal hypothyroxinemia with the manifestation

of ADHD symptoms in children (172) and a relation of low fT4 concentration levels with increased internalizing symptoms (173). Findings regarding the role of child sex on the association of maternal thyroid dysfunction and child behavioral difficulties are contradictive; a previous study has supported that maternal TSH is associated with child attention problems in females exclusively (174), but findings of another study suggested that the association is evident in males exclusively (173).

To summarize, even though there is indirect evidence pinpointing to a possible implication of maternal thyroid functioning during pregnancy in offspring behavioral and emotional development, direct findings are scarce and the role of thyroid hormones' deficiency during pregnancy in these developmental domains is still inconclusive. Furthermore, the previous findings from observational studies regarding the role of child sex on the relationship of maternal thyroid functioning with child behavioral and emotional development are in conflict.

1.3.4. Maternal thyroid functioning and offspring's obesity and cardiometabolic health

Thyroid hormones have multiple effects on the cardiovascular function and the metabolism in adults (175). Hyperthyroidism results in increased metabolic rate, tachycardia, heat intolerance, and weight loss even when food intake is increased, while hypothyroidism leads to reduced metabolic rate, bradycardia, cold intolerance, and weight gain (175-177). The cardiovascular and metabolic effects of thyroid hormones are attributed to direct effect on the heart (176) and the function of the fat tissue (178), as well as to indirect effect due to their impact on the nervous system and their regulatory role regarding sympathetic activity (178-180).

Evidence from experimental studies suggests that thyroid hormones during gestation may impact offspring cardiometabolic health through multiple pathways. An experimental study in sheep fetuses has supported that thyroid hormones directly influence cardiomyocyte maturation during gestation through the inhibition of proliferation and the promotion of cardiomyocytes' maturation (181, 182). Furthermore, experimentally induced hypothyroidism in rats during gestation has been related with offspring's hypertension, probably due to increased sympathetic modulation of vessels (183), and induced maternal hyperthyroidism alters the pattern of expression in cardiac renal-angiotensin components in pups, probably predisposing them for later cardiovascular disorders (184). In addition, an experimental study in mice has suggested that inadequate supply of maternal thyroid hormones to the fetus may increase susceptibility to metabolic disorders in adulthood due to their contribution in the determination of the metabolic set-point in the offspring (185).

The only study to date on the association between maternal thyroid hormones and offspring parameters of cardiovascular function in childhood did not identify any association of hypothyroidism and hyperthyroidism with child growth and obesity. However, the results suggested that decreased and within the normal range levels of maternal TSH are associated with reduced offspring body mass index (BMI), decreased total and abdominal fat mass, and decreased diastolic blood pressure, as well as that increased and within the normal fT4 levels are related with lower BMI and decreased abdominal fat mass at 6 years of age. (186). Another longitudinal observational study in humans did not identify any association between the concentration levels of maternal thyroid hormones in pregnancy and offspring adiposity at 20 years of age; however, a relation of maternal subclinical hypothyroidism in late pregnancy and offspring hypertension was evident (187). Findings from another observational study did not identify any association of maternal thyroid hormones and offspring obesity or cardiometabolic traits at 16 years of age, but identified a link of thyroid autoimmunity during pregnancy with metabolic syndrome and obesity (188).

To sum up, even though the relationship between thyroid hormones and metabolism in adults is well-established, the role of maternal thyroid hormones during pregnancy in offspring obesity and cardiometabolic health is understudied. However, findings from previous studies have suggested that maternal thyroid hormones and thyroid autoimmunity may play a role in offspring growth, obesity, and cardiometabolic health in later life.

1.4. Aim and specific objectives of this thesis

The link of maternal thyroid dysfunction with offspring brain development has been well-known for several decades due to the increased prevalence of cretinism and the subsequent intellectual impairment and motor development abnormalities in offspring of iodine-deficient mothers (189). Furthermore, there is strong evidence regarding the link of clinical maternal thyroid dysfunction during pregnancy and offspring impaired neurocognitive development (60) and accumulating evidence suggesting that even mild maternal thyroid dysfunction may play a role in child neuropsychological development (37). However, as it has been described in detail in the introduction, there are several inconsistencies and limitations in the previous studies, which pose limitations on the official recommendations regarding the management of mild maternal thyroid dysfunction during pregnancy and are partly responsible for the still unsettled controversy among experts regarding the necessity for a universal thyroid screening during pregnancy (190).

The main aim of the present thesis is to investigate the role of maternal mild thyroid dysfunction in early pregnancy with offspring's neuropsychological and physical development and health from infancy to early childhood. The Rhea study offers the opportunity to explore the effect of mild maternal thyroid dysfunction in pregnancy on various developmental outcomes, since it provides sufficient statistical power to examine mild effects on child development. In addition, Rhea study is longitudinal with repeated reliable and valid measures of child development in various domains. Each analysis included in the present thesis is focused on a different developmental domain (cognitive and motor development, emotional and behavioral development, obesity and cardiometabolic health). In addition, as an initial analysis, we have explored the link between early life exposures and child cognitive, motor, and behavioral development.

More specifically, the objectives of this thesis are:

- To explore how early life sociodemographic, lifestyle, and perinatal factors co-occur in patterns and to identify which of these factors are associated with child neuropsychological development.
- To explore the role of concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity in child cognitive and motor development in infancy, and early childhood.
- To explore the role of concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity in the emergence of child behavioral and emotional difficulties in early childhood.
- To explore the role of concentration levels of maternal thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity in the emergence of child obesity and cardiometabolic traits in early childhood.

2. Methods

2.1. Study design

This thesis uses data from the Rhea birth cohort which is a prospective mother-child cohort that was established in 2007 in Crete, Greece. The principal aims of the Rhea study are to identify the determinants of children's growth and development, to evaluate maternal health during and after pregnancy and to examine the interaction between environmental stressors and genetic variants in children's growth and health; the predominant outcomes of interest include offspring growth and obesity, cognitive, emotional, and behavioral development, allergies and asthma, and genotoxicity (191).

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 months period, from 02/2007 until 02/2008. The inclusion criteria for participation in the study were residency in the study area, age greater than 16 years, and ability to communicate in Greek. Participants were initially contacted at the first trimester of pregnancy, after the first major ultrasound test. Trained midwives described the study in detail to pregnant women, obtained written informed consent, completed a detailed questionnaire on diet, environmental exposures, sociodemographic and lifestyle characteristics, measured height, weight, and blood pressure, and collected urine and blood samples. The participants were contacted again at the 3rd trimester and at child birth. Mother and child pairs were invited to participate in child follow-up assessments when the children were 9 months, 18 months, 4 years, and 6 years old. Figure 1 presents the Rhea study follow-up visits and a summary of measures at each timepoint.

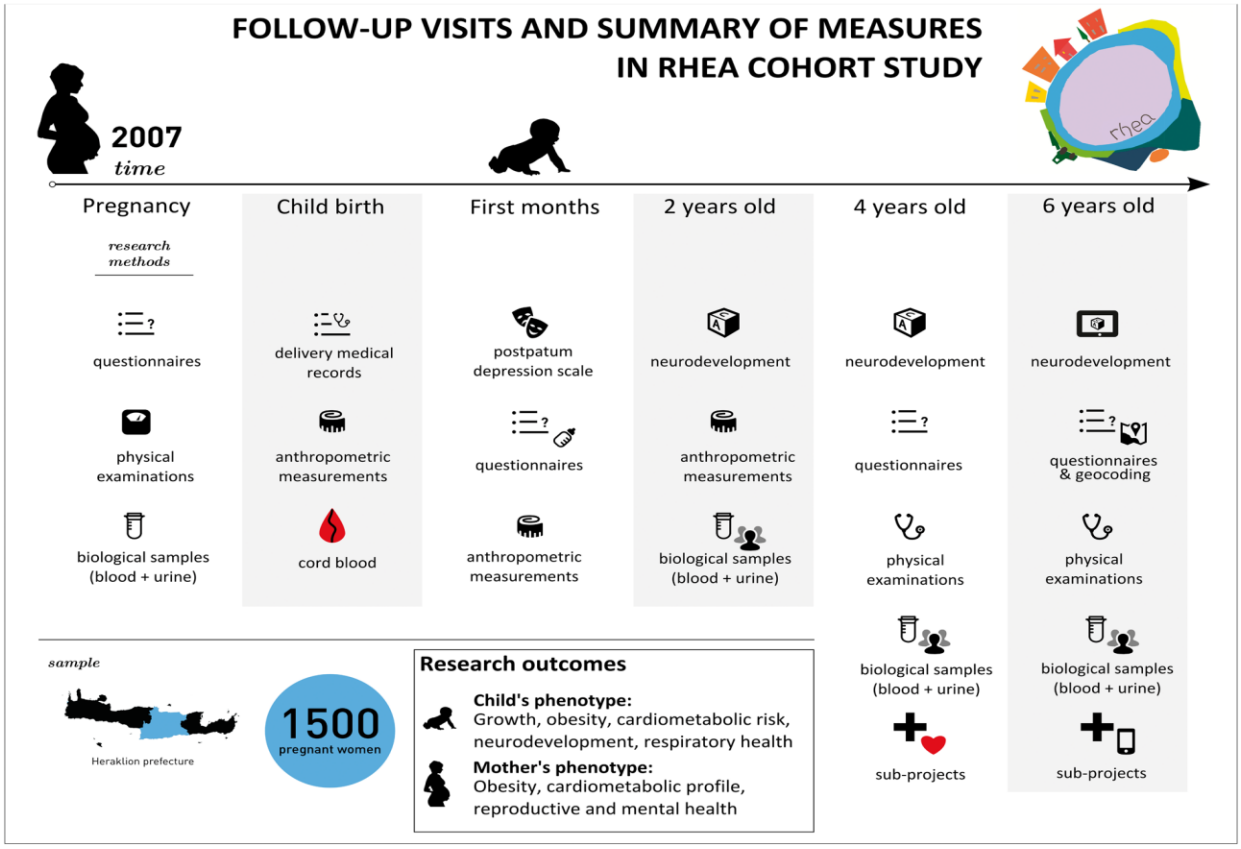


Figure 1. Follow-up visits and summary of the measures in the Rhea mother-child cohort study

2.2. Study population

During the recruitment period 1610 pregnant women agreed to participate and 1363 singleton pregnancies were followed up to delivery. A late recruitment at birth resulted 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, and at the 6-years follow-up 607 children participated. Figure 2 presents the Rhea study flow diagram. In each analysis the number of participants differentiates due to the different number of mother-child pairs with available data for the specific analysis.

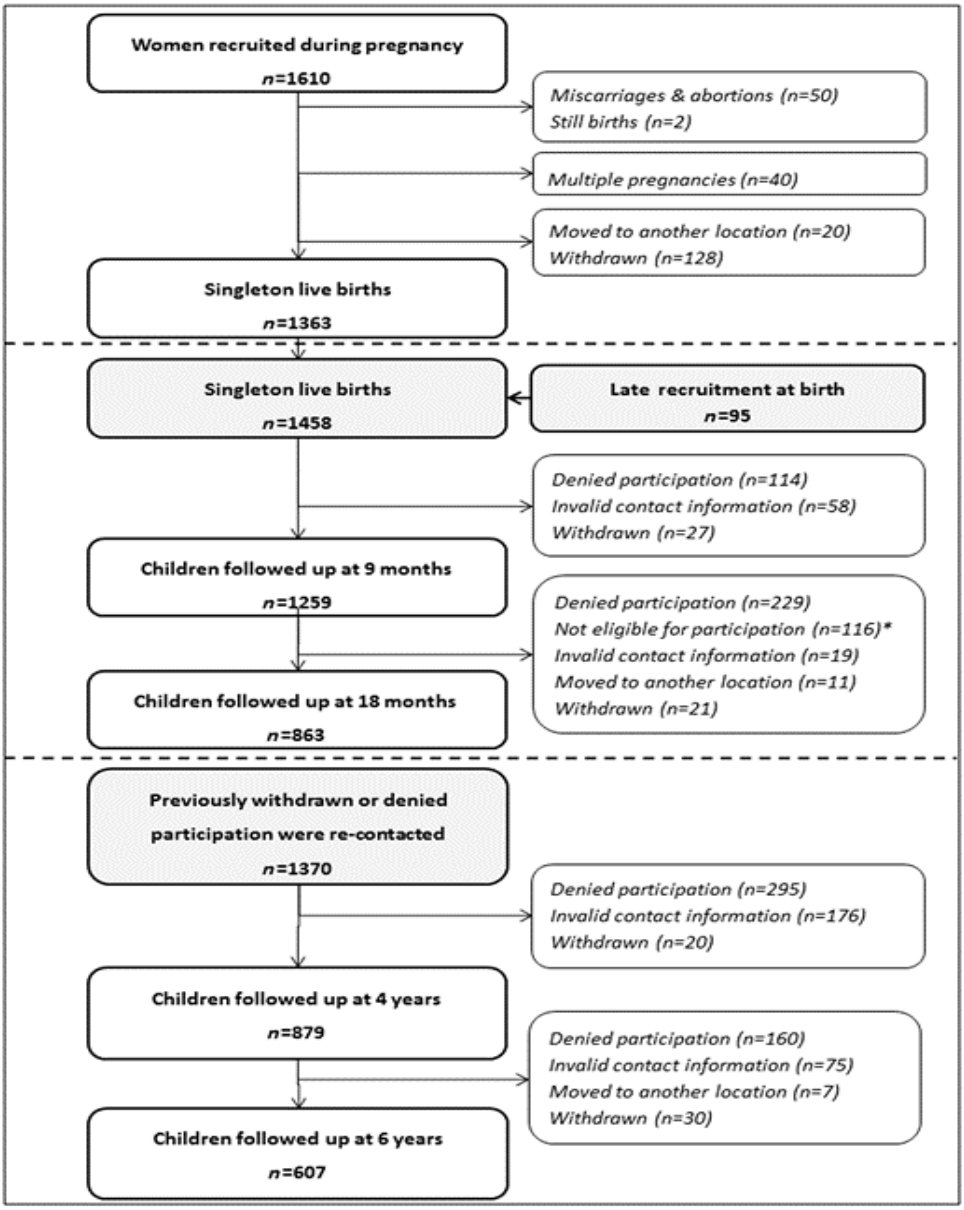


Figure 2. Flow diagram of the Rhea study population

Study population: Patterns of early-life exposures and their association with neuropsychological development in preschool age

In the analyses on the association between the patterns of early-life exposures and neuropsychological development, we had available data regarding child cognitive and motor development for 718 participants. We have excluded 18 children due to a diagnosis of a neurodevelopmental disorder,

or other medical condition (i.e. plagiocephaly, microcephaly, hydrocephaly, brain tumour) and 8 children due to incomplete neuropsychological assessment. Of the 700 participants (age M = 4.2, SD = .20), 610 provided data on ADHD difficulties. The majority of them resided in urban centres. Almost half of the children were the only or the first born child. The vast majority of the participants attended preschool at 4 years and used to spend their free time at weekends with both their parents. Participating parents were mainly Greek, married at the time of the pregnancy, while almost all fathers were employed, similarly to half of mothers. Half of the mothers had completed post-secondary education, likewise almost half of the fathers. The detailed information on baseline characteristics of the participants in the analyses are presented in Table 1.

Table 1. Study participants' characteristics

	N	Data [a]
Parental characteristics		
Maternal age at child birth		
≤ 20	28	4.0
21 – 30	347	49.6
31 – 40	315	45.1
41+	9	1.3
Paternal age at child birth		
≤ 25	32	4.6
26 – 35	406	58.1
36 – 45	239	34.2
46+	32	4.6
Maternal origin		
Greek	562	87.9
Non Greek	38	5.4
Paternal origin		
Greek	680	97.1
Non Greek	20	2.9
Parity		
Primiparous	298	43.6
Multiparous	385	56.4

Maternal education at 4 years		
Low	111	15.9
Medium	357	51
High	232	33.1
Paternal education at 4 years		
Low	227	32.4
Medium	301	43
High	172	24.6
Maternal employment status at 4 years		
Employed	409	58.4
Unemployed	291	41.6
Paternal employment status at 4 years		
Employed	648	92.6
Unemployed	49	7
Marital status at 4 years		
Married/ Engaged	690	98.6
Other	10	1.4
Residence at 4 years		
Urban	496	70.9
Rural	204	29.1
Infant characteristics		
Child's sex		
Boys	358	51.1
Girls	342	48.9
Gestational age at birth (weeks); mean, SD	38.23	1.49
Birth weight(Kg); mean, SD	3.21	0.45
Birth length (cm); mean, SD	50.51	2.33
Birth head circumference (cm); mean, SD	34.18	1.38
Breastfeeding duration (months); mean, SD	4.18	4.39
Child characteristics at 4 years		
Child age (years); mean, SD	4.2	0.2
Child Birth Order		
Only child	121	17.3

First child	204	29.1
Other	375	53.6
Preschool Attendance		
Yes	600	85.7
No	100	14.3
TV watching (minutes/per day)		
Less than 60′	190	27.1
Approximately 60′ - 120′	266	38
More than 120′	244	34.9
Parental participation in child leisure-time		
Both parents	594	84.9
Other	106	15.1

[a] Data are presented as mean and standard deviation for continuous variables and as frequency and % frequency for categorical variables; N = 700

We have compared the demographic characteristics at baseline, between participants and non-participants at the 4-year follow-up. The children who participated were more likely to have older, more educated, Greek, married parents at child-birth, and primiparous mothers in comparison with the non-participants in the follow-up assessment (Table 2).

Table 2. Comparison of parental demographic characteristics at baseline between participants and non-participants

	Participants		Non-Participants		
	N	% [a]	N	% [a]	p-value [b]
Maternal age					**<0.001**
≤ 20	28	4.0	32	5.5	
21 – 30	347	49.6	341	58.8	
31 – 40	315	45.1	198	34.1	
41+	9	1.3	9	1.6	
Paternal age					**<0.001**
≤ 25	32	4.6	46	8.6	
26 – 35	406	58.1	330	61.6	

36 – 45	239	34.2	151	28.2	
46+	22	3.1	9	1.7	
Maternal education					**<0.001**
Low	111	15.9	158	28.7	
Medium	356	50.9	275	49.9	
High	118	21.4	232	33.2	
Paternal education					**<0.001**
Low	227	32.4	242	44.7	
Medium	301	43.0	219	40.5	
High	172	24.0	80	14.8	
Maternal employment status					**<0.001**
Employed	519	75.2	355	65.9	
Unemployed	171	24.8	184	34.1	
Paternal employment status					0.216
Employed	692	99	541	98.9	
Unemployed	7	1.0	6	1.1	
Maternal origin					**<0.001**
Greek	562	87.9	661	94.6	
Non Greek	38	5.4	77	12.1	
Paternal origin					**<0.001**
Greek	680	97.1	485	86.5	
Non Greek	20	2.9	76	13.5	
Marital status					**0.003**
Married/ Engaged	624	89.4	470	84.5	
Other	86	15.5	74	10.6	
Parity					**0.004**
Primiparous	298	43.6	211	37.5	
Multiparous	385	56.4	351	62.5	

[a] Data are presented as frequency and % frequency for categorical variables.

[b] P-values obtained from chi-square test for independence of categorical data, Fisher's exact test results are presented when applicable.

The subgroups of excluded participants due to neurodevelopmental disorder diagnosis and participants included in the analyses did not differ significantly on any of the potential predictors, with the exception of breastfeeding duration, which was shorter for excluded children and preschool attendance at 4 years, which was less often for excluded children. These differences can be attributed to the specific difficulties these children face (Table 3).

Table 3. Comparison of exposure profile between excluded and non-excluded children, due to neurodevelopmental disorder diagnosis

	Non-excluded children		Excluded children		
	N	Data [a]	N	Data [a]	p-value [b]
Maternal age	827	29.7 (4.9)	17	31.4 (5.1)	0.643
Maternal education					0.397
Low	129	15.8	4	23.5	
Medium	421	51.5	6	35.3	
High	267	32.7	7	41.2	
Paternal education					0.247
Low	266	33.5	9	52.9	
Medium	331	41.7	5	29.4	
High	196	24.7	3	17.6	
Maternal employment status					0.160
Employed	464	55.8	11	61.1	
Unemployed	367	44.2	7	38.9	
Residency					
Urban	593	71	14	77.8	0.611
Rural	242	29	4	22.2	
Parity					0.476
Primiparous	380	45.6	10	55.6	
Mupltiparous	454	54.4	8	44.4	
Birth order at 4 years					0.549
Only child	149	18	5	27.8	
First Child	239	28.8	5	27.8	
Other	442	53.3	8	44.4	

Birth weight (z-adjusted)	818	.09 (.98)	18	.03 (.98)	0.778
Birth head-circumference	782	.05(1.03)	17	-.18 (.82)	0.363
Breastfeeding (mean, SD)	796	4.15(4.1)	18	1.61 (1.6)	**0.005**
Maternal smoking status					0.816
Smoking at Pregnancy	137	17.5	3	17.6	
Quit at pregnancy	138	17.6	2	11.8	
Non-smoking	509	64.9	12	70.6	
Preschool attendance					**0.014**
Yes	709	85.1	11	61.1	
No	124	14.9	7	38.9	
TV-watching					0.660
Less than 1 hour	293	35.3	8	44.4	
1-2 hours	308	37.1	5	27.8	
More than 2 hours	229	27.6	5	27.8	
Marital Status pregnancy					0.709
Married	708	88.2	16	94.1	
Non-married	95	11.8	1	5.9	
Time with both parents at weekends					0.117
Yes	691	83.4	17	94.4	
No	138	16.6	1	5.6	

[a] Data are presented as mean and standard deviation for continuous variables and as frequency and % frequency for categorical variables.

[b] P-values obtained by chi^2 or t-test for independent samples, Fisher's exact test results are presented when applicable.

Study population: Maternal thyroid mild dysfunction and offspring cognitive and motor development

In the analyses on the association between mild maternal thyroid dysfunction and child cognitive and motor development we had available data on maternal thyroid functioning from 1170 pregnant women, of which 484 provided data on child cognitive and motor development at 18 months, 695 at 4 years and 488 at 6 years of age. Approximately, 5% of the participants were hypothyroxinemic, 7% were subclinically hypothyroid, 16% were positive to thyroid antibodies (TPO-Abs and/or Tg-Abs), and 15%

were taking thyroid medication (mainly levothyroxine). The baseline characteristics of the participants included in the analyses are presented in detail in Table 4.

Table 4. Baseline characteristics of the study participants at 18 months, 4 years and 6 years of age, Rhea mother-child study, Crete, Greece

	N at 18 months [a]	N at 4 years [a]	N at 6 years [a]
Maternal Characteristics			
Age (years)	30.3 (4.7)	29.7 (5.0)	30.1 (4.8)
Educational level			
Low	61 (13.1)	110 (16.7)	70 (15.2)
Medium	228 (48.8)	335 (51.0)	232 (50.4)
High	178 (38.1)	212 (33.3)	158 (34.4)
Parity			
Primiparous	196 (42.0)	283 (43.0)	199 (43.3)
Multiparous	271 (58.0)	375 (57.0)	261 (56.7)
Origin			
Greek	449 (96.2)	617 (93.8)	435 (94.6)
Non-Greek	18 (3.9)	41 (6.2)	25 (5.4)
Smoking at early pregnancy			
Yes	161 (34.5)	233 (35.4)	159 (34.6)
No	306 (65.5)	425 (64.6)	301 (65.4)
Maternal thyroid parameters			
TSH (μIU/mL)	1.33 (1.04)	1.33 (1.41)	1.33 (1.56)
ft4 (ng/dL)	1.22 (0.20)	1.23 (0.21)	1.23 (0.21)
Maternal iodine (median) (μg/L)	178.6	172.4	168.5
Hypothyroxinemia [b]	22 (5.7)	26 (4.7)	22 (5.7)
Subclinical hypothyroidism [b]	29 (7.0)	38 (6.5)	27 (6.7)
Thyroid autoimmunity [b]	50 (13.1)	83 (15.1)	56 (14.8)
Gestational age-sampling (weeks)	14.1 (3.6)	14.1 (3.5)	14.0 (3.5)
Thyroid medication			
No medication	395 (84.6)	562 (85.4)	390 (84.8)

Thyroxine	68 (14.6)	91 (13.8)	65 (14.1)
Anti-thyroid medication	4 (0.9)	4 (0.6)	4 (0.9)
Yes, no defined	-	1 (0.2)	1 (0.2)
Child Characteristics			
Sex (female)	247 (52.9)	337 (51.2)	251 (54.6)
Birth weight (grams)	3,186.9 (435.2)	3,215.4 (450.4)	3,193.5 (448.8)
Gestational age (weeks)	38.2 (1.4)	38.2 (1.6)	38.1 (1.6)
Age at cognitive assessment (years)	1.5 (0.1)	4.2 (0.2)	6.6 (0.3)

[a] Data presented as mean (standard deviation) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned; At 18 months: N = 467, At 4 years: N = 658, At 6 years: N = 460

[b] Subclinical hypothyroidism definition: fT4 concentration levels within the population-based, trimester-specific reference ranges and TSH above the upper trimester specific limit. Hypothyroxinemia definition: TSH concentration levels within the trimester-specific reference ranges & fT4 < 5th percentile. Thyroid autoimmunity definition: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs ≥ 35 IU/mL &/or Tg-Abs > 40 IU/mL

Non-response analysis showed no differences between participants and non-participants in terms of maternal thyroid functioning (TSH, fT4), maternal smoking status during pregnancy, offspring gestational age at birth and birthweight. However, mothers who did not participate in the follow-up assessments were younger [mean difference = - 0.9 years; 95% CI (- 1.5, - 0.3) p = .004] and more often less educated [low educational level 29.8% versus 17.4%, $\chi 2 = 28.85(2)$, $p < 0.000$], multiparous [65% versus 58%, $\chi 2 = 5.14(1)$, $p = 0.023$) and non-Greek [14.3% versus 6.3%, $\chi 2 = 19.84(1)$, $p < 0.000$] compared with mothers who participated in the follow-up assessments.

Study population: Maternal thyroid mild dysfunction and offspring behavioral and emotional development

In the analyses regarding the association between mild maternal thyroid dysfunction and child behavioral and emotional development we had available data on maternal thyroid functioning from 1170 pregnant women and information on child behavioral and emotional development in 647 participants at 4 years of age, and in 489 participants at 6 years of age. The baseline characteristics of the participants in the analyses are presented in Table 5.

Table 5. Maternal and child characteristics of the study population at 4 and 6 years of age, Rhea mother-child study, Crete, Greece

	N	Data [a]	N	Data [a]
Maternal Characteristics				
Maternal age at child birth	646	29.89 (5.0)	488	30.15 (4.7)
Maternal education				
Low	105	16.4	69	14.2
Medium	329	51.3	247	50.9
High	208	32.4	169	34.9
Maternal origin				
Greek	612	95	460	95.8
Non-Greek	33	5.1	20	4.2
Parity				
Primiparous	288	44.5	218	44.9
Multiparous	359	55.5	268	55.1
Maternal smoking (early pregnancy)				
Yes	217	34.2	166	34.7
No	418	65.8	313	65.3
Maternal iodine at pregnancy (median) (μg/L)	447	168.4	366	168.4
Maternal iron at pregnancy (μg/dl)	443	72.5 (37.6)	356	72.0 (38.3)
Maternal TSH (μIU/mL)	647	1.34 (1.4)	489	1.32 (1.5)
Maternal ft4 (ng/dL)	646	1.22 (0.2)	489	1.23 (0.2)
Maternal subclinical hypothyroidism (SCH)[b]	41	7.1	30	6.9
Maternal hypothyroxinemia[c]	25	4.6	23	5.6
Maternal TPO-Abs and/or Tg-Abs+[d]	82	15.5	62	15.7
Maternal thyroid medication				
No medication	552	85.3	415	84.9
Thyroxine	90	13.9	69	14.1
Anti-thyroid medication	4	0.6	4	0.8
Yes, no defined	1	0.2	1	0.2
Child Characteristics				
Child's sex (female)	309	47.8	218	44.6

Child age at behavioral assessment	647	4.2 (0.2)	489	6.6 (0.3)
Gestational age at birth (weeks)	639	38.2 (1.6)	483	38.1 (1.6)
Birth weight (grams)	634	3,211 (446.2)	477	3,193 (452.4)
Breastfeeding duration (months)	615	4.1 (4.3)	470	4.0 (4.1)
ADHDT – total score[e]	529	14.21 (12.35)	-	-
SDQ – total score[f]	572	8.7 (4.8)	-	-
CPRS-R: S – total score[g]	-	-	471	8.9 (5.7)
CBCL – internalizing problems score[h]	-	-	423	6.2 (4.5)
CBCL – externalizing problems score[h]	-	-	462	8.7 (6.6)

[a] Data presented as mean (standard deviation) for continuous variables (unless mentioned otherwise) and as frequency (%) on each category for categorical variables; At 4 years: N = 647, At 6 years N = 489.

[b] fT4 concentration levels within the population-based, trimester-specific reference ranges and TSH above the upper TSH trimester-specific limit and below 10 µIU/mL

[c] TSH concentration levels within the trimester-specific reference ranges & fT4 < 5th percentile

[d] TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs ≥ 35 IU/mL &/or Tg-Abs > 40 IU/mL

[e] Attention Deficit Hyperactivity Disorder Test

[f] Strengths and Difficulties Questionnaire

[g] Conners' Parent Rating Scale-Revised: Short form

[h] Child Behavior Checklist, Parent report form

Non-response analyses showed no differences between participants and non-participants in terms of maternal thyroid functioning (TSH, fT4), smoking status during pregnancy, marital status, and in gestational age at birth. However, non-participants had younger [4 years assessment: mean difference = 1.1 years; 95% CI (.53, 1.69) p < .001, 6 years assessment: mean difference = 1.2 years; 95% CI (.70, 1.88) p <.001] and less educated mothers [4 years assessment: low educational level 28% versus 16%, x^2 = 25.48(2), p < .000, 6 years assessment: low educational level 27% versus 14%, x^2 = 31.74(2), p < .000] than participants. Non-participants had also lower birth weight [4 years assessment: mean difference = -58.63; 95% CI (-4.92, -112.33) p =.032] and they were breastfed for less time [4 years assessment: mean difference = -1.1 months; 95% CI (-.62, -1.62) p < .001, 6 years assessment: mean difference: -0.5 months; 95% CI (-.03, -1.03), p = .034] compared to participants.

Study population: Maternal thyroid mild dysfunction and offspring obesity and cardiometabolic traits

Of the 1170 women with a live singleton birth and available data on thyroid functioning, 735 mother-child pairs participated in the follow-up clinical assessments at 4 years and/or at 6 years of age (673 at the 4 years follow up and 477 at the 6 years follow up). Of this total, 33 mother-child pairs (4.5%) were excluded from the present analysis due to missing data on covariates. Table 6 presents the baseline characteristics of the participants in the analyses.

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

	4 years [a]	6 years [a]
Maternal Characteristics		
Age (years)	29.8 (4.9)	30.1 (4.8)
Educational level		
Low	114 (16.9)	73 (15.3)
Medium	337 (50.1)	238 (49.9)
High	222 (33.0)	166 (34.8)
Parity		
Primiparous	298 (44.3)	212 (44.4)
Multiparous	375 (55.7)	265 (55.6)
Origin		
Greek	630 (93.9)	25 (5.2)
Non-Greek	41 (6.1)	451 (94.8)
Smoking at early pregnancy		
No	437 (64.9)	312 (65.4)
Yes	236 (35.1)	165 (34.6)
TSH (μIU/mL)	1.25 (0.84)	1.22 (0.85)
ft4 (ng/dL)	1.22 (0.19)	1.23 (0.19)
Gestational age blood sampling (weeks)	14.0 (3.5)	13.9 (3.4)
Thyroid medication		
No medication	575 (85.4)	408 (85.5)
Thyroxine	93 (13.8)	64 (13.4)
Anti-thyroid medication	4 (0.6)	4 (0.8)
Yes, no defined	1 (0.2)	1 (0.2)

Child characteristics		
Sex (female)	318 (47.3)	210 (44.0)
Birth weight (grams)	3,214.95 (449.0)	3,194, 17 (444.70)
Gestational age (weeks)	38.0 (1.5)	38.2 (1.6)
Child age at follow up	4.2 (0.2)	6.6 (0.3)
Waist circumference	53.6 (4.9)	58.7 (7.0)
Overweight/Obese	147 (21.9)	151 (31.7)
Obese	47 (7.0)	53 (11.1)
Total cholesterol (mg/dl)	156.1 (27.6)	163.4 (23.8)
HDL cholesterol (mg/dl)	49.2 (11.2)	58.3 (12.3)
Systolic blood pressure (*mmHg*)	90.2 (7.5)	95.1 (9.0)
Diastolic blood pressure (*mmHg*)	53.4 (5.1)	55.0 (6.6)

Abbreviations: TSH: Thyroid Stimulating Hormone, fT4: free thyroxine, BMI: Body Mass Index, IOTF: International Obesity Task Force; HDL: High density lipoprotein; SD: Standard Deviation
BMI = weight (kg)/height (m) ^{2}
[a] Data are expressed as mean (SD) for continuous variables and as absolute frequency (%); At 4 years: N = 673, At 6 years: N = 477

Non-response analyses showed no differences between participants and non-participants in terms of maternal thyroid functioning (TSH, fT4), maternal smoking status during pregnancy and offspring gestational age at birth. However, mothers who did not participate in the follow-up assessments were younger [mean difference = - 1.1 years; 95% CI (- 1.7, - 0.4) $p < .000$] and more often they were less educated [low educational level 30% versus 17%, $\chi2 = 29.25$ (2), $p < 0.000$], multiparous [65% versus 56%, $\chi2 = 9.87(1)$, $p = 0.002$), and non-Greek [14.6% versus 6.2%, $\chi2 = 22.20(1)$, $p < 0.000$] compared to mothers who participated in the follow-up assessments.

2.3. Assessment of thyroid function

Maternal blood samples were collected at the first prenatal visit (mean gestational age 14.1 weeks, SD 3.6 weeks). Serum samples were collected in 10 ml vacutainer tubes, were centrifuged and stored in aliquots at -80° C until assayed. Maternal thyroid functioning was assessed by quantitative analysis of serum TSH, fT4, TPO-Abs, and Tg-Abs, by Immulite 2000 immunoassay system (Siemens Healthcare Diagnostics, Los Angeles, CA). For TSH, the inter- and intra-assay variability were < 5.3 and < 6.4, respectively, for levels of 0.32 – 39 mIU/mL. For fT4, the inter- and intra-assay variability were < 7.8% and

< 7.1%, respectively, for levels of 0.51–4.82 ng/dL or 6.56–62.03 pmol/L. For Tg-Abs, the inter- and intra-assay variability were < 4.9% and < 5.8% and for TPO-Abs < 7.4% and 7.2%, respectively.

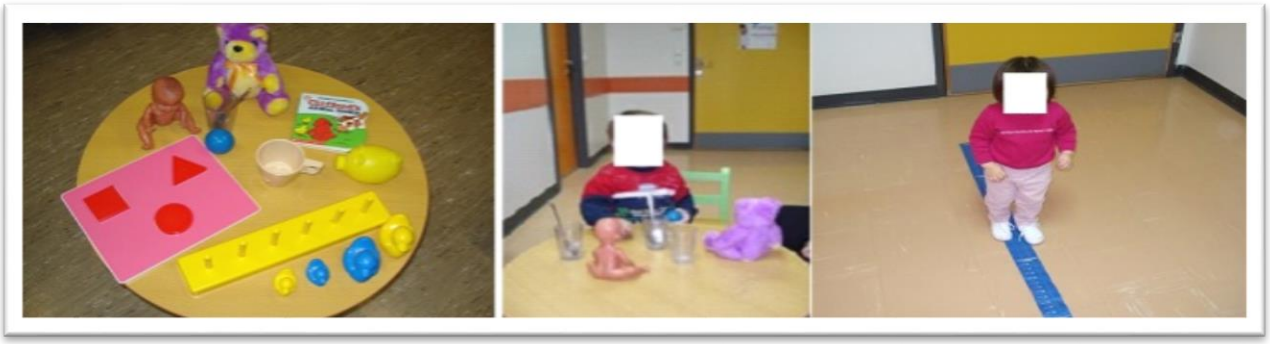
The proposed reference limits of the manufacturer for euthyroidism were: fT4: 0.89–1.76 ng/dL (11.5–22.7 pmol/L), and TSH: 0.4–4 μIU/mL. However, for the analyses included in this thesis, we used population-based, and trimester-specific reference intervals, according to the latest guidelines for thyroid dysfunction screening and management in pregnancy (37). The population-based reference ranges we used were defined using inclusion criteria regarding thyroidal, obstetric, and general medical status of the participants; detailed information on the calculation of the reference intervals have been previously published (192). The reference ranges were for the 1st trimester: TSH: 0.05 - 2.53 µIU/mL and fT4: 0.95 - 1.53 ng/dL, and for the 2nd trimester: TSH: 0.18 - 2.73 µIU/mL & fT4: 0.87 – 1.45 ng/dL.

