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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Προοπτική μελέτη του ρόλου της 25(OH) βιτD και παραγόντων του μεταβολικού συνδρόμου στο 1ο τρίμηνο της κύησης στην σωματική ανάπτυξη και νευροανάπτυξη παιδιών προσχολικής ηλικίας-Μελέτη Μητέρας Παιδιού Κρήτης, Μελέτη ΡΕΑ

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PhD Thesis

Association of maternal 25(OH)D levels and metabolic profile in early pregnancy, with child cardiometabolic traits and neurodevelopment at preschool age: the "Rhea" pregnancy cohort in Crete, Greece

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Στην οικογένεια μου!

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Abbreviations

ADHD	Attention deficit hyperactivity disorder
BIA	Bioelectric impedance analysis
BMI	Body mass index
BF	Body fat
CI	Confidence interval
DBP	Vitamin D binding protein
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
DOHaD	Developmental origins of health and disease
FFM	Fat free mass
FM	Fat mass
GAMs	Generalized additive models
GDM	Gestational diabetes mellitus
HDL	High density lipoprotein cholesterol
IQ	Intelligence quotient
LDL	Low density lipoprotein cholesterol
LGA	Large for gestational age
MSCA	McCarthy Scales of Children's Abilities
NCEP:ATP III	National Cholesterol Education Programme Adult Treatment Panel III
NHANES	National Health and Examination Survey
ICC	Intra-class correlation coefficient
IOM	Institute of Medicine
RCT	Randomized controlled studies
RR	Relative risk
SDQ	Strengths and difficulties questionnaire
SD	Standard deviation
SGA	Small for gestational age
VDR	Vitamin D receptor
WC	Waist circumference
WHO	World Health Organization
25(OH)D	25-hydroxyvitamin D

Abstract in Greek

Περίληψη

Εισαγωγή:

Τα περισσότερα όργανα και δομές του αναπτυσσόμενου εμβρύου σχηματίζονται τους πρώτους μήνες μετά τη σύλληψη. Για το λόγο αυτό, το πρώτο τρίμηνο της κύησης θεωρείται ένα κρίσιμο χρονικό διάστημα της εμβρυικής ανάπτυξης, ιδιαίτερα επιρρεπές σε επιβλαβείς παράγοντες, οι οποίοι είναι είτε ενδογενείς, είτε εξωγενείς. Εκτός από τη διατροφή και άλλους περιβαλλοντικούς παράγοντες, η παχυσαρκία της μητέρας πριν την κύηση έχει συσχετισθεί, την τελευταία δεκαετία, με αυξημένο κίνδυνο εμφάνισης μεταβολικών νοσημάτων στους απογόνους, όπως παχυσαρκία, δυσλιπιδαιμία και διαταραγμένη ανοχή γλυκόζης, αλλά και αυξημένη εμφάνιση νευροψυχιατρικών διαταραχών στην παιδική ηλικία και στην ενήλικο ζωή. Πέραν της παχυσαρκίας, κατά την κύηση δεν έχει μελετηθεί επαρκώς μέχρι σήμερα η επίδραση άλλων παραμέτρων του μεταβολικού συνδρόμου και ιδιαιτέρως, των παραγόντων εκείνων του ενδομητρίου περιβάλλοντος οι οποίοι επηρρεάζουν την ανάπτυξη του λιπώδους ιστού και του νευρικού συστήματος στο αναπτυσσόμενο έμβρυο. Παραδείγματος χάριν, αν και είναι γνωστό ότι τα επίπεδα βιταμίνης D της μητέρας αποτελούν σημαντικό παράγοντα για την ομαλή ανάπτυξη των οστών και του μυικού συστήματος του εμβρύου, λίγα είναι γνωστά για τον πιθανό ρόλο της βιταμίνης D στην εναπόθεση του λίπους και την νευροανάπτυξη των απογόνων.

Στόχος της παρούσας διατριβής ήταν να διερευνήσει το ρόλο παραγόντων του μεταβολικού συνδρόμου της μητέρας και των επιπέδων 25(OH)-βιταμίνης D στο 1ο τρίμηνο κύησης με τη σωματική ανάπτυξη και νευροανάπτυξη παιδιών ηλικίας 4 ετών στην Κρήτη, στο πλαίσιο της Μελέτης Μητέρας-Παιδιού Κρήτης, Μελέτη ΡΕΑ.

Ειδικοί στόχοι:

1. Να μελετηθεί η σχέση παραγόντων του μεταβολικού συνδρόμου στις εγκύους, στο 1ο τρίμηνο κύησης με την σωματική ανάπτυξη και την εμφάνιση παραγόντων καρδιαγγειακού κινδύνου σε παιδιά ηλικίας 4 ετών.
2. Να μελετηθεί η σχέση παραγόντων του μεταβολικού συνδρόμου στις εγκύους, στο 1ο τρίμηνο κύησης με την νευροανάπτυξη παιδιών ηλικίας 4 ετών.

3. Να μελετηθεί η σχέση των επιπέδων 25(OH)D στις εγκύους, στο 1ο τρίμηνο κύησης με την σωματική ανάπτυξη και την εμφάνιση παραγόντων καρδιαγγειακού κινδύνου σε παιδιά ηλικίας 4 ετών.
4. Να μελετηθεί η σχέση των επιπέδων 25(OH)D στις εγκύους, στο 1ο τρίμηνο κύησης με την νευροανάπτυξη παιδιών ηλικίας 4 ετών.

Μεθοδολογία:

Ο πληθυσμός της παρούσας διατριβής προέρχεται από την προοπτική μελέτη Μητέρας-Παιδιού «Ρέα», στην οποία εντάχθηκαν έγκυες κατά τη διάρκεια ενός έτους (Μάρτιος 2007-Φεβρουάριος 2008). Ο σχεδιασμός της μελέτης περιλάμβανε 3 διαδοχικές συναντήσεις με τις εγκύους μέχρι και τον τοκετό και εν συνεχεία ακολούθησε παρακολούθηση των παιδιών με συνεντεύξεις και δειγματοληψία αίματος στους 9 μήνες, στους 18 μήνες, στα 4 και στα 6 έτη ζωής. Η συλλογή των δεδομένων πραγματοποιήθηκε με τη χρήση ερωτηματολογίων, τη συλλογή και φύλαξη βιολογικών δειγμάτων και την κλινική εξέταση των εγκύων και των παιδιών τους. Οι μητέρες εκτιμήθηκαν κλινικά στο 1ο τρίμηνο της κύησης, κατά τη διάρκεια της γυναικολογικής εκτίμησης για το 1ο υπερηχογράφημα του εμβρύου, με προσδιορισμό του ύψους και του βάρους από ειδικά εκπαιδευμένους εξεταστές. Ταυτόχρονα συλλέχτηκαν πληροφορίες για το βάρος των εγκύων πριν την εγκυμοσύνη και υπολογίστηκε ο δείκτης μάζας σώματος προ της κύησης. Στην ίδια συνέντευξη εκτιμήθηκε η συστολική και διαστολική πίεση και συλλέχθηκαν βιολογικά δείγματα για τον προσδιορισμό των συγκεντρώσεων σακχάρου, λιπιδίων και 25(OH)-βιτ D των εγκύων.

Στα 4 έτη παρακολούθησης, ειδικά εκπαιδευμένοι ερευνητές πεδίου, ακολουθώντας συγκεκριμένα πρωτοκολλα, μέτρησαν το βάρος, το ύψος, την περίμετρο μέσης, τις δερματικές πτυχές και την αρτηριακή πίεση των παιδιών, ενώ ταυτόχρονα συλλέχθηκαν βιολογικά δείγματα για τον προσδιορισμό των λιπιδίων στην προσχολική ηλικία. Επιπλέον στα 6 έτη, το ποσοστό λίπους των παιδιών εκτιμήθηκε με τη μέθοδο της βιοηλεκτρικής αντίστασης (BIA). Όσον αφορά την ψυχοκινητική ανάπτυξη των παιδιών στα 4 έτη ζωής, ειδικά εκπαιδευμένοι ψυχολόγοι την αξιολόγησαν χρησιμοποιώντας τις Κλίμακες Εκτίμησης Παιδικών Δεξιοτήτων (McCarthy Scales of Children's Abilities). Επίσης η αξιολόγηση διαταραχών στη συμπεριφορά των παιδιών έγινε με τη συμπλήρωση από τις μητέρες ειδικών ερωτηματολογίων που αφορούσαν τις δυνατότητες και τις δυσκολίες συμπεριφοράς των παιδιών τους (SDQ questionnaire), καθώς και την εκδήλωση συμπτωμάτων ελείμματος

προσοχής-υπερκινητικότητας (ADHD Test) στην προσχολική ηλικία. Τα δεδομένα που συλλέχθηκαν αναλύθηκαν με πολυπαραγοντικά μοντέλα γραμμικής και λογιστικής παλινδρόμησης.

Αποτελέσματα:

1) Κατά τη διερεύνηση της επίδρασης παραγόντων του μεταβολικού συνδρόμου στο 1ο τρίμηνο κύησης με καρδιομεταβολικούς παράγοντες κινδύνου παιδιών προσχολικής ηλικίας βρήκαμε ότι ο αυξημένος δείκτης μάζας σώματος της μητέρας πριν την εγκυμοσύνη (υπέρβαρες/ παχύσαρκες) συσχετίζεται με αυξημένο κίνδυνο υπέρβαρου ή παχύσαρκου παιδιού στα 4 έτη ζωής, καθώς και αυξημένο κίνδυνο κεντρικής παχυσαρκίας και αυξημένης ποσότητας λιπώδους μάζας στην προσχολική ηλικία. Επίσης αύξηση της συγκέντρωσης της ολικής χοληστερόλης της μητέρας στο 1ο τρίμηνο κύησης κατά 40mg/dl βρέθηκε να συσχετίζεται με αυξημένο κίνδυνο υπέρβαρου/παχύσαρκου παιδιού και αυξημένο πάχος των δερματικών πτυχών στα 4 έτη ζωής. Η συσχέτιση αυτή παρέμεινε στατιστικά σημαντική και ύστερα από έλεγχο διαφόρων συνεπιδρώντων μεταβλητών, συμπεριλαμβανομένου του δείκτη μάζας σώματος της μητέρας πριν την κύηση. Τέλος, τα αυξημένα επίπεδα διαστολικής πίεσης της μητέρας, στο 1ο τρίμηνο κύησης, βρέθηκε να συσχετίζονται με αυξημένες παραμέτρους παχυσαρκίας των απογόνων στα 4 έτη ζωής.
2) Από το σύνολο των παραγόντων του μεταβολικού συνδρόμου στο 1ο τρίμηνο κύησης που μελετήσαμε, μόνο η παχυσαρκία της μητέρας πριν την κύηση βρέθηκε να συσχετίζεται με σημαντικά χαμηλότερη επίδοση στην αντιληπτική, στην αριθμητική και στη γενική γνωστική ικανότητα καθώς και στις επιτελικές λειτουργίες του μετωπιαίου λοβού των απογόνων στα 4 έτη ζωής. Επίσης η παχυσαρκία της μητέρας πριν την κύηση βρέθηκε να συσχετίζεται με αύξηση της εμφάνισης διαταραχών συμπεριφοράς και εκδήλωσης συμπτωμάτων ελλειματικής προσοχής-υπερκινητικότητας των παιδιών στα 4 έτη ζωής. Οι ανωτέρω συσχετίσεις παρέμειναν σημαντικές και μετά από τον έλεγχο διάφορων συγχυτικών παραγόντων, συμπεριλαμβανομένου του δείκτη μάζας σώματος του πατέρα.
3) Κατά τη διερεύνηση της επίδρασης των συγκεντρώσεων της 25(OH) βιτ D στο 1ο τρίμηνο κύησης στην υγεία των παιδιών προσχολικής ηλικίας, βρέθηκε ότι τα 2/3 των γυναικών που συμμετείχαν στη μελέτη μας έπασχαν από ανεπάρκεια βιταμίνης D. Οι απόγονοι γυναικών με επίπεδα 25(OH) βιτ D στο χαμηλότερο τριτημόριο είχαν

σημαντικά αυξημένο BMI SD score, αυξημένη κεντρικού τύπου παχυσαρκία και υψηλότερο ποσοστό λίπους στα 4 έτη ζωής, συγκρινόμενοι με τους απογόνους γυναικών με υψηλότερα επίπεδα 25(OH) βιτ D, στο πρώτο μισό της κύησης.

4) Σε αντίθεση, τα υψηλά επίπεδα 25(OH) βιτ D της μητέρας στο 1ο τρίμηνο της κύησης φάνηκε να προστατεύουν από την ανάπτυξη διαταραχών στη συμπεριφορά παιδιών προσχολικής ηλικίας, καθώς οι απόγονοι μητέρων με επίπεδα 25(OH) βιτ D στο υψηλότερο τριτημόριο είχαν σηματικά λιγότερα συμπτωμάτα υπερκινητικότητας-παρορμητικότητας και γενικά λιγότερα συμπτωμάτα ελλειματικής προσοχής (ADHD) καθώς και λιγότερα προβλήματα συμπεριφοράς στα 4 έτη ζωής, συγκρινόμενοι με απογόνους μητέρων με χαμηλότερα επίπεδα 25(OH) βιτ D στην κύηση. Οι ανωτέρω συσχετίσεις παρέμεναν σημαντικές ύστερα από τον έλεγχο διαφόρων συγχυτικών παραγόντων συμπεριλαμβανομένου του δείκτη μάζας σώματος της μητέρας πριν την κύηση και ήταν πιο ισχυρές στα κορίτσια από ότι στα αγόρια.

Συμπεράσματα: Συνοψίζοντας όλα τα παραπάνω, η παρούσα μελέτη έδειξε ότι τα μεταβολικά νοσήματα της μητέρας και τα επίπεδα βιταμίνης D στο 1ο τρίμηνο της κύησης, παίζουν σημαντικό ρολο στην εκδήλωση παχυσαρκίας και διαταραχών στην νευροανάπτυξης παιδιών προσχολικής ηλικίας, ανεξάρτητα από κοινωνικοδημογραφικούς και γενετικούς παράγοντες. Τα ευρήματα αυτά είναι ιδιαίτερα σημαντικά για τη δημόσια υγεία καθώς οι ανωτέρω παράγοντες είναι δυνατόν να τροποποιηθούν με τον κατάλληλο σχεδιασμό στρατηγικών πρόληψης και παρέμβασης, ιδιαίτερα σε ευπαθείς πληθυσμούς. Περαιτέρω παρακολούθηση των παιδιών σε μεγαλύτερες ηλικίες είναι απαραίτητη, προκειμένου να επιβεβαιωθούν και ισχυροποιηθούν τα παραπάνω συμπεράσματα.

Abstract in English

Introduction:

Most organs and structures of the developing fetus are formed in the first months after conception. Therefore, early pregnancy is highly recognized as a time period with significant susceptibility to an adverse intrauterine environment. Beyond nutrition and other environmental risk factors, maternal pre-pregnancy obesity has been associated with increased risk of offspring obesity, adverse lipid profile and impaired glucose tolerance as well as neurodevelopmental disorders in childhood and adulthood. However, little is known about the potential impact of other parameters of the metabolic syndrome in early pregnancy on metabolic programming and neurodevelopment in the offspring. Additionally, maternal vitamin D levels are considered an essential biological factor for intrauterine skeletal growth and muscle development, but limited data exist so far on the potential impact of circulating vitamin D on offspring adiposity and mental development.

We aimed to investigate in greater detail the effect of metabolic dysregulation and maternal 25(OH)D levels in early pregnancy with multiple offspring metabolic and neuropsychological outcomes at 4 years of age, in a prospective pregnancy cohort in Crete, Greece.

Specific Objectives:

1) To investigate the impact of components of metabolic syndrome in early pregnancy on offspring cardiometabolic traits at 4 years of age.
2) To evaluate the role of components of metabolic syndrome in early pregnancy in psychomotor development, and behavioral difficulties at 4 years of age.
3) To investigate the association of 25(OH)D levels in early pregnancy on offspring cardiometabolic traits at preschool age.
4) To examine the impact of 25(OH)D levels in early pregnancy on cognitive and psychomotor development at preschool age.

Methods: Our study population was part of the "Rhea" study, a prospective pregnancy cohort, at the prefecture of Heraklion, Crete, Greece. Pregnant women were recruited at the time of the first ultrasound examination, during the twelve-month period from February 2007 until February 2008. Women were contacted again at various times during pregnancy, at

birth, and for child's follow-up at 9th, 18th months, and at 4 and 6 years of age. Face-to face completed questionnaires together with self-administered questionnaires and medical records were used to obtain information on dietary, environmental, and psychosocial exposures during pregnancy and early childhood. Maternal pre-pregnancy BMI was calculated by maternal height, measured at the first prenatal visit, and pre-pregnancy weight, as reported at the first ultrasound visit. At the first prenatal visit trained examiners measured maternal blood pressure levels, and collected maternal serum samples for maternal glucose, lipid and 25(OH)D measurements. At 4 years of age data on child anthropometry, blood pressure measures, and serum samples for offspring lipid measurements were collected by specially trained research assistants, according to standard operating procedures. Additionally, at 6 years follow up offspring body composition was estimated, by a bioelectric impedance analysis (BIA). Moreover, children's cognitive and motor function at 4 years of age was evaluated by two trained psychologists through the McCarthy Scales of Children Abilities. Behavioral difficulties were assessed by Strengths and Difficulties Questionnaire and Attention Deficit Hyperactivity Disorder Test, completed by mothers. Adjusted associations were obtained via multivariable linear and logistic regression analyses.

Results: 1) In the analysis of the relationship between components of metabolic syndrome in early pregnancy and cardiometabolic traits at preschool age, we found that maternal overweight/obesity pre-pregnancy was associated with increased risk of offspring overweight/obesity, central adiposity, and greater fat mass at 4 years of age, predominantly in girls. In addition, an increase of 40mg/dl in fasting serum cholesterol levels in early pregnancy was also associated with increased risk of overweight/obesity and fat mass in preschoolers. The associations remained the same after adjustment for several maternal and child characteristics, including maternal BMI. Seemingly a significant positive association was observed between higher levels of maternal diastolic blood pressure and adiposity measures at 4 years of age. 2) Among maternal metabolic diseases examined, only maternal overweight/obesity pre-pregnancy was associated with a significant score reduction in perceptual performance, quantitative ability, general cognitive function, and executive functions at 4years of age. In addition maternal overweight/obesity pre-pregnancy was associated with significant increment in behavioral problems and ADHD-like symptoms at preschool age. The observed associations remained significant after adjustment for several confounders, including paternal BMI. 3) In our analysis for the impact of maternal 25(OH)D

status on offspring health outcomes at preschool age, about two-thirds of participating women suffered from vitamin D deficiency in the first half of pregnancy. Offspring of mothers with 25(OH)D levels in the lower tertile had significantly increased BMI SD score, central adiposity and body fat percentage at 4 years of age, compared to offspring of mothers with higher 25(OH)D levels. 4) In contrast increased maternal 25(OH)D levels in early pregnancy seemed to protect from behavioral problems in early childhood, as offspring of women with 25(OH)D levels in the high tertile had significantly decreased hyperactivity/impulsivity and total ADHD-like symptoms, as well as decreased total behavioral difficulties at 4 years of age, compared to offspring of women with 25(OH)D levels in the low tertile. The observed associations remained the same after adjustment for several confounders and maternal BMI, and were more pronounced in girls than in boys.

Conclusions: In summary, findings in the present thesis support the view that maternal metabolic dysregulation and vitamin D status in early pregnancy may have an important impact on offspring adiposity measures and neurodevelopmental outcomes in early childhood, independently of sociodemographic and family parameters. Our results may have important public health implications, as the examined exposures are modifiable risk factors and may be prevented with appropriate awareness and guidance. Further follow-up of this cohort will allow to determine whether our findings persist at later ages.

PhD thesis publications

The current thesis consists of a compilation of 4 scientific publications

1.**Daraki V**, Georgiou V, Papavasiliou S, Chalkiadaki G, Karahaliou M, Koinaki S, Sarri K, Vassilaki M, Kogevinas M, Chatzi L, Metabolic Profile in Early Pregnancy Is Associated with Offspring Adiposity at 4 Years of Age: The Rhea Pregnancy Cohort Crete, Greece. *PLoS One* 2015; 10(5): e0126327. Published online 2015 May 13. doi: 10.1371/journal.pone.0126327

2.**Daraki V**, Roumeliotaki T, Koutra K, Georgiou V, Kampouri M, Kyriklaki A, Vafeiadi M, Papavasiliou S, Kogevinas M, Chatzi L, Effect of parental obesity and gestational diabetes on child neuropsychological and behavioral development at 4 years of age: the Rhea mother-child cohort, Crete, Greece.
Eur Child Adolesc Psychiatry. 2017 Jan 3. doi: 10.1007/s00787-016-0934-2.

3. **Daraki V**, Roumeliotaki T, Koutra K, Chalkiadaki G, Katrinaki M, Kyriklaki A, Kampouri M, Margetaki K, Vafeiadi M, Papavasiliou S, Kogevinas M, Chatzi L, High maternal vitamin D levels in early pregnancy may protect against behavioral difficulties at preschool age: the Rhea mother-child cohort, Crete, Greece.
Eur Child Adolesc Psychiatry, 2017 July 6. doi: 10.1007/s00787-017-1023-x

4. **Daraki V**, Roumeliotaki T, Chalkiadaki G, Katrinaki M, Karachaliou M, Leventakou V, Vafeiadi M, Sarri K, Vassilaki M, Papavasiliou S, Kogevinas M, Chatzi L, Effect of very low vitamin D levels in pregnancy on offspring obesity indices and cardiometabolic traits in childhood.
Pediatric Obesity, under revision.

1. General Introduction

1.1 Early life programming /The DOHaD model and child outcomes

Over the past two decades, it has been increasingly recognized that several of the major diseases in adulthood, including obesity, coronary heart disease, and neurodevelopmental disorders, originate from impaired intrauterine growth and development[1]. These diseases may be defects in "early life programming", whereby a stimulus or insult at critical time points of early life can result in permanent effects on structure, physiology, and metabolism of the developing fetus[2,3]. Barker and collaborators were the first who supported that undernutrition result in low birthweight, which may program the onset of cardiovascular disease or diabetes mellitus in early adult life. This association has been postulated to be an adaptive response of the fetus to a suboptimal intrauterine environment to ensure survival of the organism, through a "sparing" of vital organs such as the brain at the expense of organs such as the pancreas, heart, kidney, and skeletal muscle known as the "thrifty phenotype" theory[4].

With the addition of other epidemiological findings, such as the role of preconception maternal obesity, or the role of factors in utero that do not impact on fetal weight or growth, the model has been expanded to include events beginning prior to conception as well as in early postnatal life, a model now referred to as the Developmental Origins of Health and Disease (DOHaD)[5]. Fetal adaptations are thought to vary according to the timing, type, dosage and duration of environmental exposures across early development[6] while a mismatch between pre-and postnatal factors is considered the basis of disease in later life (Figure 1)[7,8]. Since the first introduction of this concept, it has been corroborated by many animal studies, showing that environmental factors or maternal exposures may affect fetus development by disrupting the intrauterine environment or by influencing specific gene expression mainly through epigenetic changes, such as DNA methylation[7].

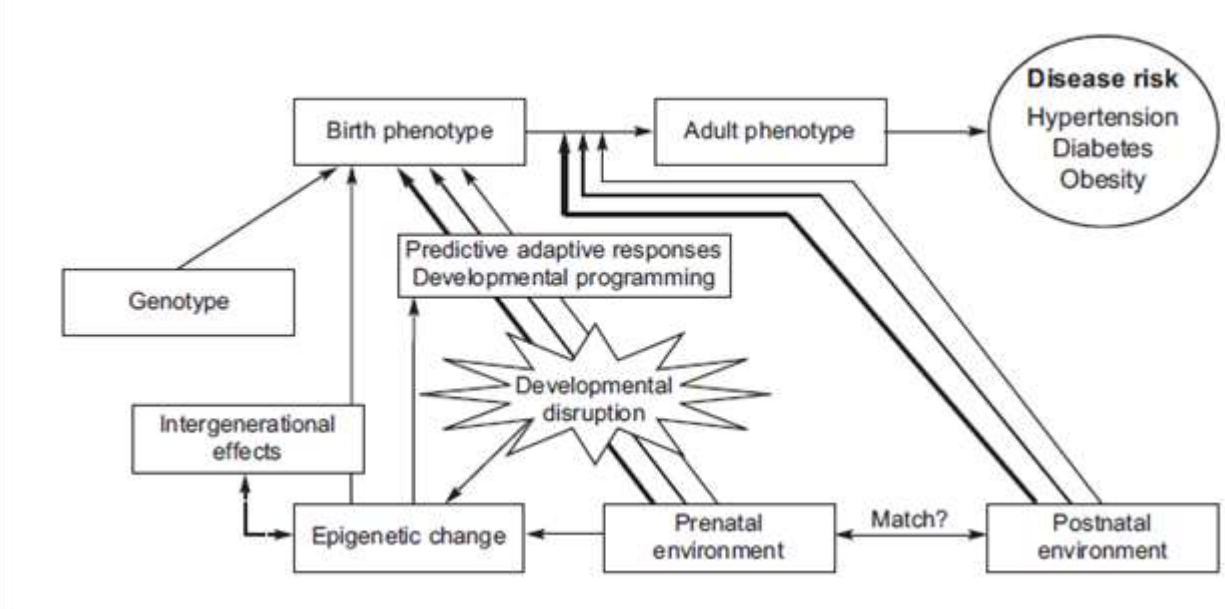

Figure 1 Effects of epigenetics and environment on birth and adult phenotypes Gluckman and Hanson [8].

Maternal metabolic diseases are of great interest in humans on their plausible effects on the developing fetus. In the last decades several epidemiological studies have investigated the association between maternal obesity pre-pregnancy and offspring outcomes supporting an inverse relationship of maternal body mass index (BMI) with increased risk of obesity, adverse lipid profile and impaired glucose tolerance in childhood and adulthood[9]. Moreover accumulating data support a link between maternal obesity and adverse fetal brain development, resulting in impaired cognitive abilities, attention deficit hyperactivity disorders (ADHD) and neuropsychiatric diseases[10,11]. Limited epidemiological data exist so far on the effect of other components of the metabolic syndrome such as maternal hyperlipidemia, hypertension or impaired glucose tolerance, in early pregnancy, on fetus metabolism or neurodevelopment.

In recent years, increased attention has also received on the impact of steroids on impaired fetal programming. Steroids cross the placenta and via their receptors are binding to DNA elements and act as transcripting factors regulating the expression of several genes[12]. Vitamin D is a secosteroid with important role on calcium homeostasis and bone metabolism, but is also recognized as a potent modulator of cellular proliferation and differentiation and a potent immunomodulator[13]. Vitamin D is able to directly or indirectly regulate up to 2,000 genes[14] and may affect maternal and perhaps fetal gene expression during pregnancy[15]. Vitamin D

deficiency is recognized as a public health problem and is common among pregnant women[16]. Several epidemiological studies have shown a link between low maternal vitamin D levels and increased risk of adverse pregnancy and birth outcomes[17], but data on child obesity and cardiometabolic outcomes or neurodevelopmental disorders are still inconsistent.

Population-based prospective birth cohorts offer an excellent opportunity to investigate associations between early life exposures and later human diseases, and to understand mechanisms involved in disease predisposition. Birth cohorts have important strengths in that they collect data prospectively on many covariates and follow children for several years after birth, thereby providing insights into developmental problems at birth as well as in the first years of life. Although association between maternal metabolic diseases and vitamin D status with pregnancy or birth outcomes have been established, large mother-child cohorts investigating their plausible impact on child metabolism, cognitive function and behavioral difficulties at preschool age are limited, with inconsistent results.

1.2 Components of metabolic syndrome and pregnancy

1.2.1 Metabolic syndrome overview

The metabolic syndrome is described as a cluster of biochemical and physiological abnormalities associated with the development of cardiovascular disease[18]. Its main components are considered dyslipidemia (elevated triglycerides and apolipoprotein B-containing lipoproteins, and low high-density lipoproteins (HDL)), elevation of arterial blood pressure, dysregulated glucose homeostasis, abdominal obesity, and/or insulin resistance[19]. Chronic proinflammatory and prothrombotic states, non-alcoholic fatty liver disease and sleep apnea have recently been added to the syndrome, making its definition even more complex[19]. However, no clearly defined diagnostic criteria exist so far.

Over the last two decades, various organizations have proposed different definitions, using varying terminologies. One of the most widely used definitions is that of the NCEP:ATP III according to which metabolic syndrome is diagnosed when 3 of these 5 components are present: 1) elevated waist circumference (≥88 cm for women and ≥102 cm for men), 2) elevated triglycerides (≥150 mg/dL) 3) low HDL cholesterol (<40 mg/dL for men and <50

mg/dL for women) 4) elevated blood pressure (systolic ≥130 mm Hg, or diastolic ≥85 mmHg, or both), and 5) elevated fasting glucose (≥110 mg/dL)[20].

The prevalence of metabolic syndrome is high due to obesity epidemic and depends on the criteria used. According to National Health and Examination Survey (NHANES) 2003-2006 approximately 34% of people studied met the NCEP:ATPIII revised criteria for metabolic syndrome[21]. Components of metabolic syndrome during pregnancy are a significant concern, in terms of global public health due to their association with gestational complications and long-term consequences for offspring.

1.2.2 Components of metabolic syndrome in pregnancy and offspring cardiometabolic traits and neurodevelopment

Pre-pregnancy maternal obesity

The prevalence of obesity, especially in women of child-bearing age, has increased worldwide to almost epidemic proportions and depending on the population, it can be as high as 34%[22,23]. The prevalence of maternal overweight and obesity in Greece has been estimated as 16.6% and 25.6 % respectively, according to the WHO cut-offs[24]. Numerous studies have reported an increased risk of gestational diabetes[25], gestational hypertension/pre-eclampsia[26], and caesarean delivery[27], among women who are overweight/obese prior to conception. Maternal pre-pregnancy obesity has also been linked with a variety of adverse fetal outcomes[9]. These include increased risk of congenital anomalies[28], large-size-for gestational age at birth[29], and infant mortality[30,31]. Furthermore, accumulating epidemiological evidence support an association between maternal pre-pregnancy obesity with increased risk of low Apgar score, neonatal hypoglycemia and referral to neonatal intensive care unit[32].

Several epidemiological studies have shown a relationship between maternal body mass index pre-pregnancy with offspring adiposity measures in childhood[33,34]. Maternal pre-pregnancy obesity has been associated with a threefold higher risk of childhood obesity[33], higher waist circumference and higher total body fat mass[35-37]. According to Generation R, a large birth-cohort in Rotterdam maternal body mass index pre-pregnancy was associated with both subcutaneous fat mass and abdominal pre-peritoneal fat mass, a measure of visceral fat mass in children at the age of 6 years[36]. A relationship of maternal obesity with adverse lipid profile and higher blood pressure, in children has also been demonstrated by several studies,

however results are still inconclusive[36,38-41]. Differences in sample size, examined outcomes, populations under study and incomplete adjustment for confounders may explain inconsistent results between studies. To our knowledge, no study has examined so far the effect of pre-pregnancy maternal obesity with child obesity and cardiometabolic traits in Greek children.

An emerging body of evidence support an association between maternal obesity during pregnancy and mental health outcomes in later life. Animal studies have shown that maternal obesity in mice impairs hippocampal progenitor cell division, neuronal production and consequently hippocampus-dependent cognitive functions in young offspring, possibly due to metabolic and oxidative changes[42]. Moreover, fetal brain inflammation produced by maternal obesity has been associated with reduced offspring brain apoptosis and increased susceptibility to mental disorders later in life[43]. Human studies support that offspring of overweight/obese women are at increased risk for cognitive deficits, attention deficit hyperactivity symptoms, eating disorders in adolescence and neyropsychiatric disorders in adulthood[10,11]; however, it is not clear if the observed relationships are mainly due to an adverse intrauterine environment or they are confounded by socioeconomic and family risk factors.

A way to control for family background is to compare the associations of maternal and paternal BMI on child neurodevelopmental outcomes, since a stronger maternal association would reflect direct intrauterine mechanisms[44]. Only four studies have examined the influence of maternal versus paternal obesity on child neurodevelopmental outcomes, with inconclusive results[45-48]. Casas et al found that maternal obesity (and not paternal) was associated with reduced neurodevelopmental scores at 2 years of age in the Rhea and the Spanish INMA birth studies [45], while Brion et al, in an analyses of two other pregnancy cohorts, found no association between maternal/paternal obesity and child verbal skills or behavioral difficulties at 3 and 8 years of age[47]. In contrast Bliddal et al have shown that both maternal and paternal BMI were associated with reduced child IQ at 5 years of age[46], while Suren et al support a strong association of paternal obesity with autism spectrum disorders at 4-13 years of age[48]. Differences in study designs, and neurodevelopmental outcomes examined by each study may explain the aforementioned discrepancies. Additionally most of these studies have examined only one neurodevelopmental outcome, making it difficult to determine whether offspring are at risk for cognitive problems or behavioral difficulties.

With the obesity epidemic in reproductive-age women, an ever-increasing number of fetuses will be at risk for large for gestational age neonates and metabolic derangements[33]; however, no data exist on the potential mediating role of macrosomia on the association of maternal obesity and child neurodevelopment.

Maternal dyslipidemia

During the first two-thirds of gestation there is an increase in body fat accumulation, associated with both hyperphagia and increased lipogenesis[49]. In late pregnancy the mother switches to a catabolic condition which is characterized by an accelerated breakdown of fat depots, necessary for normal fetal development[49,50]. As gestation progresses maternal total cholesterol and triglycerides levels are increased and taken up by the placenta, where they metabolized and transported to the fetus in various forms[51]. Although maternal cholesterol is an important source of cholesterol for the fetus during early gestation, its importance becomes minimal during late pregnancy, due to cholesterol synthesis by fetal tissues per se[52]. On the other hand maternal triglycerides do not cross the placental barrier, but the presence of lipoprotein lipase and other lipases in placenta allows their hydrolysis and the release of fatty acids and glycerol to the fetus[49]. Normal fetal development depends on the availability of both essential fatty acids and long chain polyunsaturated fatty acids, and the nutritional status of the mother during gestation has been related to fetal growth[50]. However, excessively high levels of maternal total cholesterol or triglycerides have been associated with adverse pregnancy or birth outcomes.

Maternal dyslipidemia has shown to increase the risk of gestational diabetes[53], pregnancy-induced hypertension[54], and pre-eclampsia[55,56] in some studies, although in others no association between maternal lipid profile and pregnancy outcomes has been demonstrated[57]. Epidemiological data on birth outcomes have shown an association between maternal dyslipidemia with spontaneous preterm birth[58,59]. Additionally, several studies support that high maternal triglyceride levels increase the risk of being large for gestational age[60,61], whereas low maternal cholesterol[59,62] or triglyceride levels[63] increase the risk for being small for gestational age, both are known to be associated with childhood obesity.

The potential impact of maternal lipid profile on offspring development has mainly been investigated during the second and third trimester of pregnancy, a time period that pregnancy-

related complications or placenta dysfunction may confound effect estimates. One birth-cohort, investigating the relationship between maternal non fasting lipid levels in early pregnancy with offspring adiposity measures, has shown that maternal apolipoprotein B and total cholesterol levels were positively associated with child fat percentage and maternal triglyceride levels were positively associated with child waist-to-height ratio values at preschool age[64]. These findings are supported by animal studies showing that an exaggerated lipid profile in utero can result in offspring hyperphagia, altered adipocyte function, accelerated weight gain, and adiposity through alterations in neuroendocrine system[65]. Beyond adiposity measures, no study have examined so far the plausible role of maternal dyslipidemia on other cardiometabolic risk factors such as lipid profile and blood pressure or adverse neurodevelopmental outcomes in offspring.

Maternal glucose intolerance

Glucose metabolism during pregnancy is characterized by a complex endocrine–metabolic adaptation process, including impaired insulin sensitivity, increased β-cell response, and moderately increased blood glucose levels, particularly after a meal[66]. Progressive insulin resistance, during pregnancy, may favor glucose supply to the fetus by switching the maternal energy metabolism from metabolizing carbohydrates to lipid substrates (i.e., free fatty acids), redirecting carbohydrates toward the fetal tissues[67]. Although, glucose homeostasis is maintained in normal pregnancies, gestational diabetes develops as soon as insulin secretion from β-cell is no longer sufficient to compensate for insulin resistance[68]. The degree of insulin resistance seems to be influenced by obesity pre-pregnancy, with the incidence of gestational diabetes (GDM) estimated as 1.4- to 20-fold higher in obese pregnant women, compared to their normal weight counterparts[25,69]. However, gestational hyperglycemia may have distinct effects on offspring clinical outcomes independently of obesity alone.

Women with GDM are in a higher risk of adverse pregnancy outcomes such as pre-eclampsia, pre-term delivery, and polyhydramnios, compared to normal pregnant women[70]. In addition, GDM has been associated with fetal complications including congenital anomalies, macrosomia, shoulder dystocia, stillbirth, growth restriction, and hypoglycemia, among others[70].

The association of in utero hyperglycemia with metabolic programming in fetus was first described in the Native American Pima population. Offspring born to mothers with GDM had a considerably higher risk of diabetes and obesity than those born to mothers who developed diabetes after pregnancy[71,72]. However, whether similar putative programming effects occur in mild maternal hyperglycemia in other populations remains uncertain. Several prospective cohort studies have reported an association between maternal gestational diabetes with increased risk of obesity[73] or impaired glucose tolerance in offspring[74-77], but results are not clear yet, in part due to differences in the definitions of maternal hyperglycemia and gestational diabetes and in adjustments for confounding factors. Additionally, in most studies mothers diagnosed with gestational diabetes had received interventions to normalize the glycemic level during pregnancy, which may confound the effect of this complication on offspring outcomes.

Several epidemiological studies support a possible association between GDM and increased risk of neurobehavioral abnormalities such as cognitive deficits, behavioral problems (particularly ADHD), or internalizing psychopathology[78-81]. However, results from other studies suggest negative[80,82-85] or null[86,87] associations. A recent systematic review and meta-analysis found that infants of women with GDM had a lower mental and psychomotor development, but evidence is scarce for older children[88]. Whether maternal glucose intolerance of non-diabetic women in early pregnancy-a critical time point for organ formation- affects offspring metabolic or neurodevelopmental outcomes has not been investigated so far.

Maternal blood pressure

Hypertension is the most common medical disorder of pregnancy. It complicates up to 1 in 10 gestations and affects about 240,000 women in the United States every year[89]. Hypertensive disorders of pregnancy are classified into the following categories: chronic hypertension, gestational hypertension, pre-eclampsia, and pre-eclampsia superimposed on preexisting hypertension[89]. Chronic hypertension is defined as blood pressure exceeding 140/90 mm Hg before pregnancy or before 20 weeks' gestation, while gestational hypertension or pregnancy-induced hypertension is the development of new hypertension in a pregnant woman after 20 weeks gestation without the presence of protein in the urine[90]. Almost in half of the cases of

gestational hypertension the disorder progresses into pre-eclampsia, a potential dangerous complication for expectant mothers[91].

Hypertensive disorders in pregnancy may increase morbidity during pregnancy, and they remain a leading cause of maternal mortality[92]. They could also result to major adverse effects in mothers, including central nervous system injuries such as seizures (eclampsia), hemorrhagic and ischemic strokes, hepatic disorders such as transaminase elevation, the so-called "HELLP syndrome" (hemolysis, elevated liver enzymes, and low platelets), or hepatic failure, renal dysfunction, and increased frequency of cesarean delivery. In addition, pre-eclampsia-eclampsia have been associated with preterm birth[92], and can lead to higher frequency of induced labor, fetal growth restriction, neonatal respiratory difficulties, and increased frequency admission to neonatal intensive care unit[93]. In contrast, the effects of chronic, controlled hypertension in pregnancy on the fetus are minimal, while gestational hypertension, even in its more severe forms, causes only minimal increased risk for perinatal or fetal death[93].

Beyond infancy, pregnancies complicated by pre-eclampsia have been associated with increased offspring blood pressure and BMI in childhood, although evidence are still inconclusive[94]. On the other hand, hypertension in early pregnancy has been associated with increased risk of fetal growth restriction[95,96], but its potential impact on offspring cardiometabolic traits and cognitive abilities or behavior in childhood has not been investigated so far.

Vitamin D

1.3.1 Overview

The last decade is highly acknowledged that Vitamin D is one of the essential nutrients to sustain the human health. As a member of the steroid hormone family, it regulates hundreds of genes around the body including those coding for proteins involved in cell proliferation, differentiation, apoptosis, immune regulation, genome stability, and neurogenesis as well as calcium and phosphate homeostasis[97]. Vitamin D deficiency and insufficiency is a global health issue[98]. Epidemiological studies have shown that Vitamin D levels are closely related to the occurrence and development of many chronic conditions in humans, such as bone

metabolic disorders, tumors, cardiovascular diseases, and diabetes, while vitamin D is recognized as an important risk factor for neuropsychiatric disorders and autoimmune diseases[99]. Vitamin D deficiency is highly prevalent in pregnant women, children and adolescents as well as older people[100].

The prevalence of vitamin D deficiency among pregnant women, ranges from 18 to 84%, depending on the country of residence and local clothing customs[101,102]. Accumulating evidence suggest that maternal vitamin D status may be an independent risk factor for pregnancy and birth complications, as well as, adverse offspring health outcomes later in life. However, data on the potential effect of vitamin D on offspring growth and neurodevelopment is limited with inconsistent results. As fetal vitamin D status is totally dependent on maternal supply, recognizing the adverse health effects of maternal vitamin D deficiency, in critical time points during pregnancy, is an important public health issue, to design interventions and early treatments for those who are likely to have low vitamin D levels.

1.3.2 Vitamin D metabolism and action

Vitamin D is a fat soluble vitamin, but its 4-carbon ring backbone makes it more of a steroid hormone than a vitamin[103]. Vitamin D exists in several different forms that differ primarily in their side chains. The two major forms are vitamin D2 or ergocalciferol, and vitamin D3 or cholecalciferol, known collectively as calciferol[104]. Vitamin D2 is made by plants and vitamin D3, is made by animals, including humans. Both forms require UV light, specifically UVB in the spectrum of 280–320 nm to catalyze the reaction. In humans solar ultraviolet B radiation penetrates the skin and converts 7-dehydrocholesterol to previtamin D3, which is rapidly converted to vitamin D3[105]. The synthesis in the skin is the most important source of vitamin D and depends on season and latitude[106]. Humans get also vitamin D from their diet which is absorbed with neutral lipids in the small intestine and transferred to the lymphatic system in chylomicrons. Vitamin D is present only in a few foods (oily fish, eggs, and fortified dairy products) and therefore only a small amount (<10–20%) is obtained through diet sources[107]. Vitamin D intake from diet is even too low in vegetarians or people used to have low milk consumption and low fish intake[108].

Vitamin D is cleared rapidly from the blood and lymphatics in the liver[106,109], where the first hydroxylation takes place to produce 25-hydroxyvitamin D [25(OH)D]. 25(OH)D is

considered as one of the most stable vitamin D biomarkers and under normal circumstances is used to determine a patient's vitamin D status[105,110]. 25(OH)D is transported in the blood by vitamin D binding protein (DBP; which binds vitamin D and its metabolites in serum) to the kidney, where in the proximal renal tubule the second hydroxylation to a variety of metabolites takes place. The most important and biologically active metabolite is 1,25-dihydroxyvitamin D, which is hydroxylated at the position of carbon 1 of the A ring (Figure 2)[106]. 1,25-dihydroxyvitamin D can also be locally produced in many tissues and cells in the body such as osteoblasts, monocytes, macrophages, neuron cells, pancreatic cells, breast cancer, and colon cancer cells[111].

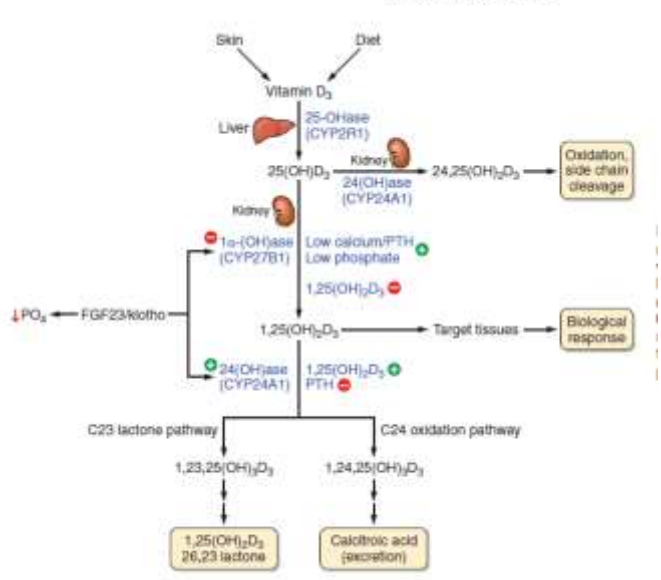

Figure 2 Vitamin D metabolism Source Christakos et al **[106]**

The biological actions of 1,25-dihydroxyvitamin D are mediated by the vitamin D receptor (VDR). VDR belongs to the steroid receptor superfamily, which include receptors for retinoic acid, thyroid hormones, sex hormones, and adrenal steroids [112]. VDR is present in the cytosol and has both ligand and DNA binding domains. Homodimerization or heterodimerization with the retinoic acid X receptor precedes the binding of the hormone receptor complex to specific vitamin D response elements in the promoter regions of genes that code for several peptides[109]. Bone and intestine are traditionally regarded as the main target organs of 1,25-dihydroxyvitamin D action, but receptors have also been identified in many tissues in the body not directly involved in calcium metabolism[109].

Over the course of the last decades, it has become increasingly clear that 1,25-dihydroxyvitamin D is necessary to maintain health and function of multiple systems beyond calcium and phosphate homeostasis including immune, reproductive, muscular, skeletal and integumentary system and can directly or indirectly regulate the expression of thousands genes[104].

1.3.3 Vitamin D deficiency in general population

The 25(OH)D levels recommended as 'cutoffs' to define vitamin D deficiency differ between the US Institute of Medicine (IOM) report and the US Endocrine Society guideline. According to US Endocrine Society guideline vitamin D deficiency is defined as 25(OH)D level less than 20 ng/ml (50 nmol/l), vitamin D insufficiency as 25(OH)D level between 21 and 29 ng/ml (52.5 to 72.5 nmol/L), while the safety margin to minimize the risk of hypercalcemia is considered as 25(OH)D level equal to 100 ng/ml (250 nmol/l)[113]. On the other hand, the US IOM report concluded that a 25(OH)D concentration equal to 20 ng/ml (50 nmol/l) covers the requirements of ⩾97.5% of the population for bone health, and 25(OH)D >50 ng/ml (125 nmol/l) should raise concerns about potential adverse health effects[114].

Vitamin D status worldwide varies according to latitude, season, skin pigmentation and diet[100]. Traditional risk groups for vitamin D deficiency include pregnant women, children, older persons, the institutionalized, and non-western immigrants[100]. Recent data suggest that about 1 billion people have low vitamin D levels and this is found in all ethnicities and age groups[13]. In Europe serum 25(OH)D are higher in Northern Europe than in Southern Europe, a phenomenon known as the vitamin D paradox in the Mediterranean region[114]. Possible reasons for this paradox could be a higher intake of fatty fish and cod liver oil in northern countries, as well as differences in maternal skin pigmentation, clothing, sunshine exposure, and prevalence of obesity[114]. In addition, preventive strategies for maternal vitamin D deficiency in the Mediterranean region are lacking so far, as hypovitaminosis D is largely unrecognized and underrated in several South European countries[114].

1.3.4 Vitamin D and pregnancy

Vitamin D metabolism in gestation

Vitamin D metabolism during pregnancy differs significantly from nonpregnant and nonfetal states[115]. 25(OH)D serum concentrations in pregnant women are either similar to or lower than those in non-pregnant women. In contrast circulating concentrations of 1,25-dihydroxyvitamin D are increased from the beginning of pregnancy. By 12 weeks of gestation, maternal circulating 1,25-dihydroxyvitamin D levels are more than twice that of a non-pregnant adult and continue to rise two to threefold from the non-pregnant baseline,

without causing hypercalciuria or hypercalcemia[116]. These circulating levels of 1,25-dihydroxyvitamin D during pregnancy are possibly of placental origin or from increased action of maternal renal 1-α-hydroxylase, that would have to be uncoupled from feedback control by classic regulators such as calcium, phosphorus and PTH[116]. Contributors to this rise in 1,25-dihydroxyvitamin D levels may be calcitonin, also known to rise during pregnancy and is capable to stimulate renal 1-a-hydroxylase gene independently of calcium levels[117,118] and prolactin, which has also been considered as a stimulator of the 1-a-hydroxylase gene[119].

During gestation, the 1,25-dihydroxyvitamin D levels rise much higher and depend on 25(OH)D availability more than ever observed in normal human physiology, driven by classical calcium homeostasis. Although, it was first thought that this increase in circulating 1,25-dihydroxyvitamin D levels during pregnancy was due to an increase in the serum vitamin D binding protein with the fracture of unbound or 'free' 1,25-dihydroxyvitamin D remaining the same, several studies demonstrated that free 1,25-dihydroxyvitamin D levels are also increased during pregnancy[116,120]. In addition, data from a randomized, controlled trial support that a circulating 25(OH)D level of approximately 100 nmol/l (40 ng/ml) is required to optimize the production of 1,25-dihydroxyvitamin D during human pregnancy through renal and/or placental production of the hormone[116]. It is hypothesized that, 1,25-dihydroxyvitamin D increment is important for maternal tolerance to the foreign fetus, whose DNA is only half that of the mother's DNA[121].

Cord concentrations of 25(OH)D are consistently lower than those measured in the mother's serum, but correlate significantly with maternal levels implying a passive diffusion of this metabolite across the placental barrier and that the 25(OH)D pool of the fetus depends entirely on maternal supply[109]. Several studies support that maternal 25(OH)D level would need to be at least 80 nmol/l to attain cord blood 25(OH)D levels of 50 nmol/l [116,122]. Relations of maternal and fetus 1,25-dihydroxyvitamin D concentrations are more complex. Accumulating evidence suggest that under physiological conditions, there is little maternal-placental crossover, while the most of the 1,25-dihydroxyvitamin D levels in fetal plasma derives from fetal kidney production, according to the fetal needs in mineral[123]. Thus, in both the mother and fetus, the rise in 1,25-dihydroxyvitamin D depends on substrate availability, in this case, 25(OH)D, and is independent of calcium homeostasis.

Vitamin D deficiency in pregnancy and maternal health outcomes

Using the 20ng/ml (50 nmol/l) cut off point, studies worldwide show high percentages of vitamin D deficiency during pregnancy ranging from 100% in Somali immigrants in Sweden to 7% in North Carolina women[124]. Several cross-sectional observational studies, conducted across Europe, have shown a very high prevalence of pregnant populations with vitamin D deficiency, including countries of Mediterranean region[114,125-127]. Yet, as mentioned earlier, the optimization of 1,25-dihydroxyvitamin D production does not occur until total circulating 25(OH)D levels are at least 40 ng/mL (100nmol/L). It is suggested that in developed countries, with the present Western diet and lifestyle, without adequate supplementation, a high percentage of un-supplemented pregnant women worldwide do not have optimal 1,25-dihydroxyvitamin D production.

Animal studies has shown that vitamin D receptor was expressed differentially throughout pregnancy in placental, decidual, and ovarian follicular tissue, suggesting a profound role of vitamin D in the physiological changes of pregnancy[128]. Gene expression profiles of pregnant women with low levels of vitamin D (<25.5ng/ml) compared to those with higher levels (>31.7ng/ml) showed significant differences in the expression of more than 300 genes known to be involved in multiple functions such as angiogenesis, carbohydrate metabolism, and immune function[129].

Accumulating epidemiological evidence associate maternal vitamin D deficiency throughout pregnancy with adverse maternal health outcomes, including glucose intolerance and gestational diabetes mellitus (GDM), pre-eclampsia, bacterial vaginosis, high rates of caesarian section and preterm birth[17,130,131]. However, randomized clinical trials on the effect of vitamin D supplementation during pregnancy on maternal outcomes are scarce with inconsistent results. Most of them support a protective association between vitamin D supplementation and pre-eclampsia risk[132]. A recent review of the Cochrane Pregnancy and Childbirth Group has also demonstrated a lower risk of pre-eclampsia in women who received vitamin D with calcium compared to those not receiving any intervention, but an increased risk of preterm birth[133], whereas a recent meta-analysis support a reduction of preterm birth risk in women receiving vitamin D supplementation alone[134]. The clinical significance of the increased serum 25(OH)D concentrations in pregnancy remains unclear, while further investigation is needed to confirm the above results.

Maternal Vitamin D deficiency and offspring cardiometabolic traits and neurodevelopment

As mentioned above the fetus is completely dependent on maternal vitamin D stores for the supply of 25(OH)D, which crosses the placenta. Therefore maternal vitamin D status may influence the offspring's health and development in various ways.

During the first and second trimesters, the fetus is developing most of its organ systems and laying down the collagen matrix for its skeleton, while in the last trimester, the fetus begins to calcify the skeleton, thereby increasing maternal demand for calcium. Early rickets and symptomatic neonatal hypocalcaemia have been reported in infants born to mothers with profound vitamin D deficiency, most commonly in offspring of mothers with dark skin pigmentation or extensive skin covering[135,136]. Vitamin D status during pregnancy appears to play a significant role in fetal skeletal development, tooth enamel formation, and general fetal growth and development[137]. Fetuses of mothers with low maternal 25(OH)D levels may have smaller femoral volumes[138] and widening of the distal femoral metaphysis relative to femur length[139], as estimated by gestational ultrasound. However, intervention studies on the relation of vitamin D nutrition status with gestational length reported no statistically significant difference in mean gestational length, between control and intervention groups[17]. Regarding neonatal birth weight, two meta-analyses of observational studies showed that women with 25(OH)D concentrations <50 nmol/l were at increased risk of small for gestational age (SGA) neonates[140,141], while others could not confirm a significant association[142]. Additionally data from randomized controlled studies (RCT) are still controversial[143].