Categorical entities of mild thyroid dysfunction were used in the analyses included in this thesis. Subclinical hypothyroidism was defined as TSH above the upper limit of the trimester-specific reference interval but below 10 mIU/mL and fT4 within the normal range, while the comparison group included women with TSH and fT4 concentration levels within the trimester-specific reference ranges (37, 64). Hypothyroxinemia was defined as TSH within the normal trimester-specific reference range and fT4 below the 5th percentile, the comparison group for hypothyroxinemia included women with TSH within the normal trimester specific reference range and fT4 above the 5th percentile (37). In addition, the 10th percentile of fT4 was used as an alternative cut-off point for the definition of hypothyroxinemia in sensitivity analyses. Maternal thyroid antibodies were considered elevated if TPO-Abs were equal or greater than 35 IU/mL, and if Tg-Abs were greater than 40 IU/mL, according to the limits proposed by the manufacturer.

2.4. Assessment of offspring development

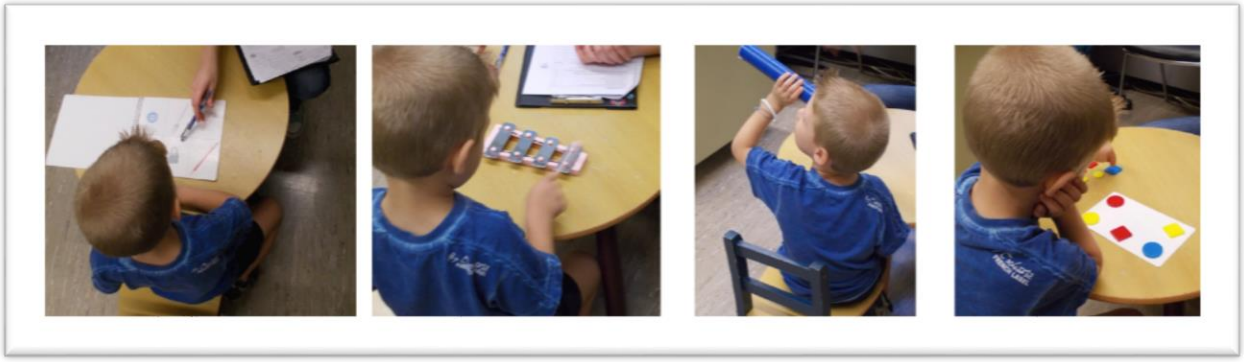
2.4.1. Cognitive and motor development

The children's mental and motor development was assessed at 18 months (±6 weeks) using the 3rd edition of Bayley Scales of Infant and Toddler Development (Bayley-III) (193). The Bayley-III scales assess infant and toddler development across three domains. The cognitive scale (COG) includes items that assess sensorimotor development, exploration and manipulation, object relatedness, concept formation, memory, and other aspects of cognitive processing. The language scale is composed of the receptive communication (RC) and the expressive communication (EC) subtest; the RC subtest includes items that assess preverbal behaviors, receptive vocabulary development, and understanding of morphological markers, children's social referencing and verbal comprehension and the EC subtest includes items that assess preverbal communication, expressive vocabulary development, and morpho-syntactic development. The motor scale is divided into the fine motor (FM) and the gross motor (GM) subtest; the FM subtest includes fine motor skills associated with perceptual-motor integration, motor planning, and motor speed; the GM subtest measures primarily the movement of the limbs and torso. For each scale, the scores were determined by the number of items for which credit was received.



Neuropsychological assessments were conducted by three trained psychologists. Total administration time was approximately 90 minutes. All testing was done at the Medical School of the University of Crete and two public hospitals in Heraklion, in the presence of the toddlers' mothers. The examiners, noted comments at the end of the assessment about the difficulties or special conditions of the process, to evaluate the ''quality of assessment'' (e.g. physical illness, tiredness, asleep, nervousness, shyness, difficulties due to behavior problems, etc.). Mothers were re-contacted by mail to get feedback on their child's performance. Raw scores were standardised for toddler's age at test administration using a parametric method for the estimation of age-specific reference intervals [29]. The parameters of the distribution are modeled as fractional polynomial (FP) function of age and estimated by maximum likelihood (ML). Standardised residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales (parameters conventionally used in psychometrics for IQs).

Preschoolers' cognitive and motor development at 4 years was assessed through the *McCarthy Scales of Children's Abilities (MSCA)* (194). The *MSCA* are developed for children of ages 2½–8½ years, and they are designed to assess children's cognitive and motor development to identify possible developmental delay. The *MSCA* include 18 tasks, which are summarized in five scales: (a) verbal (verbal expression and comprehension), (b) perceptual performance (reasoning), (c) quantitative scale (numerical aptitude and interest), (e) memory (verbal and non-verbal short-term memory), and (f) 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.



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 indicated 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 (Royston & Wright, 1998). 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.

The *MSCA* were administered individually to the participants by two trained psychologists. The inter-observer variability was < 1%. At the end of the neuropsychological assessment the examiners completed a standard form regarding the assessment conditions used to evaluate the "quality of assessment". Families received detailed feedback on their children's performance.

Cognitive and motor 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)* (195), the modified for children version of Trail Making Test parts A & B (*TMT-Part A* & *TMT-Part B*) (196), and the *Finger Tapping Test (FTT)* (196). 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 people 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 used was the standardized score of the total sum of correct responses. The *TMT* assess visual search, speed of processing, and executive functioning. The *TMT* consists of two parts; in *TMT-A* the participant has to draw lines sequentially connecting 25 framed numbers distributed on the screen, in *TMT-B* the participant has to connect numbers sequentially, while alternating between framed and encircled symbols. The outcome we used is the time spent to finish each part of the task in seconds. The *FTT* is designed to assess motor speed. The participant has 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 current analyses was the sum of hits of each hand.

2.4.2. Behavioral and emotional development

Behavioral and emotional symptoms at 4 years of age were assessed through two parent-report questionnaires: *Attention Deficit Hyperactivity Disorder Test (ADHDT)* (197) and *Strengths and Difficulties Questionnaire (SDQ)* (198). The *ADHDT* is based on ADHD criteria of DSM-IV and includes 36 items which assess ADHD related symptoms in ages 3-23 years. The instrument provides 4 indexes, corresponding to 3 subscales (hyperactivity, inattention, impulsivity) and a total ADHD difficulties score. Higher scores indicate higher and more severe ADHD related symptomatology. The *ADHDT* has been translated and adapted to the Greek population (Maniadaki and Kakouros, 2002). The *SDQ* is a brief screening questionnaire designed to assess behavioral strengths and difficulties in children 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). The *SDQ* was translated and adapted to the Greek population (Mpimpou - Nakou et al., 2001).

Behavioral and emotional problems at 6 years of age were assessed through the parent-report questionnaires *Child Behaviour Checklist – Parent Report Form (CBCL)* (199) and the *Conner's Parent Rating Scale, Revised, Short Form (CPRS-R: S)* (200). 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 (201) (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 (202). 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) (203).

Scores in each scale of *ADHDT*, *SDQ*, *CBCL*, and *CPRS-R* were treated as continuous variables. The scores indicate perceived symptoms and the severity of these symptoms and do not represent clinical disorders.

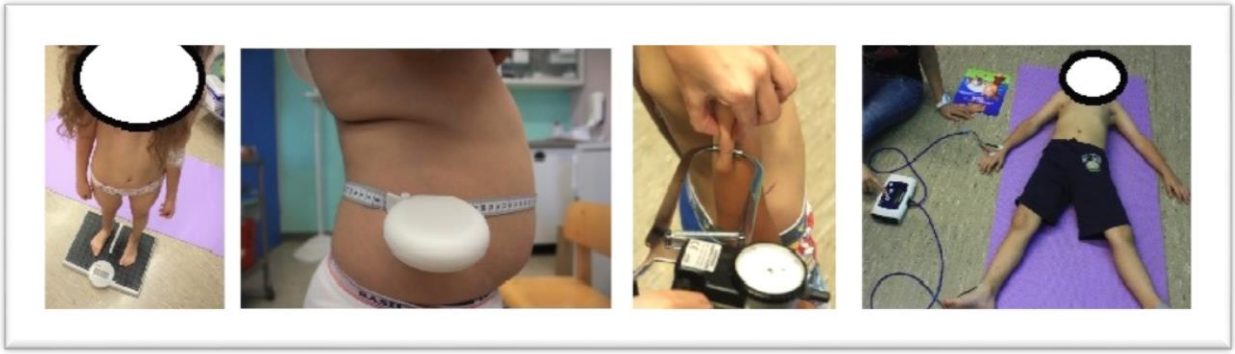
2.4.3. Anthropometry and cardiometabolic health traits

Information on birthweight and birth-length/height was obtained from medical records. Anthropometry at the follow up examinations at 4 and 6 years of age was conducted by trained research assistants according to standard protocols.

Weight (Seca Bellisima 841) and height (Seca 213) was measured on light clothing and without shoes. Height was measured in standing position while the children kept their shoulders relaxed, their arms hanging freely, and their head aligned in Frankfort horizontal plane. Since child age varied at the follow-up visits, we estimated weight and height values at 4 years and 6 years of age, using age-specific, and sex-specific growth curves fitted with multilevel models with fractional polynomial of age and random effects for the child age terms. Age-specific and sex-specific weight and BMI z-scores were estimated at

the aforementioned time-points based on World Health Organization (WHO) growth standards (204, 205). Child overweight/obesity was defined using the International Obesity Task Force cut-off values (206).

Waist circumference (WC) was measured in duplicate to the nearest 0.1 cm in standing position, at the high point of the iliac crest at the end of a gentle expiration, using a flexible tape measure (Seca 201). Skinfold thickness were measured to the nearest 0.1 mm using a calibrated Harpenden caliper (Harpenden HSK- BI, CE-0120; Baty Inernational, West Sussex, UK) at four anatomical sites (triceps, subscapular, suprailiac and quadriceps). Three complete sets of measurement were taken consecutively and the mean was used as the representative value for each site. The sum of the four skinfolds represents a general index of the subcutaneous total fat mass (207). Intra-observer reliability was > 0.98 and interobserver reliability was > 0.82 for all anthropometric measurements. In addition, at 6 years of age, hand to leg bioelectric impedance analysis (BIA) was performed using the Bodystat 1500 (Bodystat, Douglas, Isle of Man). The participants were asked to empty their bladder and remove any heavy clothing and metal jewellery. Four electrodes were placed as specified by the manufacturer, 2 on the right wrist and two on the right ankle. The measurement was taken while children were in supine position with legs abducted to 45^{o}, after 5 minutes rest in this position. Impedance measure was recorded to the nearest ohm. Trained research assistants recorded the body fat and percentage of body fat estimations to the datasheet of every participant. The BIA machine was calibrated daily using the resistor provided by the manufacturer. BIA provides a total fat mass index and a total fat mass index as proportion of total body mass, using age- and race-specific equations validated for use in children (208).



Systolic and diastolic blood pressure (SBP & DBP) levels were measured at 4 and 6 years of age using an automatic oscillometric device (Dinamap, Pro Care 400) on child's right arm with a cuff of appropriate size for arm circumference. Five measurements were made, after the participants were rested for 5 minutes in seated position, with a 1-minute interval between them and the average of all measurements

was calculated (209). At the end of the follow-up clinical examinations we collected non-fasting blood samples. Blood samples were processed within 2 hours and blood sera frozen at -80 °C until analyzed. Analysis of lipids [total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein (LDL-C), triglycerides)] was performed by standard enzymatic methods (Medicon, Greece) on an automatic analyzer (AU5400 high-volume chemistry analyzer; Olympus America, Inc., Melville, New York). All inter- and intra-assay coefficients of variation were < 5.5%. The cardiometabolic risk score is expressed in standard deviations and was calculated as the sum of the following components: sex- specific and age-specific z-scores of waist circumference and non-HDL cholesterol, and the average of age-specific, sex-specific, and height-specific z-scores for systolic and diastolic blood pressure.

2.5. Selection of Covariates

We initially selected the potential confounders based on previously described covariates of the exposure and the outcome variables. The "change in estimate", and the Directed Acyclic Graphs (DAGs) methodological approaches have been applied to determine the selected potential confounders that were inserted in the adjusted multivariate models. The cut-off point that was applied for the inclusion of the covariates in the models when the change in estimate method was followed was a change greater than the 10% of the initial values of the estimates. DAGs were also used because they represent causal relationships among variables, and they have been applied in epidemiologic research to identify variables which must be measured and controlled to obtain unconfounded effect estimates (210). More specifically, a DAG is composed of variables and arrows between the variables (directed edges); it is acyclic, therefore 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 depicts the causal assumptions behind each hypothesis and facilitates the selection of the confounders of each relationship, avoiding the risk for selection bias that is introduced when other traditional methods are used (211).

Covariate selection: Maternal thyroid mild dysfunction and offspring cognitive and motor development

We followed *the* DAGs approach to determine the potential confounders of the association between maternal thyroid mild dysfunction and child cognitive development. Figure 3 presents the causal assumptions regarding the association of maternal thyroid function and child cognition; the minimal sufficient adjustment set for estimating the total effect included maternal origin (Greek/other), parity

(primiparous/multiparous), maternal smoking at early pregnancy (yes/no), maternal BMI in early pregnancy, maternal education (low level: ≤ 9 years of school, medium level: 9 to 12 years of school, high level: university or technical college degree), maternal age at birth (years), maternal marital status at pregnancy (married/other) and gestational age at blood sampling. Quality of assessment and child sex were included a priori in all models and child age was included a priori in the models involving *TMT* and *FTT*. The total missing values of the covariates were < 6%.

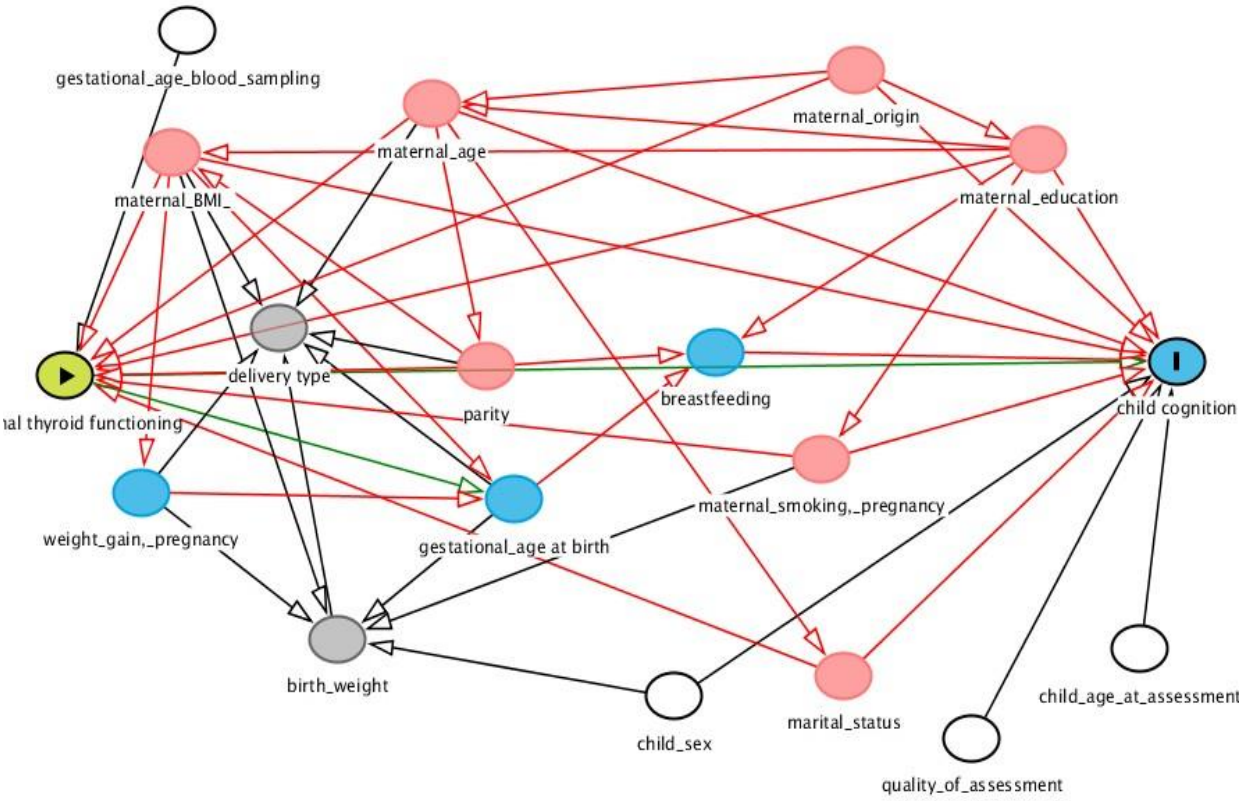


Figure 3. Causal assumptions regarding the association of maternal thyroid function and child cognition

Covariate selection: Maternal thyroid mild dysfunction and offspring behavioral and emotional development

We applied the change-in-estimate criterion to determine the confounders of the association between maternal thyroid mild dysfunction and child behavioral and emotional problems in our dataset. The potential confounders were selected on the basis of literature regarding the study's hypotheses and included maternal age, maternal educational level, maternal marital status at pregnancy, maternal origin, maternal smoking at pregnancy, maternal alcohol intake during pregnancy, maternal BMI, maternal IQ,

parity, child's gestational age and weight at birth, child's sex, and child's age at the behavioral assessment. After the application of the change-in estimate criterion, we retained the following variables as confounders: maternal age (continuous), maternal educational status (low/medium/high), parity (primiparous/multiparous), maternal smoking status during pregnancy (yes/no), and maternal marital status at pregnancy (single/married). Breastfeeding duration (continuous) and birthweight (continuous) were also included in the models, since participants and non-participants significantly differed in these covariates (according to the results of the non-response analyses). The models were also adjusted for child's sex and child's age at the behavioral assessment (a priori selection).

Covariate selection: Maternal thyroid mild dysfunction and offspring obesity and cardiometabolic traits

We applied the DAG approach to determine the potential confounders of the association between maternal thyroid mild dysfunction and offspring obesity. Figure 4 presents the causal assumptions regarding the association of maternal thyroid function and offspring obesity; the minimal sufficient adjustment set for estimating the total effect of maternal thyroid hormones on offspring obesity measures included maternal age at birth (years), maternal BMI at early pregnancy, maternal smoking during pregnancy (yes/no), maternal education (low level: ≤ 9 years of school, medium level: 9 to 12 years of school, high level: university or technical college degree), and parity (primiparous/multiparous). Gestational age at blood sampling and child sex were included a priori in all models. Child age at measurement and child sex were included in the models involving measures that were not adjusted for these variables (i.e. fat mass measurements, waist circumference, and the cardiometabolic traits). Child height was included in the models with waist circumference, diastolic and systolic blood pressure as outcomes. The percentage of combined missing values of the covariates was < 5%.

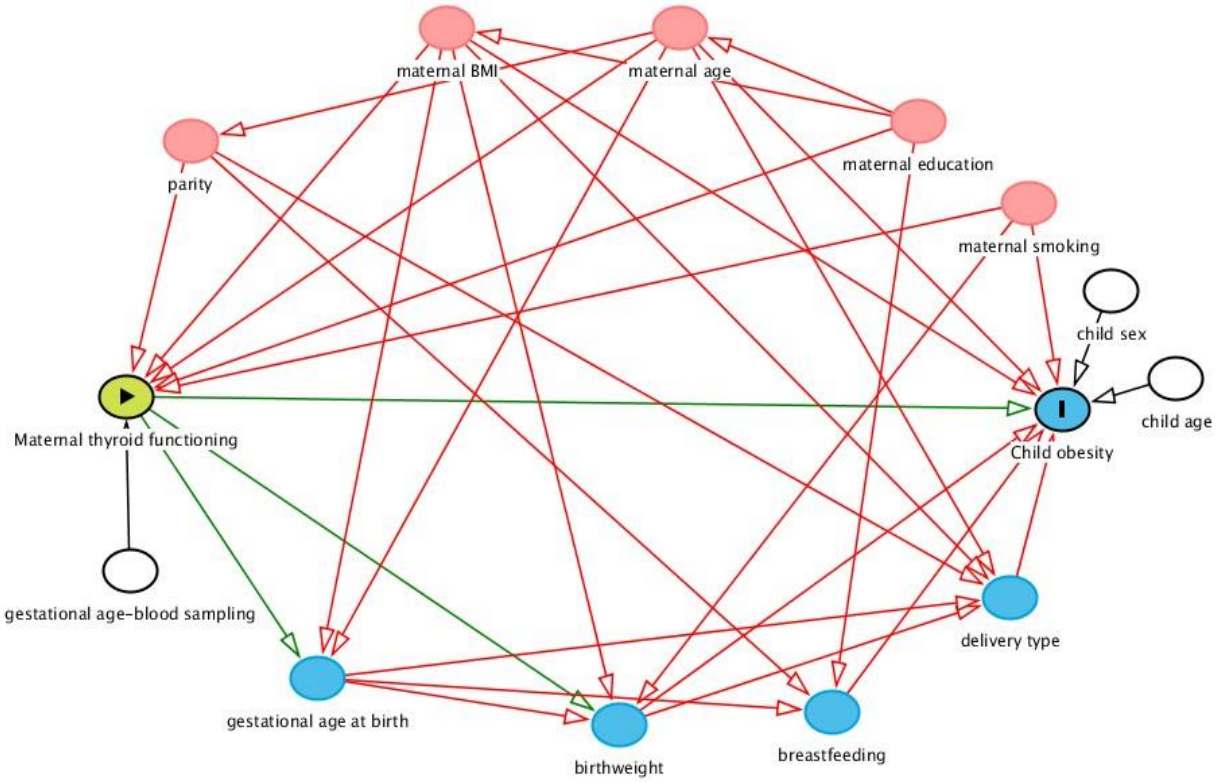


Figure 4. Causal assumptions regarding the association of maternal thyroid function and child obesity

2.6. Statistical analysis

For each analysis in this thesis, descriptive analyses of the study population characteristics, exposures, and outcomes were conducted. In brief, 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), whereas associations of categorical variables were tested using Pearson's Chi-square test.

In order to explore the linearity of the associations between maternal thyroid parameters and offspring developmental outcomes, we used generalized additive models (GAMs). Linearity was assumed if p-gain, defined as the difference in normalized deviance between the GAM and the linear model for the same exposure and outcome (212), was greater than 0.1. We categorized thyroid hormones' parameters in tertiles, when non-linearity was evident in the associations. Maternal subclinical hypothyroidism, maternal hypothyroxinemia, and thyroid autoimmunity were also examined as possible predictors of child

development. 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.

Non-response analyses were conducted for the population included in each analysis of this thesis, in order to determine the baseline differences between participants and non-participants.

Statistical analysis: Patterns of early-life exposures and their association with neuropsychological development in preschool age

We explored, as an initial analysis, the early-life social and environmental exposures that determine child neuropsychological development. Potential factors of child cognition were subjected to principal component analysis (PCA), with varimax rotation. We applied a regression based method to extract the components and used the Bartlett's test of sphericity, the Kaiser-Meyer-Olkin measure, and the correlation-matrix Determinant to examine sampling adequacy. The selection criterion for the extracted components was eigenvalues over 1, and the accepted factor loadings over 0.3.

The extracted components were inserted in multivariate linear regression models to examine their association with child neuropsychological development at 4 years of age, after they were classified into tertiles to facilitate interpretation. The reference-group was the 1st tertile, which corresponds to the lowest tertile of scores on each component. The components were entered simultaneously in each model, and adjusted for child's sex, the examiner, and quality of assessment.

Sensitivity analysis was conducted, excluding preterm births to distinguish any confounding by prematurity. The main analyses were repeated in a subsample of children (N = 346) with available maternal intelligence data [Raven SPM; (213)], to adjust for any potential confounding effect by maternal intelligence. Modification effect of child sex was examined by including an interaction term with each component and child's sex in the models. Subsequently, multivariate models stratified by child sex were implemented. Benjamini-Hochberg correction was performed post hoc to control for false discovery rate in every model included in the analyses.

Statistical analysis: Maternal thyroid mild dysfunction and offspring's cognitive and motor development

In the analyses of the association of maternal thyroid dysfunction with child cognition and motor ability, maternal thyroid data were categorized into tertiles (low/medium/high), since we found evidence of non-linearity between maternal thyroid parameters and the outcomes of interest (GAMs). The medium

tertile was set as the comparison group for the multivariate models. Maternal subclinical hypothyroidism, maternal hypothyroxinemia, and maternal euthyroidism with elevated thyroid antibodies (TPO-Abs and/or Tg-Abs) were also examined as possible predictors of offspring cognitive and motor development in multivariate models.

In addition, we constructed trajectories of longitudinal non-verbal cognitive development from infancy to early childhood using Group Based Trajectory Modeling (GBTM) (214). The distribution percentiles of the Bayley's cognitive scale, the *MSCA*'s perceptual performance scale, and the *RCPM*'s total score were calculated and used in GBTM, in order to homogenize the inserted scales. We included in this analysis participants with data on neuropsychological development in, at least, two time-points (N=586). Probability of group membership, predicted trajectory of each group and posterior probabilities of group membership were estimated. 2-5 possible cognitive development trajectories were tested and a model with 4 classes was selected based on the Bayesian information criterion (BIC), the evaluation of average posterior probability (AvePP value > 0.65), the odds of correct classification (OCC > 5), and the number of participants in each group (Table 7).

Table 7. Data for the trajectory model selection, Rhea mother-child study, Crete, Greece

N of Groups	Shape	BIC	APP G1	APP G2	APP G3	APP G4	OCC Gr1	OCC Gr2	OCC Gr3	OCC Gr4	Prob G1	Prob G2	Prob G3	Prob G4
2	0 0	-6595.4	90%	88%	-	-	7.7	9.0	-	-	54%	46%	-	-
2	0 1	-6598.3	90%	89%	-	-	7.6	9.1	-	-	54%	46%	-	-
2	2 0	-6599.7	90%	89%	-	-	7.6	9.3	-	-	54%	46%	-	-
2	1 2	-6601.6	89%	89%	-	-	7.6	9.3	-	-	54%	46%	-	-
3	0 0 0	-6600.8	72%	50%	82%	-	3.9	4.8	5.6	-	39%	17%	44%	-
3	0 1 0	-6599.4	81%	58%	87%	-	4.1	51.9	8.0	-	51%	2%	46%	-
3	2 1 0	-6597.3	65%	87%	82%	-	22.5	6.1	6.7	-	7%	51%	41%	-
3	2 1 1	-6599.2	67%	87%	82%	-	20.4	6.3	6.8	-	8%	51%	40%	-
3	2 2 0	-6598.9	68%	86%	81%	-	24.1	6.1	6.4	-	8%	51%	41%	-
4	1 0 0 0	-6604.8	51%	70%	81%	47%	19.8	3.8	5.9	5.0	4%	38%	42%	15%
4	0 1 0 0	-6604.2	70%	51%	81%	47%	3.8	19.8	5.9	5.0	38%	4%	42%	15%
4	**2 2 0 1**	**-6592.0**	**68%**	**77%**	**81%**	**70%**	**10.4**	**6.5**	**9.1**	**11.6**	**17%**	**34%**	**32%**	**19%**
4	0 2 0 2	-6587.9	74%	70%	83%	67%	6.0	11.5	10.0	8.5	32%	17%	32%	19%
4	1 2 2 2	-6600.7	69%	72%	69%	83%	11.0	5.2	12.0	9.3	17%	33%	16%	34%
4	1 2 1 0	-6593.6	68%	76%	67%	82%	11.0	6.4	9.5	8.8	16%	33%	17%	33%
4	0 1 2 0	-6587.9	76%	69%	67%	81%	6.4	11.0	9.9	8.8	34%	17%	17%	33%
4	2 2 2 2	-6599.5	69%	74%	71%	82%	10.5	5.7	11.9	9.2	18%	33%	17%	32%

Shapes: 0 = zero-order; 1 = linear, 2 = quadratic
Abbreviations: BIC = Bayesian information criterion, APP: Average posterior probability, OCC: Odds of Correct Classification

We identified 4 trajectories of non-verbal cognitive development from infancy to early childhood (continuously low, continuously high, high at 18 months-decreasing over time, low at 18 months-

increasing over time). Individual trajectories based on GBTM analysis are presented at Figure 5, and the 4 trajectories of non-verbal cognitive development at Figure 6.

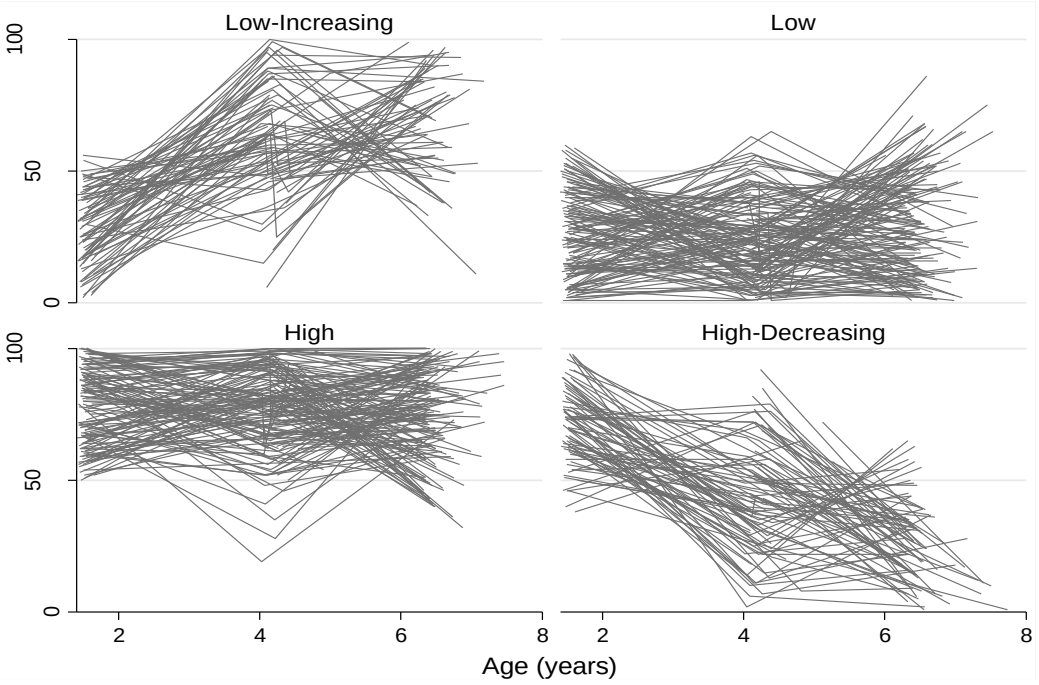


Figure 5. Individual trajectories, Group Based Trajectory Modelling, Rhea mother-child study, Crete, Greece

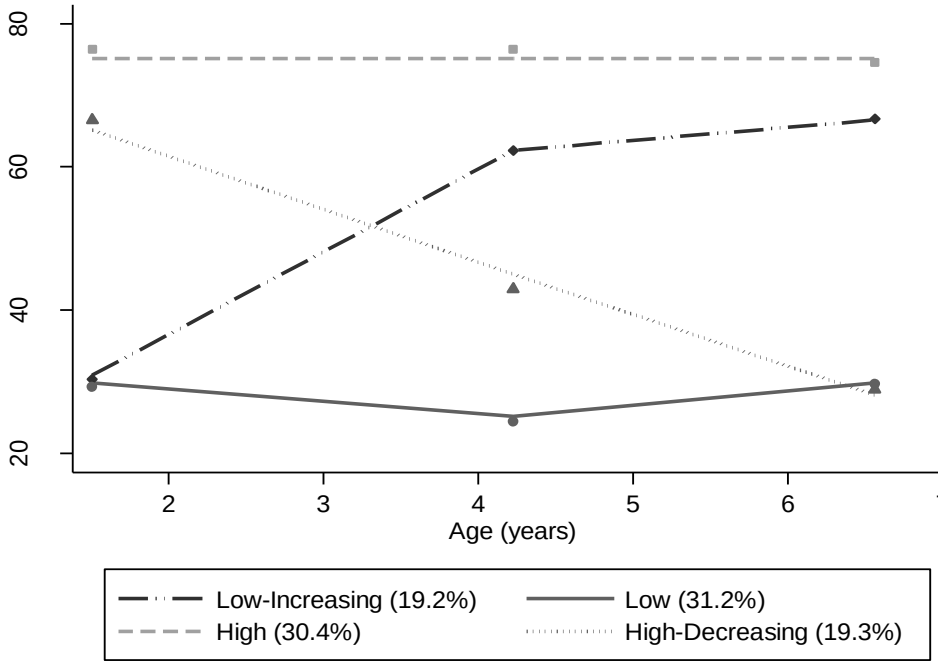


Figure 6. Non-verbal cognitive development trajectories, Group Based Trajectory Modeling, Rhea mother-child study, Crete, Greece

The trajectory of continuously high non-verbal cognitive development from infancy to early childhood, was set as the base outcome for the multinomial logistic regression models. The associations of maternal thyroid dysfunction and thyroid autoimmunity with the trajectories of non-verbal cognitive development were explored using multinomial linear regression models, weighted for each individual's posterior probability of belonging to each of the trajectories. The associations were presented in terms of Relative Risk Ratios (RRR) and 95% confidence intervals.

We performed sensitivity analyses, excluding mothers who took thyroid medication during pregnancy (N = 96). We also repeated the main analyses in children exposed to elevated levels of maternal TPO-Abs (TPO-Abs ≥ 35 IU/mL) and to elevated levels of Tg-Abs (Tg > 40 IU/mL) separately.

Statistical analysis: Maternal mild thyroid dysfunction and offspring behavioral and emotional development

In the analyses of the association of maternal thyroid dysfunction with child behavioral and emotional symptoms (*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) (215). In the imputation model, all the questionnaire items (raw data) were regressed on all the other items (216). 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 (217). 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 (218). 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.

We found evidence of non-linearity between maternal thyroid parameters and the outcomes (GAMs), thus maternal thyroid data were categorized into tertiles (low/medium/high). The medium tertile was set as the comparison group for the multivariate models. Maternal subclinical hypothyroidism,

maternal hypothyroxinemia, and maternal euthyroidism accompanied with elevated thyroid antibodies (TPO-Abs and/or Tg-Abs) were also examined as possible predictors of child behavioral and emotional symptoms at 4 and 6 years of age. In order to examine the role of thyroid-antibodies' presence in subclinical hypothyroidism, stratified analyses by maternal thyroid-antibodies' status were also conducted.

To examine a potentially modifying effect of child sex, we included the respective interaction terms in the regression models (p for interaction < .05). Sensitivity analyses were conducted excluding mothers who took thyroid medication during pregnancy. Furthermore, the analyses were repeated to a subsample with available child thyroid hormones' assessment at 4 years (TSH), to adjust for any potential effect of child thyroid functioning on the identified associations. Sensitivity analyses with additional adjustment for maternal iodine and iron status were also conducted in a sub-sample with available information on these covariates. The multivariate models regarding maternal hypothyroxinemia were repeated using an alternative cut-off point for maternal fT4 (fT4 < 10^{th} percentile).

Statistical analyses: Maternal thyroid mild dysfunction and offspring obesity and cardiometabolic traits

For the analyses between maternal thyroid function and offspring obesity and cardiometabolic traits, multivariate adjusted linear regression models were used to estimate beta coefficients with 95% confidence intervals for the associations of maternal TSH and fT4 with the continuous outcomes under study (BMI, waist circumference, fat mass measures, lipids, and blood pressure) and multinomial adjusted linear regression models were performed to estimate Odds Ratios (ORs) and 95% confidence intervals regarding the binary outcomes (overweight, and obesity). We have identified non-linear associations between maternal thyroid parameters and offspring obesity measures, thus thyroid parameters were categorized in tertiles for the respective models and the medium tertile was used as the comparison group. Maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity were also inserted in multivariate adjusted regression models, as categorical exposures. We performed sensitivity analysis excluding mothers who took thyroid medication during pregnancy (N = 98).

3. Results

3.1. Patterns of early-life social and environmental exposures and offspring neuropsychological development

Main findings

- Higher parental social status, preschool attendance and less TV watching, nonsmoking during pregnancy and breastfeeding, and parental involvement in child life were all protective factors of child cognitive and behavioral development at 4 years of age.
- Increased child birth order was associated with decreased verbal ability and general cognitive ability at 4 years of age.
- Increased child birth order was associated with decreased ADHD-related symptoms at 4 years of age.
- Offspring size at birth was not associated with any measure of child cognitive or behavioral development at 4 years of age.

Summary of the Results

A total of 15 early-life social and environmental exposures were included in Principal Component Analysis (PCA) and six components were extracted, accounting for 62.52% of the total variance. The extracted components were: (i) “Parental social status”, (ii) “Child birth order”, (iii) “Size at birth”, (iv) “Breastfeeding & Non-Smoking during pregnancy”, (v) “Preschool attendance & less TV watching”, and (vi) “Parental involvement”. Table 8 presents the variables inserted in PCA, the components and their factor loadings, as well as the percentage of variance explained by each component.