Beyond growth, recent reports have shown increased risk and severity of acute viral infections from respiratory syncytial virus in neonates of mothers with low 25(OH)D cord blood levels the first year of life[144,145]. Additionally, low maternal vitamin D concentrations during pregnancy have also been hypothesized to increase the risk of childhood asthma, wheezing, rhinitis and eczema through a U-shaped association[146]. However, the long-term metabolic effects on children born to mothers with vitamin D deficiency in pregnancy have not been widely studied. Some studies have shown an association of low maternal vitamin D status with insulin resistance and low muscle mass[147] or poorer bone mineral accrual[148], but the effect of maternal vitamin D levels on offspring adiposity and other cardiometabolic outcomes is still unclear. Epidemiological studies investigating the impact of low maternal

25(OH)D status in late pregnancy on offspring anthropometric measures and cardiometabolic risk factors support an association with offspring lower fat mass at birth[149] but greater fat mass at ages 4 and 6 years[149], and higher fat percentage at 9.5 years[147]. However, other studies have not observed these relationships[150-152]. Two birth cohorts in the first half of pregnancy found a significant inverse association of maternal 25(OH)D concentrations with offspring BMI z-score and increased odds of overweight at age 1 year[153] and increased fat percentage at 5-6 years[154]. Differences in the sample size, timing of blood collection and variation in outcome measures may partly explain the above heterogeneity. To our knowledge the impact of profound vitamin D deficiency on offspring cardiometabolic risk has not been investigated so far.

Vitamin D in utero could also affect fetal neurodevelopment by several ways including its effects on cell differentiation, neurotrophic factor expression, cytokine regulation, neurotransmitter synthesis, intracellular calcium signaling, anti-oxidant activity, and the expression of genes/proteins involved in neuronal differentiation, structure and metabolism[155]. However, few epidemiological data have investigated the impact of maternal vitamin D status on offspring cognition and behavior. Studies in the first half of pregnancy support an association of high maternal 25(OH)D levels, with improved mental and psychomotor development in infants[125], better receptive language development at 2 years of age[156], less language difficulties at 5 and 10 years of age[157], and a lower risk of developing ADHD-like symptoms in preschoolers[158]. Birth cohorts examining the impact of maternal 25(OH)D status in late pregnancy or cord blood levels on offspring neurodevelopment found very little[159,160], or no association with offspring IQ[150], and no association with behavioral difficulties[150,160] or ADHD diagnosis in mid childhood and adolescence[161,162]. Differences in the sample size, timing of blood collection and variation in outcome measures may partly explain the heterogeneity between studies. To our knowledge no study has examine so far the impact of maternal vitamin D status in early pregnancy on both cognitive function and behavioral difficulties in preschoolers.

2. Hypothesis and aims of the present thesis

Hypothesis

The main hypothesis of the present thesis is that in utero exposure to maternal modifiable factors such as the components of metabolic syndrome (maternal obesity pre-pregnancy glucose intolerenance, dyslipidemia, and hypertension) or inadequate vitamin D status in early pregnancy has a critical role on children's adiposity and cardiometabolic risk as well as neuropsychological development and behavioural difficulties later in life. The identification of such potential modifiable maternal factors are of particular importance for public health. The publications included in the present thesis are focused in adverse offspring metabolic and neurodevelopmental outcomes at 4 years of age.

Aims

The important role of maternal obesity in offspring obesity is well documented. However, findings from current literature on the relationship between maternal obesity and other cardiometabolic risk factors such as adverse lipid profile and high blood pressure in children remain inconclusive, with numerous inconsistencies and limitations. Similarly, accumulating evidence suggest a significant effect of maternal obesity on offspring cognitive abilities and behavior, but most of the studies have examined only one neurodevelopmental outcome, making it difficult to determine whether offspring are at risk for cognitive dysfunction or abnormal behavior. Additionally, it is not clear if the observed relationships are mainly due to an adverse intrauterine environment or they are confounded by socioeconomic and family risk factors. A few studies have compared so far the associations of maternal and paternal BMI on child neurodevelopmental outcomes, a way to control for family background, with conflictive results.

Although, early pregnancy is a critical time point for programming organ formation in the developing fetus, current research has mainly focused to examine the potential role of maternal dyslipidemia, glucose intolerance and hypertension on offspring metabolic and neurodevelopmental consequences, in late pregnancy. To our knowledge, no studies have investigated so far the effect of components of metabolic syndrome in early pregnancy with offspring cardiometabolic and neurodevelopmental outcomes in childhood.

Similarly, despite the well documented role of vitamin D status in utero on skeletal and muscle development in offspring, a great inconsistence exist between studies for the potential impact of maternal vitamin D levels on child metabolic and neurodevelopmental disorders. In addition, the effect of profound low concentrations of maternal vitamin D on offspring health outcomes has not been investigated in depth.

From a public health perspective, unlike other causes of child metabolic and neurodevelopmental health problems, maternal metabolic factors and inadequate vitamin D status in utero are modifiable risk factors, which can be changed with appropriate awareness and guidance. Interventions to enhance better maternal metabolic profile and sufficient vitamin D levels in early pregnancy is possible to lead to more cost-effective approaches in the prevention and management of metabolic and neuropsychiatric health disorders in later life.

The overall aim of the present thesis is to fill the aforementioned research gaps and contribute to a better scientific understanding of the impact of components of maternal metabolic syndrome and vitamin D status in early life on offspring metabolic and neurodevelopmental programming at preschool age, using data from the only Greek mother-child birth cohort study to date, the RHEA study in Crete, Greece.

The specific objectives are:

1) To investigate the role of components of metabolic syndrome in early pregnancy on multiple offspring cardiometabolic traits at 4 years of age, in the Rhea pregnancy cohort (paper 1).
2) To evaluate the role of components of metabolic syndrome in early pregnancy and to compare maternal and paternal obesity effect on psychomotor development, and behavioral difficulties at 4 years of age, in the Rhea pregnancy cohort (paper 2).
3) To evaluate 25(OH)D levels in pregnant women in Crete, Greece and to examine the impact of 25(OH)D status in early pregnancy on both cognitive and psychomotor development at preschool age, in the Rhea pregnancy cohort (paper 3).
4) To investigate for the first time the association of very low 25(OH)D levels in early pregnancy on offspring obesity measures and cardiometabolic traits at pre-school and school age, in the Rhea pregnancy cohort (paper 4).

Methods

This section provides a summary of the methods used in the research papers included in this thesis. Further details can be found in the papers presented in the results section.

3.1 Study Design

The Rhea mother-child birth cohort study

The "Rhea" mother-child study, is a prospective population-based birth cohort that started in February 2007 at the prefecture of Heraklion, Crete, Greece. Female residents (Greek and immigrants) who became pregnant within a 12-month period, starting in February 2007, were contacted and asked to participate in the study. The first contact was made before 15 weeks' gestation, at the time of the first ultrasound. Participants were invited to provide blood and urine samples and to participate in a face-to-face interview. Women were contacted again at various times during pregnancy, at birth, at 8-10 weeks after delivery and for child's follow-up at 9th, 18th months, 4 and 6 years of age (Figure 3). Face-to-face structured questionnaires together with self-administered questionnaires and medical records were used to obtain information on several dietary, environmental, and psychosocial exposures during pregnancy and early childhood. The inclusion criteria for study participants were: residents in the study area; pregnant women aged > 16 years; no communication handicap. The study has followed the guidelines of the Declaration of Helsinki. In addition it was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece), and all participants provided written informed consent after complete description of the study.

During the study recruitment period 1,765 eligible women were approached, 1,610 (91%) agreed to participate, and 1,388 (86%) were followed up until delivery. Of 1363 singleton live births in the Rhea study, 879 (65%) singletons participated at the 4-year follow-up, from October 2011 to January 2013, during which neurodevelopmental assessment was performed in 875 children (99.5%). In each research paper the number of participants differentiates according to the available data for the particular analysis.

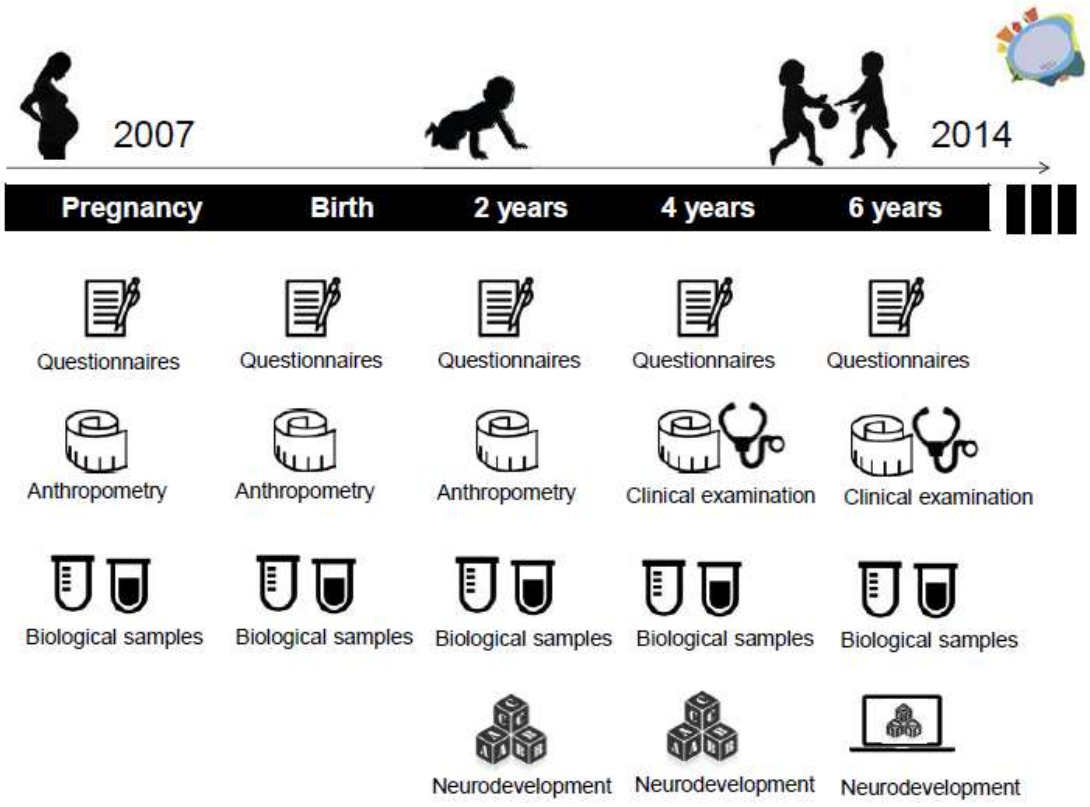

Figure 3 Overview of the data collected in the RHEA pregnancy cohort

3.2 Assessment of components of maternal metabolic syndrome in early pregnancy

Maternal pre-pregnancy overweight/obesity

Pre-pregnant body mass index (BMI) was calculated by maternal height, measured at the first prenatal visit, and pre-pregnancy weight, as reported at the first ultrasound visit, by using the formula: BMI= weight (kg)/height (m^2). Women were divided into 3 categories as follows: no excess weight (pre-pregnant BMI<25 kg/m^2), overweight (pre-pregnant BMI: 25–29.9 kg/m^2) and obese (pre-pregnant BMI >30kg/m^2) according to the definitions of the World Health Organization.

Maternal fasting glucose and lipid levels in early pregnancy

We measured maternal fasting glucose and lipid serum levels by standard enzymatic methods (Medicon, Greece) on an automatic analyzer (AU5400 high-volume chemistry analyzer; Olympus America, Inc., Melville, New York). Low density lipoprotein cholesterol (LDL) concentration was estimated by using the formula: LDL = TC-[(TG/5) + HDL]. Maternal insulin concentration was measured by IMMULITE 2000 immunoassay system (Siemens Healthcare Diagnostics, Inc., Deerfield, Illinois). The inter- and intra-assay coefficients of variation were less than 5%.

Maternal hyperglycemia in early pregnancy was defined as a maternal fasting blood glucose level ≥92 mg/dl[163]. Maternal abnormal lipid profile in early pregnancy was defined as total cholesterol levels ≥200 mg/dL, or high density lipoprotein cholesterol (HDL) levels <50 mg/dL, or low density lipoprotein cholesterol (LDL) levels ≥130 mg/dl or triglyceride levels ≥150 mg/dL[164].

Maternal blood pressure in early pregnancy

Systolic and diastolic blood pressure were measured at the ultrasound examination, after 10 minutes of rest in a sitting position. All readings were replicated 3 times in the right arm for each woman. The mean value obtained from the second and third readings was used in the analysis[165].

3.3 Assessment of maternal 25(OH)D concentrations during pregnancy

We used chemiluminescent immunoassay (CLIA) test (DiaSorin, Cat. No. 310600) to measure the total amount of 25(OH)D (both serum $25(OH)D_2$ and $25(OH)D_3$)[166] from maternal non-fasting serum samples stored at -80ºC. The analytical range for the 25(OH)D assay was 10-375 nmol/l. Inter- and intra-assay precision were <10 and <5% respectively. Maternal vitamin D concentrations were treated as categorical variable divided into tertiles.

3.4. Cardiometabolic traits assessment at preschool age

Child adiposity outcomes

Child anthropometric measures at preschool age, were taken by specially trained research assistants according to standard procedures at the University Hospital of Heraklion, Crete, Greece. Body weight and height was measured with subjects standing without shoes and in light clothing. Overweight/obesity were defined using age-and sex-specific BMI thresholds proposed by the International Obesity Task Force[167]. Waist circumference (WC) was measured in the standing position, at the high point of the iliac crest at the end of a gentle expiration. We used age-and sex-specific 90^{th} waist circumference percentiles based on national references[168], as a cut-off point to define central adiposity. Skin fold thickness was measured at four anatomical sites (triceps, thigh, subscapular and suprailiac) on the right side of the body. Body composition was estimated at 6 years follow up by a bioelectric impedance analysis (BIA) performed using a tetra-polar device (Bodystat 1500). All children had not eaten or participated in physical activity a minimum of 120 mins prior to being measured. We used pediatric specific BIA equation[169] to obtain free fat mass (FFM), fat mass (FM) and body fat percentage (%BF) at 6 years of age. As BIA was not available at the 4 years follow up, we estimated child %BF at 4 years using the Slaughter skinfold-thickness equations[170].

Child non fasting lipid profile

We measured non fasting child serum lipid levels with the same biochemical methods used for maternal lipid measurements. As there is no standard definition for lipid disorders at preschool age, we used the 75th percentile of the study cohort distribution for total cholesterol (≥173.9 mg/dL) and LDL (≥111.5 mg/dL) and the 25th percentile for HDL levels (<40 mg/dL) as a cut-off point to denote abnormal lipid levels in children[171].

Child blood pressure

Systolic and diastolic blood pressure at preschool age were measured by trained research assistants after 5 minutes rest in the seated position, at the child right arm with a cuff of appropriate size for arm circumference. We used the average of five consecutive measurements, taken with 1 minute intervals[172]. We then calculated blood pressure percentiles specific for age, sex, and height, as blood pressure measurements in children are suggested to differ according to these characteristics.

3.5 Neurodevelopmental assessment at preschool age

McCarthy Scales of Children's Abilities

Children's cognitive and motor development at preschool age was assessed using the McCarthy Scales of Children's Abilities (MSCA)[173]. The MSCA represent an age appropriate instrument, developed for children of ages 2½-8½ years, which gives standardized test scores for five domains: i) the Verbal Scale (verbal expression and verbal comprehension ability); ii) the Perceptual-Performance Scale (reasoning ability through materials manipulation); iii) the Quantitative Scale (number aptitude and numbers interest); iv) the Memory Scale (short term memory through verbal and non-verbal stimuli); v) the Motor Scale (gross and fine motor ability)[173]. A general cognitive score, which estimates global intellectual function, was calculated by combining the verbal, perceptual performance and quantitative scores.

To further improve our understanding of the specific functions associated with the exposures of interest, the MSCA items were reorganised, for tasks highly associated with specific neurocognitive functions, into the following new outcomes,: executive functions of frontal cortex and functions of posterior cortex.

The MSCA was administered individually and the administration time ranged from 40 to 60 min. All testing was done at the University Hospital of Heraklion, Crete, Greece, and Medical Health Centres in the prefecture of Heraklion, Crete, by two trained psychologists. A strict protocol was applied to avoid inter-observer variability, including two sets of quality controls undertaken during the fieldwork (one at the beginning and one in the middle of the follow up). The Intra-class correlation coefficient (ICC) was used to measure the inter-rater reliability for absolute agreement between scores in a two-way random model. The inter-rater reliability was very high for all scales at both periods of reliability assessment (ICC≥0.973). The psychologists also noted critical comments about the difficulties or special conditions of the neurodevelopmental assessment so as to evaluate the "quality of assessment" such as: no difficulties, difficulties due to physical problems (e.g. physical illness, tiredness, asleep), difficulties due to behavior problems (e.g. nervousness, shyness).

Raw scores of MSCA scales were standardized for child's age at test administration. Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales. Standardized scores were treated as continuous variables with higher scores representing better general cognition, language, or psychomotor development. MSCA translation and cross-cultural adaptation was conducted according to the internationally recommended methodology. Internal consistency (Cronbach's alpha) varied between 0.76 and 0.89, showing adequate reliability for all the scales. Confirmatory factor analysis supported good fit of the model ($\chi 2/df = 2$, CFI=.83, GFI=.97, RMSEA=.034)[174].

Behavioral difficulties

Information on children's behavior and ADHD-like symptoms at preschool age was obtained via maternal report on two standardized child behavior scales. Strengths and Difficulties Questionnaire (SDQ)[175], is a behavioral screening instrument designed for children aged 3-16 year old. It consists of five subscales generating scores for emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems and prosocial behavior. A total SDQ score can be calculated by aggregating the scores for the above subscales except prosocial behavior, with a high score being less favourable (range 0–40).Two additional scores were calculated: the internalizing problems score by adding the emotional and peer relationships subscales together (range 0–20) and the externalizing problems score by adding the conduct and hyperactivity subscales together (range 0–20). The prosocial behavior scale

provides information on protective factors of the child; a low score is less favourable. The SDQ has been translated and adapted for the Greek population[176]. The Cronbach's alpha measuring internal consistency of the total SDQ score was α=0.667.

The Attention Deficit Hyperactivity Disorder Test (ADHDT)[177], is designed to identify and evaluate ADHD symptoms in ages 3-23 years. It is composed of 36 items in three subscales; (i) Hyperactivity, (ii) Inattention, and (iii) Impulsivity. All 36 items are summed to generate an index for total ADHD difficulties (range 0-72). Higher scores indicate more intensive ADHD symptoms. The ADHDT has been translated and adapted for the Greek population[178]. The Cronbach's alpha measuring internal consistency of the total ADHD index was α=0.951.

We used the ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) to categorize ADHD-like symptoms. Three quantitative traits were generated for use in our analyses: (1) a count of the number of hyperactive–impulsive symptoms, (2) a count of the number of inattentive symptoms, and (3) a count of the total number of ADHD-like symptoms. In all three cases, a binary measure indicating the presence or absence of each symptom was measured and the totals were generated by summing over all symptoms, making the maximum number of symptoms 9, 9, and 18, respectively.

3.6 Statistical analysis

In summary, differences in distributions of normally distributed variables were tested with either Student t-test or ANOVA; non-normally distributed continuous variables were tested with non-parametric Mann–Whitney U test for two independent samples and Kruskal–Wallis test for more than two independent samples, whereas categorical variables were tested with Chi square test. Pearson's r or Spearman's rho correlation coefficient was used to estimate the strength of the association between continuous dependent and independent variables. The possibility of nonlinear associations was tested by generalized additive models (GAMs).

Analyses between components of metabolic syndrome in early pregnancy and offspring outcomes

Maternal pre-pregnancy BMI, lipid levels and blood pressure in early pregnancy were used both as categorical and continuous variables. Multivariable log-Poisson regression models with robust standard errors were used for dichotomous outcomes, as these are more

appropriate than logistic regression when the incidence of the outcome is 10% or more[179]. Linear regression models were performed for continuous outcomes. Estimated associations were described as relative risks (RR) with 95% confidence intervals (CIs) or β-coefficients with 95% CIs accordingly. Potential covariates associated with the exposures and the outcomes of interest with a $p < 0.05$, as well as a priori selected potential confounders were included in the multivariable models.

Analyses between maternal 25(OH)D during pregnancy and offspring outcomes

The distribution of mean 25(OH)D concentration was plotted by calendar month and showed a seasonal variation. As 25(OH)D concentrations followed a sinusoidal pattern, we fitted a cosinor model to the data to predict "deseasonalized" annual mean 25(OH)D concentration for each participant adjusted for month at blood collection. Maternal 25(OH)D concentration was treated as categorical divided into tertiles. In order to test the dose-response relationship of 25(OH)D concentrations and outcomes of interest, p-for-trend was assessed ($p <0.10$).

A more detailed description of the statistical methods used can be found in the following section, in each paper.

4. Results

4.1 Paper 1. Metabolic Profile in Early Pregnancy Is Associated with Offspring Adiposity at 4 Years of Age: The Rhea Pregnancy Cohort Crete, Greece

Main Findings:

Our findings support that exposure to metabolic dysregulation in early pregnancy may predict increased risk of obesity in preschool children. Most specifically:

1. Higher maternal pre-pregnancy BMI was associated with increased risk of offspring overweight/obesity, central adiposity, and fat mass in preschoolers, predominantly in girls.

2. Hypercholesterolemia in early pregnancy was associated with increased offspring overweight/obesity, greater fat mass and adverse lipid profile at preschool age.

3. Higher diastolic blood pressure in early pregnancy was associated with increased offspring fat mass at preschool age.

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PLOS ONE

RESEARCH ARTICLE

Metabolic Profile in Early Pregnancy Is Associated with Offspring Adiposity at 4 Years of Age: The Rhea Pregnancy Cohort Crete, Greece

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Data Availability Statement: Ethical restrictions prevent public sharing of data from the Rhea pregnancy cohort study, as imposed by the Research Ethics Committee of the Rhea Cohort Study. Data can be made available to all interested researchers upon request by contacting the Research Committee at rhea@med.uoc.gr or the Principal Investigators of the study (Prof. M. Kogevinas, kogevinas@creal.cat; Dr. L. Chatzi, lchatzi@med.uoc.gr).

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Abstract

Context

Maternal pre-pregnancy obesity may increase the risk of childhood obesity but it is unknown whether other metabolic factors in early pregnancy such as lipid profile and hypertension are associated with offspring cardiometabolic traits.

Objective

Our objective was to investigate whether fasting lipid, glucose, and insulin levels during early pregnancy and maternal pre-pregnancy weight status, are associated with offspring adiposity measures, lipid levels and blood pressure at preschool age.

Design and Methods

The study included 618 mother-child pairs of the pregnancy cohort "Rhea" study in Crete, Greece. Pregnant women were recruited at the first prenatal visit (mean: 12weeks, SD: 0.7). A subset of 348 women provided fasting serum samples for glucose and lipid measurements. Outcomes measures were body mass index, abdominal circumference, sum of skinfold thickness, and blood pressure measurements at 4 years of age. A subsample of 525 children provided non-fasting blood samples for lipid measurements.

Results

Pre-pregnancy overweight/obesity was associated with greater risk of offspring overweight/obesity (RR: 1.83, 95%CI: 1.19, 2.81), central adiposity (RR: 1.97, 95%CI: 1.11, 3.49), and greater fat mass by 5.10mm (95%CI: 2.49, 7.71) at 4 years of age. These associations were

ENV.2007.1.2.2.2. Project No 211250 Escape, EU FP7-2008-ENV-1.2.1.4 Envirogenomarkers, EU FP7-HEALTH-2009- single stage CHICOS, EU FP7 ENV.2008.1.2.1.6. Proposal No 226285 ENRIECO, EU- FP7-HEALTH-2012 Proposal No 308333 HELIX) and the Greek Ministry of Health (Program of Prevention of obesity and neurodevelopmental disorders in preschool children, in Heraklion district, Crete, Greece: 2011-2014; "Rhea Plus": Primary Prevention Program of Environmental Risk Factors for Reproductive Health, and Child Health: 2012-15). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

more pronounced in girls. An increase of 40mg/dl in fasting serum cholesterol levels in early pregnancy was associated with greater skinfold thickness by 3.30mm (95%CI: 1.41, 5.20) at 4 years of age after adjusting for pre-pregnancy BMI and several other confounders. An increase of 10mmHg in diastolic blood pressure in early pregnancy was associated with increased risk of offspring overweight/obesity (RR: 1.22, 95%CI: 1.03, 1.45), and greater skinfold thickness by 1.71mm (95% CI: 0.57, 2.86) at 4 years of age.

Conclusions

Metabolic dysregulation in early pregnancy may increase the risk of obesity at preschool age.

Introduction

Childhood obesity is one of the greatest public health challenges worldwide and is having a major impact on human morbidity, mortality and quality of life [1, 2]. In Europe, its prevalence has increased dramatically in last decades, while recent estimates report that Greece has the highest prevalence of childhood obesity [3]. The commonly held causes of obesity, which are over-eating, inactivity, and genetic pre-disposition, do not fully explain the current obesity epidemic [4]. According to the developmental origins of health and disease (DOHaD) hypothesis changes in the intrauterine environment at critical or sensitive periods of the developmental process could have irreversible, lifelong consequences in offspring metabolism [4, 5]. Metabolic disorders during pregnancy like obesity, gestational diabetes, and excess gestational weight gain are well known exposures that predispose offspring to obesity [6–9]. However, the role of maternal metabolism in the first trimester of pregnancy, which is a critical developmental time window for gestational programming, is unclear [10].

Epidemiological studies indicate that higher maternal pre-pregnancy body mass index is associated with increased risk of childhood obesity [7, 8]. Few studies have examined so far its association with other cardiometabolic risk factors such as lipid levels and blood pressure in children with controversial results [11–15]. Whether these associations reflect direct intrauterine causal mechanisms or are driven in a gender-related manner remains unclear. Animal studies suggest that sex-specific vulnerabilities to an altered *in utero* metabolic environment may mediate sex differences in fetal growth and predisposition to adult diseases, such as cardiovascular disease [16, 17]), however evidence from human studies is scarce [18]. Moreover, it has been suggested that maternal gestational weight gain and smoking during pregnancy can act as confounders of such associations [6, 19, 20], although it can be argued that they may also act as mediators.

Studies on other maternal cardiovascular risk factors such as dyslipidemia or hypertension in pregnancy in association with offspring cardiometabolic health are scarce with conflicting results [21, 22]. Maternal non fasting lipid levels in early pregnancy were shown to be associated with increased offspring's fat percentage and waist-to-height ratio values at preschool age [22]. Hypertension in early pregnancy has been associated with increased risk of fetal growth restriction [23, 24]), and preeclampsia [23], but there are no studies examining blood pressure in early pregnancy with offspring cardiometabolic traits.

In this study, we aimed to fill these research gaps by investigating the impact of maternal metabolic profile in early pregnancy characterized by pre-pregnancy Body Mass Index (BMI), blood pressure levels, and fasting lipids, insulin and glucose levels on offspring cardiometabolic

traits in early childhood, in a prospective pregnancy cohort in Crete, Greece, after controlling for several confounding and mediator factors.

Materials and Methods

Study design and population: Rhea cohort

The present study is part of the "Rhea" project, a pregnancy cohort which examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece [25]. We recruited pregnant women (Greek and immigrants) at the time of the first comprehensive ultrasound examination, around week 12 of gestation (mean: 12.1 weeks, SD: 0.7), from four prenatal clinics (two public and two private) in Heraklion city, during the twelve-month period from February 2007 until February 2008. The inclusion criteria for study participants were: residents in the study area; pregnant women aged > 16 years; 1st prenatal visit: hospitals or private clinics at Heraklion district; no communication handicap. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece), and all participants provided written informed consent after complete description of the study.

Of 1363 singleton live births in the Rhea study, 879 children participated at the 4 years follow up, during which anthropometry and non-fasting blood samples were obtained from 785 children. From those, complete data for maternal anthropometry, follow-up interview and child anthropometric measurements were available for 631 mother–child pairs. We excluded women who had been diagnosed with preeclampsia [n = 13 (8 in this, and 5 in previous pregnancies)], since this condition is associated with a higher blood pressure and BMI in childhood and early adult life [21]. Thus, a cohort of 618 mother–child pairs was available for the present analysis. Of them a subset of 348 women provided fasting blood samples for glucose and lipid measurements, due to the timing of enrollment in the study. A subsample of 525 children provided non-fasting blood samples at the 4 year follow up (mean: 4.2 years, SD: 0.2).

Exposures: maternal pre-pregnancy BMI and metabolic profile during early pregnancy

Maternal overweight/obesity. Maternal height, measured at the first prenatal visit, and pre-pregnancy weight, as reported at the first major ultrasound visit, were used to calculate the pre-pregnant body mass index (BMI; weight (kg)/height $(m)^2$). Women were divided into 3 categories as follows: no excess weight (pre-pregnant BMI<25 kg/m^2), overweight (pre-pregnant BMI: 25–29.9 kg/m^2) and obese (pre-pregnant BMI $\geq$30kg/m^2) according to the definitions of the World Health Organization.

Maternal fasting glucose and lipid levels in early pregnancy. We measured lipids [total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C)] and glucose by standard enzymatic methods (Medicon, Greece) on an automatic analyzer (AU5400 high-volume chemistry analyzer; Olympus America, Inc., Melville, New York). Low density lipoprotein cholesterol (LDL-C) concentration was estimated by using the formula: LDL-C = TC-[(TG/5) + HDL-C]. C-reactive protein levels were measured with a high-sensitivity homogenous immunoassay (ORS 6199, Beckman Coulter, USA) on an automatic analyzer (AU5400 high-volume chemistry analyzer; Olympus America, Inc., Melville, New York). Maternal insulin concentration was measured by IMMULITE 2000 immunoassay system (Siemens Healthcare Diagnostics, Inc., Deerfield, Illinois). The inter- and intra-assay coefficients of variation were less than 5%.

Maternal abnormal lipid profile in early pregnancy was defined as triglyceride levels ≥150 mg/dL, or total cholesterol levels ≥200 mg/dL, or high density lipoprotein cholesterol (HDL-C) levels <50 mg/dL, or low density lipoprotein cholesterol (LDL-C) levels ≥130 mg/dl [26]. Maternal hyperglycemia in early pregnancy was defined as a maternal fasting blood glucose level ≥92 mg/dl [27].

Maternal blood pressure in early pregnancy. Systolic (SBP) and diastolic (DBP) blood pressure were measured at the ultrasound examination. Measurements were taken by using an electronic blood pressure monitor after 10 minutes of rest in a sitting position. All readings were replicated 3 times in the right arm for each woman. The mean value obtained from the second and third readings was used in the analysis [25].

Outcomes: Offspring cardiometabolic traits during early childhood

Child adiposity outcomes at 4 years of age. Child anthropometric measures at 4 years of age (mean: 4.2 years, SD: 0.2), were taken by specially trained research assistants according to standard procedures at the University Hospital of Heraklion, Crete, Greece. Body weight was measured once by a digital scale (Seca Bellisima 841) to the nearest 0.1kg with subjects standing without shoes and in light clothing. Height was measured to the nearest 0.1 cm with the use of a commercial stadiometer (Seca 213). Overweight/obesity were defined using age-and sex-specific BMI thresholds proposed by the International Obesity Task Force [28].

Waist circumference (WC) was measured in duplicate to the nearest 0.1 cm in the standing position, at the high point of the iliac crest at the end of a gentle expiration, using a flexible tape measure (Seca 201). We used age-and sex-specific 90th waist circumference percentiles based on national references [29], as a cut-off point to define central adiposity.

Skin fold thickness was measured to the nearest 0.1mm at four anatomical sites (triceps, thigh, subscapular and suprailiac) on the right side of the body, using calibrated calipers (Harpenden HSK- BI, CE-0120).

Child non fasting lipid profile at 4 years of age. We measured non fasting total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) with the same biochemical methods used for maternal lipids measurements. As there is no standard definition for lipid disorders at preschool age, we used the 75th percentile of the study cohort distribution for total cholesterol (≥173.9 mg/dL), LDL-C- (≥111.5 mg/dL) and the 25th percentile for HDL-C levels (<40 mg/dL) as a cut-off point to denote abnormal lipid levels in children [30].

Child blood pressure at 4 years of age. At 4 years of age, trained research assistants measured systolic (SBP) and diastolic blood pressure (DBP) after 5 minutes rest in the seated position, at the child right arm with a cuff of appropriate size for arm circumference using a Dinamap Pro Care 400, which utilizes an oscillometric method. We used the average of five consecutive measurements, taken with 1 minute intervals [31]. We then calculated blood pressure percentiles specific for age, sex, and height, as blood pressure measurements in children are suggested to differ according to these characteristics.

Potential confounders

Potential confounders included characteristics that have an established or potential association with maternal metabolic profile in early pregnancy or cardiometabolic risk in childhood including: maternal age at delivery (years); maternal education (low level: ≤6 yrs. of school; medium level: ≤12 yrs. of school; high level: university or technical college degree); maternal origin (Greek/other); marital status (married/other); physical activity before pregnancy (yes/no); parity (primiparous/multiparous); type of delivery (vaginal/caesarean); smoking during pregnancy (yes/no); gestational weight gain, categorized according to 2009 Institute of

Medicine guidelines [32]; family history of dyslipidemia (yes/no); family history of diabetes (yes/no); gestational diabetes (yes/no); gestational hypertension (yes/no); gestational age (weeks); birth weight (kg); child's sex (male/female); duration of breastfeeding (months); day of care attendance at the first 2 years of life (yes/no); TV viewing at 4 years of age (hours/day); child's energy intake (Kcals/day) at 4 years of age based on a validated food frequency questionnaire [33].

Statistical analysis

Differences in distributions of normally distributed variables were tested with t-test, non-normally distributed continuous variables were tested by using non parametric tests (i.e., Mann-Whitney, Kruskal-Wallis, and Spearman non parametric statistical tests), whereas categorical variables were tested with chi-square test (Pearson's or Fisher exact test with Monte-Carlo correction). The possibility of nonlinear associations was tested by generalized additive models (GAMs) indicating linear relationships for all exposure-outcomes associations.

Multivariable log-Poisson regression models with robust standard errors were used for dichotomous outcomes, as these are more appropriate than logistic regression when the incidence of the outcome is 10% or more [34]. Linear regression models were performed for continuous outcomes. Estimated associations were described as relative risks (RR) with 95% confidence intervals (CIs) or β-coefficients with 95% CIs accordingly. We examined the associations of maternal metabolic profile in early pregnancy with childhood cardiometabolic traits at 4 years of age in 3 models: The first model (crude model) was adjusted for the child's sex (except models using offspring systolic and diastolic blood pressure percentiles as an outcome); the second model (confounder model) was additionally adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI (only for models using maternal fasting lipid levels or blood pressure as an exposure variable). In a third model (mediation model), we additionally adjusted for maternal weight gain during pregnancy, birth weight, breastfeeding duration, child's anthropometry at age of outcome assessment, and child lifestyle characteristics [TV viewing (hours/day)]. Because relations of pre-pregnancy BMI with offspring cardiometabolic traits could be moderated by paternal BMI we also examined associations after adjusting for paternal BMI.

We examined potential effect modification by child's sex, maternal smoking during pregnancy, and gestational weight gain by including the interaction term in the models (statistically significant effect modification if p-value$<$0.05) and stratified analyses accordingly. We also examined potential effect modification by child BMI in the models using child lipid levels as an outcome variable.

All hypotheses testing were conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. We used Stata S.E. version 11.2 for the statistical analyses (Stata Corp, Texas, USA).

Results

Participants' characteristics. Maternal and child demographic characteristics according to maternal overweight/obesity status are shown in Table 1. A total of 209 (34%) women were overweight/obese pre-pregnancy, while 77 (12.5%) women were obese prior to gestation. Overweight/obese women prior to gestation were more likely to be multiparous, less educated, to gain excessive weight during pregnancy and to breastfeed their children for shorter durations compared with women with no excess weight (Table 1). S1 Table in the supporting information material shows that mothers without offspring follow-up data were more likely to be younger, smokers, less educated, and of non-Greek ethnicity. There were no significant differences in

Table 1. Mother- child characteristics by pre-pregnancy overweight/obesity status, Rhea pregnancy cohort, Crete, Greece.

	Pre-pregnancy obesity status		
	No excess weight (n = 409)	Overweight/Obese (n = 209)	*P*- value [a]
Maternal characteristics			
Maternal age at delivery (yr), *mean (SD)*	29.87 (0.2)	29.98 (0.3)	0.891
Education, *n (%)*			<0.001
Low	49 (12.0)	54 (25.8)	
Medium	208 (50.9)	102 (48.8)	
High	152 (37.2)	53 (25.4)	
Greek origin, *n (%)*	384 (93.9)	199 (95.2)	0.499
Primiparous, *n (%)*	194 (47.4)	70 (33.5)	0.001
Smoking during pregnancy, *n (%)*	126 (30.8)	70 (33.5)	0.497
Gestational diabetes, *n (%)*	32 (8.4)	18 (9.2)	0.758
Gestational hypertension, *n (%)*	15 (4.0)	14 (7.1)	0.106
Gestational weight gain (kg), *n (%)*			<0.001
Inadequate	114 (27.9)	15 (7.2)	
Adequate	155 (37.9)	81 (38.8)	
Excessive	140 (34.2)	113 (54.1)	
Caesarian section, *n (%)*	195 (48.0)	115 (55.0)	0.100
Metabolic profile in early pregnancy (n = 348)			
Glucose (mg/dL), *mean (SD)*	74.93 (0.7)	76.10 (1.2)	0.341
Insulin (mg/dL), *mean (SD)*	9.33 (0.9)	12.75 (1.5)	<0.001
TC (mg/dL), *mean (SD)*	195.49 (2.3)	203.87 (3.7)	0.025
LDL-C (mg/dL), *mean (SD)*	113.87 (1.8)	123.14 (3.0)	0.008
HDL-C (mg/dL), *mean (SD)*	60.18 (0.9)	55.42 (1.3)	0.007
TG (mg/dL), *mean (SD)*	108.04 (2.8)	126.23 (4.8)	<0.001
SBP (mmHg), *mean (SD)*	105.06 (0.5)	109.92 (0.8)	<0.001
DBP (mmHg), *mean (SD)*	69.27 (0.5)	71.31 (0.7)	0.013
Child characteristics in infancy			
Sex, girl, *n (%)*	201 (49.1)	93 (44.5)	0.274
Birth weight (kg), *mean (SD)*	3.20 (0.02)	3.21 (0.03)	0.887
Gestational age (weeks), *mean (SD)*	38.32 (0.07)	38.08 (0.11)	0.123
Duration of breastfeeding (months), *mean (SD)*	4.69 (0.2)	3.44 (0.3)	<0.001
Day care attendance in the first 2 years of life, *n (%)*	79 (19.5)	36 (17.3)	0.518
Child characteristics at 4 years of age			
BMI (Kg/m^2), *mean (SD)*	16.11 (0.08)	16.93 (0.15)	<0.001
Overweight/obese, *n (%)*	74 (18.1)	60 (28.7)	0.002
Waist circumference (cm), *mean (SD)*	52.90 (0.2)	54.66 (0.4)	<0.001
Waist circumference ≥90th pct, *n (%)*	35 (8.7)	37 (17.9)	<0.001
Sum of skinfolds (mm), *mean (SD)*	37.78 (0.7)	43.29 (1.1)	<0.001
TC (mg/dL), *mean (SD)*	156.39 (1.5)	158.97 (2.0)	0.247
LDL-C (mg/dL), *mean (SD)*	69.98 (27.4)	69.58 (26.7)	0.856
HDL-C (mg/dL), *mean (SD)*	47.17 (0.6)	47.78 (0.8)	0.518
TG (mg/dL), *mean (SD)*	69.69 (1.5)	70.40 (2.1)	0.690
Time spent watching TV (hours/day), *n (%)*			0.019
Almost never	124 (30.5)	52 (25.0)	
1–2 hours/day	251 (61.8)	126 (60.6)	
More than 3 hours/day	31 (7.6)	30 (14.4)	

(*Continued*)

Table 1. (*Continued*)

	Pre-pregnancy obesity status		
	No excess weight (n = 409)	Overweight/Obese (n = 209)	*P*- value [a]
Energy intake (Kcals/day), *mean (SD)*	1583.5 (23.0)	1594.3 (32.1)	0.752

BMI, Body Mass Index; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; TG, Triglyceride; CRP, C-reactive protein; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile

[a] *P*-values obtained by Mann-Whitney U test for two independent samples, and χ^2 test or Fisher exact test with Monte-Carlo correction.

Numbers may not correspond to the total due to missing numbers.

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socio- demographic characteristics between women who provided fasting blood samples and those who did not (S2 Table).

In the subset of pregnant women with available fasting serum samples in early pregnancy, dyslipidemia was the most frequent metabolic disorder, as 49.7% women had total cholesterol levels ≥ 200 mg/dL, 26.8% had HDL-C levels< 50 mg/dL, and 18.7% had TG levels ≥ 150 mg/dL. Only 22 (6.3%) women were suffering from hyperglycemia in the first trimester of pregnancy. Overweight/obesity prior to gestation was associated with higher fasting total cholesterol, LDL-C, triglycerides, and insulin levels at the first trimester of pregnancy, lower HDL-C levels, and higher systolic and diastolic blood pressure (Table 1).

The prevalence of overweight/obesity and central adiposity (WC ≥ 90th percentile) at 4 years of age was 21.7% (n = 134) and 11.8% (n = 72), respectively (Table 1). Mean (±SD) non fasting total cholesterol, LDL-C and HDL-C were 198.1 (±36.2), 116.7 (±29.4), and 58.7 (±14.1) respectively. Children whose mothers were overweight/obese prior to gestation had higher BMI, waist circumference, and fat mass at 4 years of age compared to children whose mothers had no excess weight pre-pregnancy (Table 1).

Overweight/obesity pre-pregnancy in association with offspring cardiometabolic traits at 4 years of age

Generalised additive models examining the shape of the relationships of metabolic profile in early pregnancy with offspring cardiometabolic traits at 4 years of age showed no significant departures from linearity overall. Pre-pregnancy overweight/obesity showed a positive linear relationship with the probability of overweight/obesity at 4 years of age (Fig 1). Pre-pregnancy overweight/obesity was also positively associated with all other offspring adiposity outcomes at 4 years of age (Table 2). We found no association between pre-pregnancy BMI and offspring non-fasting lipid levels or blood pressure percentiles at 4 years of age (Table 2). Further adjustment for paternal BMI did not attenuate the observed associations (S3 Table).

Fasting lipid, glucose and insulin levels in early pregnancy in association with offspring cardiometabolic traits at 4 years of age

Maternal fasting cholesterol levels showed a positive linear relationship with the probability of overweight/obesity at 4 years of age (Fig 2). An increase of 40mg/dl in total cholesterol levels was associated with 42% increased risk of overweight/obesity (RR: 1.42, 95% CI: 1.03, 1.95) and greater skinfold thickness by 3.30 mm (95%CI: 1.41, 5.20) at 4 years of age after adjustment for several covariates and pre-pregnancy BMI (Model 3). A positive association was also observed between maternal fasting cholesterol levels and offspring cholesterol levels at 4 years of age, but the associations were attenuated when we further adjusted for potential mediators (Table 3,

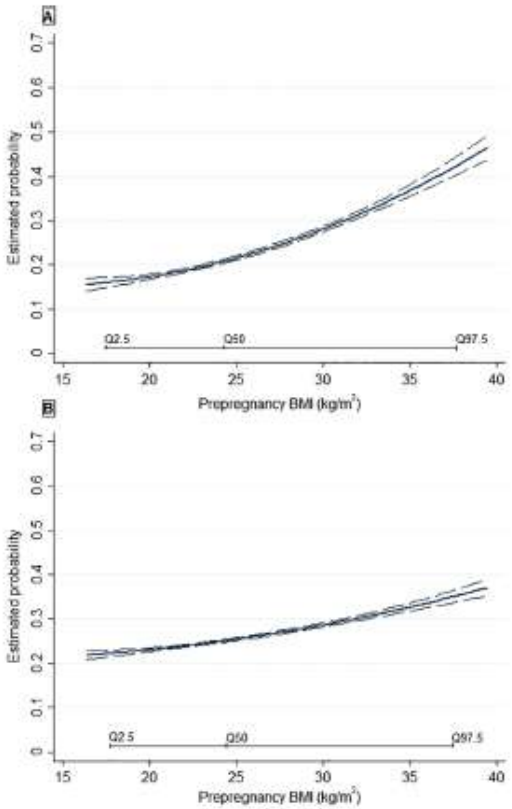

Fig 1. Relationship between pre-pregnancy BMI and the estimated probability for overweight/obesity (A) and cholesterol levels >75th percentile (B) at 4 years of age. Estimated probability is based on multivariable models adjusted for maternal age, education, parity, smoking during pregnancy, gestational weight gain, birth weight, breastfeeding duration and TV watching at 4 years of age. Q2.5, Q50, Q97.5 represent the 2.5th, 50.0th, and the 97.5th percentiles of the studied population. Long-dashes represent the 95%CIs.

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model 3). No association was found between maternal fasting cholesterol levels and offspring blood pressure percentiles at 4 years of age (Table 3). No association was also observed among maternal fasting glucose and insulin serum levels in early pregnancy and offspring cardiometabolic traits at 4 years of age (data not shown).

Maternal blood pressure levels in early pregnancy in association with offspring cardiometabolic traits at 4 years of age

An increase of 10mmHg in diastolic blood pressure in early pregnancy was associated with 23% increased risk of offspring overweight/obesity (RR: 1.22, 95%CI: 1.03, 1.45), and greater skin fold thickness by 1.71 mm (95%CI: 0.57, 2.86) in the fully adjusted model (Table 4). We found no association between maternal blood pressure levels in early pregnancy and offspring lipids and blood pressure percentiles at 4 years of age (Table 4).

Table 2. Association of maternal pre-pregnancy obesity status with offspring cardiometabolic traits at 4 years of age, Rhea pregnancy cohort Crete, Greece.

		Pre-pregnancy overweight/obese (≥25 kg/m²) (n = 209)		
Cardiometabolic traits at 4 years of age	**n**	**Model 1**	**Model 2**	**Model 3**
Adiposity outcomes		**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**
Overweight/obese	134	**1.59 (1.17, 2.15)**	**1.53 (1.11, 2.09)**	**1.83 (1.19, 2.81)**
WC (cm) ≥ 90th pct	72	**2.03 (1.32, 3.14)**	**1.89 (1.20, 2.96)**	**1.97 (1.11, 3.49)**
		β-coef. (95%CI)	**β-coef. (95%CI)**	**β-coef. (95%CI)**
Child BMI	618	**0.80 (0.45, 1.14)**	**0.78 (0.44, 1.12)**	**0.79 (0.36, 1.06)**
WC (cm)	606	**1.75 (0.87, 2.63)**	**1.76 (0.89, 2.64)**	**1.36 (0.55, 2.17)**
Sum of 4 Skinfolds (mm)	601	**5.74 (3.17, 8.30)**	**5.37 (2.75, 7.99)**	**5.10 (2.49, 7.71)**
Non-fasting lipid levels		**β-coeff. (95%CI)**	**β-coef. (95%CI)**	**β-coef. (95%CI)**
TC(mg/dl)	525	2.64 (-2.21, 7.50)	2.52 (-2.50. 7.55)	2.18 (-3.04. 7.41)
HDL-C(mg/dl)	525	0.59 (-1.38, 2.56)	0.43 (-1.63, 2.49)	0.59 (-1.54, 2.73)
Blood pressure levels		**β-coef. (95%CI)**	**β-coef. (95%CI)**	**β-coef. (95%CI)**
SBP percentiles	488	0.26 (-0.17, 0.69)	0.30 (-0.13, 0.75)	0.21 (-0.24, 0.67)
DBP percentiles	488	-0.07 (-0.49, 0.34)	-0.11 (-0.53, 0.31)	-0.10 (-0.54, 0.33)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile

Model 1: adjusted for child sex. (except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at p<0.05.

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Effect modification-Sensitivity analyses

In the mediation model, further adjustment for birth characteristics, child's anthropometry and life-style behaviors did not substantively alter the adjusted estimations for most childhood outcomes (Tables 2, 3 and 4). Further analyses showed evidence for an interaction between child sex and maternal pre-pregnancy BMI in response to offspring overweight/obesity and central adiposity (p for interaction <0.05), but not with skinfold thickness (Table 5). The greatest risk for these adiposity outcomes was observed for girls whose mothers were overweight/obese prior to gestation (RR-overweight/obesity: 3.54, 95%CI: 1.80, 6.98; RR-central adiposity: 5.33, 95%CI: 2.17, 13.07), whereas similar associations in boys were not significant (Table 5). We saw no evidence for a multiplicative interaction of maternal metabolic profile in early pregnancy with maternal smoking during pregnancy, gestational weight gain or child BMI (p for interaction >0.05).

To elucidate whether gestational diabetes modified the observed results, we performed a sensitivity analysis in which we excluded all women who were diagnosed with gestational diabetes (n = 50). Results did not differ substantially from those derived from the main analysis (S4 Table, S5 Table and S6 Table). We also found no difference in the observed estimates after excluding preterm births (data not shown).

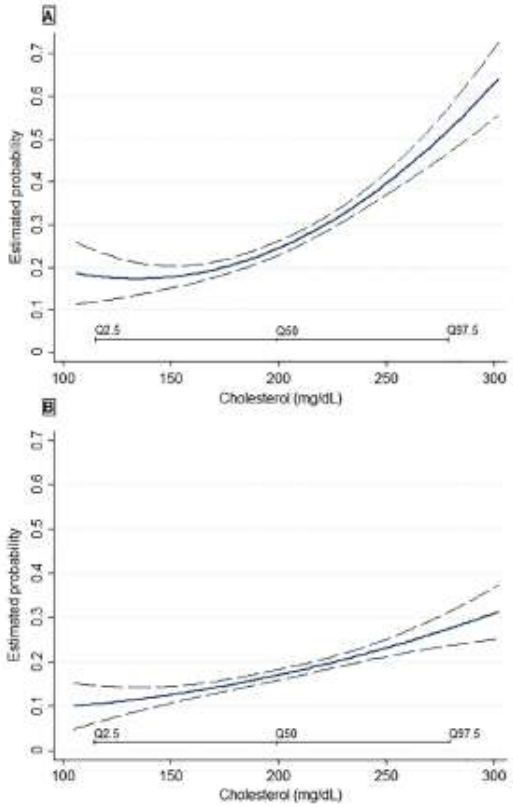

Fig 2. Relationship between first-trimester fasting maternal cholesterol levels and the estimated probability for overweight/obesity (A) and cholesterol levels >75th percentile (B) at 4 years of age. Estimated probability is based on multivariable models adjusted for maternal age, education, parity, smoking during pregnancy, BMI pre-pregnancy, gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Q2.5, Q50, Q97.5 represent the 2.5th, 50.0th, and the 97.5th percentiles of the studied population. Long-dashes represent the 95%CIs.

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Discussion

In this prospective pregnancy cohort we showed that exposure to metabolic dysregulation in early pregnancy may predict increased risk of obesity in preschool children. To our knowledge this is the first study examining maternal metabolic profile in early pregnancy with the use of fasting serum samples in association with offspring cardiometabolic risk.

Our findings are in consistence with previous epidemiological studies examining the effect of maternal pre-pregnancy BMI with child BMI measures [7, 8, 35], and fat mass [12, 14, 22, 36, 37]. The associations of maternal pre-pregnancy BMI with childhood adiposity may be explained by intrauterine mechanisms or shared environmental, life-style and genetic characteristics [9]. Animal studies suggest that epigenetic alterations induced by maternal overnutrition in pregnancy may modulate expression of genes that regulate adipogenesis, glucose homeostasis, inflammation, and/or insulin signaling, including genes encoding hormones (e.g., leptin), nuclear receptors (adipogenic and lipogenic transcription factors PPARγ and PPARα, respectively), gluconeogenic enzymes and transmembrane proteins [38]. Moreover, adverse maternal

Table 3. Association of maternal fasting lipid profile in early pregnancy with offspring cardiometabolic traits at 4 years of age, Rhea pregnancy cohort Crete, Greece.