Table 8. Principal Component Analysis of Early life Social and Environmental Exposures

	Extracted Components					
	Parental Social Status	Child Birth Order	Size at Birth	Breastfeeding & Non-Smoking	Preschool Attendance & less TV watching	Parental Involvement
Maternal education	.675	-	-	-	-	-
Paternal education	.672	-	-	-	-	-
Maternal occupational status	.612	-	-	-	-	-
Maternal age at birth	.585	-	-	-	-	-
Residence	.570	-	-	-	-	-
Maternal parity at birth	-	.963	-	-	-	-
Birth order	-	.944	-	-	-	-
Birth weight	-	-	.855	-	-	-
Birth head circumference	-	-	.843	-	-	-
Smoking during pregnancy	-	-	-	.778	-	-
Breastfeeding duration	-	-	-	.721	-	-
TV-watching	-	-	-	-	.761	-
Preschool attendance	-	-	-	-	.750	-
Parental participation in child leisure-time	-	-	-	-	-	.769
Marital status	-	-	-	-	-	.767
Variance explained	15.76%	13.63%	10.03%	8.11%	7.68%	7.31%
Cumulative variance explained	62.52%					

Only items with factor loadings > 0.30 were retained for each factor; N = 700

Analyses on the patterns of early-life exposures and child cognitive and motor development:

- Higher parental social status was associated with increased scores in verbal ability, perceptual performance, quantitative ability, memory, general cognitive development and motor ability at 4 years of age.
- Preschool attendance & less TV-watching was related with increased scores in verbal ability, perceptual performance, general cognitive development, and motor ability at 4 years of age.

- Parental involvement was associated with increased scores in verbal ability, perceptual performance, quantitative ability, general cognitive development, and motor ability at 4 years of age.
- Non-smoking and enhanced breastfeeding was associated with increased scores in perceptual performance and general cognitive development at 4 years of age.
- Having multiparous mothers and advanced birth order was associated with decreased scores in verbal ability and general cognitive development at 4 years of age.
- We did not identify any association between size at birth and child cognitive and motor development at 4 years of age.

The associations between the patterns of early life social and environmental exposures and offspring cognitive and motor development are presented in Figure 7.

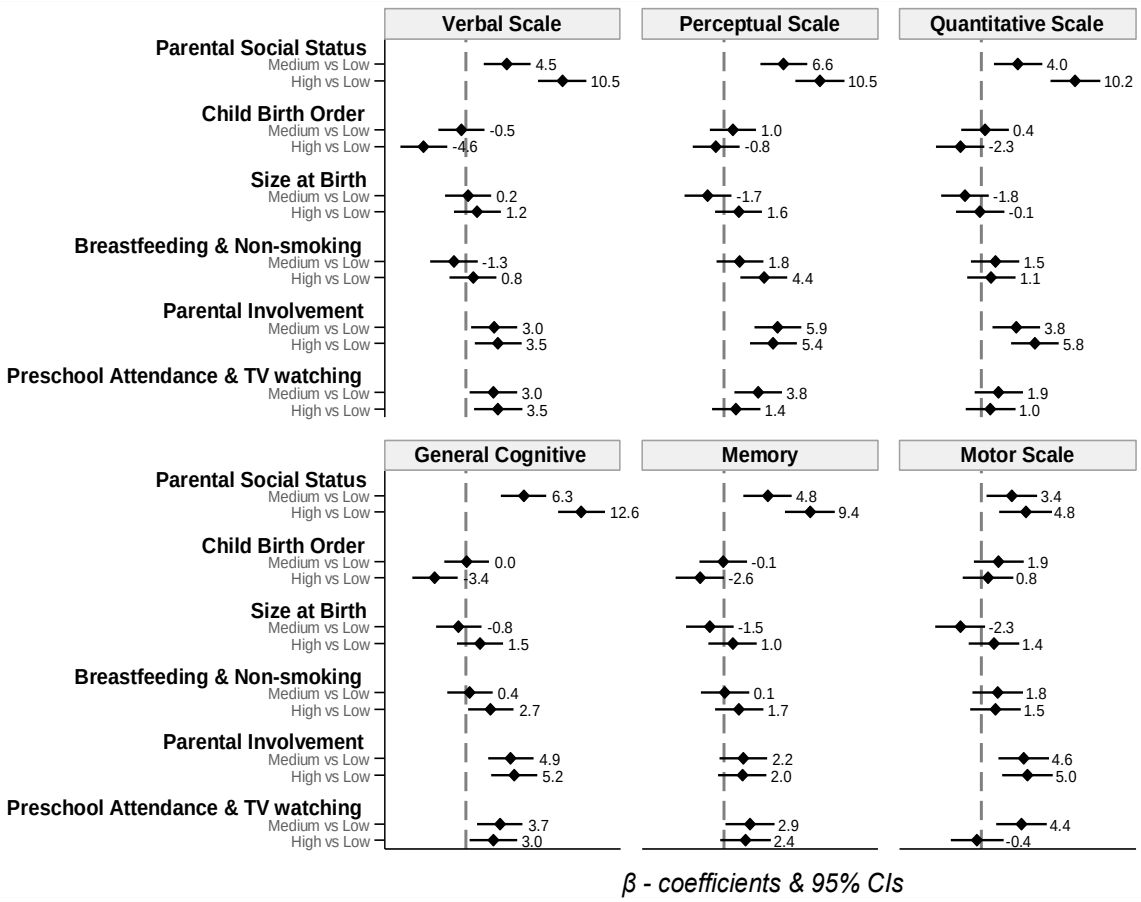


Figure 7. Associations between patterns of early-life exposures and children's cognitive and motor abilities [*McCarthy Scales of Children Abilities* (*MSCA*)] at 4 years of age (N=700). All models are adjusted for child's gender, examiner, quality of assessment and for the rest components of early-life exposures.

Analyses on patterns of early-life exposures and child ADHD-related symptoms:

- Higher parental social status was associated with decreased hyperactivity, inattention, impulsivity, and total ADHD-related symptoms at 4 years of age.
- Parental involvement was associated with decreased hyperactivity inattention, impulsivity, and total ADHD-related symptoms at 4 years of age.
- Non-smoking & enhanced breastfeeding component was associated with decreased hyperactivity inattention, impulsivity, and total ADHD-related symptoms at 4 years of age.
- Having multiparous mothers and advanced birth order was associated decreased hyperactivity inattention, impulsivity, and total ADHD-related symptoms at 4 years of age.
- Size at birth was not associated with ADHD-related symptoms at 4 years of age.

Figure 8 presents the associations between the patterns of early life social and environmental exposures and offspring ADHD-related symptoms.

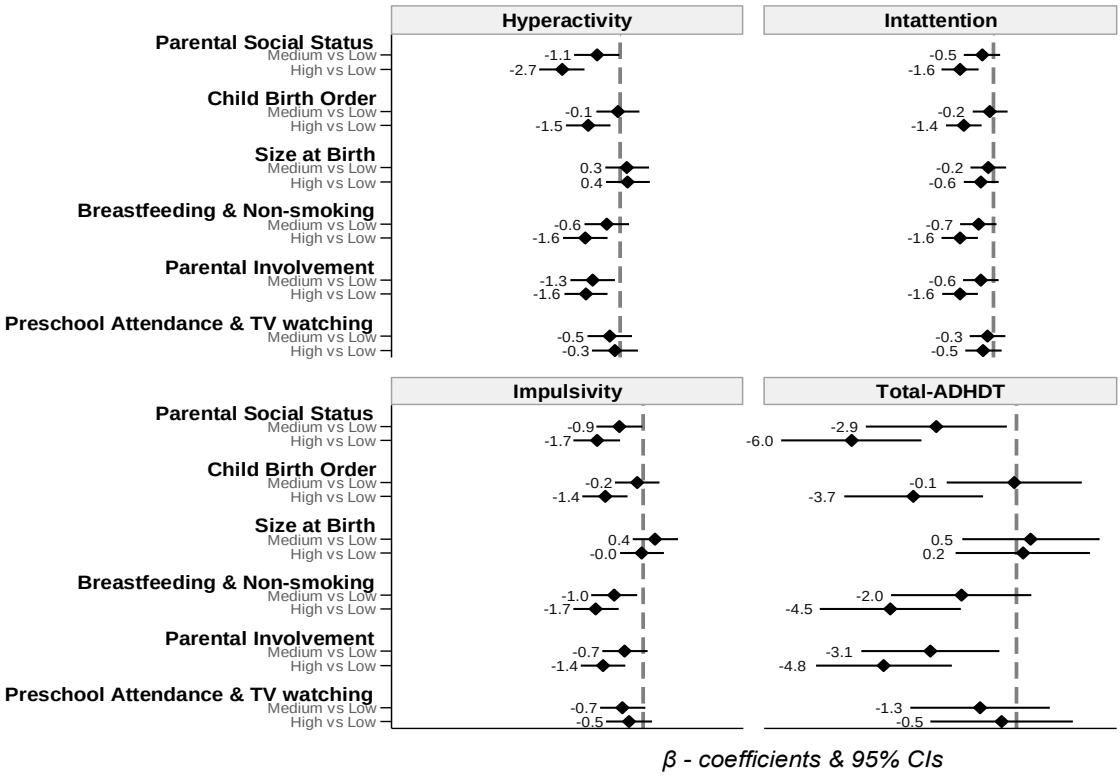


Figure 8. Associations between patterns of early-life exposures and children's ADHD-related symptoms [*Attention Deficit Hyperactivity Disorder Test (ADHDT)*], at 4 years of age (N=610). All models are adjusted for child gender, child age and for the rest components of early-life exposures.

Additional analyses:

- The results did not change substantially when the models were repeated excluding children born preterm (N = 613) (Table 9), nor when the models were repeated in a subsample of participants with available data on maternal intelligence (N = 346) (Table 10).
- Interaction effect of child gender was identified and multivariate models stratified by child sex showed that non-smoking in pregnancy and enhanced breastfeeding duration is a protective factor exclusively for perceptual performance of female-participants. Gender interaction was also found in the association of child birth order and verbal ability. Stratified analysis showed that greater birth order constitutes a stronger risk factor for females (Table 11A and Table 11B).
- Benjamini – Hochberg multiple testing correction was applied post hoc to control for the false discovery rate. The correction did not result in any important change of the initial results.

Table 9. Associations between patterns of early life characteristics and *McCarthy Scales of Children Abilities* (MSCA) at 4 years, excluding children born preterm

	Verbal		Perceptual		Quantitative		General Cognitive		Memory		Motor	
	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI
Parental Social Status												
High	**10.18**	**(7.44, 12.92)[b]**	**10.10**	**(7.32, 12.88)[b]**	**9.61**	**(6.79, 12.43)[b]**	**12.04**	**(9.36, 14.71)[b]**	**9.07**	**(6.23, 11.90)[b]**	**4.32**	**(1.32, 7.32)[b]**
Medium	**4.63**	**(1.97, 7.29)[b]**	**6.76**	**(4.04, 9.47)[b]**	**4.24**	**(1.50, 6.98)[b]**	**6.49**	**(3.89, 9.09)[b]**	**5.36**	**(2.61, 8.12)[b]**	**3.21**	**(0.29, 6.12)[b]**
P for trend		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**0.014**
Child Birth Order												
High	**-4.09**	**(-6.78, -1.40)[b]**	0.06	(-2.68, 2.79)	-2.24	(-5.00, 0.53)	**-2.76**	**(-5.38, -0.13)[b]**	-2.28	(-5.06, 0.51)	1.52	(-1.43, 4.46)
Medium	-0.42	(-3.01, 2.19)	1.19	(-1.46, 3.83)	-0.34	(-3.02, 2.33)	0.65	(-2.47, 2.60)	-0.08	(-2.78, 2.61)	2.70	(-0.15, 5.55)
P for trend		**0.005**		0.607		0.232		0.054		0.189		0.177
Size at Birth												
High	1.07	(-1.57, 3.71)	2.02	(-0.66, 4.70)	-0.08	(-2.79, 2.63)	1.56	(-1.01, 4.13)	0.87	(-1.86, 3.60)	1.93	(-0.95, 4.82)
Medium	-0.14	(-2.77, 2.49)	-1.94	(-4.61, 0.74)	-2.06	(-4.76, 0.65)	-1.15	(-3.72, 1.42)	-1.79	(-4.51, 0.94)	-2.43	(-5.31, 0.45)
P for trend		0.617		**0.015**		0.240		0.116		0.150		**0.012**
Non Smoking in Pregnancy – Breastfeeding												
High	0.77	(-1.88, 3.41)	**5.25**	**(2.56, 7.93)[b]**	1.57	(-1.15, 4.28)	**3.16**	**(0.58, 5.74)[b]**	2.40	(-0.34, 5.14)	1.40	(-1.50, 4.29)
Medium	-1.12	(-3.78, 1.54)	2.27	(-0.43, 4.97)	2.16	(-0.58, 4.89)	0.84	(-1.76, 3.43)	0.73	(-2.03 3.48)	2.02	(-0.88, 4.93)
P for trend		0.368		**0.001**		0.279		**0.045**		0.209		0.378
Preschool Attendance - less TV Watching												
High	**3.88**	**(1.26, 6.51)[b]**	**4.29**	**(1.62, 6.96)[b]**	**5.97**	**(3.27, 8.67)[b]**	**5.01**	**(2.45, 7.57)[b]**	1.67	(-1.05, 4.38)	**4.66**	**(1.78, 7.53)[b]**
Medium	**3.03**	**(0.41, 5.65)[b]**	**4.88**	**(2.22, 7.54)[b]**	**3.25**	**(0.56, 5.95)[b]**	**4.26**	**(1.71, 6.82)[b]**	1.47	(-1.25, 4.18)	**4.11**	**(1.24, 6.97)[b]**
P for trend		**0.009**		**<0.001**		**<0.001**		**<0.001**		0.419		**0.002**
Parental Involvement												
High	2.62	(-1.61, 5.39)	1.46	(-1.36, 4.28)	-0.77	(-3.62, 2.09)	2.09	(-0.62, 4.80)	0.97	(-1.91, 3.84)	-0.74	(-3.78, 2.30)
Medium	2.56	(-0.11, 5.22)	**4.73**	**(2.03, 7.43)[b]**	1.11	(-1.62, 3.85)	**3.74**	**(1.14, 6.33)[b]**	**2.82**	**(0.07, 5.57)[b]**	**4.67**	**(1.76, 7.58)[b]**
P for trend		0.097		**0.002**		0.417		**0.019**		0.124		**<0.001**

The comparison group for the models is the first tertile, which corresponds to the lowest values of the component; N=613

[a] All models are adjusted for child's gender, examiner, quality of assessment and for the rest patterns of early life characteristics.

[b] $p < 0.05$

Table 10. Associations between patterns of early life characteristics and *McCarthy Scales of Children Abilities (MSCA)* at 4 years of age, adjusted for maternal intelligence

	Verbal		Perceptual		Quantitative		General Cognitive		Memory		Motor	
	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI
Parental Social Status												
High	**9.06**	**(5.29, 12.83)[b]**	**9.40**	**(5.65, 13.16)[b]**	**7.42**	**(3.72, 11.12)[b]**	**10.72**	**(7.11, 14.33)[b]**	**7.73**	**(3.91, 11.55)[b]**	**4.89**	**(0.74, 9.03)[b]**
Medium	**4.27**	**(0.66, 7.91)[b]**	**6.38**	**(2.75, 10.00)[b]**	3.45	(-0.13, 7.02)	**5.92**	**(2.43, 9.41)[b]**	3.68	(-0.01, 7.36)	2.63	(-1.38, 6.63)
P for trend		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**<0.001**		0.070
Child Birth Order												
High	**-4.94**	**(-8.57, -1.30)[b]**	0.20	(-3.43, 3.82)	-2.22	(-5.79, 1.34)	-3.15	(-6.63, 0.33)	-1.83	(-5.50, 1.86)	1.29	(-2.71, 5.29)
Medium	-0.96	(-4.50, 2.58)	0.34	(-3.19, 3.87)	0.85	(-2.62, 4.33)	-0.14	(-3.53, 3.26)	0.61	(-2.97, 4.20)	1.55	(-2.35, 5.45)
P for trend		**0.016**		0.982		0.190		0.120		0.371		0.715
Size at Birth												
High	0.15	(-3.39, 3.69)	**3.94**	**(0.41, 7.47)[b]**	-0.47	(-3.94, 3.01)	1.80	(-1.59, 5.19)	0.37	(-3.21, 3.96)	**5.14**	**(1.25, 9.04)[b]**
Medium	-0.43	(-3.96, 3.10)	0.26	(-3.26, 3.77)	-1.39	(-4.85, 2.07)	-0.22	(-3.60, 3.16)	-1.34	(-5.12, 2.07)	-0.76	(-4.64, 3.12)
P for trend		0.943		**0.043**		0.721		0.419		0.593		**0.004**
Non Smoking in Pregnancy – Breastfeeding												
High	-1.22	(-4.77, 2.33)	1.93	(-1.60, 5.47)	**-2.99**	**(-6.47, -0.49) [b]**	-0.03	(-3.65, 3.15)	-1.53	(-5.12, 2.07)	1.16	(-2.74, 5.06)
Medium	-2.46	(-5.97, 1.06)	-1.20	(-4.70, 2.30)	-0.83	(-4.28, 2.62)	-1.92	(-5.29, 1.45)	-2.52	(-6.07, 1.04)	2.17	(-1.70, 6.03)
P for trend		0.390		0.189		0.213		0.462		0.381		0.547
Preschool Attendance - less TV Watching												
High	**5.80**	**(2.34, 9.26)[b]**	**6.13**	**(2.69, 9.58)[b]**	**6.82**	**(3.42, 10.22)[b]**	**7.05**	**(3.74, 10.37)[b]**	**3.91**	**(0.41, 7.42)[b]**	**6.83**	**(3.02, 10.64)[b]**
Medium	**6.11**	**(2.65, 9.57)[b]**	**8.87**	**(5.42, 12.32)[b]**	**7.04**	**(3.64, 10.44)[b]**	**8.36**	**(5.04, 11.68)[b]**	**4.64**	**(1.13, 8.14)[b]**	**7.00**	**(3.19, 10.81)[b]**
P for trend		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**0.020**		**<0.001**
Parental Involvement												
High	2.98	(-0.64, 6.60)	1.77	(-1.84, 5.37)	1.54	(-2.01, 5.10)	3.14	(-0.33, 6.61)	3.17	(-0.50, 6.83)	-0.01	(-3.99, 3.98)
Medium	2.47	(-0.99, 5.94)	**3.68**	**(0.23, 7.13)[b]**	1.56	(-1.83, 4.96)	**3.39**	**(0.08, 6.71)[b]**	**3.75**	**(0.25, 7.25)[b]**	**5.04**	**(1.23, 8.84)[b]**
P for trend		0.210		0.112		0.593		0.087		0.081		**0.013**

The comparison group for the models is the first tertile, which corresponds to the lowest values of the component; N = 346

[a] All models are adjusted for child's gender, examiner, quality of assessment and for the rest patterns of early life characteristics.

[b] $p < 0.05$

Table 11A. Associations between patterns of early life characteristics and *McCarthy Scales of Children Abilities (MSCA)* at 4 years, models stratified by sex

	Verbal				Perceptual				Quantitative			
	Males		Females		Males		Females		Males		Females	
	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI
Parental Social Status												
High	**8.03**	**(4.39, 11.66)[b]**	**13.40**	**(9.77, 17.02)[b]**	**9.77**	**(5.98, 13.57)[b]**	**11.23**	**(7.69, 14.77)[b]**	**8.58**	**(4.65, 12.51)[b]**	**11.76**	**(8.18, 15.35)[b]**
Medium	**5.21**	**(1.66, 8.76)[b]**	**4.47**	**(0.94, 8.00)[b]**	**6.72**	**(3.00, 10.43)[b]**	**6.36**	**(2.92, 9.80)[b]**	**3.86**	**(0.25, 7.70)[b]**	**4.46**	**(0.97, 7.95)[b]**
P for trend		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**<0.001**		**<0.001**
Child Birth Order												
High	**-4.09**	**(-7.70, -0.49)[b]**	**-5.66**	**(-9.17, -2.16)[b]**	0.61	(-3.16, 4.37)	-2.61	(-6.03, 0.81)	-1.84	(-5.73, 2.06)	**-3.25**	**(-6.72, 0.22)[b]**
Medium	2.46	(-0.97, 5.88)	**-4.23**	**(-7.75, -0.72)[b]**	3.30	(-0.29, 6.88)	-1.80	(-5.24, 1.63)	2.26	(-1.45, 5.96)	-1.83	(-5.31, 1.64)
P for trend		**0.001**		**0.004**		0.154		0.306		0.101		0.181
Size at Birth												
High	1.16	(-2.34, 4.66)	1.27	(-2.31, 4.86)	2.09	(-1.57, 5.75)	1.14	(-2.36, 4.64)	0.04	(-3.75, 3.28)	-0.19	(-3.73, 3.35)
Medium	0.18	(-5.05, 2.09)	-0.64	(-4.08, 2.79)	-1.42	(-5.15, 2.30)	-2.35	(-5.70, 1.01)	-1.69	(-5.54, 2.16)	-2.40	(-5.80, 1.00)
P for trend		0.782		0.572		0.165		0.132		0.600		0.313
Non Smoking in Pregnancy – Breastfeeding												
High	-1.70	(-5.24, 1.85)	3.47	(-0.05, 7.00)	2.20	(-1.51, 5.90)	**6.86**	**(3.42, 10.30)[b]**	-0.74	(-4.57, 3.09)	**3.55**	**(0.06, 7.04)[b]**
Medium	-1.48	(-5.05, 2.09)	-0.92	(-4.40, 2.56)	1.52	(-2.22, 5.25)	2.28	(-1.11, 5.68)	1.75	(-2.11, 5.61)	1.61	(-1.83, 5.05)
P for trend		0.599		**0.038**		0.496		**<0.001**		0.413		0.136
Preschool Attendance - less TV Watching												
High	**3.81**	**(0.27, 7.36)[b]**	2.47	(-1.04, 5.99)	**5.84**	**(2.14, 9.54)[b]**	**4.19**	**(0.76, 7.62)[b]**	**6.75**	**(2.93, 10.58)[b]**	**4.48**	**(1.01, 7.95)[b]**
Medium	**3.55**	**(0.10, 6.99)[b]**	2.72	(-0.80, 6.23)	**7.38**	**(3.78, 10.98)[b]**	**4.32**	**(0.88, 7.75)[b]**	**4.75**	**(1.02, 8.47)[b]**	3.06	(-0.42, 6.53)
P for trend		0.056		0.246		**<0.001**		**0.020**		**0.002**		**0.036**
Parental Involvement												
High	3.23	(-0.48, 6.94)	**4.56**	**(0.97, 8.15)[b]**	0.93	(-2.95, 4.80)	2.07	(-1.43, 5.57)	0.97	(-3.04, 4.98)	1.28	(-2.27, 4.82)
Medium	1.42	(-2.07, 4.90)	**4.93**	**(1.35, 8.50)[b]**	3.63	(-0.01, 7.27)	**3.87**	**(0.38, 7.36)[b]**	1.46	(-2.31, 5.23)	2.51	(-1.03, 6.04)
P for trend		0.233		**0.011**		0.130		0.094		0.743		0.380

The comparison group for the models is the first tertile, which corresponds to the lowest values of the component; Males: N = 358, Females: N = 342

a All models are adjusted for child's gender, examiner, quality of assessment and for the rest patterns of early life characteristics.

b $p < 0.05$

Table 11B. Associations between patterns of early life characteristics and *McCarthy Scales of Children Abilities (MSCA)* at 4 years, models stratified by sex

	General Cognitive				Memory				Motor			
	Males		Females		Males		Females		Males		Females	
	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI	β[a]	95% CI
Parental Social Status												
High	**10.55**	**(6.96, 14.15)[b]**	**14.86**	**(11.38, 18.34)[b]**	**8.46**	**(4.67, 12.25)[b]**	**10.79**	**(7.08, 14.50)[b]**	2.37	(-1.76, 6.50)	**7.28**	**(3.55, 11.01)[b]**
Medium	**6.81**	**(3.30, 10.33)[b]**	**6.22**	**(2.84, 9.61)[b]**	**5.81**	**(2.11, 9.52)[b]**	**4.35**	**(0.74, 7.96)[b]**	**3.84**	**(-0.20, 7.87)[b]**	2.86	(-0.77, 6.49)
P for trend		**<0.001**		**<0.001**		**<0.001**		**<0.001**		0.173		**0.001**
Child Birth Order												
High	-2.43	(-6.00, 1.13)	**-4.90**	**(-8.26, -1.54) [b]**	-1.49	(-5.25, 2.27)	**-4.37**	**(-7.95, -0.78)[b]**	1.79	(-2.31, 5.88)	-0.60	(-4.20, 3.02)
Medium	3.07	(-0.32, 6.46)	**-3.67**	**(-7.04, -0.30)[b]**	2.01	(-1.57, 5.58)	-2.43	(-6.03, 1.17)	3.57	(-0.32, 7.47)	-0.10	(-3.72, 3.52)
P for trend		0.007		**0.011**		0.165		0.057		0.198		0.944
Size at Birth												
High	1.64	(-0.32, 6.46)	1.37	(-2.07, 4.81)	0.98	(-2.68, 4.63)	0.75	(-2.92, 4.42)	1.53	(-2.45, 5.51)	1.28	(-2.41, 4.97)
Medium	-0.62	(-1.82, 5.10)	-1.79	(-5.09, 1.50)	-2.08	(-5.80, 1.65)	-1.81	(-5.33, 1.70)	-2.43	(-6.48, 1.62)	-2.37	(-5.91, 1.17)
P for trend		0.415		0.192		0.251		0.361		0.146		0.139
Breastfeeding & Non – Smoking												
High	-0.10	(-3.61, 3.40)	**5.81**	**(2.43, 9.19)[b]**	-0.87	(-4.57, 2.83)	**4.51**	**(0.90, 8.12)[b]**	-1.78	(-5.81, 2.25)	**4.62**	**(0.99, 8.24)[b]**
Medium	0.08	(-3.45, 3.61)	1.00	(-2.34, 4.34)	0.29	(-3.43, 4.02)	0.09	(-3.48, 3.65)	-0.01	(-4.07, 4.05)	**3.28**	**(0.30, 6.86)[b]**
P for trend		0.995		**0.002**		0.808		0.021		0.598		**0.036**
Preschool Attendance - less TV Watching												
High	**5.79**	**(2.29, 9.29)[b]**	**3.90**	**(0.53, 7.27)[b]**	2.25	(-1.44, 5.95)	1.31	(-2.29, 4.90)	3.58	(-0.44, 7.61)	**5.71**	**(2.09, 9.32)[b]**
Medium	**5.83**	**(2.42, 9.24)[b]**	**3.95**	**(0.58, 7.33)[b]**	2.60	(-0.99, 6.20)	1.78	(-1.82, 5.38)	**4.84**	**(0.93, 8.76)[b]**	**4.34**	**(0.72, 7.96)[b]**
P for trend		**0.001**		**0.031**		0.307		0.604		**0.042**		**0.006**
Parental Involvement												
High	2.70	(-0.97, 6.37)	**3.93**	**(0.49, 7.37)[b]**	2.09	(-1.78, 5.96)	3.20	(-0.47, 6.87)	-1.01	(-5.23, 3.21)	0.59	(-3.10, 4.28)
Medium	2.65	(-0.80, 6.10)	**4.98**	**(1.55, 8.41)[b]**	1.76	(-1.87, 5.40)	**4.16**	**(0.50, 7.82)[b]**	**4.29**	**(0.33, 8.25)[b]**	**4.65**	**(0.97, 8.33)[b]**
P for trend		0.228		**0.011**		0.502		0.066		**0.026**		**0.027**

The comparison group for the models is the first tertile, which corresponds to the lowest values of the component; Males: N = 358, Females: N = 342

[a] All models are adjusted for child's gender, examiner, quality of assessment and for the rest patterns of early life characteristics.

[b] $p < 0.05$

Discussion

The observed association of parental social status with offspring cognitive and behavioral development was robust even after controlling for several factors that may mediate or confound this association (preschool attendance and less TV watching, breastfeeding duration and non-smoking in pregnancy, birth order, parental involvement in child's life and size at birth). This finding supports previous results that have identified similar strong associations of parental socioeconomic status and offspring neurodevelopment (219-222). Moreover, previous evidence derived from brain imaging studies have shown specific neural differentiations in children of parents of low socioeconomic status, such as less grey matter development in the hippocampus region (223, 224), in temporal, occipito-temporal and anterior frontal brain regions, which are implicated in important cognitive functions for language development, including memory and executive functioning (224).

Preschool attendance was accompanied by fewer hours of TV watching in daily life; this pattern was positively associated with nearly every cognitive domain, and negatively with ADHD-related symptoms. Previous findings from cross-country comparison studies highlighted preschool education's impact on later school achievement and intelligence (225); with these results evident even at the age of three (226). Previous observational studies have not supported any relationship of early TV watching with ADHD-related difficulties (227), however our results have supported the opposite. The adverse effect of TV watching on cognitive development has been identified by a previous study (228), which showed that the relationship is observed even before the age of 3 and suggested that this outcome may be attributed to less time spent in beneficial activities (228, 229).

Children of divorced parents have been reported to exhibit poorer cognitive (230) and educational outcomes (231). It has been stated that such an association may arise due to less available parental resources (232). Our findings support this explanation of the link between parental divorce and suboptimal cognitive outcomes, since a coexistence of married parental status with the participation of both parents' at child leisure time was identified, as well the contribution of this component to optimal cognitive development.

Non-smoking during pregnancy and enhanced breastfeeding duration component was positively associated with children's motor ability and perceptual performance in females exclusively. Breastfeeding has been recently suggested to have a protective role for ADHD-related difficulties (233). Furthermore, a gender specific effect has been previously supported by a long term, follow-up study on preterm infants, in which cognitive benefits of infant formula supplementation with long-chain polyunsaturated fatty acids

(component of human breast milk) were identified only in girls (234). Enhanced breastfeeding was accompanied with non-smoking in pregnancy which has also been previously demonstrated to pose a risk for behavioral difficulties generally, as well as for ADHD-related difficulties (235-237).

Greater child birth order was found to be negatively associated with children's general cognitive ability and mainly with the verbal domain of cognition. The same association was observed at 18 months of age in our study (238). This association remained significant after adjustment for advanced parental age. Previous studies have demonstrated that home crowding is correlated with increased chaos which is linked with less parental responsiveness to children (239, 240). Even though increased child birth order has been linked with decreased risk for developmental and mental disorders, such as autism (241) and schizophrenia (242), relevant previous studies have not supported increased ADHD risk for increased child birth (243, 244). On the contrary, our results suggest an impact of greater child birth order on increased ADHD-related difficulties, likewise was suggested by one previous study (245).

Strengths and limitations

The strengths of the present study include the population-based prospective design of the study, and the large sample size included in the analyses. The use of *MSCA*, which is a reliable, valid and comprehensive tool for child neuropsychological development is another strength of the current analyses. Moreover, the application of PCA gave the opportunity for concurrent exploration of multiple variables avoiding multi-collinearity, as well as over-adjustment implications (246). An important limitation of the current results is the high attrition rate, which corresponds to 36%; even though we cannot exclude bias due to non-participation, this is an inevitable limitation in longitudinal studies that has to be taken account when considering the current findings. Residual confounding effect of unmeasured variable consists of another possible limitation of the results; we have included multiple factors in the analyses, however development is dependent on and affected by multiple exposures, thus we cannot exclude a residual confounding effect in the current results. In addition, a number of variables of these analyses are categorical, this approach has led to loss of variability that could have strengthen the presented results.

3.2. Maternal mild thyroid dysfunction and offspring cognitive and motor development

Main findings

- Exposure to maternal hypothyroxinemia is related with reduced verbal and motor ability in early childhood.
- Exposure to maternal thyroid autoimmunity is associated with decreased child perceptual performance and motor ability in early pregnancy.
- Maternal thyroid autoimmunity in pregnancy increases the risk for adverse child cognitive development from infancy to early childhood.

Summary of the results

Associations of maternal thyroid parameters and mild thyroid dysfunction with offspring's cognitive and motor ability at 18 months, 4 years, and 6 years of age:

- Children exposed to low maternal fT4 during gestation had decreased receptive communication scores at 18 months of age (Table 12).
- Children exposed to maternal hypothyroxinemia during gestation had decreased verbal ability at 4 years and reduced motor ability at 6 years of age (Table 13).
- Children exposed to maternal thyroid autoimmunity during gestation had decreased perceptual performance and reduced motor ability at 4 years of age (Table 13).
    - Mothers with thyroid autoimmunity did not differ in fT4 concentration levels (M = 1.20, SD = ± 0.1) in comparison with euthyroid mothers without thyroid autoimmunity [(M = 1.19, SD = ± 0.1): t (962) = -0.99, p = 0.32].
- We did not identify any association between maternal subclinical hypothyroidism, hyperthyroxinemia, and child cognitive and motor development (Table 13).
- Children exposed to maternal thyroid autoimmunity during gestation had increased risk for adverse non-verbal cognitive development from infancy to early childhood (Table 14).

Table 12. Maternal thyroid stimulating hormone (TSH) concentration levels and thyroxine (fT4) concentration levels during pregnancy and child neuropsychological development at 18 months, 4 years and 6 years of age, Rhea mother-child study, Crete, Greece

	TSH - Low [a]	TSH - High [a]	fT4 - Low [a]	fT4 - High [a]
	β (95% CI) [b]	β (95% CI) [b]	β (95% CI) [b]	β (95% CI) [b]
Neuropsychological development at 18 months [c]				
Bayley Scales of Infant and Toddler Development-III				
Cognitive	-1.8 (-5.1, 1.5)	-1.4 (-4.6, 1.7)	0.1 (-3.1, 3.3)	-0.5 (-3.8, 2.9)
Expressive communication	-0.7 (-4.0, 2.5)	-1.7 (-5.0, 1.5)	-0.9 (-4.1, 2.3)	-0.5 (-3.8, 2.8)
Receptive communication	0.1 (-3.1, 3.3)	-1.5 (-4.6, 1.6)	-3.3 (-6.4, -0.1)	-0.9 (-4.2, 2.3)
Gross motor	-1.1 (-5.0, 2.7)	-3.1 (-6.9, 0.6)	1.1 (-2.7, 4.9)	-0.4 (-4.3, 3.5)
Fine motor	0.7 (-2.5, 3.9)	0.0 (-3.1, 3.1)	0.0 (-3.1, 3.2)	1.2 (-2.0, 4.4)
Neuropsychological development at 4 years [c]				
McCarthy Scales of Children Abilities (MSCA)				
Verbal	0.1 (-2.5, 2.7)	0.1 (-2.5, 2.8)	--0.1 (-2.7, 2.6)	1.3 (-1.3, 3.9)
Perceptual	-0.2 (-2.9, 2.5)	-0.4 (-3.1, 2.3)	1.3 (-1.4, 4.0)	0.1 (-2.6, 2.8)
Quantitative	0.6 (-2.2, 3.4)	0.1 (-2.7, 2.9)	0.1 (-2.7, 3.0)	1.6 (-1.2, 4.4)
General Cognitive	0.1 (-2.5, 2.7)	-0.1 (-2.7, 2.6)	0.6 (-2.0, 3.3)	1.1 (-1.5, 3.8)
Memory	-0.2 (-2.9, 2.5)	-0.2 (-2.9, 2.5)	-0.4(-3.1, 2.3)	1.5 (-1.2, 4.2)
Motor	-0.4 (-3.2, 2.5)	-0.6 (-3.5, 2.3)	-0.2 (-3.1, 2.7)	-0.1 (-3.0, 2.8)
Neuropsychological development at 6 years [c]				
RCPM: Total score	1.9 (-1.4, 5.2)	0.4 (-3.0, 3.9)	1.7 (-1.8, 5.1)	3.5 (-0.1, 6.8)
TMT: Part A (log-transformed)	-0.0 (-0.1, 0.1)	-0.0 (-0.1, 0.1)	0.3 (-0.2, 0.8)	-0.1 (-0.3, 0.1)
TMT: Part B (log-transformed)	0.0 (-0.1, 0.1)	-0.1 (-0.2, 0.1)	0.4 (-0.2, 1.0)	0.0 (-0.2, 0.3)
FTT: Dominant hand	-0.4 (-4.3, 3.5)	0.5 (-3.6, 4.6)	-0.0 (-0.1, 0.1)	-0.1(-0.1, 0.1)

FTT: Non-dominant hand	2.4 (-1.5, 6.3)	4.4 (-0.4, 8.7)	-0.1 (-0.2, 0.1)	-0.1 (-0.2, 0.1)

Abbreviations: *Raven's Coloured Progressive Matrices (RCPM), Trail Making Test (TMT) Finger Tapping Test (FTT)*; At 18 months: N = 484, At 4 years: N = 695, At 6 years: N = 488

[a] Comparison group was the middle tertile

[b] $p < 0.05$

[c] Models adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at blood sampling-thyroid assessment & quality of assessment; additional adjustment for child's age was included in the models with *TMT* and *FTT* outcomes

Table 13. Maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia and thyroid autoimmunity during pregnancy and child cognitive development at 18 months, 4 years and 6 years of age, Rhea mother-child study, Crete, Greece

	Hypothyroxinemia [a]	Subclinical Hypothyroidism [a]	Hyperthyroxinemia [a]	Thyroid Autoimmunity [a]
Neuropsychological development at 18 months [c d]				
Bayley Scales of Infant and Toddler Development-III				
Cognitive	2.6 (-3.9, 9.0)	-0.9 (-6.4, 4.7)	1.2 (-5.3, 7.7)	-3.6 (-7.9, 0.7)
Expressive communication	-4.1 (-10.6, 2.5)	-1.9 (-7.4, 3.6)	4.3 (-2.5, 11.0)	1.7 (-2.8, 6.1)
Receptive communication	-3.5 (-9.9, 2.9)	-0.2 (-5.6, 5.2)	4.3 (-2.3, 10.8)	-0.7 (-5.1, 3.6)
Gross motor	2.3 (-5.5, 10.1)	-0.5 (-7.1, 6.0)	1.1 (-9.0, 6.8)	-2.3 (-7.5, 3.0)
Fine motor	-0.1 (-6.5, 6.3)	1.4 (-4.1, 6.8)	2.6 (-3.9, 9.0)	-3.1 (-7.4, 1.2)
Neuropsychological development at 4 years [c e]				
McCarthy Scales of Children Abilities (MSCA)				
Verbal	**-6.6 (-12.3, -0.9)** [b]	-0.0 (-4.6, 4.5)	3.3 (-2.2, 8.7)	0.3 (-3.0, 3.5)
Perceptual	-0.5 (-6.5, 5.5)	0.0 (-4.8, 4.8)	-0.7 (-6.4, 5.0)	**-3.6 (-7.1, -0.2)** [b]
Quantitative	-5.7 (-11.9, 0.4)	-2.5 (-7.4, 2.4)	-0.7 (-6.6, 5.1)	-1.5 (-5.1, 2.0)
General Cognitive	-4.9 (-10.6, 0.9)	-0.6 (-5.2, 3.9)	1.0 (4.4, 6.5)	-1.4 (-4.7, 1.9)
Memory	-2.6 (-8.5, 3.3)	-2.0 (-6.6, 2.7)	2.4 (-3.3, 8.0)	1.7 (-1.7, 5.0)
Motor	0.7 (-5.7, 7.1)	-0.1 (-5.2, 5.0)	-2.5 (-8.6, 3.6)	**-4.5 (-8.2, -0.8)** [b]
Neuropsychological development at 6 years [c f]				
Raven's Coloured Progressive Matrices (RCPM)				
Total score	-2.2 (-8.9, 4.5)	-0.2 (-6.2, 5.8)	-0.2 (-7.5, 7.0)	-3.3 (-7.5, 1.0)
Trail Making Test (TMT)				
Part A: log-transformed	0.2 (-0.0, 0.5)	-0.2 (-0,4, 0.0)	-0.1 (-0.4, 0.2)	-0.1 (-0.3, 0.1)
Part B: log-transformed	0.0 (-0.3, 0.3)	-0.0 (-0.3, 0.2)	0.1 (-0.2, 0.4)	-0.0 (-0.2, 0.2)
Finger Tapping Test (FTP)				
Dominant hand	-2.0 (-10.0, 6.0)	-1.5 (-8.4, 5.4)	1.8 (-7.0, 10.6)	-2.9 (-8.0, 2.2)
Non-dominant hand	**-9.6 (-17.6, -1.5)** [b]	-1.4 (-8.4, 5.6)	-3.0, (-11.7, 5.8)	-1.4 (-6.5, 3.8)

[a] Comparison group for hypothyroxinemia models: TSH concentration levels within the trimester-specific reference ranges & fT4 ≥ 5th percentile and below the upper trimester-specific limit; comparison group for subclinical hypothyroidism models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges; comparison group for hyperthyroxinemia models: TSH concentration levels within the trimester-specific reference ranges & fT4 ≤ 95th percentile; comparison group for thyroid autoimmunity models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges, TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[b] $p < 0.05$

[c] Models adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at blood sampling-thyroid assessment & quality of assessment; additional adjustment for child's age was included in the models with *TMT* and *FTP* outcomes

[d] Hypothyroxinemia: N = 22, Subclinical Hypothyroidism: N = 29, Hyperthyroxinemia: N = 20, Thyroid autoimmunity: N = 50

[e] Hypothyroxinemia: N = 26, Subclinical Hypothyroidism: N = 38, Hyperthyroxinemia: N = 28, Thyroid autoimmunity: N = 83

[f] Hypothyroxinemia: N = 22, Subclinical Hypothyroidism: N = 27, Hyperthyroxinemia: N = 19, Thyroid autoimmunity: N = 56

Table 14. Maternal hypothyroxinemia, subclinical hypothyroidism and thyroid autoimmunity during pregnancy and longitudinal trajectories of child non-verbal cognitive development (Group Based Trajectory Modelling) from 18 months to 6 years of age, Rhea mother-child study, Crete, Greece

	Non-verbal cognitive development trajectories [c d]					
	Low		High-Decreasing		Low-Increasing	
	RRR	95% CI	RRR	95% CI	RRR	95% CI
Hypothyroxinemia [a]	1.3	(0.4, 3.7)	1.7	(0.7, 4.3)	0.9	(0.4, 2.2)
Subclinical Hypothyroidism [a]	1.0	(0.5, 2.2)	0.6	(0.3, 1.3)	0.8	(0.4, 1.7)
Hyperthyroxinemia	1.4	(0.5, 3.9)	1.4	(0.6, 3.7)	1.6	(0.6, 4.2)
Thyroid Autoimmunity [a]	**2.7**	**(1.4, 5.2)** [b]	**2.2**	**(1.2, 4.0)** [b]	**1.8**	**(1.0, 3.2)** [b]

[a] Comparison group for hypothyroxinemia models: TSH concentration levels within the trimester-specific reference ranges & fT4 ≥ 5th percentile and below the upper trimester-specific limit; comparison group for subclinical hypothyroidism models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges; comparison group for hyperthyroxinemia models: TSH concentration levels within the trimester-specific reference ranges & fT4 ≤ 95th percentile and above the lower trimester-specific limit; comparison group for thyroid autoimmunity models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges, TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[b] $p < 0.05$

[c] Reference trajectory: continuously high non-verbal cognitive development from 18 months to 6 years

[d] Models adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at thyroid assessment & quality of assessment

We did not observe any significant differentiation when we excluded participants who were taking thyroid medication during pregnancy (N = 96) (Table 15), nor when we used TPO-Abs and Tg-Abs as exposures separately (Table 16).

Table 15. Maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia, and thyroid autoimmunity during pregnancy and child cognitive development at 18 months, 4 years and 6 years, excluding mothers under thyroid medication during pregnancy, Rhea mother-child study, Crete, Greece

	Hypothyroxinemia [a]	Subclinical Hypothyroidism [a]	Hyperthyroxinemia [a]	Thyroid Autoimmunity [a]
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Neuropsychological development at 18 months: Bayley Scales of Infant and Toddler Development-III [C]				
Cognitive	2.3 (-4.5, 9.1)	-1.2 (-7.9, 5.4)	3.7 (-11.2, 18.5)	-4.0 (-9.3, 1.3)
Expressive communication	-4.6 (-11.3, 2.1)	-1.6 (-8.1, 4.8)	-4.4 (-19.2, 10.4)	0.1 (-5.2, 5.3)
Receptive communication	-3.6 (-10.2, 2.9)	2.0 (-4.3, 8.3)	-2.1 (-16.5, 12.3)	-0.4 (-5.5, 4.7)
Gross motor	2.7 (-5.4, 11.0)	1.9 (-6.0, 9.7)	4.0 (-14.0, 22.0)	-2.1 (-8.6, 4.3)
Fine motor	-0.3 (-6.8, 6.2)	0.2 (-6.2, 6.5)	4.6 (-9.5, 18.8)	-3.9 (-8.9, 1.1)
Neuropsychological development at 4 years: McCarthy Scales of Children Abilities (MSCA) [C]				
Verbal	**-6.5 (-12.4, -0.6)** [b]	0.4 (-5.1, 6.0)	2.8 (-7.7, 13.2)	-0.7 (-4.6, 3.1)
Perceptual	-1.7 (-8.0, 4.5)	0.8 (-5.1, 6.6)	-0.1 (-11.0, 10.9)	**-4.9 (-9.0, -0.9)** [b]
Quantitative	-5.1 (-11.5, 1.3)	-3.4 (-9.3, 2.5)	2.2 (-9.1, 13.4)	-2.3 (-6.4, 1.9)
General Cognitive	-5.2 (-11.1, 0.8)	-0.2 (-5.8, 5.4)	2.0 (-8.5, 12.5)	-2.5 (-6.4, 1.4)
Memory	-2.1 (-8.2, 3.9)	-2.3 (-8.0, 3.3)	5.1 (-5.6, 15.9)	-0.5 (-4.4, 3.5)
Motor	-0.7 (-7.3, 5.8)	0.1 (-6.0, 6.2)	-9.0 (-20.6, 2.6)	**-5.0 (-9.3, -0.7)** [b]
Neuropsychological development at 6 years [C]				
Raven's Coloured Progressive Matrices (RCPM)				
Total score	-2.0 (-8.9, 4.9)	-2.3 (-9.3, 4.7)	-4.2 (-16.2, 7.7)	-3.4(-8.4, 1.6)
Trail Making Test (TMT)				
Part A: log-transformed	**0.3 (0.0, 0.6)** [b]	-0.2 (-0.5, 0.1)	-0.2 (-0.7, 0.2)	-0.1(-0.3, 0.1)
Part B: log-transformed	0.1 (-0.2, 0.4)	0.0 (-0.3, 0.3)	-0.1 (-0.6, 0.5)	-0.1(-0.3, 0.2)
Finger Tapping Test (FTP)				
Dominant hand	-3.8 (-11.9, 4.4)	2.2 (-5.9, 10.4)	-5.5 (-19.3, 8.4)	-5.8(-11.9, 0.2)
Non-dominant hand	**-9.5 (-17.7, -1.3)** [b]	-0.4 (-8.6, 7.8)	-8.5 (-22.4, 5.3)	-2.1(-8.3, 4.2)

[a] Comparison group for hypothyroxinemia: TSH concentration levels within the trimester-specific reference ranges & fT4 ≥ 5th percentile and below the upper trimester-specific limit; comparison group for subclinical hypothyroidism: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges; comparison group for hyperthyroxinemia: TSH concentration levels within the trimester-specific reference ranges & fT4 ≤ 95th percentile; comparison group for thyroid autoimmunity: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges, TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[b] $p < 0.05$

[c] Models are adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at thyroid assessment, quality of assessment; additional adjustment for child's age was included in the models with TMT and FTP outcomes

Table 16. Maternal thyroid peroxidase antibodies (TPO-Abs +) and maternal thyroglobulin antibodies (Tg-Abs +) during pregnancy and child cognitive development at 18 months, 4 years and 6 years of age, Rhea mother-child study, Crete, Greece

	TPO-Abs (+) [a]		Tg-Abs (+) [a]	
	β	95% CI	β	95% CI
Neuropsychological development at 18 months [c]				
Bayley Scales of Infant and Toddler Development-III				
Cognitive	-3.8	(-8.4, 0.7)	-1.1	(-7.5, 5.3)
Expressive communication	0.0	(-4.7, 4.7)	5.6	(-0.9, 12.1)
Receptive communication	-2.2	(-6.7, 2.4)	2.6	(-3.8, 9.0)
Gross motor	-2.3	(-7.8, 3.3)	2.4	(-5.3, 10.2)
Fine motor	-3.8	(-8.3, 0.7)	1.2	(-5.1, 7.5)
Neuropsychological development at 4 years [d]				
McCarthy Scales of Children Abilities (MSCA)				
Verbal	-1.1	(-4.6, 2.4)	0.7	(-3.8, 5.2)
Perceptual	**-4.5**	**(-8.1, -0.8)[b]**	**-5.2**	**(-9.9, -0.5)[b]**
Quantitative	-3.1	(-6.8, 0.7)	-1.6	(-6.4, 3.3)
General Cognitive	-2.7	(-6.2, 0.8)	-1.9	(-6.4, 2.7)
Memory	0.1	(-3.5, 3.8)	1.2	(-3.4, 5.9)
Motor	**-6.1**	**(-10.0, -2.1)[b]**	-4.7	(-9.8, 0.4)
Neuropsychological development at 6 years [e]				
RCPM: Total score	-4.2	(-8.7, 0.3)	-3.3	(-9.0, 2.4)
TMT: Part A (log-transformed)	-0.1	(-0.3, 0.1)	-0.2	(-0.4, 0.0)
TMT: Part B (log-transformed)	0.1	(-0.1, 0.3)	-0.0	(-0.3, 0.2)
FTT: Dominant hand	-2.6	(-8.0, 2.7)	2.0	(-5.0, 8.9)
FTT: Non-dominant hand	-0.9	(-6.4, 4.5)	0.6	(-6.7, 7.8)

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

Models are adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at thyroid assessment, quality of assessment; additional adjustment for child's age was included in the models with *TMT* and *FTT* outcomes

[a] Comparison group euthyroid pregnant women with TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[b] $p < 0.05$

[c] TPO-Antibodies (+) : N = 44, Tg-Antibodies (+) : N = 22

[d] TPO-Antibodies (+) : N = 70, Tg-Antibodies (+) : N= 40

[e] TPO-Antibodies (+) : N = 48, Tg-Antibodies (+) : N = 28

Discussion

Current results confirm findings of previous observational studies which supported that maternal hypothyroxinemia is associated with decreased child neuropsychological development (96, 100, 133-135, 138). Even though thyroid hormones impact on fetal neurodevelopment through the interaction of T3 with nuclear receptors of the fetal nervous system cells, T3 is formed locally through the deiodination of maternal T4 by iodothyronine deiodinase enzymes in glial cells (114). Furthermore, findings from animal studies have demonstrated that induced maternal hypothyroxinemia leads to atypical neuronal migration and structural alterations in the somatosensory cortex and hippocampus, regions which are important for learning, memory, basic perceptual skills and higher-order cognitive abilities (164, 247). However, the significance of maternal hypothyroxinemia as a marker of thyroid dysfunction in pregnancy has been challenged due to methodological issues in the relevant studies and due to concerns about the clinical relevance of hypothyroxinemia. The methodological problems that limit the interpretation of the studies include the differences in the definition of hypothyroxinemia, the diagnostic inaccuracy of fT4 measurements due to the physiological changes caused by pregnancy and due to the biases of the widely used automated immunoassays, the normal variability of fT4 concentration depending on the gestational age and the inadequate adjustment of the models for possible confounders of the explored associations (37, 93, 142, 248, 249). Furthermore, observational studies that did not identify any association of maternal hypothyroxinemia with several pregnancy outcomes (70), as well as Randomized Controlled Trials (RCTs) that did not find any cognition benefit in offspring exposed to maternal hypothyroxinemia or other mild thyroid dysfunction after levothyroxine medication (75, 93, 250, 251) have resulted in concerns about the clinical importance of maternal hypothyroxinemia and in the no treatment-recommendation by the latest guidelines for thyroid dysfunction management in pregnancy (37).

Our results also suggest that maternal thyroid autoimmunity is associated with decreased perceptual performance and motor scores at 4 years and with increased risk for disadvantageous non-verbal cognitive development from infancy to early childhood. Previous findings regarding the role of maternal thyroid autoimmunity on child neuropsychological development have been diverse (90, 91, 135, 145, 146); and it has been suggested that the different iodine status between populations may be the underlying cause of this heterogeneity (145). Current results support the adverse impact of maternal thyroid autoimmunity on child neuropsychological development in a Greek, iodine sufficient population.

The observed associations between maternal thyroid autoimmunity and offspring neuropsychological development can be explained through an impact of elevated of maternal thyroid

antibodies on the concentration levels of maternal fT4. It has previously been shown that elevated maternal TPO-antibodies impair thyroidal stimulation caused by human chorionic gonadotropin (hCG) (252). HCG is a pregnancy-specific hormone which binds to TSH receptors and ensures fT4 availability during pregnancy (145, 253). Even though, we did not identify any significant differences of maternal fT4 concentration levels between mothers with and without elevated thyroid antibodies, later fT4 insufficiency during pregnancy cannot be excluded. In addition, maternal TSH concentration levels were higher in women with thyroid autoimmunity in comparison with those with normal levels of antibodies; this difference suggests an impact of the elevated antibodies on thyroidal function. This combination of factors (i.e. elevated thyroid antibodies with high TSH concentration), regardless of the presence or the absence of subclinical hypothyroidism, may synergistically increase the risk of adverse pregnancy outcomes (253).

Findings from animal studies support the observed associations, since they have demonstrated that the primary brain regions affected by decreased availability of maternal fT4 are the hippocampus, which is involved in memory and learning, the cortex, which is involved in perceptual skills and higher-order cognitive abilities, and the cerebellum, which is involved in motor abilities and motor coordination (254). We have found no association of maternal subclinical hypothyroidism and offspring cognitive and motor development corroborating the results of previous large population-based studies (96, 133) and failing to replicate the findings of other studies (135, 136, 144). Current findings support that thyroid autoimmunity regardless the presence of subclinical hypothyroidism is linked with adverse offspring outcomes. We have also explored the role of increased maternal fT4 concentration and hyperthyroxinemia in offspring cognitive and motor development, since a recent MRI study has suggested that both low and high concentration of maternal fT4 in early pregnancy are related with decreased intelligence and decreased grey matter and cortex volume (100). We did not find any association of maternal hyperthyroxinemia with offspring cognitive and motor development. This null finding may be the consequence of low power in the specific analysis, since we have few cases with hyperthyroxinemia in our population and further research is necessary to elucidate if hyperthyroxinemia is linked with adversary offspring developmental outcomes.

Strengths and limitations

The strengths of the present study include its population-based prospective design, the long follow-up period, and the reliable valid, and comprehensive psychometric instruments that were used to assess child cognition and motor development. The use of GBTM to identify non-verbal cognitive development

trajectories and explore the longitudinal impact of mild maternal thyroid dysfunction on child non-verbal cognition is another strength and a novelty of the current analysis. Thyroid hormones were measured at a single-timepoint during gestation, therefore the measurements might reflect a transient dysfunction. However, it has been previously suggested that longitudinal and single-timepoint thyroid assessments are highly correlated (255). Bias due to non-participation and loss to follow up might influence the results; non-participants did not differ regarding maternal thyroid parameters but they were more likely to have younger, non-Greek, multiparous, and less educated mothers compared with the participants of this study. In addition, residual confounding effect of unmeasured variables cannot be excluded, even though the models have been adjusted for multiple possible confounders. Finally, mothers who took thyroid medication during pregnancy were not excluded from the main analyses, but sensitivity analyses excluding these participants did not support substantially different results.

3.3. Maternal mild thyroid dysfunction and offspring behavioral and emotional development

Main findings

- Maternal subclinical hypothyroidism in early pregnancy was associated with behavioral and emotional difficulties in early childhood.
- Maternal thyroid autoimmunity further reinforced the association of subclinical hypothyroidism with child behavioral and emotional difficulties in early childhood.
- Elevated maternal thyroid antibodies in euthyroid pregnant women were associated with adverse behavioral outcomes.
- No association between maternal hypothyroxinemia and child behavioral and emotional development was observed.
- No sex-related differences were identified in the association of mild maternal thyroid dysfunction with child behavioral and emotional development.

Summary of the results

Associations of maternal thyroid mild dysfunction with behavioral and emotional symptoms in preschool age (4 years of age):

- Children exposed to maternal subclinical hypothyroidism during pregnancy had higher hyperactivity, impulsivity, total ADHD-related symptoms, and internalizing symptoms, compared to children of euthyroid mothers.
    - Post-hoc analyses showed that increased internalizing symptoms were primarily driven by emotional problems.
- Children of subclinically hypothyroid mothers with elevated thyroid antibodies had higher hyperactivity, inattention, impulsivity, ADHD-related symptoms, externalizing symptoms, and internalizing symptoms, compared to children of euthyroid mothers with low concentration levels of thyroid antibodies.
    - Post-hoc analyses showed that internalizing symptoms were primarily driven by emotional problems and externalizing symptoms by conduct problems.
- Children of euthyroid mothers with elevated thyroid antibodies had higher inattention, compared to children of euthyroid mothers with low concentration of thyroid antibodies.
- No association of maternal hypothyroxinemia with child behavioral and emotional symptoms was observed.

Table 17 presents the estimates of the associations of maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity with offspring's behavioral end emotional symptoms at 4 years of age.

Table 17. Maternal subclinical hypothyroidism, maternal thyroid autoimmunity, and maternal hypothyroxinemia during gestation and children's behavioral symptoms at 4 years of age *[Attention Deficit Hyperactivity Disorder Test (ADHDT)* and *Strengths and Difficulties Questionnaire (SDQ)]*

	Subclinical Hypothyroidism (SCH) [a c]		SCH & Thyroid autoimmunity (+) [a c]		SCH & Thyroid autoimmunity (-) [a c]		Euthyroidism & Thyroid autoimmunity [a c]		Hypothyroxinemia [a c]	
	β	95% CI	β	95% CI	β	95% CI	β	95%CI	β	95% CI
Attention Deficit Hyperactivity Disorder Test										
Hyperactivity	**2.4**	**(0.7, 4.1)[b]**	**4.6**	**(1.8, 7.4)[b]**	0.9	(-1.2, 3.1)	-0.1	(-1.4, 1.1)	0.7	(-1.4, 2.8)
Inattention	1.0	(-0.5, 2.4)	**3.1**	**(0.7, 5.4)[b]**	0.0	(-1.7,1.8)	**1.1**	**(0.0, 2.2)[b]**	1.0	(-0.8, 2.8)
Impulsivity	**1.5**	**(0.1, 2.8)[b]**	**2.9**	**(0.7, 5.1)[b]**	0.6	(-1.1, 2.3)	0.4	(-0.6, 1.4)	1.7	(-0.0, 3.3)
ADHDT - Total	**4.8**	**(0.8, 8.8)[b]**	**10.6**	**(4.2, 17.1)[b]**	1.6	(-3.3, 6.5)	1.4	(-1.5, 4.3)	3.4	(-1.5, 8.2)
Strengths & Difficulties Questionnaire										
Internalizing score	**0.9**	**(0.1, 1.7)[b]**	**1.5**	**(0.3, 2.8)[b]**	0.5	(-0.4, 1.5)	0.3	(-0.3, 0.9)	-0.0	(-1.0, 0.9)
Externalizing score	0.8	(-0.2, 1.8)	**2.2**	**(0.5, 3.8)[b]**	0.1	(-1.2, 1.4)	0.4	(-0.4, 1.1)	0.5	(-0.8, 1.8)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy and maternal smoking status during pregnancy; Subclinical Hypothyroidism: N = 41, Subclinical Hypothyroidism & thyroid antibodies + status: N = 15, Subclinical Hypothyroidism & Thyroid antibodies – status: N = 26, Euthyroidism & Thyroid autoimmunity + status: N = 82, Hypothyroxinemia: N = 25

[b] $p < 0.05$

[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges

[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[e] Reference group: TSH concentration levels within the trimester-specific reference ranges & fT4 > 5th percentile (fT4 ≥ 0.95 ng/dL)

Behavioral and emotional symptoms in early school age (6 years of age):

- Children exposed to maternal subclinical hypothyroidism had higher oppositional-defiant problems and externalizing symptoms, compared to children of euthyroid mothers.
  - Post-hoc analyses showed that externalizing symptoms were primarily driven by oppositional-defiant problems and conduct problems.
- Children of subclinically hypothyroid mothers with elevated thyroid antibodies had higher hyperactivity, oppositional problems, internalizing symptoms and externalizing symptoms, compared to children of euthyroid mothers with low concentration levels of thyroid antibodies.
  - Post-hoc analyses showed that internalizing symptoms were primarily driven by somatic problems and externalizing symptoms were driven by hyperactivity/inattention, oppositional-defiant and conduct problems.
- Children of subclinically hypothyroid mothers with low concentration levels of thyroid antibodies had higher externalizing symptoms, compared to children of euthyroid mothers with low levels of thyroid antibodies.
  - Post-hoc analyses showed that externalizing symptoms were primarily driven by oppositional problems.
- Children of euthyroid mothers with elevated thyroid antibodies had increased externalizing symptoms, compared to children of euthyroid mothers with low levels of thyroid antibodies.
  - Post-hoc analyses showed that externalizing symptoms were primarily driven by hyperactivity/inattention.
- No association of maternal hypothyroxinemia with child behavioral and emotional symptoms was observed.

Table 18 presents the estimates of the associations of maternal subclinical hypothyroidism, maternal hypothyroxinemia, and maternal thyroid autoimmunity with offspring behavioral end emotional symptoms at 6 years of age.

Table 18. Maternal subclinical hypothyroidism, maternal autoimmunity, and maternal hypothyroxinemia during gestation and children's behavioral symptoms at 6 years of age *[Conners' Parent Rating Scale-Revised: Short form (CPRS-R:S)* and *Child Behavior Checklist (CBCL), Parent Report Form]*

	Subclinical Hypothyroidism (SCH) [a c]		SCH & Thyroid autoimmunity (+) [a c]		SCH & Thyroid autoimmunity (-) [a c]		Euthyroidism & Thyroid autoimmunity [a c]		Hypothyroxinemia [a c]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Conners' Parent Rating Scale-Revised: Short form Scales										
Oppositional	**1.2**	**(0.0, 2.5)[b]**	**3.1**	**(1.2, 4.9)[b]**	0.2	(-1.3, 1.7)	0.8	(-0.1, 1.7)	-0.8	(-2.2, 0.6)
Inattention	0.8	(-0.5, 2.0)	1.9	(-0.1, 3.9)	0.6	(-1.6, 2.9)	0.6	(-0.3, 1.6)	-0.8	(-2.2, 0.6)
Hyperactivity	0.4	(-0.7, 1.6)	**1.8**	**(0.0, 3.7)[b]**	-0.5	(-2.0, 0.9)	0.2	(-0.7, 1.0)	0.6	(-0.8, 1.9)
Total index	0.5	(-1.7, 2.6)	2.7	(-0.7, 6.0)	-0.8	(-3.4, 1.7)	0.6	(-0.9, 2.1)	-0.2	(-2.6, 2.1)
Child Behavior Checklist 's broad-band scales										
Internalizing	1.6	(-0.1, 3.2)	**2.8**	**(0.1, 5.4)[b]**	1.0	(-1.1, 3.1)	1.2	(-0.1, 2.4)	0.9	(-1.1, 2.8)
Externalizing	**4.6**	**(2.2, 6.9)[b]**	**6.5**	**(2.9, 10.2)[b]**	**3.5**	**(0.7, 6.4)[b]**	**1.8**	**(0.1, 3.5)[b]**	-0.9	(-3.4, 1.7)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy and maternal smoking status during pregnancy; Subclinical Hypothyroidism: N = 30, Subclinical Hypothyroidism & thyroid autoimmunity + status: N = 11, Subclinical Hypothyroidism & Thyroid autoimmunity – status: N = 19, Euthyroidism & Thyroid autoimmunity + status: N = 62, Hypothyroxinemia: N = 23

[b] $p < 0.05$

[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges

[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs < 35 IU/mL & or Tg ≤ 40 IU/mL

[e] Reference group: TSH concentration levels within the trimester-specific reference ranges & fT4 > 5th percentile (fT4 ≥ 0.95 ng/dL)

Additional analyses:

- We did not find any association between maternal thyroid parameters (TSH, fT4) and offspring behavioral and emotional symptoms (Table 19).
- We did not find any interaction effect of child sex on the association of maternal mild thyroid autoimmunity with offspring behavioral and emotional symptoms.
- Additional adjustment for child TSH at 4 years of age in a subsample of the population with available data (as a proxy of fetal thyroid functioning), further reinforced the associations of maternal subclinical hypothyroidism during pregnancy and child behavioral and emotional difficulties at 6 years of age (Table 20).
- The exclusion of the mothers who took thyroid medication during pregnancy did not cause any meaningful change of the results (Table 21).
- Additional adjustment for maternal iron and iodine status, measured at the 13^{th} gestational week, further reinforced the observed associations (Table 22).
- The analyses regarding maternal hypothyroxinemia were repeated applying an alternative fT4 cut-off point (10^{th} percentile) for the definition of hypothyroxinemia, no association of maternal thyroid mild dysfunction and child behavioral and emotional difficulties was found.

Table 19. Maternal thyroid hormones [Thyroid Stimulating Hormone(TSH) and free thyroxine (fT4)] during early pregnancy and children's behavioral symptoms at 4 and 6 years of age *[Attention Deficit Hyperactivity Disorder Test (ADHDT)* and *Strengths and Difficulties Questionnaire (SDQ)], [Conners' Parent Rating Scale-Revised: Short form (CPRS-R:S)* and *Child Behavior Checklist (CBCL), Parent Report Form]*

	Maternal TSH - Low [b]		Maternal TSH-High [b]		Maternal fT4 – Low [b]		Maternal fT4 – High [b]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
4 years of age								
ADHDT-total	0.3	(-2.1, 2.8)	0.2	(-2.2, 2.6)	0.9	(-1.5, 3.3)	0.9	(-1.5, 3.3)
SDQ-total	-0.4	(-1.3, 0.6)	-0.5	(-1.4, 0.5)	-0.1	(-1.0, 0.8)	0.3	(-0.6, 1.2)
6 years of age								
CPRS-R:S-total	-0.5	(-1.7, 0.8)	-0.7	(-2.0, 0.7))	0.3	(-1.0, 1.6)	1.2	(-0.1, 2.3)
CBCL-Internalizing	-0.8	(-1.8, 0.2)	-0.3	(-1.3, 0.8)	0.5	(-0.5, 1.7)	0.6	(-0.5, 1.7)
CBCL-Externalizing	-0.6	(-2.1, 0.7)	0.7	(-0.8, 2.2)	0.1	(-1.4, 1.5)	0.9	(-0.5, 2.3)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy, maternal smoking status during pregnancy; At 4 years: N = 647, At 6 years: N = 489

[b] The reference group for the models is the middle tertile

Table 20. Maternal subclinical hypothyroidism, maternal autoimmunity and maternal hypothyroxinemia during gestation and children's behavioral and emotional problems at 6 years of age [*Conners' Parent Rating Scale-Revised: Short form (CSRS-R: S), Child Behavior Checklist (CBCL), Parent report form*], models repeated to a subsample with available child thyroid data at 4 years of age

	Subclinical Hypothyroidism [a c]		Thyroid autoimmunity [a d]		Hypothyroxinemia [a e]	
	β	95%CI	β	95%CI	β	95%CI
CPRS-R: S						
Oppositional	**3.3**	**(1.3, 5.4)[b]**	0.5	(-1.0, 2.0)	-0.9	(-4.1, 2.3)
Inattention	0.4	(-1.6, 2.5)	-0.3	(-1.7, 1.0)	0.7	(-2.1, 3.4)
Hyperactivity	1.7	(-0.3, 3.8)	0.2	(-1.3, 1.6)	0.7	(-2.1, 3.6)
CPRS-R:S: total score	1.8	(-1.9, 5.4)	-0.2	(-2.5, 2.1)	0.7	(-3.7, 5.1)
CBCL						
Internalizing	2.8	(-0.1, 5.7)	-0.1	(-2.3, 2.1)	-0.5	(-4.8, 3.7)
Externalizing	**7.1**	**(2.8, 11.4)[b]**	0.9	(-2.1, 3.9)	-1.7	(-7.5, 4.1)

Abbreviations: *Conners' Parent Rating Scale-Revised: Short form Scales (CPRS-R:S), Child Behavior Checklist' broadband scales (CBCL)*

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy, maternal smoking status during pregnancy

[b] $p < 0.05$

[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges

[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[e] Reference group: TSH concentration levels within the trimester-specific reference ranges & fT4 > 5^{th} percentile (fT4 ≥ 0.95 ng/dL)

Table 21. Maternal subclinical hypothyroidism, maternal autoimmunity and maternal hypothyroxinemia during gestation and children's behavioral and emotional problems at 4 years of age [*Attention Deficit Hyperactivity Disorder Test (ADHDT), Strengths and Difficulties Questionnaire (SDQ)*] and at 6 years of age, *Conners' Parent Rating Scale-Revised: Short form (CSRS-R: S), Child Behavior Checklist (CBCL), Parent report form*], excluding participants who took thyroid medication during pregnancy

	Subclinical Hypothyroidism [a c]		Thyroid autoimmunity [a d]		Hypothyroxinemia [a e]	
	B	95%CI	B	95%CI	β	95%CI
Attention Deficit Hyperactivity Disorder Test (ADHDT)						
Hyperactivity	**3.6**	**(0.9, 6.3)[b]**	0.4	(-1.4, 2.2)	-1.3	(-4.6, 1.9)
Inattention	**2.2**	**(0.0, 4.5)[b]**	**1.9**	**(0.4, 3.3)[b]**	-0.3	(-3.0, 2.4)
Impulsivity	1.7	(-.44, 2.99)	1.0	(-0.4, 2.5)	1.8	(-0.8, 4.5)
ADHDT: total score	**7.5**	**(1.3, 13.8)[b]**	3.3	(-0.8, 7.4)	0.2	(-7.2. 7.7)
Strengths & Difficulties Questionnaire (SDQ)						
Emotional symptoms	0.6	(-0.3, 1.4)	**0.7**	**(0.1, 1.3)[b]**	-0.5	(-1.5, 0.6)
Conduct problems	0.4	(-0.4, 1.2)	**0.6**	**(0.0, 1.1)[b]**	0.0	(-1.0, 1.0)
Hyperactivity/Inattention	**1.1**	**(0.1, 2.2)[b]**	0.2	(-0.5, 0.9)	-0.5	(-1.8, 0.7)
Peer relationship problems	0.6	(-0.2, 1.3)	0.1	(-0.4, 0.6)	-0.1	(-1.0, 0.7)
Prosocial behavior	-0.2	(-1.2, 0.7)	0.3	(-0.3, 0.9)	-0.7	(-1.9, 0.4)
SDQ: total score	**2.7**	**(0.3, 5.1)[b]**	**1.6**	**(0.1, 3.2)[b]**	-1.1	(-3.9, 1.7)
Conners' Parent Rating Scale-Revised: Short form Scales (CPRS_R: S)						
Oppositional	**3.7**	**(1.6, 5.9)[b]**	0.4	(-0.9, 1.6)	0.0	(-2.2, 2.2)
Inattention	1.7	(-0.6, 3.9)	0.1	(-1.2, 1.5)	-0.8	(-3.0, 1.3)
Hyperactivity	1.7	(-0.5, 3.9)	-0.1	(-1.3, 1.2)	0.5	(-1.5, 2.6)
CPRS-R:S: total score	2.7	(-1.4, 6.9)	-0.9	(-3.0, 1.2)	-0.9	(-4.4, 2.6)
Child Behavior Checklist (CBCL)						
Internalizing score	2.9	(-0.1, 5.8)	0.8	(-1.0, 2.5)	0.9	(-2.0, 3.8)

Externalizing score	**8.7**	**(4.7, 12.7)[b]**	0.7	(-1.6, 3.0)	-0.0	(-3.8, 3.8)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy, maternal smoking status during pregnancy [b] $p < 0.05$

[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges

[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[e] Reference group: TSH concentration levels within the trimester-specific reference ranges & fT4 ≥ 0.95 ng/dL

Table 22. Maternal subclinical hypothyroidism, maternal autoimmunity and maternal hypothyroxinemia during gestation and children's behavioral symptoms at 4 years of age *[Attention Deficit Hyperactivity Disorder Test (ADHDT)* and *Strengths and Difficulties Questionnaire (SDQ)],* models repeated to a subsample with available data on maternal urinary iodine and maternal iron status during pregnancy

	Subclinical Hypothyroidism [a c]		Thyroid autoimmunity [a d]		Hypothyroxinemia [a]	
	β	95% CI	β	95%CI	β	95% CI
Attention Deficit Hyperactivity Disorder Test						
Hyperactivity	**3.3**	**(1.2, 5.5)[b]**	0.1	(-1.5.5, 1.5)	-1.5	(-4.7, 1.7)
Inattention	1.3	(-0.5, 3.1)	**1.7**	**(0.4, 2.9)[b]**	-0.5	(-3.1, 2.2)
Impulsivity	1.4	(-0.4, 3.2)	0.5	(-0.7, 1.8)	1.6	(-1.1, 4.2)
ADHDT – Total score	**6.0**	**(0.9, 11.1)[b]**	2.2	(-1.3, 5.7)	-0.4	(-7.8, 7.0)
Strengths & Difficulties Questionnaire						
Emotional	**0.8**	**(0.1, 1.5)[b]**	0.3	(-0.2, 0.8)	-0.4	(-1.4, 0.6)
Conduct	0.6	(-0.1, 1.2)	0.4	(-0.0, 0.9)	-0.1	(-1.0, 0.9)
Hyperactivity/ Inattention	0.8	(-0.0, 1.7)	0.1	(-0.5, 0.7)	-0.5	(-1.7, 0.7)
Peer relationship	**0.6**	**(0.0, 1.2)[b]**	-0.0	(-0.4, 0.4)	-0.2	(-1.1, 0.7)
Prosocial behavior	-0.1	(-0.8, 0.7)	0.1	(-0.4, 0.7)	-0.6	(-1.8, 0.5)
SDQ – Total score	**2.9**	**(0.9, 4.8)[b]**	0.8	(-0.5, 2.1)	-1.1	(-3.9, 1.6)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy, maternal smoking status during pregnancy, maternal iron and maternal iodine status during pregnancy

[b] $p < 0.05$

[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges

[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL

[e] Reference group: TSH concentration levels within the trimester-specific reference ranges & fT4 > 5th percentile (fT4 ≥ 0.95 ng/dL)

Discussion

Previous findings have indirectly supported the role of maternal thyroid dysfunction in child behavioral problems acknowledging a link between generalized resistance to thyroid hormones and increased ADHD symptoms in adults (256) as well as a greater risk for ADHD symptoms' manifestation for children exposed to mild iodine deficiency (257). More directly related results have supported that maternal mild thyroid dysfunction is associated with child behavior problems, with various indicators predicting child behavioral development (91, 170-174, 258).