		Fasting TC levels in early pregnancy (per increase in 40 mg/dL) (n = 348)			Fasting LDL-C levels in early pregnancy (per increase in 15 mg/dL) (n = 348)		
Cardiometabolic traits at 4 years of age	**n**	**Model 1**	**Model 2**	**Model 3**	**Model 1**	**Model 2**	**Model 3**
Adiposity outcomes							
		RR (95%CI)	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**
Overweight/obese	64	1.18 (0.92, 1.50)	1.27 (0.97, 1.66)	**1.42 (1.03, 1.95)**	1.03 (0.91, 1.17)	1.07 (0.93, 1.22)	1.10 (0.94, 1.29)
WC (cm) $\geq$ 90th pct	30	1.07 (0.73, 1.56)	1.07 (0.71, 1.62)	1.24 (0.74, 2.05)	1.02 (0.84, 1.23)	1.07 (0.87, 1.32)	1.12 (0.88, 1.44)
		β-coef. (95% CI)	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**
Child BMI	348	0.08 (-0.12, 0.29)	0.04 (-0.18, 0.26)	0.01 (-0.24, 0.26)	0.02 (-0.08, 0.13)	0.00 (-0.12, 0.13)	-0.01 (-0.16, 0.13)
WC (cm)	348	0.34 (-0.26, 0.95)	0.29 (-0.41, 1.00)	0.40 (-0.25, 1.07)	0.34 (-0.26, 0.95)	0.29 (-0.41, 1.00)	0.40 (-0.25, 1.07)
Sum of 4 Skinfolds (mm)	341	**2.53 (0.92, 4.14)**	**2.76 (1.00, 4.52)**	**3.30 (1.41, 5.20)**	0.77 (-0.08, 1.63)	0.80 (-0.16, 1.77)	**1.11 (0.08, 2.13)**
Non-fasting lipid levels		**β-coef. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**
TC(mg/dl)	294	**4.14 (1.00, 7.28)**	**3.43 (0.04, 6.83)**	3.25 (-0.53, 7.04)	**2.09 (0.56, 3.61)**	**1.93 (0.28, 3.58)**	1.90 (-0.04, 3.85)
HDL-C(mg/dl)	294	-0.18 (-1.47, 1.11)	-0.59 (-2.06, 0.87)	-1.04 (-2.74, 0.65)	-0.35 (-0.95, 0.24)	-0.56 (-1.24, 0.10)	-0.71 (-1.49, 0.06)
Blood pressure levels		**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**	**β-coef. (95% CI)**
SBP percentiles	284	-0.05 (-0.35, 0.24)	-0.07 (-0.40, 0.25)	-0.06 (-0.42, 0.29)	-0.04 (-0.19, 0.09)	-0.05 (-0.22, 0.10)	-0.06 (-0.24, 0.10)
DBP percentiles	284	-0.19 (-0.50, 0.12)	-0.19 (-0.52, 0.12)	-0.15 (-0.51, 0.21)	-0.08 (-0.23, 0.06)	-0.09 (-0.24, 0.06)	-0.09 (-0.27, 0.07)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile

Model 1: adjusted for child sex. (except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$

conditions such as maternal obesity, have been demonstrated in animal studies to affect placental morphology, blood flow, feto-maternal exchanges and endocrine function, which have direct consequences for fetal tissue development, and may lead to a higher offspring susceptibility to develop metabolic disorders [39]. Although adjustment for several sociodemographic and lifestyle related characteristics did not explain our findings, we cannot rule out the possibility of residual confounding mainly related to shared mother-child lifestyle. The observed effects were not mediated by pregnancy complications such as gestational diabetes, birth characteristics, and infant feeding patterns (breastfeeding), which have been associated with both maternal BMI and offspring postnatal growth. Additionally, our results remained

Table 4. Association of maternal blood pressure levels in early pregnancy with offspring cardiometabolic traits at 4 years of age, Rhea pregnancy cohort Crete, Greece.

		SBP in early pregnancy (per increase in 10 mm Hg) (n = 536)			DBP in early pregnancy (per increase in 10 mm Hg) (n = 536)		
Cardiometabolic traits at 4 years of age	**n**	**Model 1**	**Model 2**	**Model 3**	**Model 1**	**Model 2**	**Model 3**
Adiposity outcomes							
		RR (95%CI)	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**
Overweight/obese	156	**1.18 (1.01, 1.37)**	1.11 (0.95, 1.31)	1.15 (0.97, 1.36)	**1.23 (1.05, 1.45)**	**1.19 (1.01, 1.41)**	**1.22 (1.03, 1.45)**
WC (cm) $\geq$ 90th pct	60	1.16 (0.93, 1.44)	1.02 0.81, 1.29)	1.03 (0.82, 1.31)	1.19 (0.94, 1.50)	1.09 (0.84, 1.40)	1.09 (0.84, 1.41)
		β-coeff. (95% CI)	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**
Child BMI	536	0.15 (-0.00, 0.30)	0.05 (-0.10, 0.21)	0.09 (-0.06, 0.26)	0.13 (-0.03, 0.29)	0.06 (-0.09, 0.23)	0.09 (-0.06, 0.25)
WC (cm)	532	**0.38 (0.01, 0.76)**	0.20 (-0.18, 0.58)	0.22 (-0.12, 0.57)	0.36 (-0.05, 0.79)	0.21 (-0.19, 0.62)	0.26 (-0.09, 0.61)
Sum of 4 Skinfolds (mm)	524	1.59 (0.35, 2.83)	0.98 (-0.32, 2.30)	1.11 (-0.19, 2.42)	**1.96 (0.79, 3.13)**	**1.49 (0.32, 2.66)**	**1.71 (0.57, 2.86)**
Non-fasting lipid levels		**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**
TC(mg/dl)	458	-0.33 (-2.50, 1.83)	-0.81 (-3.05, 1.42)	-0.97 (-3.22, 1.27)	-0.85 (-3.50, 1.79)	-1.03 (-3.70, 1.63)	-1.12 (-3.86, 1.60)
HDL-C(mg/dl)	458	0.64 (-0.26, 1.54)	0.80 (-0.16, 1.77)	0.80 (-0.20, 1.80)	-0.21 (-1.23, 0.80)	0.04 (-1.00, 1.10)	-0.02 (-1.11, 1.05)
Blood pressure levels		**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**β-coeff. (95% CI)**
SBP percentiles	422	0.05 (-0.12, 0.22)	0.04 (-0.15, 0.23)	0.08 (-0.10, 0.27)	0.10 (-0.10, 0.31)	0.08 (-0.13, 0.30)	0.07 (-0.14, 0.29)
DBP percentiles	422	0.00 (-0.18, 0.19)	0.04 (-0.14, 0.24)	0.02 (-0.16, 0.22)	0.08 (-0.12, 0.29)	0.06 (-0.14, 0.27)	0.07 (-0.15, 0.29)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile

Model 1: adjusted for child sex. (except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$

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substantially the same after adjusting for paternal BMI, implying potential intrauterine mechanisms in the observed associations.

The greatest risk for overweight/obesity and central adiposity was observed for girls whose mothers were overweight/obese prior to gestation. The long-term effects of the same environmental insult, such as maternal obesity, can have various phenotypic effects on male and female offspring [17]. There are no consistent findings from epidemiological studies on offspring sex-specific responses to maternal weight status, while sex specificity in response to maternal anthropometry has been shown in fetal growth measures [40]. Animal studies have shown that there are sex-specific differences in the regulation and expression of placental genes, proteins,

Table 5. Association of pre-pregnancy BMI with offspring obesity measures at 4 years of age, stratified by child sex, Rhea pregnancy cohort, Crete, Greece.

	Offspring adiposity measures at 4 years of age		
	Overweight/obese (n = 134) RR (95% CI)	WC (cm) ≥ 90th percentile [a] (n = 72) RR (95% CI)	Sum of 4 skinfolds (mm)[a] (n = 601) β-coeff.(95% CI)
Maternal pre-pregnancy BMI (kg/m^2)			
All *(n = 618)*	**1.08 (1.04, 1.13)**	**1.10 (1.04, 1.16)**	**0.51 (0.25, 0.77)**
Boys *(n = 324)*	1.04 (0.98, 1.10)	1.02 (0.93, 1.11)	0.22 (-0.07, 0.52)
Girls *(n = 294)*	**1.13 (1.06, 1.20)**	**1.19 (1.10, 1.29)**	**0.79 (0.34, 1.25)**
P for interaction	**0.032**	**0.004**	**0.030**
Overweight/Obese (>25kg/m^2) prior to gestation			
All *(n = 209)*	**1.83 (1.19, 2.81)**	**1.97 (1.11, 3.49)**	**5.10 (2.49, 7.71)**
Boys *(n = 116)*	1.10 (0.61, 2.01)	0.97 (0.42, 2.21)	**3.03 (0.07, 5.99)**
Girls *(n = 93)*	**3.54 (1.80, 6.98)**	**5.33 (2.17, 13.07)**	**7.59 (3.10, 12.08)**
P for interaction	**0.007**	**0.007**	0.061

BMI, Body Mass Index; WC, Waist Circumference

All models are adjusted for maternal age, education, parity, smoking during pregnancy, gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day).

[a]Also adjusted for child height.

Bold indicated statistically significant differences at p<0.05.

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steroids and structure [41, 42]. Microarray animal experiments also showed that the response to a high-fat diet during gestation triggers sex-specific epigenetic alterations throughout the genome, together with sexually dimorphic deregulation of clusters of imprinted genes: mainly cell signaling involving immune cells, and uptake and metabolism of amino acids for females, and development and function of the vascular system, and uptake and metabolism of glucose and fatty acids for males [43]. Timing of exposure is also a critical issue in sex differences in developmental programming. Female fetuses respond more than males to the mother's preconception nutrition and metabolism, while, in contrast, male fetuses are more vulnerable to metabolic changes during gestation, especially after the first trimester of pregnancy [44, 45]. Further epidemiological studies are needed to explore the sex-specific causal variables and how females versus males respond and adapt to maternal obesity.

To our knowledge, this is the first study to investigate associations of maternal blood pressure levels at the first trimester of pregnancy with offspring cardiometabolic traits. We found that high blood pressure levels in early pregnancy were associated with increased risk of overweight/obesity and increased fat mass at 4 years of age after excluding pre-eclamptic pregnancies. The observed effects were not attenuated by pre-pregnancy BMI or weight gain during pregnancy, implying an independent role of maternal blood pressure to offspring fat distribution. Maternal hypertension in early pregnancy may disrupt the typical physiological changes in the spiral arteries of the decidua and myometrium, resulting in poor placental perfusion, early placental hypoxia and oxidative stress [46]. Therefore, it may be possible that even a small increase in blood pressure levels (10mmHg) in early pregnancy may predispose to adverse metabolic outcomes and increased fat mass later in life.

We also found that abnormal fasting cholesterol levels in early pregnancy were associated with increased risk of offspring overweight/obesity, and greater fat mass at 4 years of age. The observed associations were not attenuated by maternal BMI pre-pregnancy, gestational diabetes status, birth size, or child BMI at age of outcome assessment. Gademan et al reported

recently a positive association between maternal lipid levels and offspring fat percentage and waist-to height ratio values at 5–6 years of age children [22]. There are no other studies so far on the association of maternal lipid levels in pregnancy with offspring cardiometabolic traits other than adiposity measures. Maternal dyslipidemia could increase the oxidative stress in the fetus resulting not only in damage of the vessel wall, but also in the disruption of normal placentation. Hypercholesterolemia in early pregnancy has been associated with increased offspring atherosclerotic lesions both in animal models [47], and in human tissues [48]. One potential explanation for such increased cardiometabolic risk in children of mothers with hypercholesterolemia, is the induction of a constitutional state of overexpression of "atherogenes" in the fetal vascular wall by maternal hypercholesterolemia or the resulting fatty-streak formation [48].

Strengths and limitations

The strengths of the present study include the population-based prospective design and detailed cardiometabolic measurements in early pregnancy and in childhood. Unlike previous epidemiologic studies, blood pressure, lipids, glucose, and insulin concentrations were not collected from medical records but measured during the study follow up according to validated protocols. Moreover, fasting serum samples were available in early pregnancy that is a rather complicated goal for a cohort involving pregnant women. The exclusion of mother-child pairs with multiple pregnancies, pregnancies with preeclampsia, as well as adjustment for several sociodemographic variables, reduced the likelihood of potential confounding. However, because of the observational study design, residual confounding because of other unmeasured confounders may still occur.

The levels of attrition in the Rhea cohort is similar to those found in other birth cohort studies. Offspring of more educated women, and of older women were more likely to attend follow-up clinical assessment. Assuming that mothers and children with a higher BMI are less likely to participate in a detailed obesity follow-up, our estimates may be underestimated. A selection bias could be theoretically generated by the possibility that we included only women receiving an early ultrasound. However, all pregnant women in Greece have to attend several compulsory prenatal visits, one of which take place around 12 weeks of gestation, which is the time of our enrolment phase. Information on maternal pre-pregnancy weight was self-reported, which might have led to misclassification of maternal BMI pre-pregnancy. However, we have performed a validation study comparing self-reported pre-pregnancy weight with clinically measured weight in the first prenatal visit, which was available in our cohort, showing high correlation (r 0.93) and a fairly good agreement between self-reported and objectively measured BMI (Bland-Altman plots, data not shown). We were not able to measure fasting lipid levels to children at 4 years follow up, as expected at this age of follow up. It has been shown that children fasting serum lipids levels have small differences with non-fasting levels[49].

Conclusions

The results of the present study indicate that metabolic dysregulation in early pregnancy may determine offspring obesity at preschool age. The complex underlying mechanisms that explain these findings require additional study. Further follow-up of this cohort will allow to determine whether maternal metabolic profile in early pregnancy is associated with a broader range of offspring cardiometabolic disorders at later ages.

Supporting Information

S1 Table. Maternal and child characteristics of participants and non-participants in the childhood follow up of the Rhea pregnancy cohort Crete, Greece. [a] Statistically significant differences ($p<0.05$), based on Mann-Whitney U test for two independent samples and Pearson's $\chi 2$ test for independence.
(PDF)

S2 Table. Maternal and child characteristics of women who provided fasting blood samples in early pregnancy and those who did not, Rhea pregnancy cohort Crete, Greece. [a] Statistically significant differences ($p<0.05$), based on Mann-Whitney U test for two independent samples and Pearson's $\chi 2$ test for independence.
(PDF)

S3 Table. Association of maternal pre-pregnancy obesity status with offspring cardiometabolic traits at 4 years of age after adjustment for paternal BMI, Rhea pregnancy cohort Crete, Greece. BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure. All models were adjusted for child sex (except models using offspring systolic and diastolic blood pressure percentiles as an outcome) maternal age, education level, parity, smoking during pregnancy, gestational weight gain, birth weight, breastfeeding duration, TV watching at 4 years of age (hours/day) and paternal BMI. Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$.
(PDF)

S4 Table. Association of maternal pre-pregnancy obesity status with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n = 50), Rhea pregnancy cohort Crete, Greece. BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; Model 1: adjusted for child sex. (except models using offspring systolic and diastolic blood pressure percentiles as an outcome). Model 2: model 1 further adjusted for maternal age, education level, parity and smoking during pregnancy Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$.
(PDF)

S5 Table. Association of maternal fasting lipid profile in early pregnancy with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n = 25), Rhea pregnancy cohort Crete, Greece BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; Model 1: adjusted for child sex. (except models using offspring systolic and diastolic blood pressure percentiles as an outcome) Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI. Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an

outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$
(PDF)

S6 Table. Association of maternal blood pressure levels in early pregnancy with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n = 50), Rhea pregnancy cohort Crete, Greece. BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure;. Model 1: adjusted for child sex (except models using offspring systolic and diastolic blood pressure percentiles as an outcome). Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI. Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences $p<0.05$.
(PDF)

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Author Contributions

Conceived and designed the experiments: LC. Performed the experiments: VD M. Karahaliou SK. Analyzed the data: VD VG. Contributed reagents/materials/analysis tools: VG GC. Wrote the paper: VD VG SP LC. Supervised the data collection: MV KS LC. Provided feedback and critical revision of the manuscript: MV KS M. Kogevinas SP.

References

1. Reilly JJ, Kelly J. Long-term impact of overweight and obesity in childhood and adolescence on morbidity and premature mortality in adulthood: systematic review. Int J Obes (Lond). 2011 Jul; 35(7):891–8. doi: 10.1038/ijo.2010.222 PMID: 20975725
2. Cunningham SA, Kramer MR, Narayan KM. Incidence of childhood obesity in the United States. N Engl J Med. 2014 Jan 30; 370(5):403–11. doi: 10.1056/NEJMoa1309753 PMID: 24476431
3. OESD website. Available: http://www.oecd.org/health/obesity-update.htm. Assesed 2015 April 17.
4. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med. 2008 Jul 3; 359(1):61–73. doi: 10.1056/NEJMra0708473 PMID: 18596274
5. Wadhwa PD, Buss C, Entringer S, Swanson JM. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Semin Reprod Med. 2009 Sep; 27 (5):358–68. doi: 10.1055/s-0029-1237424 PMID: 19711246
6. Poston L. Maternal obesity, gestational weight gain and diet as determinants of offspring long term health. Best Pract Res Clin Endocrinol Metab. 2012 Oct; 26(5):627–39. doi: 10.1016/j.beem.2012.03.010 PMID: 22980045
7. Yu Z, Han S, Zhu J, Sun X, Ji C, Guo X. Pre-pregnancy body mass index in relation to infant birth weight and offspring overweight/obesity: a systematic review and meta-analysis. PLoS One. 2013; 8(4): e61627. doi: 10.1371/journal.pone.0061627 PMID: 23613888
8. Patro B, Liber A, Zalewski B, Poston L, Szajewska H, Koletzko B. Maternal and paternal body mass index and offspring obesity: a systematic review. Ann Nutr Metab. 2013; 63(1–2):32–41.

9. Lawlor DA, Relton C, Sattar N, Nelson SM. Maternal adiposity—a determinant of perinatal and offspring outcomes? Nat Rev Endocrinol. 2012 Nov; 8(11):679–88. doi: 10.1038/nrendo.2012.176 PMID: 23007319
10. Kyle UG, Pichard C. The Dutch Famine of 1944–1945: a pathophysiological model of long-term consequences of wasting disease. Curr Opin Clin Nutr Metab Care. 2006 Jul; 9(4):388–94. PMID: 16778567
11. Bekkers MB, Brunekreef B, Smit HA, Kerkhof M, Koppelman GH, Oldenwening M, et al. Early-life determinants of total and HDL cholesterol concentrations in 8-year-old children; the PIAMA birth cohort study. PLoS One. 2011; 6(9):e25533. doi: 10.1371/journal.pone.0025533 PMID: 21980486
12. Gaillard R, Steegers EA, Duijts L, Felix JF, Hofman A, Franco OH, et al. Childhood cardiometabolic outcomes of maternal obesity during pregnancy: the generation R study. Hypertension. 2014 Apr; 63 (4):683–91. doi: 10.1161/HYPERTENSIONAHA.113.02671 PMID: 24379180
13. Oostvogels AJ, Stronks K, Roseboom TJ, van der Post JA, van Eijsden M, Vrijkotte TG. Maternal prepregnancy BMI, offspring's early postnatal growth and metabolic profile at age 5–6 years: the ABCD-study. J Clin Endocrinol Metab. 2014 Jun 23:jc20141561.
14. Perng W, Gillman MW, Mantzoros CS, Oken E. A prospective study of maternal prenatal weight and offspring cardiometabolic health in midchildhood. Ann Epidemiol. 2014 Sep 3.
15. Derraik JG, Ayyavoo A, Hofman PL, Biggs JB, Cutfield WS. Increasing maternal prepregnancy body mass index is associated with reduced insulin sensitivity and increased blood pressure in their children. Clin Endocrinol (Oxf). 2014 Nov 11.
16. Gabory A, Roseboom TJ, Moore T, Moore LG, Junien C. Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. Biol Sex Differ. 2013; 4(1):5. doi: 10.1186/2042-6410-4-5 PMID: 23514128
17. Aiken CE, Ozanne SE. Sex differences in developmental programming models. Reproduction. 2013 Jan; 145(1):R1–13. doi: 10.1530/REP-11-0489 PMID: 23081892
18. Reynolds SA, Roberts JM, Bodnar LM, Haggerty CL, Youk AO, Catov JM. Newborns of preeclamptic women show evidence of sex-specific disparity in fetal growth. Gend Med. 2012 Dec; 9(6):424–35. doi: 10.1016/j.genm.2012.10.013 PMID: 23217566
19. Behl M, Rao D, Aagaard K, Davidson TL, Levin ED, Slotkin TA, et al. Evaluation of the association between maternal smoking, childhood obesity, and metabolic disorders: a national toxicology program workshop review. Environ Health Perspect. 2013 Feb; 121(2):170–80. doi: 10.1289/ehp.1205404 PMID: 23232494
20. Poston L. Gestational weight gain: influences on the long-term health of the child. Curr Opin Clin Nutr Metab Care. 2012 May; 15(3):252–7. doi: 10.1097/MCO.0b013e3283527cf2 PMID: 22406744
21. Davis EF, Lazdam M, Lewandowski AJ, Worton SA, Kelly B, Kenworthy Y, et al. Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. Pediatrics. 2012 Jun; 129(6):e1552–61. doi: 10.1542/peds.2011-3093 PMID: 22614768
22. Gademan MG, Vermeulen M, Oostvogels AJ, Roseboom TJ, Visscher TL, van Eijsden M, et al. Maternal prepregnancy BMI and lipid profile during early pregnancy are independently associated with offspring's body composition at age 5–6 years: the ABCD study. PLoS One. 2014; 9(4):e94594. doi: 10.1371/journal.pone.0094594 PMID: 24740157
23. Seed PT, Chappell LC, Black MA, Poppe KK, Hwang YC, Kasabov N, et al. Prediction of preeclampsia and delivery of small for gestational age babies based on a combination of clinical risk factors in high-risk women. Hypertens Pregnancy. 2011; 30(1):58–73. doi: 10.3109/10641955.2010.486460 PMID: 20795821
24. McCowan L, Horgan RP. Risk factors for small for gestational age infants. Best Pract Res Clin Obstet Gynaecol. 2009 Dec; 23(6):779–93. doi: 10.1016/j.bpobgyn.2009.06.003 PMID: 19604726
25. Chatzi L, Plana E, Daraki V, Karakosta P, Alegkakis D, Tsatsanis C, et al. Metabolic syndrome in early pregnancy and risk of preterm birth. Am J Epidemiol. 2009 Oct 1; 170(7):829–36. doi: 10.1093/aje/kwp211 PMID: 19713286
26. Jellinger PS, Smith DA, Mehta AE, Ganda O, Handelsman Y, Rodbard HW, et al. American Association of Clinical Endocrinologists' Guidelines for Management of Dyslipidemia and Prevention of Atherosclerosis: executive summary. Endocr Pract. 2012 Mar-Apr; 18(2):269–93. PMID: 22507559
27. Metzger BE, Gabbe SG, Persson B, Buchanan TA, Catalano PA, Damm P, et al. International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care. 2010 Mar; 33(3):676–82. doi: 10.2337/dc09-1848 PMID: 20190296
28. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000 May 6; 320(7244):1240–3. PMID: 10797032

29. Linardakis M, Sarri K, Bertsias G, Papadaki A, Kafatos A. Waist circumference and waist-to-height percentiles for the youth of Crete, Greece. International Journal of Child Health and Human Development. 2011; 3:329–42.

30. Steinberger J, Daniels SR, Eckel RH, Hayman L, Lustig RH, McCrindle B, et al. Progress and challenges in metabolic syndrome in children and adolescents: a scientific statement from the American Heart Association Atherosclerosis, Hypertension, and Obesity in the Young Committee of the Council on Cardiovascular Disease in the Young; Council on Cardiovascular Nursing; and Council on Nutrition, Physical Activity, and Metabolism. Circulation. 2009 Feb 3; 119(4):628–47. doi: 10.1161/CIRCULATIONAHA.108.191394 PMID: 19139390

31. Karachaliou M, Georgiou V, Roumeliotaki T, Chalkiadaki G, Daraki V, Koinaki S, et al. Association of trimester-specific gestational weight gain with fetal growth, offspring obesity, and cardiometabolic traits in early childhood. Am J Obstet Gynecol. 2014 Dec 31.

32. Rasmussen KM, & Yaktine, A. L.,. Institute of medicine (committee to reexamine iom pregnancy weight guidelines, food and nutritions board and board on children, youth, and families) weight gain during pregnancy: Reexamining the guidelines. Washington D.C.2009.

33. Leventakou V, Georgiou V, Chatzi L, Sarri K. Relative validity of an FFQ for pre-school children in the mother-child 'Rhea' birth cohort in Crete, Greece. Public Health Nutr. 2015 Feb; 18(3):421–7. doi: 10.1017/S1368980014000445 PMID: 24679849

34. McNutt LA, Wu C, Xue X, Hafner JP. Estimating the relative risk in cohort studies and clinical trials of common outcomes. Am J Epidemiol. 2003 May 15; 157(10):940–3. PMID: 12746247

35. Drake AJ, Reynolds RM. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction. 2010 Sep; 140(3):387–98. doi: 10.1530/REP-10-0077 PMID: 20562299

36. Gale CR, Javaid MK, Robinson SM, Law CM, Godfrey KM, Cooper C. Maternal size in pregnancy and body composition in children. J Clin Endocrinol Metab. 2007 Oct; 92(10):3904–11. PMID: 17684051

37. Blair NJ, Thompson JM, Black PN, Becroft DM, Clark PM, Han DY, et al. Risk factors for obesity in 7-year-old European children: the Auckland Birthweight Collaborative Study. Arch Dis Child. 2007 Oct; 92(10):866–71. PMID: 17855436

38. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. Int J Obes (Lond). 2015 Feb 2.

39. Tarrade A, Panchenko P, Junien C, Gabory A. Placental contribution to nutritional programming of health and diseases: epigenetics and sexual dimorphism. J Exp Biol. 2015 Jan 1; 218(Pt 1):50–8.

40. Lampl M, Gotsch F, Kusanovic JP, Gomez R, Nien JK, Frongillo EA, et al. Sex differences in fetal growth responses to maternal height and weight. Am J Hum Biol. 2010 Jul-Aug; 22(4):431–43. doi: 10.1002/ajhb.21014 PMID: 19950190

41. Clifton VL. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta. 2010 Mar; 31 Suppl:S33–9. doi: 10.1016/j.placenta.2009.11.010 PMID: 20004469

42. Mao J, Zhang X, Sieli PT, Falduto MT, Torres KE, Rosenfeld CS. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. Proc Natl Acad Sci U S A. 2010 Mar 23; 107(12):5557–62. doi: 10.1073/pnas.1000440107 PMID: 20212133

43. Gabory A, Ferry L, Fajardy I, Jouneau L, Gothie JD, Vige A, et al. Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta. PLoS One. 2012; 7(11):e47986. doi: 10.1371/journal.pone.0047986 PMID: 23144842

44. Eriksson JG, Kajantie E, Osmond C, Thornburg K, Barker DJ. Boys live dangerously in the womb. Am J Hum Biol. 2010 May-Jun; 22(3):330–5. doi: 10.1002/ajhb.20995 PMID: 19844898

45. Regnault N, Gillman MW, Rifas-Shiman SL, Eggleston E, Oken E. Sex-specific associations of gestational glucose tolerance with childhood body composition. Diabetes Care. 2013 Oct; 36(10):3045–53. doi: 10.2337/dc13-0333 PMID: 23877978

46. Bennett S, Bourgeois C, Cassidy A, Cloutier-Holtz K, Corbin A, Darling E, et al. Clinical Practice Guideline No.15 Hypertensive Disorders of Pregnancy. Association of Ontario Midwives. 2012.