Several previous studies regarding maternal thyroid function and child behavioral development have supported that maternal TSH is a valid predictor of child behavioral problems (170, 173, 174). Our findings suggest that subclinical hypothyroidism predicts child behavioral and emotional problems, which indirectly supports that TSH is a sensitive indicator of maternal thyroid dysfunction. It has been previously suggested that although TSH is not the biologically active hormone in the fetal brain, it might be a reliable indicator of thyroid dysfunction because of the pituitary feedback mechanism and its regulatory role for thyroid hormones secretion (170). Furthermore, the detected associations were stronger and evident in more scales in children of subclinically hypothyroid mothers with elevated thyroid antibodies. These findings might be attributed to the additional increase of TSH concentration, which was evident in the present results. However, an additional direct impact of thyroid antibodies cannot be excluded, since there is evidence that TPO-Abs positivity impairs thyroid stimulation by hCG (100).

Maternal thyroid autoimmunity (elevated TPO-antibodies) has been previously related with decreased motor and intellectual development (34, 90), greater autism risk (259), and increased attention deficit/hyperactivity problems at 3 years of age (91). The current findings support an association of elevated thyroid antibodies in euthyroid women with child inattention problems at 4 years and hyperactivity/inattention at 6 years. These associations might be attributed to further alterations of thyroid hormones' levels between the two groups in later pregnancy, which were small but already

evident in the present results. Additional possible explanations involve an adverse effect of maternal thyroid antibodies on fetal thyroid functioning that may result in transient fetal hypothyroidism (260), or the impact of a preexisting maternal subclinical autoimmune condition, which results in elevated thyroid antibodies and child behavioral problems development.

Interestingly, the observed associations of this study involve both externalizing and internalizing symptoms. Deficits in cognitive control, behavioral inhibition, and emotional regulation are common in clinical disorders that are relevant with the detected symptoms (261-265). These cognitive abilities implicate the prefrontal cortex, the hippocampus, and the cerebellum (262, 266-268); neural regions that are morphologically affected by insufficient levels of thyroid hormones during gestation (148, 163, 164). Moreover, insufficiency of thyroid hormones may result in long-lasting neurophysiological changes in the dopaminergic and the noradrenergic systems, which are implicated in emotional and behavioral manifestation (143, 269-271).

The null findings regarding the association between maternal hypothyroxinemia and child behavioral development are consistent with several previous studies (170, 174) but don't replicate the findings of others (171, 172, 258). Current null findings might be the result of the small number of mothers with hypothyroxinemia in our population. We did not observe any sex specific effect on the association of maternal thyroid hormones and child behavioral development, supporting the findings of a previously conducted study (170) and not confirming the sex specific associations that have been detected by others (173, 174). However, the several methodological differences between the aforementioned studies and the current one (e.g. different age of the participants, outcome in clinical categories/outcome continuous, trajectories of thyroid hormones/single time-point measurement) may have caused this difference in the specific findings.

Strengths and limitations

The strengths of the present study include the population-based, prospective design of the study and the opportunity to control for the potential confounding effect of several maternal and child factors. Maternal thyroid antibodies were assessed as an important cause of thyroid dysfunction in iodine sufficient populations. A possible limitation of this study is that thyroid hormones were measured at a single early point during pregnancy; as a result these measurements might reflect a transient thyroid dysfunction. However, there is evidence that longitudinal and single-point thyroid assessments are highly correlated (255). Although the selection of reliable and valid questionnaires to assess child behavioral and

emotional development is considered another strength of this study, it also consists of a limitation of the findings, since questionnaires cannot replace direct developmental assessment and clinical diagnosis of any specific disorder. Moreover, we cannot interpret differences in scores between the two time-points as developmental changes, since differences between the questionnaires may be the explanation of the observed variations. Imputation of the missing values of *ADHDT*, *SDQ*, and *CBCL* questionnaires were applied in order to avoid any bias due to selective response to specific items of the questionnaires. Participants and non-participants did not differ in relevance with the exposure status but they differed in other socio-demographic characteristics. Therefore, bias due to non-participation and loss to follow up cannot be excluded. In addition, we had no available information on offspring congenital hypothyroidism, and although sensitivity analyses with adjustment for child thyroid functioning at 4 years was conducted and no significant change in the results was evident, we have to consider this as a possible limitation of the present results. Furthermore, residual confounding cannot be excluded, although multiple confounders were included in the analyses and additional sensitivity analyses were conducted to control for a potential confounding effect of several factors.

3.4. Maternal mild thyroid dysfunction and offspring obesity and cardiometabolic health traits

Main findings

- Maternal subclinical hypothyroidism was associated with increased offspring BMI and total fat mass at 4 years of age.
- Maternal hypothyroxinemia was associated with increased waist circumference at 4 years of age.
- Maternal thyroid mild dysfunction was not associated with any measure of offspring cardiometabolic health at 4 and 6 years of age.

Summary of the results

We found evidence of non-linear associations between maternal thyroid hormones concentration levels and the outcomes of interest (GAMs). Therefore, we categorized maternal thyroid hormones in tertiles and we used the medium tertile as a comparison group for the respective models.

Associations of maternal thyroid functioning with offspring obesity measures:

- No associations were found between maternal thyroid parameters and offspring obesity measures at 4 and 6 years of age (Table 23).
- Maternal subclinical hypothyroidism during pregnancy was associated with increased offspring BMI and total fat mass at 4 years of age (Table 24).
- Maternal hypothyroxinemia during pregnancy was associated with increased offspring waist circumference at 4 years of age (Table 24).
- We did not identify any association of maternal thyroid mild dysfunction and offspring obesity at 6 years of age

Associations of maternal thyroid functioning with offspring cardiometabolic traits:

- Decreased maternal TSH levels (lowest tertile) were associated with decreased HDL at 4 years of age (Table 25).
- No association was identified between maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity and offspring cardiometabolic traits (Table 26).

Additional analyses

- We did not observe any substantial change when we repeated the analyses excluding participants who were taking thyroid medication during pregnancy (Table 27 – Table 30).

Table 23. Associations of maternal thyroid stimulating hormone (TSH) and free thyroxine (fT4) concentration levels with offspring Body Mass Index (BMI) and obesity measures at 4 and 6 years of age, Rhea mother-child study, Crete, Greece

	Low TSH		High TSH		Low fT4		High fT4	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age								
BMI z-scores [a]	0.0 (-0.2, 0.2)	0.881	0.1 (-0.1, 0.3)	0.488	-0.1 (-0.3, 0.2)	0.641	0.1 (-0.1, 0.3)	0.394
Fat mass [b]	0.1 (-0.2, 0.3)	0.640	0.1 (-0.2, 0.3)	0.608	0.1 (-0.2, 0.3)	0.621	0.2 (-0.1, 0.4)	0.160
Fat proportion [b]	0.0 (-1.0, 1.1)	0.939	-0.1(-1.1, 1.1)	0.915	0.3 (-0.8, 1.4)	0.634	0.6 (-0.5, 1.6)	0.313
Waist circumference	0.0 (-0.1, 0.2)	0.868	0.1 (-0.1, 0.2)	0.486	0.1 (-0.1, 0.2)	0.389	0.0 (-0.1, 0.2)	0.586
6 years of age								
BMI z-scores [a]	-0.1 (-0.4, 0.2)	0.678	-0.1 (-0.4, 0.2)	0.555	0.0 (-0.3, 0.3)	0.945	0.2 (-0.1, 0.5)	0.115
Fat mass [b]	0.0 (-0.4, 0.5)	0.874	-0.0 (-0.5, 0.4)	0.927	0.2 (-0.3, 0.7)	0.387	0.2 (-0.3, 0.6)	0.467
Fat proportion [b]	-0.1 (-1.4, 1.2)	0.883	-0.2 (-1.6, 1.1)	0.716	0.6 (-0.8, 1.9)	0.400	0.4 (-0.9, 1.7)	0.567
Fat mass (BIA)[b]	-0.1 (-0.8, 0.5)	0.707	-0.2 (-0.9, 0.5)	0.486	0.4 (-0.3, 1.1)	0.297	0.7 (-0.0, 1.3)	0.053
Fat % (BIA) [b]	-0.5 (-2.0, 1.0)	0.530	-0.4 (-2.0, 1.2)	0.640	0.9 (-0.7, 2.5)	0.278	1.2 (-0.4, 2.7)	0.132
Waist circumference								
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
4 years of age								
Overweight [c]	0.9 (0.7, 1.3)	0.637	0.9 (0.6, 1.3)	0.572	1.2 (0.8, 1.7)	0.303	1.1 (0.8, 1.6)	0.572
Obesity [c]	1.4 (0.7, 2.6)	0.310	0.9 (0.4, 1.8)	0.667	1.5 (0.7, 3.2)	0.314	1.7 (0.8, 3.3)	0.142
6 years of age								
Overweight [c]	0.9 (0.6, 1.2)	0.399	0.8 (0.6, 1.1)	0.236	1.3 (0.9, 1.8)	0.168	1.2 (0.9, 1.7)	0.214
Obesity [c]	1.0 (0.6, 1.7)	0.951	0.6 (0.3, 1.3)	0.197	1.1 (0.5, 2.3)	0.738	1.4 (0.7, 2.5)	0.317

β-coefficients and their 95% CIs were estimated using linear regression models. Odds ratios (ORs) and 95% CIs were calculated using generalized linear models for binary outcomes. Models were adjusted for maternal age, maternal BMI at early pregnancy, maternal smoking in pregnancy, maternal education, parity, gestational age at blood sampling; models of

waist circumference were additionally adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models.

[a] BMI z-scores represent the difference from the mean sex-specific and age-specific BMI value for the World Health Organization reference population and is expressed in standard deviations

[b] Fat mass and fat proportion were obtained through skinfolds; fat mass (BIA) and fat % (BIA) were obtained through bioelectrical impedance assessments

[c] Overweight and obesity were defined based on the International Obesity Task Force IOTF) definition

Table 24. Associations of maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity with offspring BMI and obesity at 4 and 6 years of age, Rhea mother-child study, Crete, Greece

	Subclinical Hypothyroidism		Hypothyroxinemia		Thyroid Autoimmunity	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age						
BMI z-scores [a]	**0.41 (0.04, 0.78)[b]**	**0.030**	0.41 (-0.04, 0.86)	0.074	0.14 (-0.12, 0.40)	0.294
Fat mass [b]	**0.43 (0.01, 0.86)[b]**	**0.045**	0.41 (-0.10, 0.91)	0.113	0.12 (-0.17, 0.40)	0.422
Fat proportion [b]	0.26 (-1.59, 2.11)	0.782	1.50 (-0.81, 3.80)	0.202	0.45 -0.87, 1.76)	0.503
Waist circumference	1.04 (-2.23, 2.32)	0.108	**1.77 (0.23, 3.31)[b]**	**0.024**	0.52 (-0.37, 1.41)	0.253
6 years of age						
BMI z-scores [a]	-0.12 (-0.63, 0.39)	0.642	0.19 (-0.39, 0.77)	0.523	-0.07 (-0.44, 0.31)	0.728
Fat mass [b]	-0.19 (-0.95, 0.57)	0.623	0.33 (-0.53, 1.19)	0.452	-0.18 (-0.75, 0.38)	0.521
Fat proportion [b]	-0.91 (-3.09, 1.28)	0.416	0.91 (-1.57, 3.40)	0.470	-0.32 (-1.95, 1.31)	0.700
Fat mass (BIA)[b]	-0.46 (-1.60, 0.68)	0.430	0.50 (-0.80, 1.79)	0.449	0.14 (-0.69, 0.97)	0.739
Fat % (BIA) [b]	-0.49 (-3.12, 2.14)	0.715	1.26 (-1.70, 4.23)	0.404	0.59 (-1.31, 2.48)	0.543
Waist circumference	-0.97 (-3.56, 1.61)	0.459	0.68 (-2.25, 3.61)	0.647	-0.27 (-2.15, 1.62)	0.780
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
4 years of age						
Overweight [c]	0.97 (0.44, 2.15)	0.939	2.20 (0.93, 5.23)	0.074	0.98 (0.55, 1.75)	0.952
Obesity [c]	1.64 (0.51, 5.24)	0.405	2.78 (0.86, 8.98)	0.087	1.67 (0.68, 4.11)	0.261
6 years of age						
Overweight [c]	0.80 (0.33, 1.96)	0.627	1.61 (0.65, 4.03)	0.306	1.12 (0.61, 2.05)	0.717
Obesity [c]	0.56 (0.12, 2.53)	0.449	0.79 (0.20, 3.20)	0.745	1.00 (0.41, 2.40)	0.993

β-coefficients and their 95% CIs were estimated using linear regression models. Odds ratios (ORs) and their 95% CIs were calculated using generalized linear models for binary outcomes. Models were adjusted for maternal age, maternal BMI at early pregnancy, maternal smoking in pregnancy, maternal education, parity, gestational age at blood sampling; models of waist

circumference were additionally adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models.

[a] BMI z-scores represent the difference from the mean sex-specific and age-specific BMI value for the World Health Organization reference population and is expressed in standard deviations

[b] Fat mass and fat proportion were obtained through skinfolds & fat mass (BIA) and fat % (BIA) were obtained through bioelectrical impedance assessments

[c] Overweight and obesity were defined based on the International Obesity Task Force IOTF) definition

Table 25. Associations of maternal thyroid stimulating hormone (TSH) and free thyroxine (fT4) concentration levels with offspring Body Mass Index (BMI) and obesity measures at 4 and 6 years of age, Rhea mother-child study, Crete, Greece

	Low TSH		High TSH		Low fT4		High fT4	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β2 (95% CI)	*P*
4 years of age								
Cardiometabolic risk score [a]	-0.1 (-0.4, 0.3)	0.679	-0.0 (-0.4, 0.4)	0.996	0.1 (-0.3, 0.5)	0.660	0.1 (-0.3, 0.4)	0.677
HDL, mg/dl	**-2.9 (-5.1, -0.6)[b]**	**0.012**	-0.1 (-2.3, 2.1)	0.933	0.7 (-1.6, 3.0)	0.562	-0.0 (-2.3, 2.2)	0.968
LDL, mg/dl	-2.7 (-7.5, 2.0)	0.260	-0.7 (-5.5, 4.0)	0.769	3.1 (-1.8, 7.9)	0.211	-0.1 (-4.9, 4.7)	0957
Total cholesterol, mg/dl	-5.4 (-11.0, 2.9)	0.059	-1.4 (-6.9, 4.2)	0.630	3.6 (-2.1, 9.3)	0.213	-0.6 (-6.2, 5.0)	0.831
Triglycerides, mg/dl	0.5 (-5.2, 6.2)	0.853	-3.3 (-9.0, 2.4)	0.252	-1.5 (-7.2, 4.3)	0.619	-2.6 (-8.3, 3.1)	0.367
Systolic blood pressure, mm Hg	0.0 (-0.1, 0.1)	0.990	0.0 (-0.1, 0.2)	0.867	0.0 (-0.1, 0.2)	0.524	0.0 (-0.1, 0.2)	0.862
Diastolic blood pressure, mm Hg	-0.1 (-0.2, 0.0)	0.185	-0.0 (-0.1, 0.1)	0.874	-0.0 (-0.1, 0.1)	0.785	-0.0 (-0.1, 0.1)	0.602
6 years of age								
Cardiometabolic risk score [a]	-0.2 (-0.6, 0.2)	0.289	-0.3 (-0.7, 0.1)	0.163	0.2 (-0.2, 0.6)	0.319	0.2 (-0.2, 0.6)	0.383
HDL, mg/dl	-2.0 (-4.8, 0.7)	0.145	-1.6 (-4.5, 1.3)	0.275	-2.7 (-5.7, 0.2)	0.068	-0.3 (-3.1, 2.5)	0.832
LDL, mg/dl	-2.2 (-6.8, 2.5)	0.365	-0.7 (-5.6, 4.3)	0.787	-0.9 (-6.0, 4.1)	0.718	02.8 (-7.6, 2.0)	0.250
Total cholesterol, mg/dl	-4.3 (-9.7, 1.0)	0.110	-1.3 (-7.0, 4.3)	0.645	-3.7 (-9.5, 2.0)	0.206	-3.3 (-8.7, 2.2)	0.239
Triglycerides, mg/dl	-4.0 (-11.4, 3.6)	0.304	1.8 (-6.1, 9.8)	0.649	-3.9 (-12.0, 4.1)	0.338	-5.2 (-12.9, 2.4)	0.181
Systolic blood pressure, mm Hg	-0.2 (-0.3, 0.0)	0.053	-0.1 (-0.3, 0.0)	0.142	0.2 (-0.0, 0.4)	0.075	0.1 (-0.1, 0.2)	0.535

Diastolic blood pressure, mm Hg	-0.1 (-0.3, 0.0)	0.056	-0.1 (-0.2, 0.1)	0.223	0.1 (-0.0, 0.2)	0.120	-0.0 (-0.1, 0.1)	0.741

Beta coefficients and 95% CIs were estimated using linear regression models adjusted for maternal age, maternal BMI at study entry, maternal smoking during pregnancy, maternal education, parity. Models of individual cardiometabolic risk factors were additionally adjusted for child sex and age at outcome assessment. Models of systolic and diastolic blood pressure were also adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models.

[a] The cardiometabolic risk score is expressed in standard deviations and was derived as the sum of the following components: sex- specific and age-specific z-scores of waist circumference and non-HDL cholesterol, and the average of age-specific, sex-specific, and height-specific z-scores for systolic and diastolic blood pressure

Table 26. Associations of maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity with offspring cardiometabolic traits at 4 and 6 years of age, Rhea mother-child study, Crete, Greece

	Subclinical Hypothyroidism		Hypothyroxinemia		Thyroid Autoimmunity	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age						
Cardiometabolic risk score [a]	0.14 (-0.04, 0.32)	0.131	0.12 (-0.03, 0.28)	0.124	-0.02 (-0.21, 0.17)	0.855
HDL, mg/dl	0.43 (-0.80, 1.66)	0.495	-0.64 (-1.67, 0.40)	0.228	-0.84 (-2.07, 0.40)	0.183
LDL, mg/dl	0.64 (-1.93, 3.22)	0.623	0.74 (-1.39, 2.87)	0.496	0.65 (-1.89, 3.19)	0.615
Total cholesterol,	1.21 (-1.83, 4.25)	0.434	0.54 (-2.01, 3.09)	0.677	-0.52 (-3.54, 2.50)	0.735
Triglycerides, mg/dl	0.63 (-2.47, 3.72)	0.691	2.07 (-0.50, 4.63)	0.114	-1.73 (-4.80, 1.33)	0.267
Systolic blood pressure, mm Hg	0.24 (-0.50, 0.97)	0.527	0.12 (-0.56, 0.80)	0.728	0.41 (-0.37, 1.19)	0.306
Diastolic blood pressure, mm Hg	0.06 (-0.44, 0.56)	0.826	-0.18 (-0.66, 0.29)	0.446	-0.05 (-0.59, 0.48)	0.841
6 years of age						
Cardiometabolic risk score [a]	0.04 (-0.16, 0.24)	0.702	0.06 (-0.11, 0.240	0.474	-0.11 (-0.33, 0.11)	0.323
HDL, mg/dl	-0.95 (-2.42, 0.52)	0.206	-1.05 (-2.28, 0.17)	0.092	-1.24 (-2.73, 0.26)	0.106
LDL, mg/dl	1.45 (-1.05, 3.96)	0.255	0.74 (-1.35, 2.83)	0.485	-1.19 (-3.75, 1.38)	0.363
Total cholesterol,	1.30 (-1.57, 4.17)	0.372	0.14 (-2.24, 2.53)	0.905	-2.31(-5.21, 0.59)	0.118
Triglycerides, mg/dl	3.73 (-0.20, 7.66)	0.062	1.55 (-1.66, 4.77)	0.342	0.27 (-3.64, 4.18)	0.892

Systolic blood pressure, mm Hg	-0.28 (-1.24, 0.67)	0.561	0.03 (-0.80, 0.85)	0.950	0.26 (-0.75, 1.27)	0.611
Diastolic blood pressure, mm Hg	0.10 (-0.63, 0.83)	0.785	0.25 (-0.38, 0.88)	0.436	0.14 (-0.63, 0.91)	0.715

Beta coefficients and 95% CIs were estimated using linear regression models adjusted for maternal age, maternal BMI at study entry, maternal smoking during pregnancy, maternal education, parity. Models of individual cardiometabolic risk factors were additionally adjusted for child sex and age at outcome assessment. Models of systolic and diastolic blood pressure were also adjusted for child height.

[a] The cardiometabolic risk score is expressed in standard deviations and was derived as the sum of the following components: sex-specific and age-specific z-scores of waist circumference and non-HDL cholesterol, and the average of age-specific, sex-specific, and height-specific z-scores for systolic and diastolic blood pressure

Table 27. Associations of maternal thyroid stimulating hormone (TSH) and free thyroxine (fT4) concentration levels with offspring Body Mass Index (BMI) and obesity measures at 4 and 6 years of age, excluding participants who took thyroid medication during pregnancy, Rhea mother-child study, Crete, Greece

	Low TSH		High TSH		Low fT4		High fT4	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age								
BMI z-scores [a]	0.0 (-0.2, 0.3)	0.847	0.1 (-0.1, 0.3)	0.447	-0.1 (-0.3, 0.2)	0.651	-0.0(-0.3, 0.2)	0.889
Fat mass [b]	0.1 (-0.2, 0.4)	0.471	0.1 (-0.1, 0.4)	0.342	0.1 (-0.2, 0.3)	0.562	0.1 (-0.2, 0.3)	0.603
Fat proportion [b]	0.1 (-1.0, 1.3)	0.808	0.2 (-0.9, 1.3)	0.720	0.4 (-0.7, 1.5)	0.527	0.1 (-1.0, 1.3)	0.832
Waist circumference	0.0 (-0.2, 0.1)	0.923	0.1 (-0.1, 0.2)	0.384	0.1 (-0.1, 0.2)	0.438	-0.1 (-0.2, 0.1)	0.439
6 years of age								
BMI z-scores [a]	0.0 (-0.3, 0.3)	0.895	-0.1 (-0.4, 0.3)	0.701	-0.0 (-0.3, 0.3)	0.920	0.2 (-0.2, 0.5)	0.334
Fat mass [b]	0.2 (-0.3, 0.6)	0.511	-0.0 (-0.5, 0.5)	0.955	0.2 (-0.3, 0.7)	0.450	0.2 (-0.3, 0.7)	0.489
Fat proportion [b]	0.1 (-1.3, 1.4)	0.896	-0.2 (-1.6, 1.2)	0.748	0.5 (-0.9, 1.9)	0.503	0.4 (-1.0, 1.8)	0.609
Fat mass (BIA)[b]	0.2 (-0.5, 0.9)	0.536	0.0 (-0.7, 0.7)	0.987	0.4 (-0.3, 1.1)	0.256	0.4 (-0.3, 1.1)	0.263
Fat % (BIA) [b]	0.1 (-1.5, 1.6)	0.941	-0.0 (-1.7, 1.7)	0.992	0.8 (-0.8, 2.4)	0.318	0.6 (-1.1, 2.2)	0.480
Waist circumference	-0.0 (-0.2, 0.1)	0.669	-0.2 (-0.4, 0.0)	0.073	0.0 (-0.2, 0.2)	0.896	0.0 (-0.2, 0.2)	0.957
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
4 years of age								
Overweight [c]	0.9 (0.6, 1.4)	0.660	0.9 (0.6, 1.3)	0.623	1.2 (0.9, 1.8)	0.246	1.0 (0.6, 1.5)	0.944
Obesity [c]	2.0 (0.9, 4.4)	0.091	1.1 (0.5, 2.6)	0.823	1.8 (0.8, 3.8)	0.144	1.2 (0.5, 2.8)	0.667
6 years of age								
Overweight [c]	0.9 (0.7, 1.3)	0.712	0.8 (0.6, 1.2)	0.288	1.3 (0.9, 1.9)	0.141	1.1 (0.8, 1.7)	0.475
Obesity [c]	1.2 (0.6, 2.2)	0.614	0.8 (0.4, 1.8)	0.606	1.3 (0.6, 2.7)	0.551	1.3 (0.6, 2.6)	0.485

β-coefficients and their 95% CIs were estimated using linear regression models. Odds ratios (ORs) and 95% CIs were calculated using generalized linear models for binary outcomes. Models were adjusted for maternal age, maternal BMI at early pregnancy, maternal smoking in pregnancy, maternal education, parity, gestational age at blood sampling; models of

waist circumference were additionally adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models. At 4 years: N = 647, At 6 years N = 489.

[a] BMI z-scores represent the difference from the mean sex-specific and age-specific BMI value for the World Health Organization reference population and is expressed in standard deviations

[b] Fat mass and fat proportion were obtained through skinfolds; fat mass (BIA) and fat % (BIA) were obtained through bioelectrical impedance assessments

[c] Overweight and obesity were defined based on the International Obesity Task Force IOTF definition

Table 28. Associations of maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity with offspring BMI and obesity at 4 and 6 years of age, excluding participants who took thyroid medication during pregnancy, Rhea mother-child study, Crete, Greece

	Subclinical Hypothyroidism		Hypothyroxinemia		Thyroid Autoimmunity	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age						
BMI z-scores [a]	**0.72 (0.26, 1.17)[b]**	**0.002**	0.44 (-0.02, 0.89)	0.062	0.03 (-0.27, 0.34)	0.807
Fat mass [b]	**0.82 (0.33, 1.31)[b]**	**0.001**	0.50 (-0.01, 1.00)	0.054	-0.07 (-0.40, 0.25)	0.652
Fat proportion [b]	1.39 (-0.77, 3.56)	0.206	1.79 (-0.55, 4.13)	0.133	-0.10 -1.64, 1.44)	0.897
Waist circumference	**1.03 (0.27, 3.39)[b]**	**0.021**	**2.07 (0.51, 3.64)[b]**	**0.010**	0.36 (-0.69, 1.41)	0.498
6 years of age						
BMI z-scores [a]	-0.04 (-0.64, 0.56)	0.891	0.18 (-0.43, 0.78)	0.567	-0.32 (-0.76, 0.12)	0.159
Fat mass [b]	-0.08 (-0.98, 0.82)	0.863	0.33 (-0.56, 1.21)	0.471	-0.30 (-0.97, 0.36)	0.368
Fat proportion [b]	-0.60 (-3.18, 1.97)	0.645	0.91 (-1.64, 3.45)	0.483	-0.47 (-2.38, 1.43)	0.628
Fat mass (BIA)[b]	-0.28 (-1.61, 1.05)	0.679	0.58 (-0.73, 1.88)	0.384	-0.37 (-1.33, 0.60)	0.457
Fat % (BIA) [b]	-0.08 (-3.16, 3.0)	0.961	1.41 (-1.58, 4.41)	0.354	-0.19 (-1.41, 2.04)	0.869
Waist circumference	-0.93 (-3.95, 2.08)	0.543	1.00 (-1.98, 3.97)	0.510	-1.44 (-3.65, 0.77)	0.202
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
4 years of age						
Overweight [c]	1.81 (0.75, 4.36)	0.188	2.35 (0.95, 5.83)	0.064	0.98 (0.55, 1.75)	0.952

Obesity [c]	2.65 (0.73, 9.62)	0.138	**4.34 (1.23, 15.33)[b]**	**0.022**	1.67 (0.68, 4.11)	0.261
6 years of age						
Overweight [c]	1.02 (0.37, 2.80)	0.968	1.58 (0.61, 4.09)	0.347	1.12 (0.61, 2.05)	0.717
Obesity [c]	0.76 (0.16, 3.66)	0.732	0.85 (0.21, 3.54)	0.828	1.00 (0.41, 2.40)	0.993

β-coefficients and their 95% CIs were estimated using linear regression models. Odds ratios (ORs) and their 95% CIs were calculated using generalized linear models for binary outcomes. Models were adjusted for maternal age, maternal BMI at early pregnancy, maternal smoking in pregnancy, maternal education, parity, gestational age at blood sampling; models of waist circumference were additionally adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models.

[a] BMI z-scores represent the difference from the mean sex-specific and age-specific BMI value for the World Health Organization reference population and is expressed in standard deviations

[b] Fat mass and fat proportion were obtained through skinfolds & fat mass (BIA) and fat % (BIA) were obtained through bioelectrical impedance assessments

[c] Overweight and obesity were defined based on the International Obesity Task Force IOTF definition

Table 29. Associations of maternal thyroid stimulating hormone (TSH) and free thyroxine (fT4) concentration levels with offspring Body Mass Index (BMI) and obesity measures at 4 and 6 years of age, excluding participants who took thyroid medication during pregnancy, Rhea mother-child study, Crete, Greece

	Low TSH		High TSH		Low fT4		High fT4	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age								
Cardiometabolic risk score [a]	-0.0 (-0.4, 0.4)	0.856	-0.0 (-0.4, 0.4)	0.982	0.0 (-0.3, 0.4)	0.825	0.0 (-0.4, 0.4)	0.905
HDL, mg/dl	**-3.1 (-5.5, -0.7)[b]**	**0.012**	-0.5 (-3.0, 1.9)	0.661	0.4 (-1.9, 2.8)	0.718	-0.9 (-3.4, 1.7)	0.505
LDL, mg/dl	-3.0 (-8.2, 2.1)	0.247	-0.8 (-5.9, 4.3)	0.761	2.1 (-2.9, 7.2)	0.412	0.2 (-5.2, 5.6)	0.938
Total cholesterol, mg/dl	-6.0 (-12.0, 0.1)	0.054	-1.9 (-8.0, 4.1)	0.530	2.4 (-3.6, 8.4)	0.429	-0.7 (-7.1, 5.6)	0.823
Triglycerides, mg/dl	0.2 (-5.9, 6.4)	0.946	-3.6 (-9.7, 2.5)	0.252	-1.4 (-7.4, 4.6)	0.654	-0.9 (-7.3, 5.5)	0.776
Systolic blood pressure, mm Hg	-0.0 (-0.2, 0.1)	0.885	0.0 (-0.1, 0.2)	0.672	0.1 (-0.1, 0.2)	0.317	-0.0 (-0.2, 0.2)	0.952
Diastolic blood pressure, mm Hg	-0.1 (-0.2, 0.0)	0.248	-0.0 (-0.1, 0.1)	0.506	-0.0 (-0.1, 0.1)	0.955	-0.0 (-0.1, 0.1)	0.682
6 years of age								
Cardiometabolic risk score [a]	-0.1 (-0.4, 0.4)	0.930	-0.2 (-0.6, 0.3)	0.390	0.2 (-0.3, 0.6)	0.419	0.0 (-0.4, 0.5)	0.848
HDL, mg/dl	-2.3 (-5.2, 0.6)	0.126	-2.6 (-5.7, 0.4)	0.093	-3.7 (-6.7, -0.7)	0.015	-2.1 (-5.2, 0.9)	0.171
LDL, mg/dl	-1.6 (-6.6, 3.5)	0.537	0.1 (-5.2, 5.4)	0.971	-1.9 (-7.1, 3.3)	0.465	-4.4 (-9.7, 0.9)	0.101
Total cholesterol, mg/dl	-3.6 (-9.4, 2.2)	0.225	-1.7 (-7.7, 4.4)	0.595	-5.4 (-11.4, 0.5)	0.075	**-6.6 (-12.6, -0.6)[b]**	**0.032**
Triglycerides, mg/dl	-1.9 (-9.9, 6.1)	0.645	1.3 (-7.1, 9.7)	0.766	-3.9 (-12.0, 4.1)	0.338	-5.2 (-12.9, 2.4)	0.181
Systolic blood pressure, mm Hg	-0.2 (-0.4, 0.0)	0.101	-0.1 (-0.3, 0.1)	0.233	0.2 (-0.0, 0.4)	0.063	-0.0 (-0.2, 0.2)	0.975
Diastolic blood pressure, mm Hg	-0.1 (-0.3, 0.0)	0.050	-0.1 (-0.3, 0.0)	0.095	0.1 (-0.0, 0.3)	0.078	-0.0 (-0.1, 0.1)	0.925

Beta coefficients and 95% CIs were estimated using linear regression models adjusted for maternal age, maternal BMI at study entry, maternal smoking during pregnancy, maternal education, parity. Models of individual cardiometabolic risk factors were additionally adjusted for child sex and age at outcome assessment. Models of systolic and diastolic blood pressure were also adjusted for child height. Thyroid parameters are categorized in tertiles; the middle tertile is set as the comparison group for the models.

[a] The cardiometabolic risk score is expressed in standard deviations and was derived as the sum of the following components: sex- specific and age-specific z-scores of waist circumference and non-HDL cholesterol, and the average of age-specific, sex-specific, and height-specific z-scores for systolic and diastolic blood pressure

Table 30. Associations of maternal subclinical hypothyroidism, hypothyroxinemia, and thyroid autoimmunity with offspring cardiometabolic traits at 4 and 6 years of age, excluding participants who took thyroid medication during pregnancy, Rhea mother-child study, Crete, Greece

	Subclinical Hypothyroidism		Hypothyroxinemia		Thyroid Autoimmunity	
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
4 years of age						
Cardiometabolic risk score [a]	0.12 (-0.04, 0.28)	0.131	0.13 (-0.04, 0.29)	0.124	-0.02 (-0.19, 0.15)	0.855
HDL, mg/dl	0.38 (-0.71, 1.46)	0.495	-0.66 (-1.73, 0.41)	0.228	-0.75 (-1.85, 0.35)	0.183
LDL, mg/dl	0.57 (-1.70, 2.83)	0.623	0.76 (-1.44, 2.97)	0.496	0.58 (-1.69, 2.85)	0.615
Total cholesterol,	1.07 (-1.61, 3.74)	0.434	0.56 (-2.07, 3.19)	0.677	-0.46 (-3.17, 2.24)	0.735
Triglycerides, mg/dl	0.55 (-2.17, 3.27)	0.691	2.13 (-0.51, 4.78)	0.114	-1.55 (-4.30, 1.19)	0.267
Systolic blood pressure, mm Hg	0.21 (-0.44, 0.86)	0.527	0.12 (-0.58, 0.83)	0.728	0.36 (-0.33, 1.06)	0.306
Diastolic blood pressure, mm Hg	0.05 (-0.39, 0.49)	0.826	-0.19 (-0.68, 0.30)	0.446	-0.05 (-0.53, 0.43)	0.841
6 years of age						
Cardiometabolic risk score [a]	0.04 (-0.14, 0.21)	0.702	0.07 (-0.12, 0.25)	0.474	-0.10 (-0.30, 0.10)	0.323
HDL, mg/dl	-0.83 (-2.12, 0.46)	0.206	-1.09 (-2.36, 0.18)	0.092	-1.11 (-2.45, 0.23)	0.106
LDL, mg/dl	1.28 (-0.93, 3.48)	0.255	0.76 (-1.39, 2.92)	0.485	-1.06 (-3.36, 1.23)	0.363
Total cholesterol,	1.15 (-1.38, 3.67)	0.372	0.15 (-2.31, 2.61)	0.905	-2.07(-4.66, 0.53)	0.118
Triglycerides, mg/dl	3.28 (-0.17, 6.73)	0.062	1.60 (-1.72, 4.92)	0.342	0.24 (-3.26, 3.74)	0.892
Systolic blood pressure, mm Hg	-0.25 (-1.09, 0.59)	0.561	0.03 (-0.83, 0.88)	0.950	0.23 (-0.67, 1.13)	0.611
Diastolic blood pressure, mm Hg	0.09 (-0.55, 0.73)	0.785	0.26 (-0.39, 0.90)	0.436	0.13 (-0.56, 0.82)	0.715

Beta coefficients and 95% CIs were estimated using linear regression models adjusted for maternal age, maternal BMI, maternal smoking during pregnancy, maternal education, parity. Models of individual cardiometabolic risk factors were additionally adjusted for child sex and age at outcome assessment. Models of systolic and diastolic blood pressure were also adjusted for child height.

[a] The cardiometabolic risk score is expressed in standard deviations and was derived as the sum of the following components: sex-

specific and age-specific z-scores of waist circumference and non-HDL cholesterol, and the average of age-specific, sex-specific, and height-specific z-scores for systolic and diastolic blood pressure

Discussion

It is well established that thyroid hormones control energy balance and body weight in adults; more specifically, hypothyroidism promotes hypometabolism (reduced resting energy expenditure and lipolysis, weight gain, and increased cholesterol levels), while hyperthyroidism promotes hypermetabolism (increased resting energy expenditure and lipolysis, weight loss, and decreased cholesterol levels) (176, 272-274). In addition, even subtle changes in circulating levels of thyroid hormones can affect cardiac contractility, influence various hemodynamic parameters (blood pressure, blood volume, heart rate), and increase the occurrence of other cardiovascular risk factors (dyslidaemia and endothelial dysfunction) (275-277). However, the role of maternal thyroid hormones during gestation on offspring body composition, adiposity and cardiometabolic risk remains unknown.

Thyroid hormones are crucial for the development of several offspring tissues and may also be involved in adipose tissue development (278, 279). Previous animal studies have suggested that abnormal maternal thyroid hormones may increase the risk for offspring cardiovascular dysfunction in later life due to increase of blood pressure (183), altered genetic expression in the cardiomyocytes that can cause anatomical and functional differentiations (181, 280, 281), and altered genetic expression in hypothalamic regions that are responsible for cardiovascular function, body weight regulation and metabolism (185). Previous findings derived from studies in human populations have suggested that maternal subclinical hypothyroidism is associated with offspring hypertension but not related with any obesity measure at 20 years of age (187). Similarly, no association was found between maternal thyroid deficiencies and offspring adiposity nor with any other cardiometabolic health parameter at 16 years of age, even though maternal thyroid autoimmunity was linked to metabolic syndrome and obesity in the same study (188). The only study to date in children has found associations of normal and decreased TSH concentration levels with decreased BMI, reduced total fat mass, abdominal subcutaneous fat mass, and systolic blood pressure, as well as of normal and increased fT4 concentration levels with decreased child BMI, reduced abdominal subcutaneous and pre-peritoneal fat mass, at 6 years of age (186).

Current results suggest that maternal thyroid hormones may be involved in offspring body weight and body composition, since maternal subclinical hypothyroidism and hypothyroxinemia were associated with increased BMI, and total fat mass, and increased waist circumference. These relationships were observed at 4 years of age, however they were not evident at 6 years of age. The null findings at 6 years of age may be the result of loss in power analysis due to loss to follow-up.

Strengths and limitations

The strengths of the present study include its population-based prospective design and the long follow-up period. Although thyroid hormones were measured at a single-timepoint in gestation, it has been previously suggested that longitudinal and single-timepoint thyroid assessments are highly correlated (255). Bias due to loss to follow up may have impact on the results; non-participants did not differ from participants regarding maternal thyroid parameters, but they were more likely to have younger, non-Greek, multiparous, and less educated mothers compared with the participants of this study. In addition, residual confounding effect of unmeasured variables cannot be excluded, even though the models have been adjusted for multiple possible confounders. Mothers who were taking thyroid medication during pregnancy were not excluded from the main analyses, however sensitivity analyses excluding these participants did not support substantially different results. Further studies are needed in order to replicate current findings and explore how offspring cardiovascular profile is developed in later childhood and adulthood.

4. General Discussion

This thesis includes studies that aim to explore the role of the maternal concentration levels of thyroid hormones, maternal mild thyroid dysfunction (subclinical hypothyroidism, hypothyroxinemia), and maternal thyroid autoimmunity during pregnancy on various domains of offspring's development (cognitive, motor, emotional, behavioral, physical, cardiometabolic) from infancy to early childhood. The impact of maternal thyroid hormones on child neurodevelopment has been well-established due to the detrimental effect of severe iodine deficiency on offspring cognitive and motor ability (cretinism). In addition, during the last decades, evidence from observational and experimental studies has supported that even non-clinical variations of maternal thyroid hormones impact on offspring development and as a result relevant guidelines regarding thyroid management during pregnancy have been updated. Current analyses aim to extend understanding regarding the role of maternal thyroid function on offspring development and provide evidence that can support appropriate public health policies for ensuring optimal early life conditions and in result beneficial developmental outcomes in offspring. The Rhea mother-child cohort study gives the opportunity to explore even subtle variations in offspring's phenotypes longitudinally, since it offers comprehensive, reliable, and valid data on multiple domains of child development from infancy to early childhood.

The following section of the general discussion provides a broad and brief discussion and interpretation of the findings, which are discussed in detail in the results section.

4.1 What this study adds and future research

- We have explored early life sociodemographic, environmental, life style, and perinatal factors using principal component analysis to identify patterns of early-life exposures and examine their association with child cognitive and motor development and with child behavioral difficulties in preschool age. We have found that parental social status, breastfeeding & non-smoking in pregnancy, preschool attendance and less TV-watching and parental involvement are all protective factors of child neuropsychological development in preschool age, while increased child birth order was linked with decreased verbal development and decreased behavioral symptoms in preschool age. This analysis aimed to explore the combined effect of various early-life factors on child cognition and behavioral development. This is the first study, to our knowledge, that attempted to integrate multiple early life exposures and explore their associations with child cognition and behavior at preschool age. Such holistic approaches that highlight the common characteristics of single exposures are appropriate for studying development, since cognitive and behavioral development are rather complex and multi-faceted and they are based on multiple overlapping and interacting factors. Furthermore, this approach can be used to suggest a new way of objects' and target groups' selection depending on the identification of multiple, coexisting exposures for intervention designing.

- We have provided further evidence of the link of maternal hypothyroxinemia with suboptimal offspring neuropsychological development, since our results supported that hypothyroxinemia is related with decreased offspring verbal and motor ability in early childhood. We have also demonstrated that maternal thyroid autoimmunity is associated with impaired perceptual performance and motor ability in preschool age and increases the risk for adverse non-verbal cognitive development longitudinally, from infancy to early childhood. The use of GBTM to identify non-verbal cognitive development trajectories and explore the longitudinal impact of mild maternal thyroid dysfunction on offspring non-verbal cognition is another strength and a novelty of the current thesis. Further studies are needed to explore the association of maternal thyroid autoimmunity and child neuropsychological development in order to evaluate which factors impact on thyroid autoimmunity and which modify its relation with child cognitive development, as well as to identify the biological mechanisms of the observed associations.

- Our results have supported that maternal subclinical hypothyroidism in early pregnancy is associated with externalizing and internalizing problems in early childhood and that maternal thyroid autoimmunity further reinforces the aforementioned associations. Additionally, it is supported that elevated thyroid antibodies in euthyroid pregnant women are associated with adverse behavioral outcomes. Current findings extend previous evidence on the impact of maternal non-clinical thyroid-hormone dysfunction on later child neuropsychological development and suggest that subclinical hypothyroidism and thyroid antibodies concentration levels need to be considered as predictors of child behavioral and emotional development. Future studies that will combine neuropsychological assessment in multiple domains and neuroimaging techniques are necessary to explore the role of thyroid mild dysfunction in child development, to pinpoint the exact mechanisms underlying such associations, and explore whether the effect of maternal thyroid dysfunction is area specific within the brain.

- Our findings also suggest that maternal thyroid hormones may be implicated in offspring's body composition and obesity, since subclinical hypothyroidism and hypothyroxinemia were associated with increased offspring BMI, waist circumference, and total fat mass in preschool age. Further studies are needed in order to replicate current findings and to explore how offspring cardiovascular profile is developed in later childhood and adulthood.

4.2 Strengths, limitations, and general conclusion

The main strength of the analyses in the current thesis is the use of data derived from the Rhea study, a population-based sample of pregnant women and their children. Due to the longitudinal design of the study repeated assessments of offspring development from infancy to childhood were available for the analyses; this provided the opportunity to consider the maturational aspect of the developmental process. In addition, phenotyping assessment was conducted by trained and expert staff with standardised protocols, comprehensive questionnaires and psychometric tools. Importantly, population-based and trimester specific reference intervals were applied for the definition of clinical, subclinical and normal ranges of thyroid hormones. The main weakness of the current analyses is the high sample attrition, which is an important problem in most prospective longitudinal studies; furthermore, the participants differed significantly compared to those who were lost in follow up; participants tended to be older, more educated, they breastfed more, and were more often primiparous, than non-participants.

We cannot exclude the possibility of residual confounding, even though we have adjusted for multiple available possible confounders, following procedures to avoid over-adjustment.

In conclusion, the present thesis supports and extends previous knowledge regarding the link of maternal thyroid dysfunction on offspring development using Rhea study's longitudinal design and its valid, multi-domain, and comprehensive developmental assessment. Current findings support that both maternal hypothyroxinemia and subclinical hypothyroidism during early pregnancy are linked with reduced offspring developmental outcomes. Furthermore, the present findings provide evidence that maternal thyroid autoimmunity is implicated in adverse offspring development and it may also strengthen the negative effect of maternal subclinical hypothyroidism on offspring development. Taking into account that the prevalence of mild maternal thyroid dysfunction is high in pregnancy, current findings, alongside previous evidence on the link of mild maternal thyroid dysfunction with negative pregnancy outcomes and adverse offspring development, highlight the need for evidence on the effectiveness of medical treatment on mild thyroid dysfunction. The appropriate next research step is cautiously designed RCTs, initiated in early pregnancy and avoiding overtreatment, in order to respond correctly to the still unresolved conflict regarding the necessity of universal thyroid hormones' screening in pregnancy and the appropriate management of mild thyroid dysfunction in pregnant women.

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Patterns of Early-Life Social and Environmental Exposures and Child Cognitive Development, Rhea Birth Cohort, Crete, Greece

Mariza Kampouri, Andriani Kyriklaki, Theano Roumeliotaki, Katerina Koutra, Despoina Anousaki, Katerina Sarri, and Maria Vassilaki
University of Crete

Manolis Kogevinas
Centre for Research in Environmental Epidemiology (CREAL)

Leda Chatzi
University of Crete

Early-life exposures are critical for later child cognitive development. McCarthy Scales of Children's Abilities (MSCA) were used to assess cognitive development of 700 preschoolers (M_{age} = 4.2 years), derived from the "Rhea" birth cohort, in Greece. Principal component analysis (PCA) was applied on prospectively collected exposure data. Six components were extracted; five of them were associated with child cognition. Higher parental social status, preschool attendance and less TV watching, nonsmoking during pregnancy and breastfeeding, and parental involvement in child life were protective factors of child cognition at 4 years. Increased child birth order was negatively associated with child cognition. Offspring's size at birth was not associated with any cognitive outcome. These findings reveal the importance of early-life exposures to child cognitive development.

Events occurring in early human development influence health in later life, shaping and determining the risk for chronic diseases (DOHaD, Developmental Origins of Health and Disease hypothesis, Hanson & Gluckman, 2014). The development of the central nervous system constitutes a long-lasting process, thus central nervous system exhibits developmental plasticity for a long period of time (de Graaf-Peters & Hadders-Algra, 2006) during which environmental conditions influence its development, predisposing to specific cognitive and behavioral phenotypes (Meaney, 2010).

Over the past decades, epidemiological studies have evaluated the effects of several single, early-life exposures on child cognition and behavior. More specifically, higher parental social status has been associated with advantageous cognitive (Hackman & Farah, 2009; Hackman, Farah, & Meaney, 2010; Hanson & Gluckman, 2014; Wong & Edwards, 2013) and behavioral outcomes in childhood (Russell, Ford, Williams, & Russell, 2015). Nursery and preschool attendance have also been reported to be beneficial for child cognition (Côté, Doyle, Petitclerc, & Timmins, 2013; Sylva, Stein, Leach, Barnes, & Malmberg, 2011), likewise enhanced breastfeeding duration (Belfort et al., 2013; Leventakou et al., 2015; Victora et al., 2015).

On the contrary, maternal smoking during pregnancy has been associated with decreased cognition and greater risk for attention deficit hyperactivity disorder (ADHD; Linnet et al., 2003), similarly with single parenthood (Oliver, Kretschmer, & Maughan, 2014), prematurity and low birth weight (Aarnoudse-Moens, Weisglas-Kuperus, van Goudoever, & Oosterlaan, 2009), as well as younger maternal age (Chang et al., 2014; Chudal et al., 2015) and

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Correspondence concerning this article should be addressed to Mariza Kampouri, Department of Social Medicine, Faculty of Medicine, University of Crete, Heraklion, 71003, Crete, Greece. Electronic mail may be sent to m.kambouri@med.uoc.gr.

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lower maternal education (Gurevitz, Geva, Varon, & Leitner, 2014).

To our knowledge, there have been no studies exploring the association of multiple, coexisting, and overlapping early-life exposures with child cognitive development at preschool years. The need for a more integrated and comprehensive study of multiple exposures on human development has been recently highlighted (Vrijheid et al., 2014; Wild, 2012). Principal component analysis (PCA) enables the exploration of multiple exposures, identifying potential groups of variables; therefore, giving the opportunity to expose the dominating characteristics of single exposures (Wold, Esbensen, & Geladi, 1987).

The "Rhea" study is a prospective, birth cohort in Crete, Greece, which has information on multiple exposures from the prenatal period through the preschool age. The aim of the present study is to identify patterns of early-life social and environmental exposures, and examine their association with child development at preschool age.

Method

Study Population

The "Rhea study" is an ongoing prospective birth cohort based in Heraklion, Crete. Pregnant women, living in the prefecture of Heraklion, were recruited within 1 year (February 2007–January 2008; Chatzi et al., 2009). The inclusion criteria were residence in the study area, good understanding of Greek, and maternal age above 16 years. The first contact with the families was made at the 15th gestational week (first major ultrasound examination) and the second at the 24th gestational week. The participants were invited again at child follow-up assessments when the children were 9 and 18 months old and 4 years old. Participants' assessments, at the 4-year follow-up, took place at the University Hospital of Heraklion or at health centers for the families residing in rural areas. 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. Written informed consent was obtained from the participants of the study.

Study Participants

Of the 1,363 live singleton births, 875 children participated in the 4-year follow-up, 18 children were excluded due to neurodevelopmental disorder or other medical condition (i.e., plagiocephaly, microcephaly, hydrocephaly, brain tumor) and 8 children due to incomplete neuropsychological assessment, resulting in a cohort of 849 children with valid outcome data at 4 years. Of the 849 children with valid data on cognitive development, 700 had available data on multiple early-life exposures (M_{age} = 4.2, SD = 0.20). Of these participants, 358 were male (M_{age} = 4.2, SD = 0.19) and 342 female (M_{age} = 4.2, SD = 0.21). Of the 700 participants, 610 provided data on ADHD difficulties, of which 300 were female and 310 male. The total of the 700 children were Greek speaking, and the majority of them resided in urban centers (71%). Almost half of the children were the only or the firstborn child. The vast majority of the participants (85.7%) attended preschool at 4 years and used to spend their free time at weekends with both their parents (84.9%). Participating parents were mainly Greek (fathers 97% and mothers 87%), married at the time of the pregnancy, and living in urban areas, and almost all fathers were employed, similarly to half of the mothers. Half of the mothers had completed postsecondary education, likewise 43% of the fathers. We have compared the demographic characteristics at baseline between participants and nonparticipants at the 4-year follow-up (Table S1). Parents of children who participated were more likely to be older, more educated, and married at child birth, in comparison with nonparticipants. No statistically significant differences have been identified between the excluded participants due to neurodevelopmental disorder diagnosis and nonexcluded children, in relevance with the exposure variables, with the exception of breastfeeding duration and preschool attendance at 4 years, which can be attributable to the specific difficulties these children face (Tables 1 and S2).

Exposure Assessment

Parental and child sociodemographic characteristics were collected through questionnaires administered by interviewers. The variables were entered in PCA, after they were coded as it is referred below. Information on maternal age at child birth (in years), maternal parity at birth (primiparous, multiparous), and maternal smoking status during pregnancy (smoking, quit during pregnancy, no smoking) was collected during pregnancy. Information on breastfeeding duration (in months) was collected at 9 months postpartum and was updated at 18 months. Information regarding maternal and paternal education (low: mandatory schooling

Table 1
Study Participants' Characteristics

	N (%)
Parental characteristics	
Maternal age at child birth	
≤ 20	28 (4.0)
21–30	347 (49.6)
31–40	315 (45.1)
41+	9 (1.3)
Paternal age at child birth	
≤ 25	32 (4.6)
26–35	406 (58.1)
36–45	239 (34.2)
46+	32 (4.6)
Maternal origin	
Greek	562 (87.9)
Non-Greek	38 (5.4)
Paternal origin	
Greek	680 (97.1)
Non-Greek	20 (2.9)
Parity	
Primiparous	298 (43.6)
Multiparous	385 (56.4)
Maternal education at 4 years	
Low	111 (15.9)
Medium	357 (51)
High	232 (33.1)
Paternal education at 4 years	
Low	227 (32.4)
Medium	301 (43)
High	172 (24.6)
Maternal employment status at 4 years	
Employed	409 (58.4)
Unemployed	291 (41.6)
Paternal employment status at 4 years	
Employed	648 (92.6)
Unemployed	49 (7)
Marital status at 4 years	
Married/engaged	690 (98.6)
Other	10 (1.4)
Residence at 4 years	
Urban	496 (70.9)
Rural	204 (29.1)
Infant characteristics	
Child's sex	
Boys	358 (51.1)
Girls	342 (48.9)
Gestational age at birth (weeks), *M* (*SD*)	38.23 (1.49)
Birth weight (kg), *M* (*SD*)	3.21 (0.45)
Birth length (cm), *M* (*SD*)	50.51 (2.33)
Birth head circumference (cm), *M* (*SD*)	34.18 (1.38)
Breastfeeding duration (months), *M* (*SD*)	4.18 (4.39)
Child characteristics at 4 years	
Child age (years), *M* (*SD*)	4.2 (0.2)
Child birth order	
Only child	121 (17.3)
First child	204 (29.1)
Other	375 (53.6)
Preschool attendance	
Yes	600 (85.7)
No	100 (14.3)
TV watching (min/day)	
Less than 60	190 (27.1)
Approximately 60–120	266 (38)
More than 120	244 (34.9)
Parental participation in child leisure time	
Both parents	594 (84.9)
Other	106 (15.1)

[9 years], medium: postsecondary education [12 years], high: university/technical college degree), residence (rural, urban), maternal occupational status (unemployed, employed), birth order (only child, first child, other), and maternal marital status (single, married) was obtained during the first trimester of pregnancy and updated at 4 years. Data on child preschool attendance (yes, no), television watching during weekdays (less than 1 hr, 1–2 hr, more than 2 hr), and parental participation at leisure time during weekends (both parents, one parent, other person) were obtained at the 4-year follow-up assessment. Gestational age and anthropometric measurements at birth were collected from clinical records at delivery (used as *z* scores, adjusted for gestational age and gender).

Neuropsychological Assessment

McCarthy Scales of Children's Abilities

Children's cognitive and motor development at 4 years was assessed through the McCarthy Scales of Children's Abilities (MSCA; MacCarthy, 1972). The MSCA represent an age-appropriate instrument, developed for children of ages 2½–8½ years, which assesses children's present cognitive development to identify possible developmental delay in different domains. The MSCA contain 18 subscales, which provide six scales: (a) verbal scale (verbal expression and comprehension), (b) perceptual performance (reasoning), (c) quantitative scale (numerical aptitude and interest), (d) general cognitive scale (overall cognitive ability), (e) memory (verbal and nonverbal short-term memory), and (f) motor scale (gross and fine motor ability).

The MSCA were administered individually to the participants by two trained psychologists. The participants were randomly distributed between them. The interobserver variability was < 1%. At the end of the neuropsychological assessment the examiners completed a standard form regarding the assessment's conditions used to evaluate the "quality of assessment" (excellent, bad, very bad). Families received detailed feedback on their children's performance.

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 (χ^2/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 (Royston & Wright, 1998). 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* = 15 to homogenize the scales.

Attention Deficit Hyperactivity Disorder Test

Information on children's ADHD difficulties was gathered through the ADHD test (ADHDT; Gilliam, 1995), which was completed by the participants' mothers. The questionnaire is based on *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.) criteria and designed to identify and evaluate ADHD symptoms at ages 3–23 years. It includes 36 items, providing three subscales (hyperactivity, inattention, and impulsivity) and a total ADHD difficulties index. The ADHDT has been translated and adapted for the Greek population (Maniadaki & Kakouros, 2002).

Statistical Analyses

Potential factors affecting cognitive development were subjected to PCA, with varimax rotation, and the score loadings to extract the components were derived using a regression based method. Bartlett's test of sphericity (p < .001), the Kaiser–Meyer–Olkin measure (KMO = .596), and the correlation matrix determinant (determinant = .049) supported sampling adequacy for PCA. The selection criterion for the extracted components was eigenvalues > 1, and the accepted factor loadings over 0.3.

Multivariable linear regression models were implemented to examine the associations between the extracted components and each outcome, after the components were classified in tertiles to facilitate interpretation. The reference group was the first tertile, which corresponds to the lowest tertile of scores on each component. The components were entered simultaneously in each model and adjusted for confounding variables (child's gender, examiner, quality of assessment). Sensitivity analysis was conducted excluding preterm births to distinguish confounding by prematurity. The main analyses was repeated in a subsample of children (N = 346) with available maternal intelligence data (Raven's Standard Progressive Matrices; Raven & Court, 1998) to adjust for any confounding effect by maternal intelligence. The potential modification effect of child sex was examined by including an interaction term with each component and child's sex in the models. Subsequently, multivariate models stratified by child sex were implemented.

Estimated associations are described in terms of β coefficients and 95% confidence intervals (CIs). All hypothesis testing was conducted assuming a .05 significance level and a two-sided alternative hypothesis. Benjamini–Hochberg correction was performed post hoc to control for false discovery rate. The statistical analyses were performed using SPSS 22 (IBM Corporation, Armonk, NY) and Stata 13 (Stata Corp. LP, College Station, TX) statistical software.

Results

Patterns of Early-Life Social and Environmental Exposures

A total of 15 potential factors affecting cognitive development were included in this analysis and six components were extracted, accounting for 62.52% of the total variance; the correlation between variables and each component are presented in Table 2: (a) *parental social status*, which accounted for 15.76% of the variance, includes paternal and maternal education, maternal occupational status, maternal age at birth, and residence; (b) *child birth order*, which accounted for 13.63% of the variance, includes birth order at 4 years and parity; (c) *size at*

Table 2
Principal Component Analysis of Early-Life Social and Environmental Exposures (N = *700*)

	Extracted components					
	Parental social status	Child birth order	Size at birth	Breastfeeding and nonsmoking	Preschool attendance and less TV watching	Parental involvement
Maternal education	.675					
Paternal education	.672					
Maternal occupational status	.612					
Maternal age at birth	.585					
Residence	.570					
Maternal parity at birth		.963				
Birth order		.944				
Birth weight			.855			
Birth head circumference			.843			
Smoking during pregnancy				.778		
Breastfeeding duration				.721		
TV watching					.761	
Preschool attendance					.750	
Parental participation in child leisure time						.769
Marital status						.767
Variance explained	15.76%	13.63%	10.03%	8.11%	7.68%	7.31%
Cumulative variance explained	62.52%					

Note. Only items with factor loadings > 0.30 were retained for each factor.

birth, which accounted for 10.03% of the variance, includes birth weight and birth head circumference; (d) *breastfeeding and nonsmoking during pregnancy*, which accounted for 8.11% of the variance, includes smoking during pregnancy and breastfeeding duration; (e) *preschool attendance and less TV watching*, which accounted for 7.68% of the variance, includes preschool attendance and hours of daily TV watching at 4 years; and (f) *parental involvement*, which accounted for 7.31% of the variance, includes parental marital status and parental participation at child leisure time during weekends at 4 years.

Early-Life Exposures and Child Cognitive Development

Figure 1 presents the associations of the six components with child's *cognitive* and *motor* abilities. Benjamini–Hochberg multiple testing correction was applied post hoc to control for false discovery rate. Higher parental social status was associated with increased scores in both *general cognitive* scale (β coefficient = 12.55, 95% CI [10.03, 15.08) and *motor* scale (β coefficient = 4.83, 95% CI [2.03, 7.63]), as well as with every other cognitive scale. Similarly, children who attended preschool at 4 years and watched less TV had higher scores in *general cognitive* scale (β coefficient = 5.25, 95% CI [2.80, 7.69]) and *motor* scale (β coefficient = 5.03, 95% CI [2.32 7.74]), as well as in specific cognitive scales. Children with married parents, who both participated in child leisure time during weekends, scored higher in the motor scale (β coefficient = 4.37, 95% CI [1.65, 7.09]) as well as in specific cognitive scales. Having multiparous mothers and more siblings was associated with lower scores in general cognitive scale (β coefficient = −3.40, 95% CI [−5.86, −0.94]), and particularly in the verbal domain (β coefficient = −4.61, 95% CI [−6.79, −1.24]), which remained significant after additional adjustment for advanced parental age.

Additional analyses were performed (Table S6) for children with low cognitive performance (verbal/perceptual scores < 80). Higher parental social status was associated with increased scores in the verbal domain (β coefficient = 3.49, 95% CI [0.02, 6.96]). Breastfeeding and nonsmoking during pregnancy were associated with increased perceptual performance scores (β coefficient = 5.59, 95% CI [0.61, 10.56]).

Sensitivity Analyses

Sensitivity analysis was conducted, excluding children born preterm, in order to examine any related confounding effect that was not identified previously (Table S3). Sensitivity analysis was conducted to test

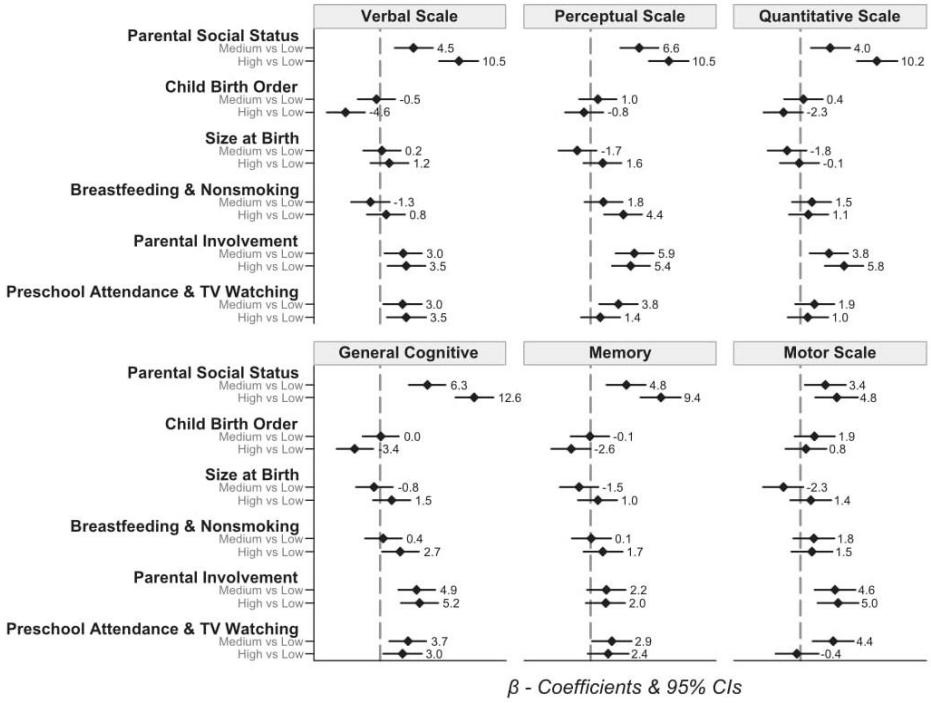

Figure 1. Associations between early-life exposures and McCarthy Scales of Children's Abilities (MSCA) at 4 years of age (N = 700). All models are adjusted for child's gender, examiner, quality of assessment, and for the rest components of early-life exposures.

for any confounding effect of maternal intelligence (available data for N = 346; Table S4). Corresponding results did not substantially differ.

Interaction and Stratified Analyses

Interaction effect of child gender was identified affecting the association between breastfeeding and nonsmoking component and perceptual performance (β coefficient = 8.27, $p < .001$), general cognitive scale (β coefficient = 7.35, $p < .001$), memory (β coefficient = 5.18, $p = .005$), and motor scale (β coefficient = 8.46, $p < .001$). Multivariate models stratified by child sex showed that nonsmoking in pregnancy and enhanced breastfeeding duration component constitutes a protective factor only for girls (Tables S5A and S5B). Gender interaction was also found to affect the association of child birth order and the verbal scale (β coefficient = 5.26, $p = .003$). Stratified analysis showed that greater birth order constitutes a stronger risk factor for girls (Tables S5A and S5B).

Early-Life Exposures and ADHD Difficulties

Figure 2 presents the results of multivariate analyses between the six components of PCA and ADHDT at 4 years. Lower scores in ADHDT subscales were identified in children of higher parental social status (total ADHD index: β coefficient = −5.80, 95% CI [−8.28, −3.31]), in children who attended preschool at 4 years and watched less TV during weekdays (total ADHD index: β coefficient = −4.65, 95% CI [−7.07, −2.23]), in children who were breastfed for enhanced time and their mothers did not smoke in pregnancy (total ADHD index: β coefficient = −4.54, 95% CI [−7.02, −2.05]), and in children with older siblings (total ADHD index: β coefficient = −3.68, 95% CI [−6.15, −1.22]).

Additional analysis was performed (Table S6) for children with high scores in ADHDT (ADHD index > 90th percentile). Breastfeeding and nonsmoking during pregnancy was associated with lower ADHDT scores (β coefficient = −7.52, 95% CI [−13.55, −1.50]) as well as preschool attendance and less TV watching (β coefficient = −4.94, 95% CI [−9.42, −0.46]).

Discussion

The results of this population-based, mother–child cohort study demonstrated that patterns of early-life characteristics such as higher parental social

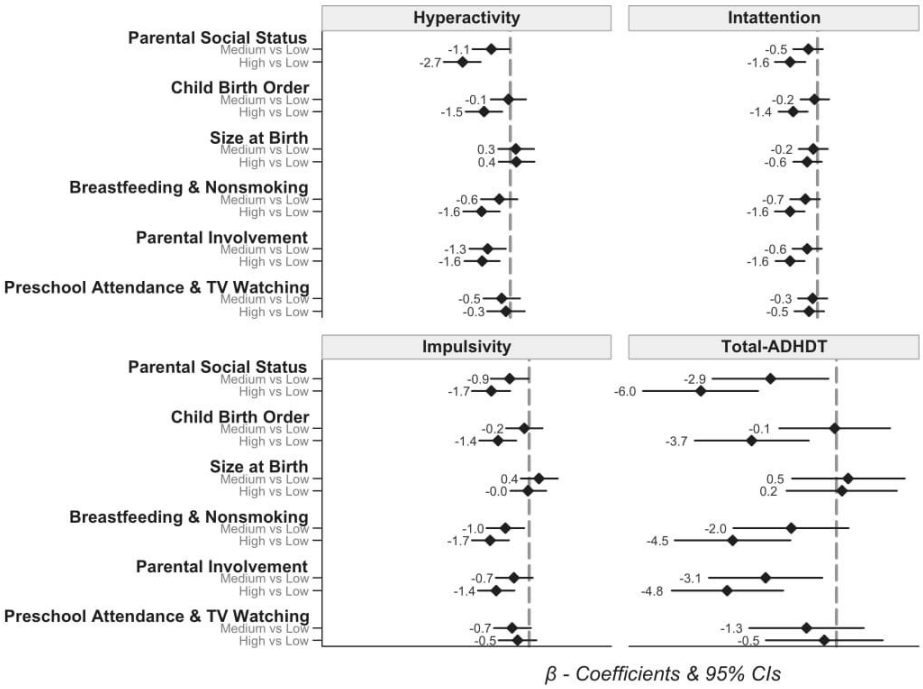

Figure 2. Associations between early-life exposures and attention deficit hyperactivity disorder test (ADHDT) at 4 years of age (N = 610). All models are adjusted for child's gender, child age, and for the rest components of early-life exposures.

status, preschool attendance along with TV watching, nonsmoking during pregnancy and enhanced breastfeeding and married, more participative in child-life parents are protective factors of cognitive and behavioral development at 4 years. Greater child birth order is a risk factor for child verbal development, whereas child size at birth has no association with cognition and behavior.

Higher parental social status was associated with children's optimal cognitive and behavioral development, supporting previous findings (Hackman et al., 2010; Kishiyama, Boyce, Jimenez, Perry, & Knight, 2009; Mezzacappa, 2004; Noble, McCandliss, & Farah, 2007). Such an association is supported by results showing less gray matter development in hippocampus region (Hanson, Chandra, Wolfe, & Pollak, 2011; Jednoróg et al., 2012) and in temporal, occipitotemporal, and anterior frontal regions in children with parents of lower social status (Jednoróg et al., 2012).

Preschool attendance was accompanied by fewer hours of TV watching in daily life; this pattern was positively associated with nearly every cognitive domain and negatively with ADHD difficulties. Previous findings from cross-country comparison studies highlighted preschool education's impact on school achievement and intelligence (Rindermann & Ceci, 2009) even at the age of 3 (National Institute of Child Health and Human Development Early Child Care Research Network, 2000). There are studies supporting no association of early TV watching ADHD difficulties (Foster & Watkins, 2010), however, our results support the opposite. The adverse effect of TV watching on the subsequent cognitive development has been identified by Zimmerman and Christakis (2005), even before the age of 3, and is possibly attributable to less time spent in beneficial activities (Koolstra, Van, & Voopt, 1996; Zimmerman & Christakis, 2005).

Children of divorced parents have been reported to exhibit poorer cognitive (Carlson & Corcoran, 2001) and educational outcomes (Ginther & Pollak, 2004). It has been stated that such an association may arise due to more available parental resources (Brown, 2004). Our findings support and extend the reported results since a coexistence of married parental status with the participation of both parents' at child leisure time was identified, as well its contribution to optimal cognitive outcomes.

Nonsmoking during pregnancy and enhanced breastfeeding duration factor was positively associated with children's motor ability and perceptual performance in girls. Breastfeeding has been recently suggested to have a protective role for

ADHD-related difficulties (Mimouni-Bloch et al., 2013). Furthermore, a gender-specific effect has been previously supported by a long-term, follow-up study on preterm infants, by which cognitive benefits of infant formula supplementation with long-chain polyunsaturated fatty acids (component of human breast milk) were identified only for girls (Isaacs et al., 2011). On the contrary, maternal smoking during pregnancy has been demonstrated to pose a risk for behavioral difficulties generally, as well as for ADHD (Langley, Heron, Smith, & Thapar, 2012; Linnet et al., 2005; Obel et al., 2010).

Greater child birth order was found to be negatively associated with children's general cognitive ability and especially with the verbal domain of cognition, which was also evident at 18 months of age (Koutra et al., 2012). This association remained significant after adjustment for advanced parental age. Previous studies have demonstrated that home crowding is correlated with increased chaos, which is linked with less parental responsiveness to children (Corapci & Wachs, 2002; Evans, Maxwell, & Hart, 1999). Even though increased child birth order has been linked with less risk for mental disorders development, such as autism (Gardener, Spiegelman, & Buka, 2009) and schizophrenia (Haukka, Suvisaari, & Lönnqvist, 2004), most studies do not identify such a link with ADHD risk (Berger & Felsenthal-Berger, 2009; Ghanizadeh, Abotorabi-Zarchi, Mohammadi, & Firoozabadi, 2012). On the contrary, our results suggest an impact of greater child birth order on greater maternal report of children's ADHD difficulties likewise was suggested by a previous study (Marín et al., 2012).

The strengths of the present study include the population-based prospective design, as well as the large sample size. The attrition rate of this study corresponds to 36%, and even though we cannot exclude bias due to nonparticipation, this is an inevitable limitation in longitudinal studies that has to be taken into account considering the presented findings. The use of MSCA, an internationally recognized psychometric instrument for its reliability, validity, and comprehensiveness, is another strength of the presented analyses. Moreover, the application of PCA gave the opportunity for concurrent exploration of multiple variables avoiding multicollinearity as well as overadjustment implications (Liu, Kuang, Gong, & Hou, 2003). However, even though multiple factors were included in the analyses, development is dependent on and affected by multiple exposures; thus, we cannot exclude the possibility of residual confounding effect of unmeasured variables on the results. In addition, a number of variables in the analyses are categorical, which is inevitably leading to loss of variability and could violate the assumptions of PCA.