47. Napoli C, de Nigris F, Welch JS, Calara FB, Stuart RO, Glass CK, et al. Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray. Circulation. 2002 Mar 19; 105(11):1360–7. PMID: 11901049

48. Napoli C, Glass CK, Witztum JL, Deutsch R, D'Armiento FP, Palinski W. Influence of maternal hypercholesterolaemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of Early Lesions in Children (FELIC) study. Lancet. 1999 Oct 9; 354(9186):1234–41. PMID: 10520631

49. Steiner MJ, Skinner AC, Perrin EM. Fasting might not be necessary before lipid screening: a nationally representative cross-sectional study. Pediatrics. 2011 Sep; 128(3):463–70. doi: 10.1542/peds.2011-0844 PMID: 21807697

S1 Table. Maternal and child characteristics of participants and non-participants in the childhood follow up of the Rhea pregnancy cohort Crete, Greece

	Participants (n=698)	**Non participants (n=610)**	
Maternal characteristics			***P*- value[a]**
Maternal age (years), *mean(SD)*	29.83±4.8	28.88±5.2	<0.001
Maternal education, *n (%)*			<0.001
Low	117 (16.8)	153 (26.5)	
Medium	351 (50.3)	294 (51.0)	
High	230 (33.0)	130 (22.5)	
Mother's origin, *n (%)*			<0.001
Greek	660 (94.7)	516 (86.1)	
Non greek	37 (5.3)	83 (13.9)	
Smoking status, *n (%)*			<0.001
Smoker	218 (31.2)	226 (41.0)	
Non-smoker	480 (68.8)	325 (59.0)	
Parity, *n (%)*			0.065
Primiparous	304 (43.6)	217 (38.4)	
Multiparous	394 (56.4)	348 (61.6)	
Pre-pregnancy BMI (kg/m^2), *mean(SD)*	24.51±4.8	23.69±4.6	<0.001
Pre-pregnancy BMI categories			0.284
< 25 kg/m^2	471 (67.5)	402 (70.3)	
≥ 25 kg/m^2	227 (32.5)	170 (29.7)	
Gestational weight gain (kg), *n (%)*			0.210
Inadequate	130 (22.1)	106 (25.2)	
Adequate	203 (34.5)	124 (29.5)	
Excessive	256 (43.50	191 (45.4)	
Delivery type, *n (%)*			0.888
Vaginal	350 (50.4)	303 (50.8)	
Caesarean	345 (49.6)	294 (49.2)	
Child characteristics			
Child gender, *n (%)*			0.239
Male	362 (51.9)	292 (48.6)	
Female	336 (48.1)	309 (51.4)	
Breastfeeding duration (months), *mean(SD)*	4.19±4.4	3.08±3.6	<0.001
Birth weight (kg), *mean(SD)*	3.21±0.4	3.14±0.5	0.016
Gestational age (weeks), *mean(SD)*	38.24±1.5	38.21±1.6	0.971

[a] Statistically significant differences ($p<0.05$), based on Mann-Whitney U test for two independent samples and Pearson's $\chi 2$ test for independence.

S2 Table. Maternal and child characteristics of women who provided fasting blood samples in early pregnancy and those who did not in the Rhea pregnancy cohort Crete, Greece.

	Fasting samples (n=348)	**Non fasting samples (n=145)**	
Maternal characteristics			***P*- value[a]**
Maternal age (years), mean (SD)	29.90 (4.7)	30.50 (4.7)	0.160
Maternal education, *n(%)*			0.552
Low	58 (16.7)	23 (15.9)	
Medium	178 (51.1)	68 (46.9)	
High	112 (32.2)	54 (37.2)	
Mother's origin, *n (%)*			0.193
Greek	328 (94.2)	132 (91.0)	
Non greek	20 (5.8)	13 (9.0)	
Smoking status, *n (%)*			0.178
Smoker	101 (29.0)	51 (35.2)	
Non-smoker	24 7(71.0)	94 (64.8)	
Parity, *n (%)*			0.619
Primiparous	138 (39.7)	61 (42.1)	
Multiparous	210 (60.3)	84 (57.9)	
Pre-pregnancy BMI (kg/m^2), mean (SD)	24.62 (4.8)	24.67 (4.8)	0.957
Pre-pregnancy BMI categories			0.768
< 25 kg/m^2	228 (65.5)	97 (66.9)	
≥ 25 kg/m^2	120 (34.5)	48 (33.1)	
Gestational weight gain (kg), *n (%)*			0.204
Inadequate	67 (19.3)	34 (23.4)	
Adequete	134 (38.5)	62 (42.8)	
Excessive	147 (42.2)	49 (33.8)	
Delivery type, *n (%)*			0.564
Vaginal	166 (47.8)	73 (50.7)	
Caesarean	181 (52.2)	71 (49.3)	
Child characteristics			
Child gender, *n (%)*			0.087
Male	195 (56.0)	69 (47.6)	
Female	153 (44.0)	76 (52.4)	
Breastfeeding duration (months), mean(SD)	4.26 (4.3)	4.67 (4.6)	0.586
Birth weight (kg), mean (SD)	3.20 (0.4)	3.24 (0.4)	0.406
Gestational age (weeks), mean (SD)	38.25 (1.5)	38.39 (1.5)	0437

[a] Statistically significant differences ($p<0.05$), based on Mann-Whitney U test for two independent samples and Pearson's $\chi 2$ test for independence.

S3 Table. Association of maternal pre-pregnancy obesity status with offspring cardiometabolic traits at 4 years of age after adjustment for paternal BMI in the Rhea pregnancy cohort Crete, Greece.

Offspring cardiometabolic traits at 4 years of age	n	**Pre-pregnancy overweight/obese (≥25 kg/m^2) (n=206)**
Adiposity outcomes		
		RR (95%CI)
Overweight/obese	133	**1.31 (0.94, 1.81)**
WC (cm) ≥ 90th pct	71	1.40 (0.90, 2.18)
		β-coeff. (95%CI)
Child BMI	609	**0.58 (0.24, 0.93)**
WC (cm)	601	**1.14 (0.34, 1.93)**
Sum of 4 Skinfolds (mm)	592	**4.34 (1.71, 6.97)**
Non-fasting lipid levels		***β-coeff. (95%CI)***
TC (mg/dl)	518	2.37 (-2.87, 7.62)
HDL-C (mg/dl)	518	0.82 (-1.31, 2.96)
Blood pressure levels		***β-coeff. (95%CI)***
SBP percentiles	482	0.12 (-0.33, 0.58)
DBP percentiles	482	-0.12 (-0.56, 0.31)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressucte; pct, percentile;

All models were adjusted for child sex (except models using offspring systolic and diastolic blood pressure percentiles as an outcome) maternal age, education level, parity, smoking during pregnancy, gestational weight gain, birth weight, breastfeeding duration TV watching at 4 years of age (hours/day) and paternal BMI. Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI.

Bold indicated statistically significant differences at $p<0.05$.

S4 Table. Association of maternal pre-pregnancy obesity status with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n=50), Rhea pregnancy cohort Crete, Greece.

		Pre-pregnancy overweight/obese (≥25 kg/m^2) (n=209)		
Offspring cardiometabolic traits at 4 years of age	n	Model 1	Model 2	Model 3
Adiposity outcomes				
		RR (95%CI)	***RR (95%CI)***	***RR (95%CI)***
Overweight/obese	122	**1..65 (1.21, 2.26)**	**1.57 (1.14, 2.18)**	1.39 (0.98, 1.97)
WC (cm) ≥ 90th pct	63	**2.07 (1.32, 3.25)**	**1.90 (1.19, 3.04)**	1.51 (0.92, 2.46)
		β-coeff. (95%CI)	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
Child BMI	568	**0.81 (0.44, 1.17)**	**0.78 (0.42, 1.14)**	**0.71 (0.32, 1.10)**
WC (cm)	556	**1.77 (0.84, 2.70)**	**1.76 (0.84, 2.68)**	**1.35 (0.45, 2.25)**
Sum of 4 Skinfolds (mm)	551	**5.52 (2.85, 8.19)**	**5.05 (2.34, 7.78)**	**4.38 (1.42, 7.35)**
Non-fasting lipid levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
TC(mg/dl)	475	2.73 (-2.28, 7.74)	2.74 (-2.47, 7.96)	1.84 (-3.87, 7.55)
HDL-C(mg/dl)	475	0.43 (-1.64, 2.50)	0.39 (-1.78, 2.55)	0.22 (-2.13, 2.57)
Blood pressure levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
SBP percentiles	438	0.35 (-0.11, 0.81)	0.40 (-0.07, 0.88)	0.32 (-0.16, 0.81)
DBP percentiles	438	-0.02 (-0.46, 0.42)	-0.07 (-0.51, 0.40)	-0.05 (-0.50, 0.40)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile;

Model 1: adjusted for child sex.(except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity and smoking during pregnancy

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$.

S5 Table. Association of maternal fasting lipid profile in early pregnancy with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n=25), Rhea pregnancy cohort Crete, Greece.

		Fasting TC levels in early pregnancy (per increase in 40 mg/dL) (n=323)			Fasting LDL-C levels in early pregnancy (per increase in 15 mg/dL) (n=323)		
Offspring cardiometabolic traits at 4 years of age	n	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Adiposity outcomes							
		RR (95%CI)	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***
Overweight/obese	59	1.18 (0.92, 1.53)	1.23 (0.95, 1.61)	1.37 (0.99, 1.89)	1.05 (0.93, 1.19)	1.06 (0.93, 1.21)	1.08 (0.92, 1.27)
WC (cm) ≥ 90th pct	27	1.05 (0.71, 1.56)	1.04 (0.70, 1.57)	1.24 (0.72, 2.11)	1.04 (0.86, 1.27)	1.06 (0.86, 1.30)	1.11 (0.87, 1.41)
		β-coeff. (95%CI)	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
Child BMI	323	0.06 (-0.16, 0.29)	0.08 (-0.16, 0.32)	0.03 (-0.22, 0.28)	0.01 (-0.10, 0.13)	0.02 (-0.11, 0.16)	-0.02 (-0.17, 0.12)
WC (cm)	323	0.29 (-0.37, 0.95)	0.30 (-0.42, 1.01)	0.42 (-0.26, 1.11)	0.10 (-0.25, 0.44)	0.11 (-0.27, 0.50)	0.14 (-0.25, 0.53)
Sum of 4 Skinfolds (mm)	316	2.52 (0.78, 4.26)	2.82 (0.98, 4.65)	**3.19 (1.22, 5.15)**	0.78 (-0.15, 1.71)	0.85 (-0.15, 1.86)	1.05 (-0.02, 2.13)
Non-fasting lipid levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
TC(mg/dl)	269	**3.45 (0.10, 6.81)**	3.44 (-0.12, 7.00)	3.68 (-0.38, 7.75)	2.28 (0.60, 3.96)	2.20 (0.44, 3.95)	**2.34 (0.22, 4.47)**
HDL-C(mg/dl)	269	-0.47 (-1.89, 0.94)	-0.83 (-2.32, 0.67)	-1.15 (-2.93, 0.62)	-0.43 (-1.10, 0.24)	-0.68 (-1.28, 0.12)	-0.70 (-1.56, 0.14)
Blood pressure levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
SBP percentiles	259	-0.08 (-0.42, 0.25)	-0.12 (-0.47, 0.23)	-0.14 (-0.52, 0.24)	-0.06 (-0.22, 0.10)	-0.08 (-0.25, 0.09)	-0.09 (-0.27, 0.09)
DBP percentiles	259	-0.12 (-0.32, 0.08)	-0.16 (-0.36, 0.05)	-0.16 (-0.39, 0.05)	-0.04 (-0.14, 0.05)	-0.07 (-0.17, 0.03)	-0.07 (-0.18, 0.03)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile;

Model 1: adjusted for child sex.(except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$

S6 Table. Association of maternal blood pressure levels in early pregnancy with offspring cardiometabolic traits at 4 years of age, after excluding women with gestational diabetes (n=48), Rhea pregnancy cohort Crete, Greece

		SBP in early pregnancy (per increase in 10 mm Hg) (n=488)			**DBP in early pregnancy (per increase in 10 mm Hg) (n=488)**		
Offspring cardiometabolic traits at 4 years of age	**n**	**Model 1**	**Model 2**	**Model 3**	**Model 1**	**Model 2**	**Model 3**
Adiposity outcomes							
		RR (95%CI)	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***	***RR (95%CI)***
Overweight/obese	103	**1.23 (1.04, 1.44)**	**1.22 (1.04, 1.44)**	**1.22 (1.02, 1.45)**	**1.25 (1.06, 1.47)**	**1.23 (1.04, 1.46)**	**1.23 (1.03, 1.47)**
WC (cm) ≥ 90th pct	54	1.22 (0.97, 1.54)	1.18 (0.93, 1.51)	1.07 (0.84, 1.36)	1.22 (0.96, 1.56)	1.18 (0.92, 1.53)	1.14 (0.88, 1.48)
		β-coeff. (95%CI)	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
Child BMI	462	**0.18 (0.00, 0.36)**	**0.18 (0.00, 0.35)**	0.13 (-0.05, 0.32)	0.15 (-0.03, 0.33)	0.15 (-0.02, 0.32)	0.12 (-0.04, 0.30)
WC (cm)	458	**0.48 (0.05, 0.92)**	**0.48 (0.05, 0.92)**	0.27 (-0.13, 0.68)	0.44 (-0.02, 0.91)	0.40 (-0.05, 0.86)	0.32 (-0.05, 0.71)
Sum of 4 Skinfolds (mm)	451	**1.85 (0.40, 3.30)**	**1.74 (0.31, 3.18)**	1.43 (-0.09, 2.95)	**2.30 (1.02, 3.57)**	**2.16 (0.93, 3.39)**	**1.93 (0.71, 3.16)**
Non-fasting lipid levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
TC(mg/dl)	395	-0.70 (-3.07, 1.67)	-0.67 (-3.07, 1.72)	-1.29 (-3.76, 1.17)	-1.62 (4.50, 1.25)	-1.36 (-4.20, 1.47)	-1.79 (-4.77, 1.19)
HDL-C(mg/dl)	395	0.51 (-0.46, 1.48)	0.54 (-0.43, 1.51)	0.72 (-0.34, 1.79)	-0.49 (-1.60, 0.62)	-0.24 (-1.37, 0.88)	-0.22 (-1.40, 0.95)
Blood pressure levels		***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***	***β-coeff. (95%CI)***
SBP percentiles	360	**0.18 (0.00, 0.37)**	**0.19 (0.00, 0.38)**	**0.23 (0.02, 0.43)**	0.20 (-0.01, 0.41)	0.17 (-0.04, 0.39)	0.17 (-0.04, 0.39)
DBP percentiles	360	0.07 (-0.04, 0.19)	0.07 (-0.04, 0.19)	0.09 (-0.02, 0.21)	0.10 (-0.03, 0.23)	0.08 (-0.05, 0.21)	0.08 (-0.05, 0.21)

BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol;; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; pct, percentile;

Model 1: adjusted for child sex.(except models using offspring systolic and diastolic blood pressure percentiles as an outcome)

Model 2: model 1 further adjusted for maternal age, education level, parity, smoking during pregnancy and pre-pregnancy BMI

Model 3: model 2 additionally adjusted for gestational weight gain, birth weight, breastfeeding duration, and TV watching at 4 years of age (hours/day). Models using offspring WC and sum of skinfolds as an outcome variable were also adjusted for child height, while those using offspring non-fasting lipid levels as an outcome were also adjusted for child BMI. Bold indicated statistically significant differences at $p<0.05$

4.2 Paper 2. Effect of parental obesity and gestational diabetes on child neuropsychological and behavioral development at 4 years of age: the Rhea mother–child cohort, Crete, Greece

Main Findings:

1) Maternal obesity pre-pregnancy was associated with reduced child cognitive development at preschool age, independently of paternal obesity status and shared family characteristics.

2) Born large for gestational age may modify the association between maternal overweight/obesity and child cognitive function at preschool age.

3) Glucose metabolism in early pregnancy was not associated with child neurodevelopment.

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ORIGINAL CONTRIBUTION

Effect of parental obesity and gestational diabetes on child neuropsychological and behavioral development at 4 years of age: the Rhea mother–child cohort, Crete, Greece

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Abstract Studies have suggested an association between maternal obesity pre-pregnancy and gestational diabetes (GDM) with impaired offspring neurodevelopment, but it is not clear if these associations are explained by shared familiar characteristics. We aimed to assess the associations of maternal and paternal obesity, maternal glucose intolerance in early pregnancy and GDM, with offspring neurodevelopment at 4 years of age. We included 772 mother–child pairs from the "Rhea" Mother–Child cohort in Crete, Greece. Data on maternal/paternal body mass index (BMI) and maternal fasting serum samples for glucose and insulin measurements were collected at 12 weeks of gestation. GDM screening was performed at 24–28 weeks. Neurodevelopment at 4 years was assessed using the McCarthy Scales of Children's Abilities. Behavioral difficulties were assessed by Strengths and Difficulties Questionnaire and Attention Deficit Hyperactivity Disorder Test. Multivariate linear regression analyses showed that maternal obesity was associated with a significant score reduction in general cognitive ability (β-coeff −4.03, 95% CI: −7.08, −0.97), perceptual performance (β-coeff −4.60, 95% CI: −7.74, −1.47), quantitative ability (β-coeff −4.43, 95% CI: −7.68, −1.18), and executive functions (β-coeff −4.92, 95% CI: −8.06, −1.78) at 4 years of age, after adjustment for several confounders and paternal BMI. Maternal obesity was also associated with increased behavioral difficulties (β-coeff 1.22, 95% CI: 0.09, 2.34) and ADHD symptoms (β-coeff 4.28, 95% CI: 1.20, 7.36) at preschool age. Paternal obesity maternal glucose intolerance in early pregnancy and GDM was not associated with child neurodevelopment. These findings suggest that maternal obesity may impair optimal child neurodevelopment at preschool age independently of family shared characteristics.

Keywords Obesity · Gestational diabetes · Neuropsychological development · Behavior · Preschool age · Longitudinal study

Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
AGA	Appropriate for gestational age
BMI	Body mass index
CI	Confidence interval
GAMs	Generalized additive models
GDM	Gestational diabetes mellitus
IOM	Institute of Medicine
IQ	Intelligence quotient
LGA	Large for gestational age
MSCA	McCarthy Scales of Children's Abilities
SDQ	Strengths and Difficulties Questionnaire
SD	Standard deviation
SGA	Small for gestational age
TSH	Thyroid stimulating hormone

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Introduction

The prevalence of obesity in pregnant women is increasing worldwide at an alarming rate [1]. In a recent study for the prevalence of maternal overweight and obesity in Greece 16.6% of participating pregnant women in 6 Greek counties were classified as overweight and 25.6% as obese, according to the WHO cut-offs [2]. Maternal obesity is a critical public health problem because of its association with multiple adverse health outcomes for the child including neurodevelopmental disorders [3].

Recent data suggest that offspring of overweight/obese women are at increased risk for cognitive deficits, externalizing problems, and internalizing psychopathology in childhood and adolescence [3, 4]; however, it is not clear if the effect of maternal obesity on offspring neurodevelopment is mainly due to an adverse intrauterine environment or if it is confounded by socioeconomic and family risk factors. A way to control for family background is to compare the associations of maternal and paternal BMI on child neurodevelopment, since a stronger maternal association would reflect direct intrauterine mechanisms [5]. However, only four studies have assessed so far the influence of maternal versus paternal obesity on child neurodevelopment with conflicting results [6–9]. In addition, most of these studies have examined only one neurodevelopmental outcome, making it difficult to determine whether offspring are at risk for cognitive dysfunction or abnormal behavior. With the obesity epidemic in reproductive-age women, an ever-increasing number of fetuses will be at risk for large for gestational age neonates and metabolic derangements [10]; however, no data exist on the potential mediating role of macrosomia on the association of maternal obesity and child neurodevelopment.

A common characteristic of overweight/obese women is the development of gestational diabetes (GDM) which is commonly diagnosed in mid-gestation [11]. Several epidemiological studies suggest a possible association between GDM and neurobehavioral abnormalities including cognitive deficits, behavioral problems (particularly ADHD), or internalizing psychopathology [12–14]. However, results are inconclusive suggesting negative [14–18], null [19, 20] or positive associations [21]. A recent systematic review and meta-analysis found that infants of women with GDM had a lower mental and psychomotor development; however, evidence is scarce for older children [22]. There are no studies so far evaluating the effect of maternal glucose intolerance in early pregnancy on offspring neurodevelopment of non-diabetic women.

We aimed to add to this research more detailed data by investigating the associations of maternal and paternal obesity status, as well as maternal glucose intolerance in early pregnancy and GDM, with multiple offspring neurodevelopmental outcomes including cognitive and behavioral development, at 4 years of age, in a prospective pregnancy cohort in Crete, Greece.

Methods

Study design and participants

The present study is part of the "Rhea" study, a prospective pregnancy cohort, at the prefecture of Heraklion, Crete, Greece [23]. A detailed description is provided elsewhere [23]. In brief female residents (Greek and immigrants) were enrolled at the time of the first comprehensive ultrasound examination [mean (SD): 12.4 (1.6) weeks] from February 2007 to February 2008 [23]. Women were contacted again at various times during pregnancy, at birth, at 8–10 weeks after delivery, and for child's follow-up at 9th, 18th months, and at 4 years of age. Face-to face completed questionnaires together with self-administered questionnaires and medical records were used to obtain information on dietary, environmental, and psychosocial exposures during pregnancy and early childhood. The inclusion criteria were the following: residents in the study area; pregnant women aged >16 years; no communication handicap. The study has followed the guidelines of the Declaration of Helsinki. In addition it was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece), and all participants provided written informed consent after complete description of the study.

Of 1363 singleton live births in the Rhea study, neurodevelopmental assessment at 4 years of age was performed in 875 children from October 2011 to January 2013. We excluded 26 children with diagnosed neurodevelopmental disorders (i.e. Pervasive Developmental Disorder), other severe medical disorders (i.e. plagiocephalus, microcephalus, hydrocephalus, brain tumor) and/or incomplete examination, as well as pregnant women with pre-gestational diabetes ($n = 6$). Thus 843 mother–child pairs were available for our analysis. From those, complete data for maternal/paternal obesity was available for 783 mother–child pairs. We further excluded 11 mothers with missing data for possible confounders. Thus, a cohort of 772 mother–child pairs (98% of the children with maternal/paternal pre-pregnancy BMI data and neurodevelopmental assessment at 4 years of age) participated in the present analysis. Information on behavioral difficulties and ADHD symptoms was available for 633 and 581 children, respectively, due to incomplete maternal report. Data for GDM was available for 691 pregnant women, while 452 mothers provided fasting glucose and insulin serum measurements in early pregnancy due to the time of their

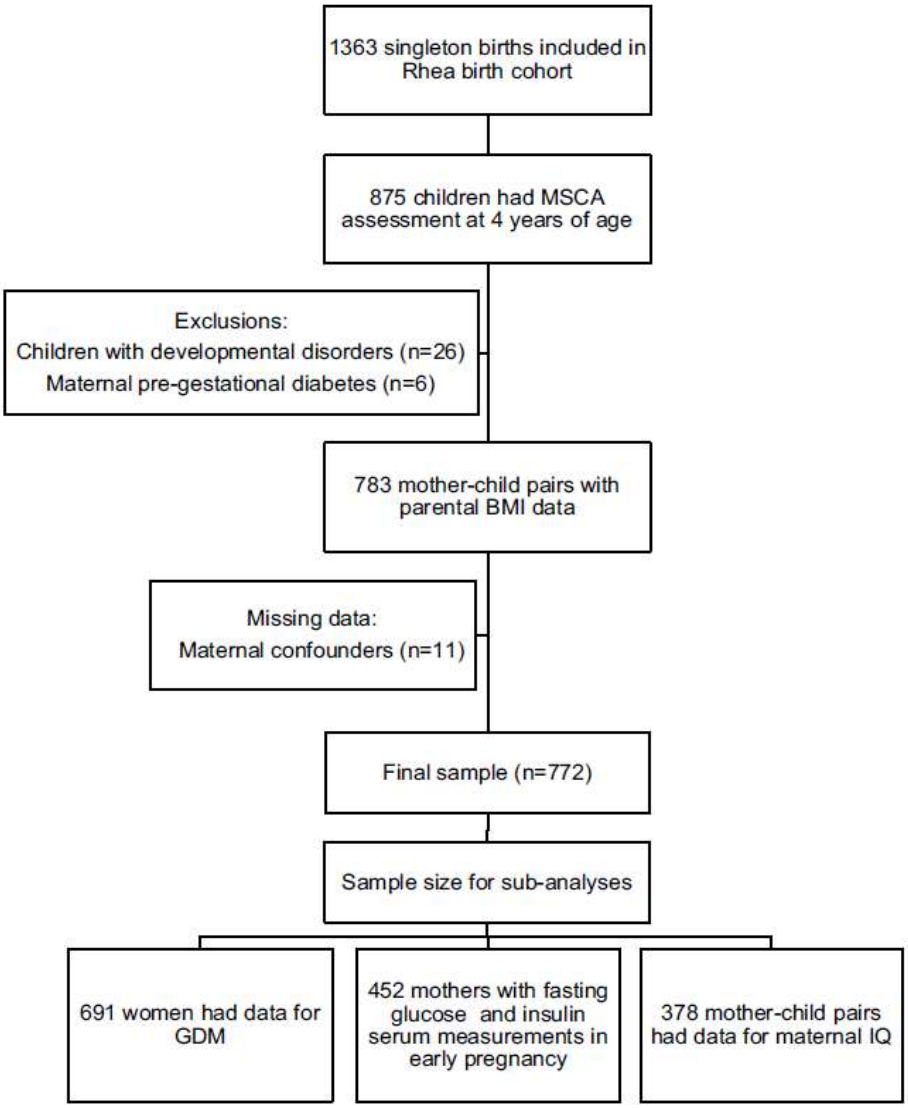

Fig. 1 Flow chart of the study population

ultrasound appointment, where enrolment to the study took place. We present this information in a flow diagram (Fig. 1).

Measures

Parental overweight/obesity

We calculated maternal pre-pregnancy BMI (kg/m^2) based on maternal height measured at the first prenatal visit [mean (SD): 12.4 (1.6) weeks] and pre-pregnancy weight reported by the mother. Paternal BMI was based on paternal weight and height reported by the mother, since the study protocol did not include contact with the father of the child. BMI was categorized according to the WHO classification: underweight (BMI < 18.5), normal weight (18.5 ≤ BMI < 25), overweight (25 ≤ BMI < 30), and obese (BMI ≥ 30). For our analysis, we have grouped together maternal and paternal underweight and normal weight due to the limited number of underweight participants ($n = 28$ mothers and $n = 1$ father, respectively).

Maternal glucose intolerance in early pregnancy and GDM

Maternal fasting serum samples were collected at the first prenatal visit [mean (SD): 12.4 (1.6) weeks]. Maternal glucose concentration was measured by standard enzymatic methods (Medicon, Greece) on an automatic analyser (AU5400 high-volume chemistry analyser; Olympus America, Inc., Melville, New York, USA). Maternal insulin concentration was measured by IMMULITE 2000

immunoassay system (Siemens Healthcare Diagnostics, Inc., Deerfield, Illinois, USA). The inter- and intra-assay coefficients of variation were less than 5%.

Pregnant women were screened for GDM between 24 and 28 weeks of gestation and GDM was defined by the two-step approach according to the criteria proposed by Carpenter and Coustan [24].