To our knowledge, this is the first study that attempts to integrate multiple early-life exposures and explore their associations with child cognition and behavior at preschool age. Such holistic approaches that highlight the common characteristics of single exposures are crucial to expand knowledge in this field, as cognitive and behavioral development are rather complex and multifaceted, and they are based on multiple overlapping and interacting factors. Furthermore, this approach suggests a new way of objects' and target groups' selection for intervention designing, depending on the identification of multiple, coexisting exposures. Future research should implement innovative methods to incorporate multiple early-life exposures in order to define profiles of exposures and identify possible additive effects of them on child cognitive development.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website:

Table S1. Comparison of Parental Demographic Characteristics at Baseline Between Participants and Nonparticipants Excluding Multiple Pregnancies

Table S2. Comparison of Exposure Profile Between Excluded and Nonexcluded Children Due to Neurodevelopmental Disorder Diagnosis

Table S3. Associations Between Patterns of Early-Life Characteristics and McCarthy Scales of Children's Abilities at 4 Years ($N = 613$), Excluding Children Born Preterm

Maternal mild thyroid dysfunction and offspring cognitive and motor development from infancy to childhood: the Rhea mother–child cohort study in Crete, Greece

Mariza Kampouri,[1] Katerina Margetaki,[1] Katerina Koutra,[1,2] Andriani Kyriklaki,[1] Polyxeni Karakosta,[1] Despoina Anousaki,[1] Georgia Chalkiadaki,[3] Marina Vafeiadi,[1] Manolis Kogevinas,[4,5,6] Leda Chatzi[1,7,8]

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For numbered affiliations see end of article.

Correspondence to
Mariza Kampouri, Department of Social Medicine, Faculty of Medicine, University of Crete, Heraklion 71003, Crete, Greece; mar.kabouri@gmail.com

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ABSTRACT

Background Maternal thyroid hormones' supply is crucial for fetal neurodevelopment; however, the role of maternal mild thyroid dysfunction is not clear. We aimed to assess the association of maternal mild thyroid dysfunction with child neuropsychological development from infancy to early childhood.

Methods We included 757 mother–child pairs from the prospective 'Rhea' cohort on Crete, Greece. Maternal thyroid functioning was assessed by quantitative analysis of serum thyroid-stimulating hormone, free thyroxine, thyroid peroxidase antibodies and thyroglobulin antibodies at early gestation (mean=14 weeks). Neuropsychological assessment was based on Bayley Scales of Infant Development (18 months of age), McCarthy Scales of Children's Abilities (4 years of age), Raven's Coloured Progressive Matrices, Trail Making Test and Finger Tapping Test (6 years of age).

Results In multivariate adjusted linear regression analyses, maternal hypothyroxinemia was associated with decreased verbal scores at 4 years and reduced motor speed at 6 years of age. Maternal thyroid autoimmunity was associated with decreased child perceptual and motor ability at 4 years of age. Four trajectories of longitudinal non-verbal cognitive development were identified and children exposed to maternal thyroid autoimmunity had increased risk for belonging to an adverse trajectory ('low': adjusted relative risk ratio (RRR) = 2.7 95% CI: (1.4, 5.2), 'high-decreasing': adjusted RRR = 2.2 95% CI: (1.2, 4.0), 'low-increasing': adjusted RRR = 1.8 95% CI: (1.0, 3.2)).

Conclusion Maternal hypothyroxinemia is associated with reduced offspring verbal and motor ability. Maternal thyroid autoimmunity is associated with decreased offspring perceptual performance and motor ability and increased risk for adverse non-verbal cognitive development from infancy to childhood.

INTRODUCTION

Brain development begins shortly after conception and occurs in a chain of developmental events, which correspond to periods of increased vulnerability to environmental stressors.[1] During these critical periods, thyroid hormones are essential for normal neural network construction since the interactions of the active thyroid hormone triiodothyronine with nuclear receptors in the central nervous system regulate the expression of genes involved in cell differentiation, migration, signalling and myelination.[2]

Thyroxine of maternal origin represents the primary source of thyroid hormones until fetal thyroid gland functional maturation (18th–20th gestational week) and remains a complementary source of circulating thyroxine for the fetus until birth.[3] MRI studies have shown that children of mothers with overt hypothyroidism during pregnancy have morphological alterations in the cortex[4] and the hippocampal volume[5], brain regions that support various cognitive functions like perception, analytical thinking, executive functioning and memory.[6]

Evidence from observational studies suggest that low maternal free thyroxine (fT4) levels are associated with decreased mental performance[7] and intelligence,[8] and maternal hypothyroxinemia (thyroid-stimulating hormone (TSH) within the normal interval and decreased thyroxine (fT4)) is associated with decreased intelligence,[9–11] decreased quantitative and general cognitive development,[12] reduced cognitive ability and perceptual performance and memory,[13] decreased response speed[14] and increased risk for delay in expressive language and non-verbal cognition.[15] Furthermore, findings from a recent study have supported that both low and high concentrations of maternal thyroxine are associated with reduced intelligence and decreased grey and total cortex volume[8]; however, the role of increased thyroxine levels in child cognitive development has not been studied in other populations and the role of hyperthyroxinemia is still unclear. Previous findings regarding subclinical hypothyroidism (TSH above the normal interval and fT4 within the normal interval) have been less conclusive. More specifically, subclinical hypothyroidism has been associated with neurodevelopmental delays in infancy,[16] decreased intelligence in toddlerhood[10] and reduced verbal, memory and cognitive scores in prematurely born infants.[12] Conversely, results from two large observational studies did not support any association of maternal subclinical hypothyroidism or decreased levels of TSH with offspring cognitive development.[7 15] Furthermore, the heterogeneity of the findings regarding the role of maternal thyroid autoimmunity[10 17–20] has

underlined the need for further studies in order to clarify if there is any effect of maternal thyroid autoimmunity on child cognition, if the effect is transient[19] or whether it is dependent on the specific characteristics of the examined population (eg, iodine status).[17]

In the present study, we evaluated the impact of maternal mild thyroid dysfunction on child neuropsychological development. We hypothesised that maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia and thyroid autoimmunity during early pregnancy are associated with decreased neuropsychological development from infancy to early childhood.

METHODS

Participants

This study is part of the 'Rhea' study, a mother–childbirth cohort on Crete, Greece, which follows up children from fetal life onward. Participants were recruited in early pregnancy, at the time of the first ultrasound examination. The inclusion criteria were residency within the study area, good understanding of the Greek language and maternal age greater than 16 years. Participants were invited to child neuropsychological follow-up assessments when the children were 18 months, 4 years and 6 years old. Data on maternal thyroid parameters were available for 1170 women with a live singleton birth. Of this population, 484, 695 and 488 participants provided data on neuropsychological development at 18 months, 4 years and 6 years of age, respectively (online supplemental figure 1), while 757 children participated in at least one of these follow-up assessments. Further details on participant recruitment and follow-up procedures have been previously described in detail.[21]

The present study was conducted according to the principles of the Helsinki Declaration. All procedures were approved by the Ethics Committee of the University Hospital of Heraklion. Written informed consent was obtained from all adult participants.

Maternal thyroid parameters

Maternal blood samples were collected at the first prenatal visit (mean gestational age 14.12 weeks, SD 3.6 weeks). Serum samples were collected in vacutainer tubes (10 mL), were centrifuged and stored in aliquots at −80°C until assayed. Maternal thyroid functioning was assessed by quantitative analysis of serum TSH, fT4, thyroid peroxidase antibodies (TPO-Abs) and thyroglobulin antibodies (Tg-Abs), by Immulite 2000 immunoassay system (Siemens Healthcare Diagnostics, Los Angeles, California, USA). The interassay and intra-assay coefficients of variation were as follows: TSH <5.3 and <6.4 (0.32–39 mIU/mL), fT4 <7.8% and <7.1% (0.51–4.82 ng/dL or 6.56–62.03 pmol/L), Tg-Abs <4.9% and <5.8% and TPO-Abs <7.4% and 7.2%.

Population-based and trimester-specific reference intervals were applied for participants' assignment to subclinical hypothyroidism, hyperthyroxinemia and hypothyroxinemia categories. Further details regarding the estimation of the reference intervals are provided elsewhere.[22] Subclinical hypothyroidism was defined as TSH above the normal, trimester-specific reference interval but below 10 mIU/mL and fT4 within the normal range and the respective comparison group included women with TSH and fT4 levels within the normal trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL and fT4: 0.95–1.53 ng/dL; 2nd trimester: TSH: 0.18–2.73 μIU/mL and fT4: 0.87–1.45 ng/dL). Hypothyroxinemia was defined as TSH within the normal trimester-specific reference range and fT4 below the 5th percentile (corresponding to values of fT4 <0.95 ng/dL); the respective comparison group included women with TSH within the normal trimester-specific reference range and fT4 above the 5th percentile and below the upper trimester-specific fT4 limit. Hyperthyroxinemia was defined as TSH within the normal trimester-specific reference range and fT4 above the 95th percentile (corresponding to values of fT4 >1.54 ng/dL); the respective comparison group included women with TSH within the normal trimester-specific reference range and fT4 below the 95th percentile and above the lower trimester-specific fT4 limit. Thyroid autoimmunity was defined as elevated thyroid antibodies and TSH and fT4 within the normal range; thyroid-antibodies' status was considered elevated if the concentration of TPO-Abs was ≥35 IU/mL and/or the concentration of Tg-Abs was >40 IU/mL.

Child neuropsychological development

Child neuropsychological development was assessed by internationally validated and standardised scales administered by trained psychologists. Neuropsychological development assessment at 18 months (mean (SD): 1.5 (0.1) years) was conducted using the Bayley Scales of Infant and Toddler Development (Bayley-III).[23] The Bayley-III provides indexes on five developmental domains (cognitive, receptive communication, expressive communication, fine motor and gross motor). Raw scores were standardised for child's age and homogenised with a mean (SD) of 100 (15).[24] Further information is provided elsewhere.[25] Cognitive and motor development assessment at 4 years of age (mean (SD): 4.3 (0.2) years) was conducted using McCarthy Scales of Children's Abilities (MCSA).[26] The MCSA provide indexes on five developmental domains (verbal, quantitative, memory, perceptual performance and motor) and a general cognitive development index. MCSA raw scores were standardised for child's age and homogenised with a mean (SD) of 100 (15).[24] Further details are provided elsewhere.[27] Cognitive development assessment at 6 years of age (mean (SD): 6.6 (0.3) years) was computerised and included the Raven's Coloured Progressive Matrices (RCPM),[28] which assess non-verbal intelligence, the Finger Tapping Test (FTT),[29] which assesses motor speed, and the Trail Making Test (TMT-Part A & Part B),[29] which assesses processing speed, mental flexibility and executive functioning.

Covariates

We selected the covariates of the analyses based on the Directed Acyclic Graphs (DAGs) approach (online supplemental figure 2). The minimal sufficient adjustment set for estimating the total effect of maternal thyroid hormones on child cognition included maternal origin (Greek/other), parity (primiparous/multiparous), maternal smoking at early pregnancy (yes/no), maternal BMI in early pregnancy, maternal education (low level: ≤9 years of school, medium level: 9–12 years of school, high level: university or technical college degree), maternal age at birth (years), maternal marital status at pregnancy (married/other) and gestational age at blood sampling. Quality of assessment and child sex were included a priori in all models and child age was included a priori in the models involving TMT and FTT. The combined missing values of the covariates were <6%.

Statistical analysis

We conducted descriptive analyses on the characteristics of the study population and the distribution of the exposure. Multivariate linear regression models were used to estimate

beta coefficients with 95% CIs for the associations of maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia and thyroid autoimmunity as well as the concentration levels of maternal TSH and fT4 with each score of offspring neuropsychological development. In addition, we constructed trajectories of longitudinal non-verbal cognitive development from infancy to early childhood using Group-Based Trajectory Modelling (GBTM). The distribution percentiles of the Bayley's cognitive scale, the MSCA's perceptual performance and the RCPM's total score were calculated and used in GBTM, in order to homogenise the scales. The Stata traj-plugin was used to estimate the group-based trajectory model. Participants with data on neuropsychological development in, at least, two timepoints were included (N=586). Probability of group membership, predicted trajectory of each group and posterior probabilities of group membership were estimated. Two to five possible cognitive development trajectories were tested and a model with four classes was selected based on the Bayesian information criterion, the evaluation of average posterior probability (value >0.65), the odds of correct classification (>5) and the number of participants in each group (online supplemental table 1). The associations between maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia and thyroid autoimmunity and the non-verbal cognitive trajectories of development were explored using multinomial linear regression models, weighted for each individual's posterior probability of belonging to each of the trajectories. The estimated associations derived from the multinomial linear regression models are presented in terms of relative risk ratios and 95% CIs.

We performed sensitivity analyses, excluding mothers under thyroid medication during pregnancy (N=96). We also repeated the main analyses in children exposed to elevated levels of maternal TPO-Abs ≥35 IU/mL and to elevated levels of Tg -Abs>40 IU/mL separately.

The statistical analyses were conducted using STATA 13.1 (Statacorp, College Station, Texas, USA) and the DAGs were designed using DAGitty-v. 3.0. All associations were tested assuming a p value <0.05 significance level.

RESULTS

Maternal and child characteristics at 18 months, 4 years and 6 years of age are presented in table 1. Non-response analyses showed no differences between participants and non-participants in terms of maternal thyroid functioning (TSH and fT4), maternal smoking status during pregnancy, offspring gestational age at birth and birth weight. However, mothers who did not participate in the follow-up assessments were younger (mean difference=−0.9 years; 95% CI (−1.5, −0.3) p=0.004) and more often less educated (low educational level 29.8% vs 17.4%, χ^2=28.85(2), p<0.001), multiparous (65% vs 58%, χ^2=5.14(1), p=0.023)) and non-Greek (14.3% vs 6.3%, χ^2=19.84(1), p<0.001) compared with mothers who participated in the follow-up assessments (online supplemental table 2). We identified four trajectories of non-verbal cognitive development from infancy to early childhood (continuously low, continuously high, high at 18 months-decreasing over time and low at 18 months-increasing over time) (figure 1). Individual trajectories based on GBTM analysis are presented in online supple mental figure 3. The background characteristics between exposed and non-exposed participants are presented in online supplemental table 4.

Multivariate linear regression models showed that maternal hypothyroxinemia during pregnancy was associated with decreased verbal score at 4 years and motor score at 6 years of age (table 2). Children exposed to maternal thyroid autoimmunity during gestation had decreased perceptual performance and motor score at 4 years (table 2). We did not identify any association between maternal subclinical hypothyroidism or hyperthyroxinemia and child neuropsychological development. Children exposed to decreased concentration levels of maternal fT4 had decreased receptive communication scores at 18 months (online supplemental table 3). Maternal concentration levels of TSH

Table 1 Baseline characteristics of the study participants at 18 months, 4 years and 6 years of age, Rhea mother–child study, Crete, Greece

	18 months† (N=467)	4 years† (N=658)	6 years† (N=460)
Maternal characteristics			
Age (years)	30.3 (4.7)	29.7 (5.0)	30.1 (4.8)
Educational level			
Low	61 (13.1)	110 (16.7)	70 (15.2)
Medium	228 (48.8)	335 (51.0)	232 (50.4)
High	178 (38.1)	212 (33.3)	158 (34.4)
Parity			
Primiparous	196 (42.0)	283 (43.0)	199 (43.3)
Multiparous	271 (58.0)	375 (57.0)	261 (56.7)
Origin			
Greek	449 (96.2)	617 (93.8)	435 (94.6)
Non-Greek	18 (3.9)	41 (6.2)	25 (5.4)
Smoking at early pregnancy			
Yes	161 (34.5)	233 (35.4)	159 (34.6)
No	306 (65.5)	425 (64.6)	301 (65.4)
Maternal thyroid parameters			
TSH (median (IQR)) (μIU/mL)	1.09 (0.95)	1.08 (0.95)	1.04 (0.91)
ft4 (ng/dL)	1.22 (0.20)	1.23 (0.21)	1.23 (0.21)
Maternal iodine (median) (μg/L)	178.6	172.4	168.5
Hypothyroxinemia‡	22 (5.7)	26 (4.7)	22 (5.7)
Subclinical hypothyroidism‡	29 (7.0)	38 (6.5)	27 (6.7)
Hyperthyroxinemia‡	20 (4.8)	28 (4.6)	19 (4.5)
Thyroid autoimmunity‡	50 (13.1)	83 (15.1)	56 (14.8)
Gestational age-sampling (weeks)	14.1 (3.6)	14.1 (3.5)	14.0 (3.5)
Thyroid medication			
No medication	395 (84.6)	562 (85.4)	390 (84.8)
Thyroxine	68 (14.6)	91 (13.8)	65 (14.1)
Anti-thyroid medication	4 (0.9)	4 (0.6)	4 (0.9)
Yes, no defined	–	1 (0.2)	1 (0.2)
Child characteristics			
Female sex	247 (52.9)	337 (51.2)	251 (54.6)
Birth weight (g)	3186.9 (435.2)	3215.4 (450.4)	3193.5 (448.8)
Gestational age (weeks)	38.2 (1.4)	38.2 (1.6)	38.1 (1.6)
Age at cognitive assessment (years)	1.5 (0.1)	4.2 (0.2)	6.6 (0.3)

†Data presented as mean (SD) for continuous variables and as frequency (%) on each category for categorical variables, unless otherwise mentioned.
‡Subclinical hypothyroidism definition: fT4 concentration levels within the population-based, trimester-specific reference ranges and TSH above the upper trimester-specific limit and below 10 mIU/mL; hypothyroxinemia definition: TSH concentration levels within the trimester-specific reference ranges and fT4 <5th percentile; hyperthyroxinemia definition: TSH concentration levels within the trimester-specific reference ranges and fT4 >95th percentile and above the lower trimester-specific limit; thyroid autoimmunity: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges and TPO-Abs ≥35 IU/mL and/or Tg-Abs >40 IU/mL.
fT4, free thyroxine; Tg-Abs, thyroglobulin antibodies; TPO-Abs, thyroid peroxidase antibodies; TSH, thyroid-stimulating hormone.

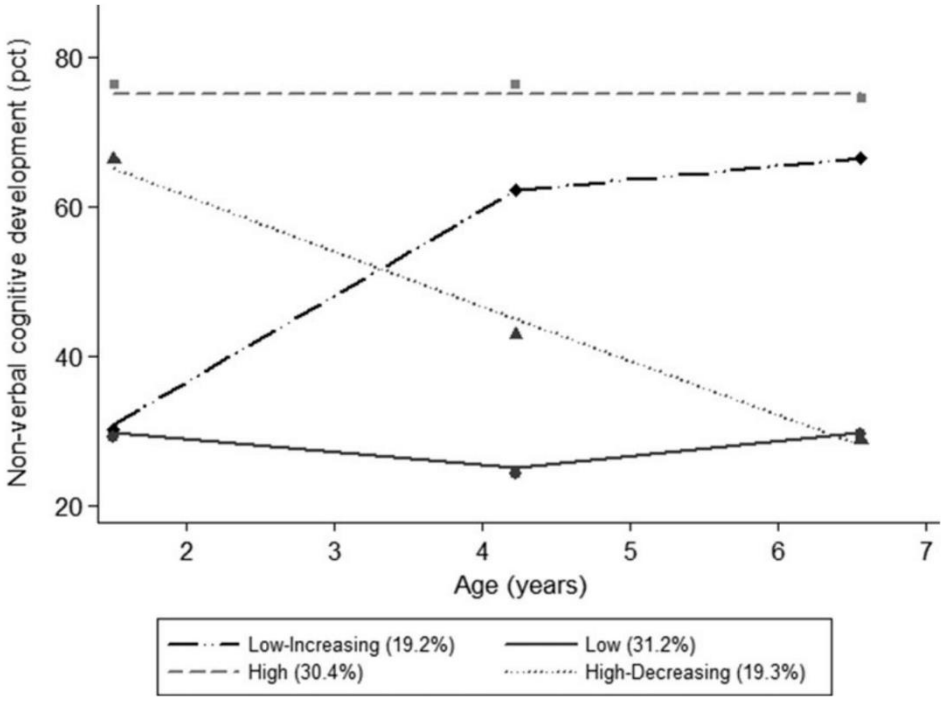

Figure 1 Non-verbal cognitive development trajectories, Group-Based Trajectory Modelling, Rhea mother–child study, Crete, Greece.

were not associated with any of the outcomes. The trajectory of continuously high non-verbal cognitive development from infancy to early childhood was set as the base outcome for the multinomial logistic regression models. Children exposed to maternal thyroid autoimmunity during gestation had increased risk for belonging to suboptimal non-verbal cognitive development trajectories (table 3).

In sensitivity analyses, we repeated the main models excluding mothers who took thyroid medication during pregnancy (N=96). We did not observe substantial differences between the results of sensitivity analyses and the results derived from the main analyses regarding the statistical significance of the associations and the magnitude of the associations (online supplemental table 5). We also repeated the analyses in children exposed to elevated levels of maternal TPO-Abs and children exposed to elevated levels of maternal Tg-Abs, separately (online supplemental table 6); we did not observe significant differences in comparison to the relevant main analyses (thyroid autoimmunity defined as elevated TPO-Abs and/or elevated Tg-Abs).

DISCUSSION

In this longitudinal cohort study, we found that maternal hypothyroxinemia is associated with decreased offspring verbal and motor ability scores. In addition, exposure to maternal thyroid autoimmunity was related to decreased perceptual performance and motor scores at 4 years of age. We also identified four trajectories of longitudinal non-verbal child cognitive development from infancy to early childhood and we demonstrated that maternal thyroid autoimmunity during pregnancy is associated with increased risk for children to belong in the 'low', 'high-decreasing' and 'low-increasing' trajectories, when compared to the continuously 'high' non-verbal cognitive trajectory.

Current results confirm findings of previous observational studies which supported that maternal hypothyroxinemia is associated with decreased child neuropsychological development.[7–10 14 15] Even though thyroid hormones impact on fetal neurodevelopment through the interaction of T3 with nuclear receptors of the fetal nervous system cells, T3 is formed locally through the deiodination of maternal T4 by iodothyronine deiodinase enzymes in glial cells.[2] Furthermore, findings from animal studies have demonstrated that induced maternal hypothyroxinemia leads to atypical neuronal migration and structural alterations in the somatosensory cortex and hippocampus, regions which are important for learning, memory, basic perceptual skills and higher-order cognitive abilities.[30 31]

However, the significance of maternal hypothyroxinemia as a marker of thyroid dysfunction in pregnancy has been challenged due to methodological issues in the relevant studies and due to concerns about the clinical relevance of hypothyroxinemia. The methodological problems that limit the interpretation of the studies include the differences in the definition of hypothyroxinemia, the diagnostic inaccuracy of fT4 measurements due to the physiological changes caused by pregnancy and due to the biases of the widely used automated immunoassays, the normal variability of fT4 concentration depending on the gestational age and the inadequate adjustment of the models for possible confounders of the explored associations.[32–34] Furthermore, observational studies that did not identify any association of maternal hypothyroxinemia with several pregnancy outcomes[35] as well as randomised controlled trials that did not find any cognition benefit in offspring exposed to maternal hypothyroxinemia or other mild thyroid dysfunction after levothyroxine medication[34 36–38] have resulted in concerns about the clinical importance of maternal hypothyroxinemia and in the

Table 2 Maternal hypothyroxinemia, subclinical hypothyroidism, hyperthyroxinemia and thyroid autoimmunity during pregnancy and child cognitive development at 18 months, 4 years and 6 years of age, Rhea mother–child study, Crete, Greece

	Hypothyroxinemia†	Subclinical hypothyroidism†	Hyperthyroxinemia†	Thyroid autoimmunity†
Neuropsychological development at 18 months§,¶				
Bayley Scales of Infant and Toddler Development-III				
Cognitive	2.6 (−3.9, 9.0)	−0.9 (−6.4, 4.7)	1.2 (−5.3, 7.7)	−3.6 (−7.9, 0.7)
Expressive communication	−4.1 (−10.6, 2.5)	−1.9 (−7.4, 3.6)	4.3 (−2.5, 11.0)	1.7 (−2.8, 6.1)
Receptive communication	−3.5 (−9.9, 2.9)	−0.2 (−5.6, 5.2)	4.3 (−2.3, 10.8)	−0.7 (−5.1, 3.6)
Gross motor	2.3 (−5.5, 10.1)	−0.5 (−7.1, 6.0)	1.1 (−9.0, 6.8)	−2.3 (−7.5, 3.0)
Fine motor	−0.1 (−6.5, 6.3)	1.4 (−4.1, 6.8)	2.6 (−3.9, 9.0)	−3.1 (−7.4, 1.2)
*Neuropsychological development at 4 years§,***				
McCarthy Scales of Children Abilities (MSCA)				
Verbal	**−6.6 (−12.3, −0.9)‡**	−0.0 (−4.6, 4.5)	3.3 (−2.2, 8.7)	0.3 (−3.0, 3.5)
Perceptual	−0.5 (−6.5, 5.5)	0.0 (−4.8, 4.8)	−0.7 (−6.4, 5.0)	**−3.6 (−7.1, −0.2)‡**
Quantitative	−5.7 (−11.9, 0.4)	−2.5 (−7.4, 2.4)	−0.7 (−6.6, 5.1)	−1.5 (−5.1, 2.0)
General cognitive	−4.9 (−10.6, 0.9)	−0.6 (−5.2, 3.9)	1.0 (4.4, 6.5)	−1.4 (−4.7, 1.9)
Memory	−2.6 (−8.5, 3.3)	−2.0 (−6.6, 2.7)	2.4 (−3.3, 8.0)	1.7 (−1.7, 5.0)
Motor	0.7 (−5.7, 7.1)	−0.1 (−5.2, 5.0)	−2.5 (−8.6, 3.6)	**−4.5 (−8.2, −0.8)‡**
Neuropsychological development at 6 years§,†,†				
Raven's Coloured Progressive Matrices (RCPM)				
Total score	−2.2 (−8.9, 4.5)	−0.2 (−6.2, 5.8)	−0.2 (−7.5, 7.0)	−3.3 (−7.5, 1.0)
Trail Making Test (TMT)				
Part A: log-transformed	0.2 (−0.0, 0.5)	−0.2 (−0,4, 0.0)	−0.1 (−0.4, 0.2)	−0.1 (−0.3, 0.1)
Part B: log-transformed	0.0 (−0.3, 0.3)	−0.0 (−0.3, 0.2)	0.1 (−0.2, 0.4)	−0.0 (−0.2, 0.2)
Finger Tapping Test (FTP)				
Dominant hand	−2.0 (−10.0, 6.0)	−1.5 (−8.4, 5.4)	1.8 (−7.0, 10.6)	−2.9 (−8.0, 2.2)
Non-dominant hand	**−9.6 (−17.6, −1.5)‡**	−1.4 (−8.4, 5.6)	−3.0, (−11.7, 5.8)	−1.4 (−6.5, 3.8)

†Comparison group for hypothyroxinemia models: thyroid-stimulating hormone (TSH) concentration levels within the trimester-specific reference ranges and free thyroxine (fT4) ≥5th percentile and below the upper trimester-specific limit; comparison group for subclinical hypothyroidism models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges; comparison group for hyperthyroxinemia models: TSH concentration levels within the trimester-specific reference ranges and fT4 ≤95th percentile; comparison group for thyroid autoimmunity models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges, thyroid peroxidase antibodies <35 IU/mL and thyroglobulin antibodies ≤40 IU/mL.
‡P<0.05.
§Models adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at blood sampling-thyroid assessment and quality of assessment; additional adjustment for child's age was included in the models with *TMT* and *FTP* outcomes.
¶Hypothyroxinemia: N=22, subclinical hypothyroidism: N=29, hyperthyroxinemia: N=20, thyroid autoimmunity: N=50.
**Hypothyroxinemia: N=26, subclinical hypothyroidism: N=38, hyperthyroxinemia: N=28, thyroid autoimmunity: N=83.
††Hypothyroxinemia: N=22, subclinical hypothyroidism: N=27, hyperthyroxinemia: N=19, thyroid autoimmunity: N=56.

no treatment recommendation by the latest guidelines for thyroid dysfunction management in pregnancy.[32]

Our results also suggest that maternal thyroid autoimmunity is associated with decreased perceptual performance and motor scores at 4 years and with increased risk for disadvantageous non-verbal cognitive development from infancy to early childhood. Previous findings regarding the role of maternal thyroid autoimmunity on child neuropsychological development have been diverse,[10 17–20] and it has been suggested that the different iodine status between populations may be the underlying cause of this heterogeneity.[17] Current results support the adverse impact of maternal thyroid autoimmunity on child neuropsychological development in a Greek, iodine-sufficient population.

The observed associations between maternal thyroid autoimmunity and offspring neuropsychological development can be explained through an impact of elevated maternal thyroid antibodies on the concentration levels of maternal fT4. It has previously been shown that elevated maternal TPO-Abs impair thyroidal stimulation caused by human chorionic gonadotropin, which is a pregnancy-specific hormone which binds to TSH receptors and ensures fT4 availability during pregnancy.[17 39] Even though we did not identify any significant differences of maternal fT4 concentration levels between mothers with and without elevated thyroid antibodies, later fT4 insufficiency during pregnancy cannot be excluded. In addition, maternal TSH concentration levels were higher in women with thyroid autoimmunity in comparison with those with normal levels of antibodies; this difference suggests an impact of the elevated antibodies on thyroidal function. This combination of factors (ie, elevated thyroid antibodies with high TSH concentration), regardless of the presence or the absence of subclinical hypothyroidism, may synergistically increase the risk of adverse pregnancy outcomes.[39]

Findings from animal studies support the observed associations, since they have demonstrated that the primary brain regions affected by decreased availability of maternal fT4 are the hippocampus, which is involved in memory and learning, the cortex, which is involved in perceptual skills and higher-order cognitive abilities, and the cerebellum, which is involved in motor abilities and motor co-ordination.[40] We have found no association of maternal subclinical hypothyroidism and offspring cognitive and motor development corroborating the results of previous large population-based studies[7 15] and failing to replicate the findings of other studies.[10 12 16] Current findings support that thyroid autoimmunity regardless of the presence of subclinical hypothyroidism are linked with adverse offspring outcomes. We have also explored the role of increased maternal fT4 concentration and hyperthyroxinemia in offspring cognitive

Table 3 Maternal hypothyroxinemia, subclinical hypothyroidism and thyroid autoimmunity during pregnancy and longitudinal trajectories of child non-verbal cognitive development (Group-Based Trajectory Modelling) from 18 months to 6 years of age, Rhea mother–child study, Crete, Greece

	Non-verbal cognitive development trajectories§,¶					
	Low		High-decreasing		Low-increasing	
	RRR	95% CI	RRR	95% CI	RRR	95% CI
Hypothyroxinemia†	1.3	(0.4, 3.7)	1.7	(0.7, 4.3)	0.9	(0.4, 2.2)
Subclinical Hypothyroidism†	1.0	(0.5, 2.2)	0.6	(0.3, 1.3)	0.8	(0.4, 1.7)
Hyperthyroxinemia	1.4	(0.5, 3.9)	1.4	(0.6, 3.7)	1.6	(0.6, 4.2)
Thyroid autoimmunity†	**2.7**	**(1.4, 5.2)‡**	**2.2**	**(1.2, 4.0)‡**	**1.8**	**(1.0, 3.2)‡**

†Comparison group for hypothyroxinemia models: TSH concentration levels within the trimester-specific reference ranges and fT4 ≥5th percentile and below the upper trimester-specific limit; comparison group for subclinical hypothyroidism models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges; comparison group for hyperthyroxinemia models: TSH concentration levels within the trimester-specific reference ranges and fT4 ≤95th percentile and above the lower trimester-specific limit; comparison group for thyroid autoimmunity models: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges, TPO-Abs <35 IU/mL and Tg-Abs ≤40 IU/mL.
‡P<0.05.
§Reference trajectory: continuously high non-verbal cognitive development from 18 months to 6 years.
¶Models adjusted for maternal age, maternal BMI, maternal origin, maternal educational level, maternal smoking status, maternal marital status at pregnancy, parity, child sex, gestational age at thyroid assessment and quality of assessment.
fT4, free thyroxine; RRR, relative risk ratio; Tg-Abs, thyroglobulin antibodies; TPO-Abs, thyroid peroxidase antibodies; TSH, thyroid-stimulating hormone.

and motor development since a recent MRI study has suggested that both low and high concentrations of maternal fT4 in early pregnancy are related to decreased intelligence and decreased grey matter and cortex volume.[8] We did not find any association of maternal hyperthyroxinemia with offspring cognitive and motor development. This null finding may be the consequence of low power in the specific analysis since we have few cases with hyperthyroxinemia in our population, and further research is necessary to elucidate if hyperthyroxinemia is linked with adversary offspring developmental outcomes.

The strengths of the present study include its population-based prospective design, the long follow-up period, and the reliable valid and comprehensive psychometric instruments that were used to assess child cognition. The use of GBTM to identify non-verbal cognitive development trajectories and explore any longitudinal impact of mild maternal thyroid dysfunction on child non-verbal cognition is another strength and a novelty of this study. Thyroid hormones were measured at a single timepoint during gestation for the current analysis; therefore, the measurements might reflect a transient dysfunction, even though single timepoint and longitudinal assessments are highly correlated. Bias due to non-participation and loss to follow-up might influence the results; non-participants did not differ regarding maternal thyroid parameters but they were more likely to have younger, non-Greek, multiparous, and less educated mothers compared with the participants of this study. In addition, residual confounding effect of unmeasured variables cannot be excluded, even though the models have been adjusted for multiple possible confounders. Mothers who took thyroid medication during pregnancy were not excluded from the main analyses; however, sensitivity analyses excluding these participants did not support substantially different results.

In conclusion, our findings support that maternal hypothyroxinemia during pregnancy is associated with decreased offspring verbal and motor ability in early childhood. We also demonstrated that maternal thyroid autoimmunity is associated with impaired perceptual performance and motor ability in preschool age and increases the risk for adverse non-verbal cognitive development longitudinally, from infancy to early childhood, in a Greek, iodine-sufficient population. Further studies are needed to explore the association of maternal thyroid autoimmunity and child neuropsychological development in other populations to evaluate the factors that impact thyroid autoimmunity and the factors that modify its relation with child neuropsychological development as well as to identify the biological mechanisms of the observed associations.

What is already known on this subject

- Maternal mild thyroid dysfunction during pregnancy is associated with adverse offspring neuropsychological development. However, previous research is focused on maternal hypothyroxinemia; hence, the information regarding the relation of maternal subclinical hypothyrodism, hyperthyroxinemia and thyroid autoimmunity with offspring development is relatively scarce and still inconclusive.

What this study adds

- Current findings replicate previous research results regarding the association of maternal hypothyroxinemia with decreased child cognitive and motor development.
- We also demonstrate that maternal thyroid autoimmunity is associated with decreased perceptual performance and motor development in preschoolers as well as with increased risk for suboptimal non-verbal cognitive development longitudinally, from infancy to early childhood, in an iodine-sufficient population.
- We did not find any association between maternal subclinical hypothyroidism or hyperthyroxinemia with offspring cognitive and motor development.

Author affiliations
[1]Department of Social Medicine, University of Crete School of Medicine, Heraklion, Greece
[2]Department of Psychology, University of Crete School of Social Sciences, Rethimno, Greece
[3]Department of Social Medicine, University of Crete - Voutes Campus, Heraklion, Greece
[4]Instituto de Salud Global Barcelona, Barcelona, Spain
[5]Hospital del Mar Institute for Medical Research, Barcelona, Spain
[6]Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública, Madrid, Spain
[7]Preventive Medicine, University of Southern California, Los Angeles, California, USA
[8]Department of Genetics and Cell Biology, Maastricht University Faculty of Health Medicine and Life Sciences, Maastricht, Netherlands

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ORCID iD
Mariza Kampouri http://orcid.org/0000-0002-3359-5691

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Maternal mild thyroid dysfunction and child behavioral and emotional difficulties at 4 and 6 years of age: The Rhea mother-child cohort study, Crete, Greece

Mariza Kampouri[a,*], Katerina Margetaki[a], Katerina Koutra[a], Andriani Kyriklaki[a], Polyxeni Karakosta[a], Katerina Sarri[a], Despoina Anousaki[a], Georgia Chalkiadaki[a], Marina Vafeiadi[a], Manolis Kogevinas[b,c,d], Leda Chatzi[e,f]

[a] Department of Social Medicine, Faculty of Medicine, University of Crete, Greece
[b] Instituto de Salud Global Barcelona, Spain
[c] Hospital del Mar Medical Research Institute (IMIM), 08003 Barcelona, Spain
[d] Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Spain
[e] Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, USA
[f] Department of Genetics & Cell Biology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, the Netherlands

1. Introduction

Thyroid hormones are essential for the development of the central nervous system, since they regulate neurodevelopmental processes such as proliferation, migration, synaptogenesis, and myelination (Howdeshell, 2002). The actions of thyroid hormones in the fetal central nervous system occur due to interactions of triiodothyronine with nuclear receptors, a process that triggers signaling cascades and regulates genetic expression of multiple genes involved in corticogenesis (Bernal, 2017). Since fetal thyroid hormones' secretion starts at the 10th gestational week and becomes sufficient between the 18th and 20th gestational week, the fetus depends, almost exclusively, on maternal thyroid hormones' supply until midgestation in order to meet the determinative hormone-dependent neurodevelopmental events of this period (Obregon et al., 2007).

MRI studies have supported that children of mothers with overt hypothyroidism during pregnancy have altered cortical morphology (Lischinsky et al., 2016), abnormal corpus callosum development (Samadi et al., 2015), and smaller hippocampus (Willoughby et al., 2014). Furthermore, animal studies have supported that even subclinically decreased thyroid hormones' concentration during early gestation alters histogenesis and the cytoarchitecture of the somatosensory cortex, the hippocampus, and the cerebellum (Lavado-Autric et al., 2003). While MRI-studies focused on clinical groups with behavioral problems have pinpointed structural and functional alterations in the aforementioned brain regions (Carmona et al., 2015; Duerden et al., 2012; Mous et al., 2014; Uytun et al., 2017; Vieira de Melo et al., 2018; Wang et al., 2018; Wu et al., 2017).

Moreover, a large body of observational studies has demonstrated that even subtle impairments of maternal thyroid function during pregnancy can impede child cognitive development (Finken et al., 2013; Ghassabian et al., 2014; Henrichs et al., 2010; Julvez et al., 2013; Korevaar et al., 2016a; Li et al., 2010; Pop et al., 2003; Pop et al., 1995; Pop et al., 1999; Williams et al., 2012), while at the same time evidence suggests that difficulties in specific cognitive domains underpin behavioral and emotional difficulties. For instance, increased externalizing symptoms have been associated with poor executive functioning (Schoemaker et al., 2013; Woltering et al., 2016), impaired general cognitive ability, and difficulties in learning and memory (Thompson et al., 2018). Conversely, increased internalizing symptoms have been linked with impairments in verbal fluency and memory (Blanken et al., 2017), problems in verbal processing (Toren et al., 2000), and poor executive functioning (Thompson et al., 2018). Even though there is considerable evidence supporting the aforementioned links, studies on the association between maternal thyroid hormones and child externalizing and internalizing problems are relatively scarce.

Findings of a previous population-based study have supported that maternal thyroid stimulating hormone (TSH) concentration levels are positively associated with child externalizing symptoms and that maternal thyroid autoimmunity increases the risk for offspring ADHD symptoms at 3 years of age (Ghassabian et al., 2011; Ghassabian et al., 2012). Results from the same study have detected an association of maternal hypothyroxinemia with child ADHD symptoms at 8 years and no association of maternal TSH with child behavioral development (Modesto et al., 2015). Other observational studies have shown that maternal hypothyroxinemia is associated with child externalizing and internalizing symptoms (Andersen et al., 2017), and child ADHD symptoms' manifestation at 5–6 years of age (Oostenbroek et al., 2017). In addition, low maternal thyroxine (fT4) concentration levels have been related with increased internalizing symptoms at 4 years of age

* Corresponding author at: Department of Social Medicine, Faculty of Medicine, University of Crete, Heraklion 71003, Crete, Greece.
E-mail address: m.kambouri@med.uoc.gr (M. Kampouri).

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(Endendijk et al., 2017).

The objective of this study was to evaluate the impact of maternal thyroid parameters during early pregnancy on child behavioral and emotional development in early childhood, in a population of an iodine sufficient country (Koutras et al., 2003), with high prevalence of iron deficiency (Karanikolaou et al., 1992; Krokidas et al., 1992; Tympa-Psirropoulou et al., 2008). Previous studies have identified various and different indicators of maternal mild thyroid dysfunction as predictors of child behavioral difficulties at different time-points of childhood. To our knowledge, this is the first study that provides a comprehensive behavioral and emotional development assessment in two different time-points of early childhood taking into account the maturational aspect of child development. The assessments were performed at preschool age and at early elementary school age. These are critical periods due to advances in cognitive and language development that enable emotional and behavioral regulation and due to the increasing academic and social demands that often mark the onset of behavioral difficulties (Steinberg and Drabick, 2015).

We also aim to examine whether child sex moderates the association between maternal thyroid functioning and child behavioral and emotional development. It has been extensively reported that males have increased vulnerability to externalizing symptoms and females to internalizing symptoms (Martel, 2013). Moreover, it has been previously proposed that different prenatal hormonal influences may explain these sex-specific findings (Endendijk et al., 2017). Previous studies regarding the role of child sex on the association of maternal thyroid dysfunction and child behavioral and emotional problems are contradictory. More specifically, results from a previous study supported that maternal TSH is associated with child attention problems in females exclusively (Päkkilä et al., 2013), but other findings suggested that this association is evident in males exclusively (Endendijk et al., 2017).

We hypothesized that increased maternal TSH and decreased maternal fT4 are associated with increased behavioral and emotional symptoms in early childhood. We also hypothesized that maternal subclinical hypothyroidism (TSH above the normal, trimester specific reference interval but below 10 mIU/mL and fT4 within the normal range), maternal hypothyroxinemia (TSH within the normal trimester specific reference range and fT4 below the 5th percentile), and elevated maternal thyroid antibodies (TSH and fT4 within normal trimester specific reference ranges and elevated TPO-Antibodies or/and Tg-Antibodies) during early pregnancy are associated with increased child behavioral and emotional problems. In addition, based on the contradictory previous findings regarding the effect of child sex on the association of maternal thyroid dysfunction and child behavioral development, we aimed to examine whether there are sex differences in the aforementioned associations.

2. Materials and methods

2.1. Participants

The population of this study is derived from the “Rhea” study, a mother-child, birth cohort in Crete, Greece. “Rhea” study was established at 2007 and follows up children from fetal life, in order to explore nutritional, environmental, and psychosocial determinants of children's health, growth, and development. Between February 2007 and February 2008, pregnant women were recruited at the time of the first comprehensive ultrasound examination (12th gestational week approximately) from 4 antenatal clinics (2 public & 2 private) covering the wider Heraklion region. The inclusion criteria were being a resident of the area, older than 16 years, and able to communicate in the Greek language. We contacted two times with the participants during pregnancy (12th and 24th gestational week) to collect information on several psycho-social, dietary, and environmental exposures through face-to-face structured questionnaires and self-administered questionnaires. We obtained additional information from medical records. Parents were invited to participate at child follow up assessments, when the children were 9 months, 18 months, 4 years, and 6 years old. Details on participant recruitment and follow up procedures have been described in detail elsewhere (Chatzi et al., 2017).

The 4- and the 6-year follow-up assessments were completed at the University Hospital of Heraklion, or at health centers for the families residing in rural areas. 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. Written informed consent was obtained from all the participants. Of 1363 live singleton births, data on maternal thyroid hormones during pregnancy were available in 1170 women. Information on child behavioral and emotional symptoms were available in 647 participants at 4 years and 489 at 6 years of age. Mothers receiving thyroid medication (N = 95) were included in the main analyses.

2.2. Maternal thyroid hormones' assessment

Maternal blood samples were collected at the first prenatal visit (mean gestational age 14.12 weeks, SD 3.6 weeks). Serum samples were collected in 10 mL vacutainer tubes, were centrifuged and stored in aliquots at −80 °C until assayed. Maternal thyroid functioning was assessed by quantitative analysis of serum thyroid stimulating hormone (TSH), free thyroxine (fT4), thyroid peroxidase antibodies (TPO-Ab), and thyroglobulin antibodies (Tg-Ab), by Immulite 2000 immunoassay system (Siemens Healthcare Diagnostics, Los Angeles, CA). The inter- and intra-assay variability were: TSH < 5.3 and < 6.4 (0.32–39 mIU/mL), fT4 < 7.8% and < 7.1% (0.51–4.82 ng/dL or 6.56–62.03 pmol/L), anti-Tg < 4.9% and < 5.8%, and anti-TPO < 7.4% and 7.2%.

Published, population-based, and trimester-specific reference intervals were used to assign participants into the categories of subclinical hypothyroidism and hypothyroxinemia (Karakosta et al., 2011). Subclinical hypothyroidism was defined as TSH above the normal, trimester specific reference interval but below 10 mIU/mL and fT4 within the normal range (Stagnaro-Green et al., 2002); the comparison group included women with TSH and fT4 concentration levels within the normal trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL; 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL). Hypothyroxinemia was defined as TSH within the normal trimester specific reference range and fT4 below the 5th percentile (corresponding to fT4 = 0.95 ng/dL); (Stagnaro-Green et al., 2002); the comparison group for hypothyroxinemia included women with TSH within the normal trimester specific reference range and fT4 above the 5th percentile. The 10th percentile of fT4 (corresponding to fT4 = 0.99 ng/dL) was used as an alternative cut-off point for the definition of hypothyroxinemia, in sensitivity analyses. Euthyroid women (TSH and fT4 within the normal population based and trimester-specific reference ranges) with elevated thyroid antibodies were compared to euthyroid women with low concentration levels of thyroid antibodies; thyroid-antibodies' status was considered elevated if thyroid peroxidase antibodies ≥ 35 IU/mL and/or thyroglobulin antibodies > 40 IU/mL.

2.3. Behavioral and emotional symptoms' assessment

Parents reported children's behavioral symptoms through validated and widely used questionnaires. The mean age of the participating children at the first behavioral assessment was 4.2 years and at the second 6.6 years. At the 4-year follow-up parents completed the Attention Deficit Hyperactivity Disorder Test (ADHDT) (Gilliam, 1995) and the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 1997). The ADHDT is based on ADHD criteria of DSM-IV. It is consisted of 36 items and constructed to identify and assess ADHD related symptoms in ages 3–23 years. The instrument provides 4 indexes, one for each of its three subscales (Hyperactivity, Inattention, and Impulsivity) and an index for total ADHD difficulties (possible range

0–72), for which higher scores indicate more and more severe ADHD symptoms. The ADHDT has been translated and adapted for the Greek population (Maniadaki and Kakouros, 2002). The SDQ is a brief screening questionnaire designed to assess behavioral strengths and difficulties in children from 3 to 16 years of age (Goodman, 1997). The questionnaire includes 25 items that are divided to five subcales: (i) Emotional symptoms, (ii) Conduct problems, (iii) Hyperactivity/Inattention, (iv) Peer-relationship problems, and (v) Prosocial behavior. The two broad band scales of Internalizing problems (emotional symptoms + peer-relationship problems) and Externalizing problems (conduct problems + hyperactivity/inattention) were also used in the current analyses. The SDQ was translated and adapted to the Greek population (Mpimpou-Nakou et al., 2001).

At the 6-year follow up assessment, parents completed the Child Behavior Checklist – Parent Report Form (CBCL) and the Conner's Parent Rating Scale, Revised, Short Form (CPRS-R: S) (Conners et al., 1998). 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 these analyses, include 6 scales that correspond to different diagnostic categories of the DSM-IV (American Psychiatric Association, 1994) (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 these analyses. The CBCL is translated, adapted, and standardized for the Greek population (Roussos et al., 1999). The *CPRS-R: S* is designed to assess ADHD symptoms and is composed by 27 items, resulting in 3 subscales (Oppositional problems, Cognitive problems/Inattention, Hyperactivity) and an index for total ADHD symptoms (possible range 0–36), for which higher scores indicate more and more severe symptoms. The translation and cross-cultural adaptation of the CPRS-R: S was performed according to the recommended methodology, including the stages of 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 to conclude to the final version of the questionnaire (Beaton et al., 2000).

2.4. Covariates

Potential covariates were selected on the basis of literature regarding the study's hypotheses (Ghassabian et al., 2012; Ghassabian et al., 2011; Modesto et al., 2015; Oostenbroek et al., 2017) and included maternal age, maternal educational level, maternal marital status at pregnancy, maternal origin, maternal smoking at pregnancy, maternal alcohol intake during pregnancy, maternal BMI, maternal IQ, parity, child's gestational age and weight at birth, child's sex, and child's age at the behavioral assessment. After the application of the change-in-estimate method (applied cut-off point: estimates' change > 10%) the following variables were retained as confounders: maternal age (continuous), maternal educational status (low/medium/high), parity (primiparous/multiparous), maternal smoking during pregnancy (yes/no), and maternal marital status at pregnancy (single, married). Breastfeeding duration (continuous) and birthweight (continuous) were also included in the models, since participants and non-participants significantly differed in these covariates (non-response analyses). This information was collected through questionnaires administered by interviewers at pregnancy and through medical records. The models were also adjusted for child's sex and child's age at the behavioral assessment (a priori selection).

2.5. Statistical analysis

Descriptive analyses on the characteristics of the study population, and the distribution of the exposures and the outcomes of interest were conducted. Multiple imputations were conducted to handle missing data in the ADHDT, SDQ, and CBCL questionnaires. Due to high item non-response, the percentage of missing values in the ADHDT, SDQ, and CBCL scales reached 24%. Thus, 20 complete data sets were generated using multiple imputations with chained equations (MICE) (White et al., 2011). In the imputation model, all the questionnaire items (raw data) were regressed on all the other items (Shrive et al., 2006). 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 PMM can yield plausible inference for ordered categorical data as well (Vink et al., 2015). 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 (Rubin, 2004). 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.

Generalized additive models (GAMs) were applied to test for the linearity of the association between maternal thyroid hormones and ADHDT, SDQ, CPRS-R: S, and CBCL scores. Linearity was assumed if p-gain, defined as the difference in normalized deviance between the GAM model and the linear model for the same exposure and outcome (Royston and Ambler, 1998) was > 0.1. Since there was evidence for non-linearity between maternal thyroid data and the outcome (Supplementary Figs. 1–4), maternal thyroid data were categorized into tertiles (low/medium/high). Maternal subclinical hypothyroidism, maternal hypothyroxinemia, and maternal euthyroidism accompanied with elevated thyroid antibodies (TPO-antibodies and/or Tg-antibodies) were also examined as possible predictors of child behavioral and emotional symptoms at 4 and 6 years of age. In order to examine the role of thyroid-antibodies' presence in subclinical hypothyroidism, stratified analyses by maternal thyroid-antibodies' status were also conducted. 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 order to assess the potential modifying effect of child sex, additional analyses were conducted. We included interaction terms in the respective regression models (p for interaction < 0.05). Sensitivity analyses were conducted excluding mothers who were under thyroid medication during pregnancy. Furthermore, the analyses were repeated to a subsample with available child thyroid hormones' assessment at 4 years (TSH), to adjust for any potential effect of child thyroid functioning on the identified associations. Sensitivity analyses with additional adjustment for maternal iodine and iron status was also conducted in a sub-sample with available information on these covariates. The multivariate models regarding maternal hypothyroxinemia were repeated using an alternative cut-off point for maternal fT4 (fT4 < 10th percentile).

The statistical analyses were conducted using STATA 13.1 (Statacorp, College Station, TX) and the threshold for statistical significance was set at the 5% level.

2.6. Non-response analysis

Non-response analyses showed no differences between participants and non-participants in terms of maternal thyroid functioning (TSH, fT4), smoking status during pregnancy, marital status, and in gestational age at birth. However non-participant mothers were younger

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

	Population at 4 years (N = 647)		Population at 6 years (N = 489)	
	N	Data[a]	N	Data[a]
Maternal characteristics				
Maternal age at child birth	646	29.89 (5.0)	488	30.15 (4.7)
Maternal education				
Low	105	16.4	69	14.2
Medium	329	51.3	247	50.9
High	208	32.4	169	34.9
Maternal origin				
Greek	612	95	460	95.8
Non-Greek	33	5.1	20	4.2
Parity				
Primiparous	288	44.5	218	44.9
Multiparous	359	55.5	268	55.1
Maternal smoking (early pregnancy)				
Yes	217	34.2	166	34.7
No	418	65.8	313	65.3
Maternal iodine at pregnancy (median) (μg/L)	447	168.4	366	168.4
Maternal iron at pregnancy (μg/dl)	443	72.5 (37.6)	356	72.0 (38.3)
Maternal TSH (μIU/mL)	647	1.34 (1.4)	489	1.32 (1.5)
Maternal ft4 (ng/dL)	646	1.22 (0.2)	489	1.23 (0.2)
Maternal subclinical hypothyroidism (SCH)[b]	41	7.1	30	6.9
Maternal hypothyroxinemia[c]	25	4.6	23	5.6
Maternal TPO-Abs and/or Tg-Abs +[d]	82	15.5	62	15.7
Maternal thyroid medication				
No medication	552	85.3	415	84.9
Thyroxine	90	13.9	69	14.1
Anti-thyroid medication	4	0.6	4	0.8
Yes, no defined	1	0.2	1	0.2
Child characteristics				
Child's sex (female)	309	47.8	218	44.6
Child age at behavioral assessment	647	4.2 (0.2)	489	6.6 (0.3)
Gestational age at birth (weeks)	639	38.2 (1.6)	483	38.1 (1.6)
Birth weight (grams)	634	3211 (446.2)	477	3193 (452.4)
Breastfeeding duration (months)	615	4.1 (4.3)	470	4.0 (4.1)
ADHDT – total score[e]	529	14.21 (12.35)	–	–
SDQ – total score[f]	572	8.7 (4.8)	–	–
CPRS-R: S – total score[g]	–	–	471	8.9 (5.7)
CBCL – internalizing problems score[h]	–	–	423	6.2 (4.5)
CBCL – externalizing problems score[h]	–	–	462	8.7 (6.6)

[a] Data presented as mean (standard deviation) for continuous variables (unless mentioned otherwise) and as frequency (%) on each category for categorical variables.
[b] fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: fT4: 0.95–1.53 ng/dL; 2nd trimester & fT4: 0.87–1.45 ng/dL) and TSH above the upper TSH trimester-specific limit (TSH > 2.53 μIU/mL & TSH > 2.73 μIU/mL for the 1st and the 2nd trimester respectively) and below 10 μIU/mL.
[c] TSH concentration levels within the trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4 < 5th percentile (fT4 < 0.95 ng/dL)).
[d] TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL) & TPO-Abs ≥ 35 IU/mL &/or Tg-Abs > 40 IU/mL.
[e] Attention Deficit Hyperactivity Disorder Test
[f] Strengths and Difficulties Questionnaire.
[g] Conners' Parent Rating Scale-Revised: Short form.
[h] Child Behavior Checklist, Parent report form.

[4 years assessment: mean difference = 1.1 years; 95% CI (0.53, 1.69) p < .001, 6 years assessment: mean difference = 1.2 years; 95% CI (0.70, 1.88) p < .001] and less educated [4 years assessment: low educational level 28% versus 16%, $x^2 = 25.48(2)$, p < .000, 6 years assessment: low educational level 27% versus 14%, $x^2 = 31.74(2)$, p < .000] while their children had lower birth weight in comparison with participants [4 years assessment: mean difference = −58.63; 95% CI (−4.92, −112.33) p = .032] and were breastfed for less time [4 years assessment: mean difference = −1.1 months; 95% CI (−0.62, −1.62) p < .001, 6 years assessment: mean difference: −0.5 months; 95% CI (−0.03, −1.03), p = .034].

3. Results

Table 1 presents participants' characteristics at 4 and 6 years of age, respectively. The median urine iodine was 168.4 μg/L, which indicates adequate iodine intake (WHO, 2001; Zimmermann and Andersson, 2012) and the mean serum iron was 72 μg/dl, which indicates marginally normal iron status (Abbassi-Ghanavati et al., 2009). No associations were found between maternal TSH and fT4 concentration levels and child behavioral and emotional difficulties at 4 and 6 years of age (Supplementary Table 1).

3.1. Subclinical hypothyroidism

Children exposed to maternal subclinical hypothyroidism during pregnancy had higher hyperactivity, impulsivity, and internalizing problems at 4 years of age, compared to children of euthyroid mothers (Table 2). Post-hoc analyses showed that increased internalizing scores were primarily driven by emotional problems: SDQ-Emotional problems: adjusted-β coefficient 0.6, 95%CI [0.0, 1.1]. In addition, children exposed to maternal subclinical hypothyroidism had higher oppositional-defiant symptoms and externalizing scores at 6 years of age, compared to children of euthyroid mothers (Table 3). Post-hoc analyses showed that increased externalizing scores were primarily driven by oppositional-defiant and conduct problems: CBCL-Oppositional-Defiant problems: adjusted-β coefficient 1.3, 95%CI [0.6, 2.1] CBCL-Conduct problems: adjusted-β coefficient 1.6, 95%CI [0.6, 2.6].

3.2. Subclinical hypothyroidism & thyroid autoimmunity

Children of subclinically hypothyroid mothers with elevated thyroid antibodies had higher hyperactivity, inattention, impulsivity, externalizing, and internalizing symptoms at 4 years, compared to children of euthyroid mothers with low concentration levels of thyroid antibodies (Table 2). Post-hoc analyses showed that increased internalizing scores were primarily driven by emotional problems: SDQ-Emotional problems: adjusted-β coefficient 1.3, 95%CI [0.4, 2.1] and increased externalizing scores by conduct problems: SDQ-Conduct problems: adjusted-β coefficient 1.1, 95%CI [0.2, 1.9]. In addition, children of subclinically hypothyroid mothers with elevated thyroid antibodies had higher hyperactivity, oppositional, internalizing, and externalizing problems at 6 years, compared to children of euthyroid mothers with low concentration levels of thyroid antibodies (Table 3). Post-hoc analyses indicated that increased externalizing symptoms were primarily the result of hyperactivity/inattention, oppositional-defiant, and conduct problems: CBCL-Hyperactivity/Inattention: adjusted-β coefficient 1.8, 95%CI [0.3, 3.3], CBCL-Oppositional-Defiant: adjusted-β coefficient 2.0, 95%CI [0.9, 3.2], CBCL Conduct: adjusted-β coefficient 2.4, 95%CI [0.8, 4.0] and increased internalizing scores the result of somatic symptoms: CBCL-Somatic Symptoms: adjusted-β coefficient 2.0, 95%CI [0.9, 3.2]. Children of subclinically hypothyroid mothers with low concentration of thyroid antibodies had higher externalizing scores at 6 years, compared to children of euthyroid mothers (Table 3). Post-hoc analyses showed that increased externalizing problems score was primarily driven by oppositional problems: CBCL-

Table 2
Maternal subclinical hypothyroidism, maternal autoimmunity, and maternal hypothyroxinemia during gestation and children's behavioral symptoms at 4 years of age [*Attention Deficit Hyperactivity Disorder Test* (*ADHDT*) and *Strengths and Difficulties Questionnaire* (*SDQ*)]

	Subclinical hypothyroidism (SCH)[a,c] (N = 41)		SCH & thyroid antibodies + status[a,c] (N = 15)		SCH & thyroid antibodies − status[a,c] (N = 26)		Euthyroidism & Thyroid antibodies + status[a,d] (N = 82)		Hypothyroxinemia (N = 25)[a,e]	
	β	95% CI	β	95% CI	β	95% CI	β	95%CI	β	95% CI
Attention Deficit Hyperactivity Disorder Test										
Hyperactivity	**2.4**	**(0.7, 4.1)**[b]	**4.6**	**(1.8, 7.4)**[b]	0.9	(−1.2, 3.1)	−0.1	(−1.4, 1.1)	0.7	(−1.4, 2.8)
Inattention	1.0	(−0.5, 2.4)	**3.1**	**(0.7, 5.4)**[b]	0.0	(−1.7,1.8)	**1.1**	**(0.0, 2.2)**[b]	1.0	(−0.8, 2.8)
Impulsivity	**1.5**	**(0.1, 2.8)**[b]	**2.9**	**(0.7, 5.1)**[b]	0.6	(−1.1, 2.3)	0.4	(−0.6, 1.4)	1.7	(−0.0, 3.3)
ADHDT - total	**4.8**	**(0.8, 8.8)**[b]	**10.6**	**(4.2, 17.1)**[b]	1.6	(−3.3, 6.5)	1.4	(−1.5, 4.3)	3.4	(−1.5, 8.2)
Strengths and Difficulties Questionnaire										
Internalizing score	**0.9**	**(0.1, 1.7)**[b]	**1.5**	**(0.3, 2.8)**[b]	0.5	(−0.4, 1.5)	0.3	(−0.3, 0.9)	−0.0	(−1.0, 0.9)
Externalizing score	0.8	(−0.2, 1.8)	**2.2**	**(0.5, 3.8)**[b]	0.1	(−1.2, 1.4)	0.4	(−0.4, 1.1)	0.5	(−0.8, 1.8)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy and maternal smoking status during pregnancy.
[b] $p < .05$.
[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL; 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL).
[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL) & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL.
[e] Reference group: TSH concentration levels within the trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4 > 5th percentile (fT4 ≥ 0.95 ng/dL)).

Oppositional-Defiant: adjusted-β coefficient 1.0, 95%CI [0.1, 1.9]. Subclinically hypothyroid women with elevated thyroid antibodies had higher TSH concentration levels (M = 4.23, SD = ± 1.60) compared to subclinically hypothyroid mothers with low thyroid antibodies concentration levels [(M = 3.44, SD = ± 0.62): t (39) = −2.23, $p < .032$].

3.3. Thyroid autoimmunity

Children of euthyroid mothers with elevated thyroid antibodies had higher inattention scores at 4 years and externalizing scores at 6 years, compared to children of euthyroid mothers with low concentration of thyroid antibodies (Tables 2 & 3). Post-hoc analyses showed that increased externalizing scores were primarily driven by hyperactivity/inattention symptoms: CBCL-Hyperactivity/Inattention score: adjusted-β coefficient 0.8, 95%CI [0.1, 1.5].

3.4. Hypothyroxinemia

No associations of maternal hypothyroxinemia with child behavioral and emotional symptoms at 4 and 6 years of age were observed (Tables 2 & 3); the same results were obtained applying an alternative

Table 3
Maternal subclinical hypothyroidism, maternal autoimmunity, and maternal hypothyroxinemia during gestation and children's behavioral symptoms at 6 years of age [*Conners' Parent Rating Scale-Revised: Short form* (*CPRS-R:S*) and *Child Behavior Checklist* (*CBCL*), *Parent Report Form*].

	Subclinical hypothyroidism (SCH)[a,c] (N = 30)		SCH & thyroid antibodies + status[a,c] (N = 11)		SCH & thyroid antibodies − status[a,c] (N = 19)		Euthyroidism & thyroid antibodies + status[a,d] (N = 62)		Hypothyroxinemia[a,e] (N = 23)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Conners' Parent Rating Scale-Revised: Short form scales										
Oppositional	**1.2**	**(0.0, 2.5)**[b]	**3.1**	**(1.2, 4.9)**[b]	0.2	(−1.3, 1.7)	0.8	(−0.1, 1.7)	−0.8	(−2.2, 0.6)
Inattention	0.8	(−0.5, 2.0)	1.9	(−0.1, 3.9)	0.6	(−1.6, 2.9)	0.6	(−0.3, 1.6)	−0.8	(−2.2, 0.6)
Hyperactivity	0.4	(−0.7, 1.6)	**1.8**	**(0.0, 3.7)**[b]	−0.5	(−2.0, 0.9)	0.2	(−0.7, 1.0)	0.6	(−0.8, 1.9)
CPRS-R:S: total	0.5	(−1.7, 2.6)	2.7	(−0.7, 6.0)	−0.8	(−3.4, 1.7)	0.6	(−0.9, 2.1)	−0.2	(−2.6, 2.1)
Child Behavior Checklist - Parent report form										
Internalizing score	1.6	(−0.1, 3.2)	**2.8**	**(0.1, 5.4)**[b]	1.0	(−1.1, 3.1)	1.2	(−0.1, 2.4)	0.9	(−1.1, 2.8)
Externalizing score	**4.6**	**(2.2, 6.9)**[b]	**6.5**	**(2.9, 10.2)**[b]	**3.5**	**(0.7, 6.4)**[b]	**1.8**	**(0.1, 3.5)**[b]	−0.9	(−3.4, 1.7)

[a] Adjusted for child age at the behavioral assessment, child sex, birthweight, breastfeeding duration, maternal age, maternal education, parity, marital status during pregnancy and maternal smoking status during pregnancy.
[b] $p < .05$.
[c] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL; 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL).
[d] Reference group: TSH and fT4 concentration levels within the population-based, trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL & fT4: 0.95–1.53 ng/dL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4: 0.87–1.45 ng/dL) & TPO-Abs < 35 IU/mL & Tg ≤ 40 IU/mL.
[e] Reference group: TSH concentration levels within the trimester-specific reference ranges (1st trimester: TSH: 0.05–2.53 μIU/mL 2nd trimester: TSH: 0.18–2.73 μIU/mL & fT4 > 5th percentile (fT4 ≥ 0.95 ng/dL)).

fT4 cut-off point (10th percentile) for the definition of hypothyroxinemia (data not presented).

3.5. Additional analyses

No interaction effect of child sex was identified. The exclusion of the mothers who took thyroid medication during pregnancy did not cause any meaningful change of the results (Supplementary Table 2). Sensitivity analyses were conducted to a subsample with available child thyroid data (assessed at 4 years of age), including an additional adjustment for child TSH in the models; the results further supported the associations of maternal subclinical hypothyroidism during pregnancy and child behavioral and emotional difficulties at 6 years of age (Supplementary Table 3). The analyses were also repeated on a subsample with available data on maternal iron and iodine status at the 13th gestational week; the additional adjustments for maternal iron and iodine status further strengthened the identified associations (Supplementary Table 4 & Supplementary Table 5).

4. Discussion

In this prospective, population-based, mother-child cohort study maternal subclinical hypothyroidism during early pregnancy predicted increased hyperactivity, impulsivity, and emotional difficulties in children at 4 years and increased oppositional-defiant and conduct symptoms at 6 years of age. Maternal thyroid autoimmunity was associated with increased inattention at 4 years and increased hyperactivity & inattention at 6 years of age. We did not identify any association between maternal hypothyroxinemia and child behavioral and emotional symptoms at 4 or at 6 years of age. We did not find any child sex effect in the association between maternal thyroid functioning and child behavioral and emotional development at 4 and 6 years of age.

Findings that indirectly support the role of maternal thyroid dysfunction in child behavioral problems acknowledged a link between generalized resistance to thyroid hormones and more ADHD symptoms in adults (Hauser et al., 1993) as well as a greater risk for ADHD symptoms' manifestation for children exposed to mild iodine deficiency (Vermiglio et al., 2004). More directly related results have supported that maternal mild thyroid dysfunction is associated with child behavior problems, with various indicators as predictors of child behavioral development (Andersen et al., 2017; Endendijk et al., 2017; Ghassabian et al., 2012; Ghassabian et al., 2011; Modesto et al., 2015; Oostenbroek et al., 2017; Päkkilä et al., 2013)

Several previous studies regarding maternal thyroid function and child behavioral development have supported that maternal TSH is a valid predictor of child behavioral problems (Endendijk et al., 2017; Ghassabian et al., 2011; Päkkilä et al., 2013). Our findings suggest that subclinical hypothyroidism predicts child behavioral and emotional problems, which indirectly supports that TSH is a sensitive indicator of maternal thyroid dysfunction. It has been previously suggested that although TSH is not the biologically active hormone in the fetal brain, it might be a reliable indicator of thyroid dysfunction because of the pituitary feedback mechanism and its regulatory role for thyroid hormones secretion (Ghassabian et al., 2011). In addition, the detected associations were stronger and evident in more scales in children of subclinically hypothyroid mothers with elevated thyroid antibodies. Although these findings might be attributed to the additional increase of TSH concentration, which was evident in the present results, an additional direct impact of thyroid antibodies cannot be excluded, since there is evidence that TPO-Abs positivity impairs thyroid stimulation by hCG (Korevaar et al., 2016b)

Maternal thyroid autoimmunity (elevated TPO-antibodies) has been previously related with decreased motor and intellectual development (Li et al., 2010; Pop et al., 1995), greater autism risk (Brown et al., 2015), and more attention deficit/hyperactivity problems at 3 years of age (Ghassabian et al., 2012). The present findings support an association of elevated thyroid antibodies in euthyroid women with child inattention problems at 4 years and hyperactivity/inattention at 6 years. These associations might be attributed to further alterations of thyroid hormones' levels between the two groups in later pregnancy, which were small but already evident in the present results. Additional possible explanations involve an adverse effect of maternal thyroid antibodies on fetal thyroid functioning that may result in transient fetal hypothyroidism (Dussault and Fisher, 1999) and an impact of a pre-existing maternal subclinical autoimmune condition, which is indicated by elevated thyroid antibodies, on child behavioral problems development (Ghassabian et al., 2012).

Interestingly, the observed associations of this study involve both externalizing and internalizing symptoms. Deficits in cognitive control, behavioral inhibition, and emotional regulation are common in clinical disorders that are relevant with the detected symptoms (Amstadter, 2008; Arnsten and Rubia, 2012; Barkley, 2011; Mullin and Hinshaw, 2007; Steinberg and Drabick, 2015; Suveg and Zeman, 2004). These cognitive abilities implicate the prefrontal cortex, the hippocampus, and the cerebellum (Arnsten and Rubia, 2012; Miller and Cohen, 2001; Phillips et al., 2008; Schutter and van Honk, 2009); neural regions that are morphologically affected by insufficient levels of thyroid hormones during gestation (Lavado-Autric et al., 2003; Lischinsky et al., 2016; Willoughby et al., 2014). Moreover, insufficiency of thyroid hormones may result in long-lasting neurophysiological changes in the dopaminergic and the noradrenergic systems, which are implicated in emotional and behavioral problems manifestation (Anand and Charney, 2000; Dunlop and Nemeroff, 2007; Moog et al., 2017; Solanto, 2002).

The null findings regarding the association between maternal hypothyroxinemia and child behavioral development are consistent with several previous studies (Ghassabian et al., 2011; Päkkilä et al., 2013) but don't replicate the findings of others (Andersen et al., 2017; Modesto et al., 2015; Oostenbroek et al., 2017). Although it has been previously suggested that the thyroid stimulating effect of hCG during the first trimester of pregnancy makes maternal TSH the most sensitive predictor of child behavioral development (Endendijk et al., 2017), the evidence provided by this study is not strong enough to support this assumption, since our null findings might be the result of the small number of mothers with hypothyroxinemia in our population.

We did not observe any sex specific effect on the association of maternal TSH and child behavioral development, supporting the findings of one previous study (Ghassabian et al., 2011) but not confirming sex specific associations that have been previously detected by others (Endendijk et al., 2017; Päkkilä et al., 2013). However, there are several methodological differences between the aforementioned studies and the current one that may cause the difference in the results (e.g. different age of the participants, outcome in clinical categories/outcome continuous, trajectories of thyroid hormones/single time-point measurement).

The strengths of the present study include the population-based, prospective design of the study and the opportunity to control for the potential confounding effect of several maternal and child factors. Maternal thyroid antibodies were assessed as an important cause of thyroid dysfunction in iodine sufficient populations. A possible limitation of this study is that thyroid hormones were measured at a single early point during pregnancy; as a result these measurements might reflect a transient thyroid dysfunction. However, there is evidence that longitudinal and single-point thyroid assessments are highly correlated (Ekinci et al., 2013). Although the selection of reliable and valid questionnaires to assess child behavioral and emotional development is considered another strength of this study, it also consists of a limitation of the findings, since questionnaires cannot replace direct developmental assessment and clinical diagnosis of any specific disorder. Moreover, we cannot interpret differences in scores between the two time-points as developmental changes, since differences between the questionnaires may be the cause of the observed variations. Imputation of the missing values of ADHDT, SDQ, and CBCL questionnaires were

applied in order to avoid any bias due to selective response to specific items of the questionnaires. Participants and non-participants did not differ in relevance with the exposure status but they differed in other socio-demographic characteristics. Therefore, bias due to non-participation and loss to follow up cannot be excluded. In addition, we had no available information on offspring congenital hypothyroidism, and although sensitivity analyses with adjustment for child thyroid functioning at 4 years did not significantly affect the identified associations, we have to consider this as a possible limitation of the present results. Furthermore, residual confounding cannot be excluded, although multiple confounders were included in the analyses and additional sensitivity analyses were conducted to control for the potential confounding effect.

5. Conclusions

The results support the conclusion that maternal subclinical hypothyroidism in early pregnancy is associated with externalizing and internalizing problems in early childhood and that maternal thyroid autoimmunity further strengthens the aforementioned associations. Additionally, it is supported that elevated thyroid antibodies in euthyroid pregnant women are associated with adverse behavioral outcomes. However, no association between maternal hypothyroxinemia and child behavioral development was observed. Current findings extend previous evidence on the impact of maternal non-clinical thyroid-hormone dysfunction on later child neuropsychological and suggest that subclinical hypothyroidism and thyroid antibodies concentration levels need to be considered as predictors of child behavioral and emotional development. Future studies combining neuropsychological assessment in multiple domains and neuroimaging techniques are necessary to explore the role of thyroid mild dysfunction in child development, to pinpoint the exact mechanisms underlying such associations, and explore whether the effect of maternal thyroid dysfunction is area specific within the brain.

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Declaration of competing interest

The authors have no potential conflicts of interest to disclose.

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Appendix A. Supplementary data

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