Neuropsychological assessment at 4 years of age

Children's cognitive and motor development at 4 years of age [mean (SD): 4.3 (0.2) years] was assessed using the McCarthy Scales of Children's Abilities (MSCA) [25]. The MSCA represents an age-appropriate instrument, developed for children of ages 2½–8½ years, which gives standardized test scores for five domains: (1) the Verbal Scale (verbal expression and verbal comprehension ability); (2) the Perceptual-Performance Scale (reasoning ability through materials manipulation); (3) the Quantitative Scale (number aptitude and numbers interest); (4) the Memory Scale (short-term memory through verbal and non-verbal stimuli); (5) the Motor Scale (gross and fine motor ability) [25]. A general cognitive score, which estimates global intellectual function, was calculated by combining the verbal, perceptual performance and quantitative scores. Raw scores of MSCA scales were standardized for child's age at test administration. Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales. Standardized scores were treated as continuous variables with higher scores representing better general cognition, language, or psychomotor development. MSCA translation and cross-cultural adaptation were conducted according to the internationally recommended methodology. Internal consistency (Cronbach's alpha) varied between 0.76 and 0.89, showing adequate reliability for all the scales. Confirmatory Factor Analysis supported good fit of the model ($\chi^2/df = 2$, CFI = 0.83, GFI = 0.97, RMSEA = 0.034) [26].

To further improve our understanding of the specific functions associated with the exposures of interest, the MSCA items were reorganized, for tasks highly associated with specific neurocognitive functions, into the following new outcomes: executive functions of frontal cortex and functions of posterior cortex.

Behavioral difficulties at 4 years of age

Information on children's behaviour at 4 years of age was obtained via two standardized child behavior scales, which delineate symptoms and may detect children at high risk of mental health problems. Strengths and Difficulties Questionnaire (SDQ) [27] is a behavioral screening instrument designed for children aged 3–16 years. It consists of five subscales generating scores for emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems, and prosocial behavior. One-point increase in the above scores corresponds to an increased rate of disorder. A total SDQ score can be calculated by aggregating the scores for the above subscales except prosocial behavior (range 0–40). The SDQ has been translated and adapted for the Greek population [28]. The Cronbach's alpha measuring internal consistency of the total SDQ score was $\alpha = 0.667$.

The Attention Deficit Hyperactivity Disorder Test (ADHDT) [29] is designed to identify and evaluate ADHD symptoms in ages 3–23 years. It is composed of 36 items in three subscales; (1) Hyperactivity, (2) Inattention, and (3) Impulsivity. All 36 items are summed to generate an index for total ADHD difficulties (range 0–72). Higher scores indicate more intensive ADHD symptoms. The ADHDT has been translated and adapted for the Greek population [30]. The Cronbach's alpha measuring internal consistency of the total ADHD index was $\alpha = 0.951$.

Procedure

Women were invited to provide blood and urine samples and to participate in a face-to-face interview at the first prenatal visit [mean (SD): 12.4 (1.6) weeks]. At the same time maternal height and weight were measured by trained nurses. Maternal intelligence quotient (IQ) was measured using the Raven's Standard Progressive Matrices test at 4-year follow-up [31]. Offspring neurodevelopmental assessments at 4 years of age were conducted by two trained psychologists via a strict protocol to avoid inter-observer variability, which was <1%. The MSCA scales were administered individually and the administration time ranged from 40 to 60 min. The examiners, also, noted critical comments about the difficulties or special conditions of the neurodevelopmental assessment so as to evaluate the "quality of assessment" such as no difficulties, difficulties due to physical problems (e.g. physical illness, tiredness, asleep, etc.), and difficulties due to behavior problems (e.g. nervousness, shyness, etc.). Information on children's behavior was obtained via maternal report on the SDQ and ADHDT questionnaires. All testing was done at the University Hospital of Heraklion, and Medical Health Centers in the prefecture of Heraklion, Crete, Greece.

Potential covariates

As potential covariates were considered characteristics that have an established or potential association with exposures or outcomes of interest, including (A) Parental characteristics: maternal and paternal age (years); education at recruitment (low level: ≤6 years of school; medium level: 7–12 years of school; high level: university or technical

college degree) and origin (Greek/non-Greek); maternal smoking during pregnancy (yes/no); parity (primiparous/multiparous); gestational weight gain based on US IOM guidelines; maternal first-trimester serum TSH levels (measured by IMMULITE 2000 immunoassay system (Siemens Healthcare Diagnostics, IL60015-0778, USA); maternal IQ (B) Child characteristics: sex (male/female); birthweight (g); gestational age, preterm birth (<37 weeks of gestation; yes/no); growth pattern at birth [Small for Gestational Age (SGA); appropriate for gestational Age (AGA); large for gestational age (LGA)] [23]; breastfeeding duration (months); preschool attendance (yes/no); child BMI (kg/m^2) calculated using measured child weight and height at 4-year follow-up.

Statistical analysis

The baseline characteristics of our study population were examined with descriptive statistics. Differences in distributions of normally distributed variables were tested with *t* test; non-normally distributed continuous variables were tested with non-parametric Mann–Whitney U test for two independent samples and Kruskal–Wallis test for more than two independent samples, whereas categorical variables were tested with Pearson's Chi square test. The main outcome variables were neurodevelopmental scores in MSCA scales including executive functions of frontal cortex and functions of posterior cortex, as well as total scores in SDQ and ADHDT at 4 years of age. The primary exposures of interest were maternal/paternal pre-pregnancy BMI, maternal fasting glucose and insulin levels in early pregnancy and GDM. Maternal and paternal pre-pregnancy BMI and GDM were used as categorical variables. All other measures were used as continuous variables. The possibility of nonlinear associations was tested by generalized additive models (GAMs) indicating linear relationships for all exposure variables ($p > 0.10$).

Linear regression was used to assess the association between exposures and outcomes of interest. We created three different multivariable-adjusted models, in which we included covariates associated with the exposures and the outcomes of interest with a $p < 0.05$, as well as a priori selected potential confounders such as child sex and age, examiner, and quality of assessment. The first minimally adjusted model included the set of a priori confounders. The second model was additionally adjusted for maternal characteristics: age at enrolment, education, origin, parity, and smoking during pregnancy. Models using paternal BMI as an exposure variable were adjusted for paternal characteristics: age at enrolment, education, and origin. The third model included the second model additionally adjusted for paternal BMI. Models using paternal BMI, glucose/insulin levels and GDM as an exposure variable were adjusted for maternal BMI.

To examine the potential confounding role of maternal IQ in the observed associations we repeated the analysis after adjusting for maternal IQ, in the subsample for which information was available ($n = 378$). We further examined potential heterogeneity in associations related to maternal educational level, TSH levels in early pregnancy, gestational weight gain, child's sex, fetal macrosomia breastfeeding duration, preschool attendance and child's BMI *z*- score at age of assessment by including the interaction term in the fully adjusted models (statistically significant effect modification if *p* value <0.10) and stratified analyses, accordingly.

Estimated associations are described in terms of β-coefficients and their 95% confidence intervals (CI). All hypotheses testing were conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. We used Stata S.E. version 13 for the statistical analyses (Stata Corp, Texas, USA).

Results

Socio-demographic characteristics

A total of 173 (22%) mothers and 397 (51%) fathers were categorized as overweight, while 101 (13%) mothers and 172 (22%) fathers were categorized as obese. Obese mothers had higher insulin levels in early pregnancy and were more likely to be less educated, multiparous, to gain less weight during pregnancy and to breastfeed their children for shorter durations compared to underweight/normal weight mothers (Table 1). Offspring of obese women were more likely to have a higher BMI at preschool age (Table 1). GDM was diagnosed in 55 (8%) pregnant women of our study population. Of them 14 (25%) were obese, and 12 (22%) received insulin therapy. GDM women were more likely to be older and to gain inadequate weight during pregnancy, but there were no relevant differences in other sociodemographic or child characteristics compared to normoglycemic women (Table S1).

Parental overweight/obesity in association with offspring neurodevelopmental outcomes at 4 years of age

Multivariate linear regression analyses showed that maternal obesity was associated with a significant score reduction in general cognitive ability (β-coeff −4.03, 95% CI: −7.08, −0.97), perceptual performance (β-coeff −4.60, 95% CI: −7.74, −1.47), quantitative ability (β-coeff -4.43, 95% CI:

Table 1 Parental and child characteristics by maternal pre pregnancy overweight/obesity, Rhea pregnancy cohort, Crete, Greece

	Maternal obesity status			
	Normal weight ($n = 498$)	Overweight ($n = 173$)	Obese ($n = 101$)	p value[a]
Maternal and paternal characteristics ($n = 772$)				
Maternal age at delivery (years), mean (SD)	29.5 (4.8)	30.2 (5.4)	30.2 (4.8)	0.15
Paternal age at delivery (years), mean (SD)	33.4 (5.3)	34.8 (6.6)	34.2 (5.3)	0.11
Maternal Raven score, median (range)	47 (13–58)	46.5 (12–57)	41 (15–57)	0.02
Maternal education, *n* (%)				<0.01
Low	59 (11.8)	32 (18.5)	35 (34.7)	
Medium	273 (54.8)	81 (46.8)	45 (44.6)	
High	166 (33.3)	60 (34.7)	21 (20.8)	
Paternal education, *n* (%)				<0.01
Low	136 (27.5)	68 (39.5)	58 (57.4)	
Medium	225 (45.5)	66 (38.4)	27 (26.7)	
High	133 (26.9)	38 (22.1)	16 (15.8)	
Primiparous, *n* (%)	246 (49.4)	68 (39.3)	32 (31.7)	<0.01
Smoking during pregnancy, *n* (%)	171 (34.4)	53 (30.6)	42 (41.6)	0.18
Maternal pre-pregnancy BMI (kg/m^2), mean (SD)	21.9 (1.9)	27.0 (1.5)	34.4 (4.1)	<0.01
Paternal BMI (kg/m^2), mean (SD)	26.9 (3.8)	28.3 (4.1)	28.3 (4.2)	<0.01
Gestational weight gain (kg), mean (SD)	14.5 (5.4)	13.8 (5.4)	10.7 (6.8)	<0.01
Gestational Diabetes, *n* (%)	35 (7.9)	7 (3.9)	14 (14.7)	<0.01
Maternal TSH (μIU/mL), mean (SD)	1.3 (1.5)	1.4 (1.6)	1.4 (0.8)	0.15
Maternal fasting serum samples in early pregnancy ($n = 452$)				
Maternal Glucose (mg/dl), mean (SD)	73.5 (11.9)	75.3 (12.8)	77.2 (14.2)	0.07
Maternal Insulin (mU/mL), mean (SD)	8.9 (14.2)	12.2 (15.2)	10.8 (20.4)	<0.01
Child characteristics ($n = 772$)				
Sex, girl, *n* (%)	251 (50.4)	72 (41.6)	55 (54.5)	0.07
Growth pattern, *n* (%)				0.92
SGA	22 (4.6)	6 (3.6)	3 (3.2)	
AGA	373 (77.2)	129 (77.3)	72 (75.8)	
LGA	88 (18.2)	32 (19.2)	20 (21.1)	
Prematurity (<37 weeks of gestation), *n* (%)	52 (10.6)	20 (11.7)	19 (19.0)	0.06
Duration of breast feeding (months), mean (SD)	4.6 (4.5)	3.3 (3.6)	3.6 (4.7)	<0.01
BMI (kg/m^2) at 4 years of age, mean (SD)	16.2 (1.7)	16.7 (2.0)	17.1 (2.3)	<0.01
Preschool attendance, *n* (%)	433 (87.1)	139 (80.4)	82 (81.2)	0.06

BMI body mass index, *SGA* small for gestational age (<10th percentile birthweight for gestational age), *AGA* appropriate for gestational age (≥10th percentile and ≤90th percentile birthweight for gestational age), *LGA* large for gestational age (>90th percentile birthweight for gestational age)

[a] p values obtained by Kruskal–Wallis test for more than two independent samples, and χ^2 test or Fisher exact test

−7.68, −1.18), and executive functions (β-coeff −4.92, 95% CI: −8.06, −1.78) after adjustment for several confounders and paternal BMI. Maternal obesity was also associated with increased behavioral difficulties (β-coeff 1.22, 95% CI: 0.09, 2.34) and ADHD symptoms (β-coeff 4.28, 95% CI: 1.20, 7.36) at preschool age. Maternal overweight was associated with a significant score reduction in the scale of perceptual performance (β-coeff −2.90, 95% CI: −5.40, −0.40) (Table 2). Paternal overweight/obesity was not associated with child neurodevelopment at 4 years of age (Table S2).

Fasting glucose and insulin levels in early pregnancy and GDM status in association with offspring neurodevelopmental outcomes at 4 years of age

GDM was associated with a positive trend in all MSCA scores except motor scale, at 4 years of age, though CIs included the null value (Table 3). Adjustment for several covariates, including maternal pre-pregnancy BMI did not change the results (Table 3). We found no association between GDM status and SDQ or ADHD total score

Table 2 Association of maternal pre-pregnancy overweight/obesity with offspring neurodevelopmental outcomes at age 4 years

	Maternal obesity status											
	Model 1				Model 2				Model 3			
	Overweight		Obese		Overweight		Obese		Overweight		Obese	
	β	95% CI	*β*	95% CI	*β*	95% CI	*β*	95% CI	*β*	95% CI	*β*	95% CI
MSCA scales ($n = 772$)												
Verbal scale	−0.71	−3.29, 1.86	**−3.83**	**−7.00, −0.66**	−0.54	−2.98, 1.90	−1.89	−4.96, 1.17	−0.88	−3.34, 1.58	−2.24	−5.32, 0.84
Perceptual performance scale	**−2.71**	**−5.29, −0.14**	**−6.19**	**−9.36, −3.03**	**−2.77**	**−5.25, −0.30**	**−4.47**	**−7.58, −1.36**	**−2.90**	**−5.40, −0.40**	**−4.60**	**−7.74, −1.47**
Quantitative scale	−0.70	−3.34, 1.94	**−5.76**	**−9.01, −2.51**	−0.69	−3.26, 1.87	**−4.30**	**−7.52, −1.07**	−0.82	−3.42, 1.77	**−4.43**	**−7.68, −1.18**
General Cognitive scale	−1.62	−4.19, 0.95	**−5.85**	**−9.01, −2.69**	−1.57	−3.98, 0.85	**−3.74**	**−6.78, −0.71**	−1.84	−4.28, 0.60	**−4.03**	**−7.08, −0.97**
Memory scale	0.17	−2.41, 2.75	**−3.80**	**−6.98, −0.63**	0.10	−2.41, 2.62	−2.43	−5.59, 0.72	−0.28	−2.82, 2.25	−2.83	−6.00, 0.34
Motor scale	−0.55	−3.23, 2.14	−2.98	−6.29, 0.32	−0.66	−3.32, 2.00	−2.38	−5.72, 0.97	−0.65	−3.35, 2.04	−2.37	−5.74, 1.00
Executive functions	−2.42	−5.05, 0.21	**−6.86**	**−10.09, −3.63**	−2.27	−4.75, 0.21	**−4.72**	**−7.84, −1.60**	−2.47	−4.97, 0.04	**−4.92**	**−8.06, −1.78**
Functions of posterior cortex	−0.86	−3.40, 1.68	**−4.32**	**−7.45, −1.20**	−0.89	−3.31, 1.54	−2.50	−5.54, 0.54	−1.20	−3.65, 1.24	−2.83	−5.89, 0.23
SDQ ($n = 633$)												
Total score	−0.07	−0.97, 0.83	**1.21**	**0.09, 2.32**	0.05	−0.83, 0.92	**1.17**	**0.06, 2.29**	0.87	−0.81, 0.97	**1.22**	**0.09, 2.34**
ADHD ($n = 581$)												
Total score	−0.98	−3.31, 1.35	**4.73**	**1.69, 7.77**	−0.59	−2.90, 1.72	**4.42**	**1.38, 7.47**	−0.69	−3.03, 1.64	**4.28**	**1.20, 7.36**

Model 1: adjusted for child sex, examiner, and quality of assessment. Models using Total SDQ and Total ADHD as an outcome were adjusted for child sex and age

Model 2: model 1 further adjusted for maternal age, maternal origin, maternal education level, parity and maternal smoking during pregnancy

Model 3: model 2 also adjusted for paternal body mass index

Ref category: Women with normal weight pre pregnancy (BMI < 25)

Bold values indicate statistically significant differences at $p < 0.05$

BMI Body Mass Index, *MSCA* McCarthy Scales of Children's Abilities, *SDQ* Strengths and Difficulties, *ADHD* Attention Deficit Hyperactivity Disorder

Table 3 Association of GDM status with offspring neurodevelopmental outcomes at 4 years of age

	Gestational diabetes ($n = 55$)					
	Model 1		Model 2		Model 3	
	β-coeff	95% CI	β-coeff	95% CI	β-coeff	95% CI
MSCA scales ($n = 691$)						
Verbal scale	2.30	−1.77, 6.37	2.15	−1.72, 6.02	2.35	−1.52, 6.22
Perceptual performance scale	1.64	−2.54, 5.81	1.22	−2.79, 5.23	1.67	−2.32, 5.66
Quantitative scale	1.58	−2.62, 5.79	1.71	−2.40, 5.82	2.00	−2.11, 6.11
General Cognitive scale	2.54	−1.58, 6.66	2.36	−1.52, 6.24	2.72	−1.15, 6.59
Memory scale	3.58	−0.51, 7.68	3.50	−0.51, 7.50	3.78	−0.23, 7.79
Motor scale	−1.30	−5.62, 3.02	−1.82	−6.12, 2.48	−1.50	−5.80, 2.79
Executive functions	2.26	−1.95, 6.47	2.06	−1.92, 6.03	2.45	−1.51, 6.42
Functions of posterior cortex	2.02	−2.04, 6.07	1.86	−2.02, 5.74	2.15	−1.73, 6.03
SDQ ($n = 583$)						
Total score	0.37	−1.05,1.78	0.63	−0.75, 2.02	0.59	−0.78, 1.98
ADHD ($n = 523$)						
Total score	1.75	−2.14, 5.66	2.41	−1.45, 6.28	2.32	−1.52, 6.16

Model 1: adjusted for child sex, examiner, and quality of assessment. Models using Total SDQ and Total ADHD as an outcome were adjusted for child sex and age

Model 2: model 1 further adjusted for maternal age, maternal origin, maternal education level, parity, and maternal smoking during pregnancy

Model 3: model 2 additionally adjusted for maternal pre-pregnancy body mass index

Ref category: Pregnant women with normal glucose tolerance during pregnancy

MSCA McCarthy Scales of Children's Abilities, *SDQ* Strengths and Difficulties, *ADHD* Attention Deficit Hyperactivity Disorder

(Table 3). We also observed no associations between fasting glucose and insulin serum levels in early pregnancy and child neuropsychological and behavioral development at 4 years of age (Table S3).

Interaction effect and sensitivity analyses

Further analyses showed evidence for a significant interaction between maternal pre-pregnancy overweight/obesity and birth weight in response to offspring cognitive functions (p for interaction <0.10). Preschool children of overweight and obese mothers born large for gestational age exhibited significantly reduced neurodevelopmental scores in most MSCA scales, compared to those born appropriate for gestational age (Fig. 2). We saw no evidence for a multiplicative interaction of maternal pre-pregnancy overweight/obesity with maternal educational level, TSH levels in early pregnancy, gestational weight gain, child sex, breastfeeding duration, preschool attendance, and child's BMI z- score at 4 years of age (p for interaction >0.10).

We repeated our analysis after excluding preterm births and results remained unchanged (data not shown). To elucidate whether GDM modified the associations between maternal pre-pregnancy overweight/obesity and offspring neurodevelopmental outcomes, we performed a sensitivity analysis in which we excluded all women diagnosed with GDM ($n = 55$). Inverse effect estimates became stronger but did not differ substantially from those derived from the main analysis (data not shown). Adjustment for maternal intelligence in the subsample ($n = 378$) for which maternal IQ data were available did not change the direction of associations, though confidence intervals were wider, probably due to small sample size (Table S4).

Discussion

In this prospective pregnancy cohort we provide evidence about the different domains of child neuropsychological and behavioral development at preschool age affected by components of maternal metabolism in early and late pregnancy. To our knowledge, this is the first study to examine the associations of maternal/paternal obesity pre-pregnancy as well as glucose intolerance in early pregnancy and GDM with multiple neurodevelopmental outcomes in preschoolers. Our findings support an inverse association between maternal pre-pregnancy obesity and offspring cognitive scores at 4 years of age, which persisted after adjustment for paternal BMI, maternal intelligence, and several other family and child characteristics. We also, found that maternal obesity was associated with increased behavioral problems and ADHD symptoms at preschool age. Additionally,

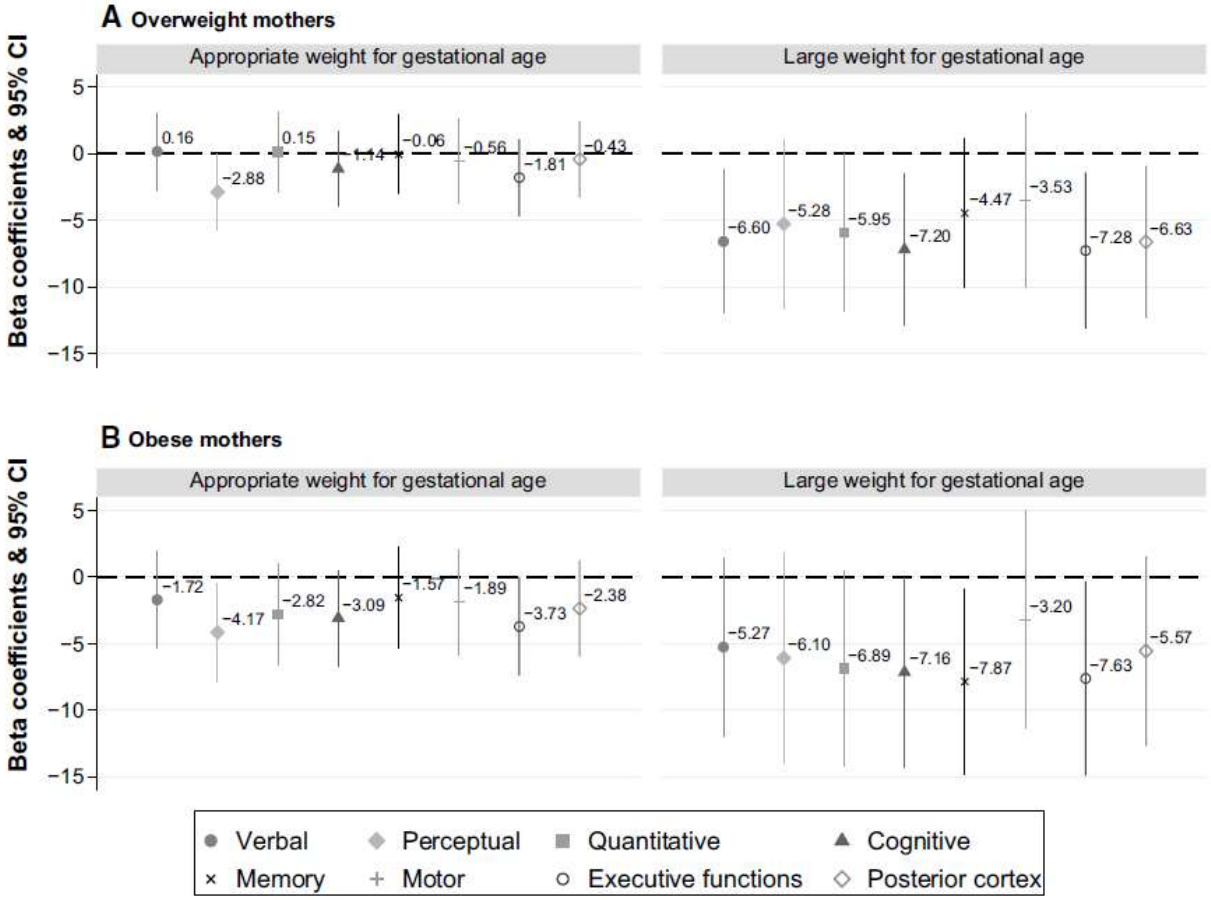

Fig. 2 Association (b coefficient with 95% confidence interval) of maternal overweight and obesity pre-pregnancy with offspring cognitive neurodevelopmental scores at 4 years of age stratified by fetal macrosomia. All models were adjusted for examiner, quality of assessment, child sex, maternal age, maternal education, maternal origin, parity, maternal smoking during pregnancy and paternal BMI

we showed for the first time that birth weight may modify the impact of maternal overweight/obesity on offspring neurodevelopment at 4 years of age. We did not find evidence of an association between paternal overweight/obesity and maternal glucose intolerance in early pregnancy with child neurodevelopment or behavioral difficulties, while exposure to GDM had a potential positive impact on child cognitive scores at preschool age.

Few epidemiological studies have examined so far maternal and paternal obesity with child neurodevelopment with inconclusive results [6–9]. Casas et al. found inverse associations of maternal obesity (and not paternal) with infant neurodevelopment (up to 2 years of age) in the Rhea and the Spanish INMA birth studies [6]. In our study we found that the aforementioned associations remained at preschool age in the Rhea birth cohort. Brion et al. [8] in an analysis of two pregnancy cohorts found no association between maternal/paternal obesity and child verbal skills or behavioral outcomes. We also found no impact of maternal and paternal obesity on child verbal ability at preschool age. However, we examined a greater board of neurodevelopmental outcomes and we found an important impact of maternal obesity on different cognitive abilities, including perceptual performance, quantitative ability, and general cognitive ability. We also found that maternal, but not paternal obesity, was associated with increased behavioral difficulties and ADHD symptoms at preschool age. Further adjustment for maternal IQ did not change the direction of the associations, suggesting a limited role of maternal genetic confounding. Our results on the impact of paternal BMI on child neurodevelopment are in contrast with the findings of Bliddal et al. [7] who support an inverse association between maternal but also paternal BMI with child IQ at 5 years of age, and the findings of Suren et al. [9] who support a strong association of paternal obesity with autism spectrum disorders at 4–13 years of age.

The aforementioned discrepancies may be explained by different study designs, and neurodevelopmental outcomes examined by each study.

Maternal obesity pre-pregnancy has been linked to an inflammatory in utero environment, resulting in dysregulation of hormonal or immune system, placental transport of excess nutrients or micronutrient deficiencies (Vitamin D, folate, iron), and increased oxidative stress [13, 32] which may impair fetal neurodevelopment [33]. Animal studies have shown that maternal obesity in mice can affect oxidative status and progenitor cell division in offspring brain, resulting in decreased hippocampal neurogenesis [34], and consequently impaired hippocampus-dependent cognitive functions in offspring. Moreover, fetal brain inflammation produced by maternal obesity has been associated with reduced offspring brain apoptosis and increased susceptibility to mental disorders later in life [35]. However, plausible biological mechanisms are not clear yet.

The effect of maternal overweight/obesity pre-pregnancy, in our study, was more pronounced in children born large for gestational age. Previous studies have already demonstrated the metabolic impact of over nutrition on the growing fetus [36], but little is known for its neurologic impact. Plausible mechanisms could be developmental adaptations to mild maternal hyperglycemia, hormonal changes, and increased pre-inflammatory cytokines [10]. However, further studies are needed to replicate the above results.

Although we did not find an association between maternal fasting glucose and insulin levels in early pregnancy and offspring neurodevelopment at preschool age, exposure to GDM had a positive effect in almost all the examined cognitive scores at 4 years of age. A sibling study suggests that the association between GDM and offspring cognition may be explained by shared familial characteristics [19]. Adjustment for several sociodemographic characteristics including maternal education in our analysis could not explain our results, although we cannot rule out the possibility of residual confounding. The lack of significant differences in offspring metabolic outcomes, including LGA and prematurity between women exposed to GDM and women with normal glucose metabolism, indicated a good diabetic control in our study population, which may explain our positive results. A recent review also supported that offspring cognitive performance could be within normal limits in well-controlled GDM women [14].

The strengths of the present study include its prospective population-based study design, well-established neurodevelopmental outcome measures by using standardized neurodevelopmental scales (mean of 100 points with a 15 SD), and control for several family and social characteristics, including paternal BMI. We used the McCarthy Scales of Children's Abilities for assessing children's neurodevelopment because they represent a valid, standardized psychometric test which provides both a general level of child's intellectual functioning as well as an assessment of specific neurodevelopmental domains. However, comparison of our results with other studies using different psychometric scales would be rather difficult. The inclusion of maternal intelligence, although available for a subsample of the total population, should be considered as an additional strength of the present study, while the exclusion of pregnancies with pre-diabetes reduced the likelihood of potential confounding by this variable.

The study has some limitations. A significant limitation of our study is the self-reported information on maternal pre-pregnancy weight, which might have led to misclassification of maternal BMI pre-pregnancy. However, we have performed a validation study comparing self-reported maternal pre-pregnancy weight with clinically measured weight in the first prenatal visit, showing high correlation ($r = 0.93$) between self-reported and objectively measured BMI. We assessed children's ADHD symptoms and behavioral difficulties using parent-reported measures, which could be different from assessments made by a health care professional. However, both ADHDT and SDQ scales are well-established and widely used screening tools with high specificity and sensitivity. Finally, although we incorporated extensive information on potential social and environmental factors that are associated with child neurodevelopment, we acknowledge that residual confounding may still occur.

In conclusion, the present study provides evidence that maternal, but not paternal, pre-pregnancy obesity may impair multiple domains of child cognitive development at preschool age supporting as a plausible mechanism the adverse intrauterine environment of obese pregnant women. This association is more pronounced in children born macrosomic and it is independent of the effect of GDM on child cognition. Whether these findings translate into long-term increased risk of neurodevelopmental disorders for the offspring of obese women is unknown. However, there is extensive literature on the public health impact of a 1-point loss of a neuropsychological scale [37] which might not be relevant at the individual level, but at the population level is possible to increase the number of persons below the normal range. Unlike other causes of neurodevelopmental disorders, maternal pre-pregnancy obesity may be prevented with appropriate awareness and guidance. Therefore, improving strategies to reduce obesity among young women during family planning is highly recommended.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

1. Guelinckx I, Devlieger R, Beckers K, Vansant G (2008) Maternal obesity: pregnancy complications, gestational weight gain and nutrition. Obes Rev 9(2):140–150. doi:10.1111/j.1467-789X.2007.00464.x
2. Grammatikopoulou MG, Pritsa AA, Badeka S, Aggelaki I, Giantsiou I, Houta A, Zeibekoglou V, Kyriazi M, Papanastasiou P, Perdiki E, Gkipatidou D, Tsigga M (2013) A pilot study on the prevalence of maternal obesity in selected Greek counties. Endocrinol Nutr 60(9):507–512. doi:10.1016/j.endonu.2013.03.009
3. Van Lieshout RJ (2013) Role of maternal adiposity prior to and during pregnancy in cognitive and psychiatric problems in offspring. Nutr Rev 71(Suppl 1):S95–S101. doi:10.1111/nure.12059
4. Van Lieshout RJ, Taylor VH, Boyle MH (2011) Pre-pregnancy and pregnancy obesity and neurodevelopmental outcomes in offspring: a systematic review. Obes Rev 12(5):e548–e559. doi:10.1111/j.1467-789X.2010.00850.x
5. Smith GD (2008) Assessing intrauterine influences on offspring health outcomes: can epidemiological studies yield robust findings? Basic Clin Pharmacol Toxicol 102(2):245–256. doi:10.1111/j.1742-7843.2007.00191.x
6. Casas M, Chatzi L, Carsin AE, Amiano P, Guxens M, Kogevinas M, Koutra K, Lertxundi N, Murcia M, Rebagliato M, Riano I, Rodriguez-Bernal CL, Roumeliotaki T, Sunyer J, Mendez M, Vrijheid M (2013) Maternal pre-pregnancy overweight and obesity, and child neuropsychological development: two Southern European birth cohort studies. Int J Epidemiol 42(2):506–517. doi:10.1093/ije/dyt002
7. Bliddal M, Olsen J, Stovring H, Eriksen HL, Kesmodel US, Sorensen TI, Nohr EA (2014) Maternal pre-pregnancy BMI and intelligence quotient (IQ) in 5-year-old children: a cohort based study. PLoS One 9(4):e94498. doi:10.1371/journal.pone.0094498
8. Brion MJ, Zeegers M, Jaddoe V, Verhulst F, Tiemeier H, Lawlor DA, Smith GD (2011) Intrauterine effects of maternal prepregnancy overweight on child cognition and behavior in 2 cohorts. Pediatrics 127(1):e202–e211. doi:10.1542/peds.2010-0651
9. Suren P, Gunnes N, Roth C, Bresnahan M, Hornig M, Hirtz D, Lie KK, Lipkin WI, Magnus P, Reichborn-Kjennerud T, Schjolberg S, Susser E, Oyen AS, Smith GD, Stoltenberg C (2014) Parental obesity and risk of autism spectrum disorder. Pediatrics 133(5):e1128–e1138. doi:10.1542/peds.2013-3664
10. Yu Z, Han S, Zhu J, Sun X, Ji C, Guo X (2013) Pre-pregnancy body mass index in relation to infant birth weight and offspring overweight/obesity: a systematic review and meta-analysis. PLoS One 8(4):e61627. doi:10.1371/journal.pone.0061627
11. Chu SY, Callaghan WM, Kim SY, Schmid CH, Lau J, England LJ, Dietz PM (2007) Maternal obesity and risk of gestational diabetes mellitus. Diabetes Care 30(8):2070–2076. doi:10.2337/dc06-2559a
12. Ornoy A, Reece EA, Pavlinkova G, Kappen C, Miller RK (2015) Effect of maternal diabetes on the embryo, fetus, and children: congenital anomalies, genetic and epigenetic changes and developmental outcomes. Birth Defects Res C Embryo Today 105(1):53–72. doi:10.1002/bdrc.21090
13. Georgieff MK (2006) The effect of maternal diabetes during pregnancy on the neurodevelopment of offspring. Minn Med 89(3):44–47
14. Ornoy A (2005) Growth and neurodevelopmental outcome of children born to mothers with pregestational and gestational diabetes. Pediatr Endocrinol Rev 3(2):104–113
15. Dahlquist G, Kallen B (2007) School marks for Swedish children whose mothers had diabetes during pregnancy: a population-based study. Diabetologia 50(9):1826–1831. doi:10.1007/s00125-007-0744-7
16. Fraser A, Nelson SM, Macdonald-Wallis C, Lawlor DA (2012) Associations of existing diabetes, gestational diabetes, and glycosuria with offspring IQ and educational attainment: the Avon Longitudinal Study of Parents and Children. Exp Diabetes Res 2012:963735. doi:10.1155/2012/963735
17. Nielsen GL, Andersen E, Lundbye-Christensen S (2010) Maternal blood glucose in diabetic pregnancies and cognitive performance in offspring in young adulthood: a Danish cohort study. Diabet Med 27(7):786–790. doi:10.1111/j.1464-5491.2010.03024.x
18. Nielsen GL, Dethlefsen C, Sorensen HT, Pedersen JF, Molsted-Pedersen L (2007) Cognitive function and army rejection rate in young adult male offspring of women with diabetes: a Danish population-based cohort study. Diabetes Care 30(11):2827–2831. doi:10.2337/dc07-1225
19. Fraser A, Almqvist C, Larsson H, Langstrom N, Lawlor DA (2014) Maternal diabetes in pregnancy and offspring cognitive ability: sibling study with 723,775 men from 579,857 families. Diabetologia 57(1):102–109. doi:10.1007/s00125-013-3065-z
20. Bonilla C, Lawlor DA, Ben-Shlomo Y, Ness AR, Gunnell D, Ring SM, Smith GD, Lewis SJ (2012) Maternal and offspring fasting glucose and type 2 diabetes-associated genetic variants and cognitive function at age 8: a Mendelian randomization study in the Avon Longitudinal Study of Parents and Children. BMC Med Genet 13:90. doi:10.1186/1471-2350-13-90
21. Veena SR, Krishnaveni GV, Srinivasan K, Kurpad AV, Muthayya S, Hill JC, Kiran KN, Fall CH (2010) Childhood cognitive ability: relationship to gestational diabetes mellitus in India. Diabetologia 53(10):2134–2138. doi:10.1007/s00125-010-1847-0
22. Camprubi Robles M, Campoy C, Garcia Fernandez L, Lopez-Pedrosa JM, Rueda R, Martin MJ (2015) Maternal diabetes and cognitive performance in the offspring: a systematic review and meta-analysis. PLoS One 10(11):e0142583. doi:10.1371/journal.pone.0142583
23. Chatzi L, Plana E, Daraki V, Karakosta P, Alegkakis D, Tsatsanis C, Kafatos A, Koutis A, Kogevinas M (2009) Metabolic syndrome in early pregnancy and risk of preterm birth. Am J Epidemiol 170(7):829–836. doi:10.1093/aje/kwp211
24. American Diabetes Association (2008) Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 31 (Supplement 1):S55–60. doi:10.2337/dc08-S055
25. McCarthy D (1972) Manual for the McCarthy scales of children's abilities. The Psychological Corporation, New York
26. Kampouri M, Kyriklaki A, Roumeliotaki T, Koutra K, Anousaki D, Sarri K, Vassilaki M, Kogevinas M, Chatzi L (2016) Patterns of early-life exposures and neuropsychological development, Rhea birth cohort, Greece. Child Development (**in press**)
27. Goodman R (1997) The Strengths and Difficulties Questionnaire: a research note. J Child Psychol Psychiatry 38(5):581–586
28. Mpipou-Nakou I, Stogiannidou A, Kisseoglou G (2001) Strengths and difficulties of school-age children in the family and school context. Psychologia 8(4):506–525
29. Gilliam JE (1995) Examiners manual for the Attention-Deficit/Hyperactivity Disorder Test: A method for identifying individuals with ADHD. Pro-Ed, Austin, TX

30. Maniadaki K, Kakouros E (2002) Translation and adaptation of the attention deficit hyperactivity disorder test (ADHDT; Giliam, 1995). In: Stalikas A, Triliva S, Roussi P (eds) The psychometric instruments in Greece. Ellinika Grammata, Athens, pp 102–103
31. Raven JC, Court JH (1996) Manual for Raven's progressive matrices and vocabulary scales. Oxford Psychologists Press, Oxford
32. Bolton JL, Bilbo SD (2014) Developmental programming of brain and behavior by perinatal diet: focus on inflammatory mechanisms. Dialogues Clin Neurosci 16(3):307–320
33. Van der Burg JW, Sen S, Chomitz VR, Seidell JC, Leviton A, Dammann O (2016) The role of systemic inflammation linking maternal BMI to neurodevelopment in children. Pediatr Res 79(1–1):3–12. doi:10.1038/pr.2015.179
34. Tozuka Y, Wada E, Wada K (2009) Diet-induced obesity in female mice leads to peroxidized lipid accumulations and impairment of hippocampal neurogenesis during the early life of their offspring. FASEB J 23(6):1920–1934. doi:10.1096/fj.08-124784
35. Edlow AG, Vora NL, Hui L, Wick HC, Cowan JM, Bianchi DW (2014) Maternal obesity affects fetal neurodevelopmental and metabolic gene expression: a pilot study. PLoS One 9(2):e88661. doi:10.1371/journal.pone.0088661
36. Boney CM, Verma A, Tucker R, Vohr BR (2005) Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115(3):e290–e296. doi:10.1542/peds.2004-1808
37. Grandjean P, Landrigan PJ (2006) Developmental neurotoxicity of industrial chemicals. Lancet 368(9553):2167–2178. doi:10.1016/S0140-6736(06)69665-7

Online Supplemental Material

TABLE S1 Maternal and child characteristics by Gestational Diabetes

TABLE S2 Association of paternal overweight/obesity with offspring neurodevelopmental outcomes at 4 years of age

TABLE S3 Association of fasting glucose and insulin serum levels in early pregnancy with offspring neurodevelopmental outcomes at 4 years of age

TABLE S4 Association of maternal pre-pregnancy overweight/obesity with offspring neurodevelopmental outcomes at 4 years of age in the subsample (n=378) for which maternal IQ data was available

TABLE S1 Maternal and child characteristics by Gestational Diabetes

	Normal glucose tolerance (n=636)	**Gestational Diabetes (n=55)**	**p-value**
Maternal characteristics			
Maternal age at delivery (years), *mean (SD)*	29.6 (4.9)	31.5 (4.4)	0.01
Maternal Raven score, *median (range)*	46 (12-58)	46 (25-57)	0.94
Maternal Education, *n (%)*			0.43
Low	104 (16.4)	8 (14.6)	
Medium	325 (51.1)	33 (60.0)	
High	207 (32.5)	14 (25.4)	
Primiparous, *n (%)*	283 (44.5)	28 (50.9)	0.36
Smoking during pregnancy, *n (%)*	214 (33.6)	18 (32.7)	0.89
Maternal pre-pregnancy BMI (kg/m^2), *mean (SD)*	24.7 (4.7)	25.9 (6.7)	0.62
Gestational weight gain (kg), *mean (SD)*	14 (5.7)	12 (6.2)	0.02
Maternal fasting serum samples in early pregnancy (n=408)			
Maternal Glucose (mg/dl), *mean (SD)*	74.3 (11.8)	79 (16.3)	0.21
Maternal Insulin (mU/mL), *mean (SD)*	10.0 (14.7)	22.1 (56.4)	0.05
Child characteristics			
Sex, girl, *n (%)*	311 (48.9)	22 (40.0)	0.21
Growth pattern, *n (%)*			0.32
SGA	24 (3.9)	4 (7.6)	
AGA	477 (77.2)	37 (69.8)	
LGA	117 (18.9)	12 (22.6)	
Prematurity (<37 weeks of gestation), *n (%)*	76 (12.1)	9 (16.4)	0.35
Duration of breast feeding (months), *mean (SD)*	4.2 (4.4)	4.0 (3.9)	0.82
BMI (kg/m^2) at 4years of age, *mean (SD)*	16.5 (1.9)	16.4 (1.8)	0.89
Preschool attendance, *n (%)*	539 (84.9)	49 (89.1)	0.39

BMI, Body Mass Index; SGA, small for gestational age (<10th percentile birthweight for gestational age); AGA, appropriate for gestational age (≥10th percentile and ≤ 90th percentile birthweight for gestational age); LGA, large for gestational age (>90th percentile birthweight for gestational age);

[a] *P*-values obtained by Mann-Whitney U test for two independent samples, and χ^2 test or Fisher exact test.

TABLE S2 Association of paternal overweight/obesity with offspring neurodevelopmental outcomes at 4 years of age

	Paternal obesity status											
	Model 1				Model 2				Model 3			
	Overweight		Obese		Overweight		Obese		Overweight		Obese	
	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI
MSCA scales (n=772)												
Verbal scale	1.74	-0.74, 4.23	2.71	-0.30, 5.71	1.79	-0.64, 4.23	2.54	-0.40, 5.47	1.92	-0.53, 4.36	2.75	-0.21, 5.70
Perceptual performance scale	1.11	-1.40, 3.62	0.75	-2.29, 3.78	0.97	-1.44, 3.38	0.96	-1.95, 3.86	1.29	-1.12, 3.69	1.29	-1.12, 3.69
Quantitative scale	0.08	-2.48, 2.65	0.02	-3.08, 3.13	0.19	-2.32, 2.69	0.22	-2.79, 3.24	0.37	-2.14, 2.88	0.54	-2.49, 3.57
General Cognitive scale	1.34	-1.16, 3.83	1.95	-1.08, 4.97	1.33	-1.07, 3.73	1.96	-0.93, 4.85	1.57	-0.83, 3.97	2.37	-0.53, 5.28
Memory scale	1.06	-1.43, 3.55	2.59	-0.43, 5.60	1.18	-1.28	2.63	-0.34, 5.60	1.34	-1.13, 3.81	2.91	-0.08, 5.89
Motor scale	1.09	-1.50, 3.68	0.25	-2.88, 3.39	0.85	-1.71, 3.41	0.48	-2.60, 3.56	1.02	-1.54, 3.58	0.77	-2.33, 3.88
Executive functions	1.09	-1.47, 3.65	1.08	-2.02, 4.18	1.12	-1.34, 3.59	1.11	-1.86, 4.08	1.40	-1.06, 3.86	1.58	-1.40, 4.56
Functions of posterior cortex	1.40	-1.05, 3.86	2.40	-0.57, 5.38	1.30	-1.08, 3.69	2.37	-0.50, 5.25	1.48	-0.91, 3.87	2.67	-0.22, 5.56
SDQ (n=633)												
Total score	-0.35	-1.21, 0.52	-0.57	-1.63, 0.48	-0.42	-1.27, 0.43	-0.55	-1.58, 0.47	-0.51	-1.37, 0.35	-0.72	-1.75, 0.32
ADHD (n=581)												
Total score	-1.48	-3.77, 0.82	-0.64	-3.38, 2.11	-1.42	-3.69, 0.85	-0.53	-3.24, 2.18	-1.74	-4.02, 0.53	-1.11	-3.83, 1.62

BMI, Body Mass Index; MSCA, McCarthy Scales of Children's Abilities; SDQ, Strengths and Difficulties; ADHD, Attention Deficit Hyperactivity Disorder;

Model 1: adjusted for child sex, examiner and quality of assessment. Models using Total SDQ and Total ADHD as an outcome were adjusted for child sex and age.

Model 2: model 1 further adjusted for paternal age, paternal origin and paternal education level

Model 3: model 2 additionally adjusted for maternal pre-pregnancy body mass index

Ref category: Fathers with normal weight (BMI<25)

TABLE S3 Association of fasting glucose and insulin serum levels in early pregnancy with offspring neurodevelopmental outcomes at 4 years of age

	Fasting glucose levels (per 15 mg/dL increase)						Fasting insulin levels (per 20 mU/mL increase)					
	Model 1		Model 2		Model 3		Model 1		Model 2		Model 3	
	β-coeff	95%CI	β-coeff	95%CI	β-coeff	95%CI	β-coeff	95%CI	β-coeff	95%CI	β-coeff	95%CI
MSCA scales												
(n=452)												
Verbal scale	0.43	-1.17, 2.02	0.23	-1.28, 1.73	0.48	-1.03, 1.99	-0.41	-1.82, 1.01	-0.21	-1.54, 1.12	-0.06	-1.40, 1.28
Perceptual performance scale	0.71	-0.95, 2.37	0.72	-0.91, 2.34	1.15	-0.45, 2.75	-0.03	-1.51, 1.45	0.35	-1.09, 1.78	0.70	-0.73, 2.12
Quantitative scale	0.08	-1.59, 1.74	0.01	-1.63, 1.66	0.28	-1.38, 1.93	-0.43	-1.90, 1.04	-0.22	-1.67, 1.23	-0.01	-1.46, 1.45
General Cognitive scale	0.50	-1.12, 2.11	0.37	-1.18, 1.92	0.73	-0.80, 2.27	-0.35	-1.79, 1.08	-0.03	-1.40, 1.34	0.23	-1.14, 1.60
Memory scale	0.85	-0.76, 2.45	0.70	-0.88, 2.28	0.99	-0.59, 2.57	0.03	-1.40, 1.46	0.24	-1.17, 1.64	0.43	-0.98, 1.83
Motor scale	-0.35	-2.13, 1.43	-0.31	-2.08, 1.46	0.01	-1.76, 1.78	-0.26	-1.83, 1.30	-0.04	-1.60, 1.52	0.22	-1.34, 1.78
Executive functions	0.68	-0.98, 2.34	0.58	-1.02, 2.18	0.95	-0.63, 2.54	-0.39	-1.87, 1.09	-0.12	-1.53, 1.29	0.15	-1.25, 1.56
Functions of posterior cortex	0.32	-1.28, 1.92	0.19	-1.35, 1.73	0.51	-1.03, 2.04	-0.281.	-1.69, 1.14	0.04	-1.32, 1.40	0.27	-1.09, 1.63
SDQ (n=366)												
Total score	0.54	-1.14, 0.06	-0.42	-1.01, 0.18	-0.45	-1.04, 0.14	0.52	-0.78, 1.83	-0.42	-0.91, 0.08	-0.48	-0.97, 0.01
ADHD (n=340)												

Total score	-0.47	-2.06, 1.11	-0.23	-1.82, 1.35	-0.51	-2.08, 1.05	0.52	-0.78, 1.82	0.35	-0.96, 1.65	0.01	-1.28, 1.29

MSCA, McCarthy Scales of Children's Abilities; SDQ, Strengths and Difficulties; ADHD, Attention Deficit Hyperactivity Disorder;

Model 1: adjusted for child sex, examiner and quality of assessment. Models using Total SDQ and Total ADHD as an outcome were adjusted for child sex and age.

Model 2: model 1 further adjusted for maternal age, maternal origin, maternal education level, parity and maternal smoking during pregnancy

Model 3: Model 2 additionally adjusted for maternal pre-pregnancy body mass index

TABLE S4 Association of maternal pre-pregnancy overweight/obesity with offspring neurodevelopmental outcomes at 4 years of age in the subsample (n=378) for which maternal IQ data was available

	Maternal obesity status											
	Crude Model				**Fully Adjusted Model**							
					Without adjustment for maternal IQ				**With adjustment for maternal IQ**			
	Overweight		**Obese**		**Overweight**		**Obese**		**Overweight**		**Obese**	
	β-coeff	**95%CI**	**β-coeff**	**95%CI**	**β-coeff**	**95%CI**	**β-coeff**	**95%CI**	**β-coeff**	**95%CI**	**β-coeff**	**95%CI**
MSCA scales (n=378)												
Verbal scale	-1.14	-4.72, 2.45	-2.33	-6.64, 1.98	-1.57	-4.95, 1.82	-1.45	-5.65, 2.75	-1.28	-4.64, 2.08	-1.02	-5.19, 3.14
Perceptual performance scale	**-4.80**	**-8.45, -1.15**	**-4.65**	**-9.04, -0.25**	**-5.16**	**-8.71, -1.62**	-3.74	-8.14, 0.65	**-4.62**	**-8.04, -1.20**	-2.94	-7.18, 1.30
Quantitative scale	-2.88	-6.50, 0.74	**-4.88**	**-9.24, -0.53**	-2.90	-6.42, 0.61	-3.60	-7.96, 0.76	-2.42	-5.84, 1.00	-2.89	-7.13, 1.36
General Cognitive scale	-3.15	-6.75, 0.45	-4.23	-8.56, 0.10	**-3.59**	**-6.98, -0.20**	-3.14	-7.35, 1.06	-3.11	-6.40, 0.17	-2.44	-6.52, 1.64
Memory scale	-1.76	-5.33, 1.80	-3.49	-7.77, 0.80	-2.49	-5.94, 0.95	-3.55	-7.82, 0.73	-2.11	-5.49, 1.28	-2.98	-7.18, 1.22
Motor scale	-2.79	-6.63, 1.05	-1.40	-6.02, 3.22	-2.88	-6.72, 0.96	-1.00	-5.77, 3.76	-2.54	-6.34, 1.27	-0.50	-5.22, 4.22
Executive functions	**-3.77**	**-7.47, -0.06**	-4.39	-8.84, 0.07	**-3.96**	**-7.47, -0.44**	-2.93	-7.29, 1.43	**-3.52**	**-6.96, -0.08**	-2.30	-6.56, 1.97
Functions of posterior cortex	-2.31	-5.83, 1.20	-3.33	-7.56, 0.90	-2.93	-6.29, 0.42	-2.66	-6.82, 1.50	-2.50	-5.77, 0.77	-2.01	-6.07, 2.04
SDQ (n=325)												
Total score	0.06	-1.26, 1.39	**1.59**	**0.01, 3.18**	0.48	-0.81, 1.77	**2.02**	**0.43, 3.61**	0.34	-0.94, 1.62	**1.81**	**0.23, 3.39**

ADHD (n=297)												
Total score	-2.52	-5.91, 0.88	3.89	-0.35, 8.13	-1.80	-5.20, 1.59	**4.71**	**0.34, 9.08**	-1.93	-5.32, 1.45	**4.49**	**0.13, 8.86**

MSCA, McCarthy Scales of Children's Abilities; SDQ, Strengths and Difficulties; ADHD, Attention Deficit Hyperactivity Disorder; IQ, Intelligence Quotient

Crude Model: adjusted for child sex, examiner and quality of assessment. Models using Total SDQ and Total ADHD as an outcome were adjusted for child sex and age.

Fully Adjusted Model: crude model additionally adjusted for maternal age, maternal origin, maternal education level, parity, maternal smoking during pregnancy and paternal body mass index

Ref category: Mothers with normal weight (BMI<25)

4.3 Paper 3. High maternal vitamin D levels in early pregnancy may protect against behavioral difficulties at preschool age: the Rhea mother-child cohort, Crete, Greece

Main Findings:

1) High maternal 25(OH)D levels in early pregnancy were associated with decreased behavioral difficulties and externalizing symptoms at preschool age.

2) High maternal 25(OH)D levels in early pregnancy were associated with decreased number of hyperactivity and total ADHD-like symptoms at preschool age.

3) The observed associations were more pronounced in girls than in boys.

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ORIGINAL CONTRIBUTION

High maternal vitamin D levels in early pregnancy may protect against behavioral difficulties at preschool age: the Rhea mother–child cohort, Crete, Greece

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Abstract Animal studies suggest that prenatal vitamin D status may affect fetal brain growth. However, human studies are scarce with conflicting results. We aimed to investigate the association of maternal 25-hydroxyvitamin D [25(OH) D] levels with multiple neurodevelopmental outcomes at 4 years of age. We included 487 mother–child pairs from the prospective pregnancy cohort, "Rhea" in Crete, Greece. Maternal serum 25(OH) D concentrations were measured at the first prenatal visit (13 ± 2.4 weeks). Cognitive functions at 4 years were assessed by means of the McCarthy Scales of Children's Abilities. Behavioral difficulties were assessed by means of Strengths and Difficulties Questionnaire and Attention Deficit Hyperactivity Disorder Test. Children of women in the high 25(OH) D tertile (>50.7 nmol/l) had 37% decreased number of hyperactivity–impulsivity symptoms (IRR 0.63, 95% CI 0.39, 0.99, $p_{trend} = 0.05$) and 40% decreased number of total ADHD-like symptoms (IRR 0.60, 95% CI 0.37, 0.95, $p_{trend} = 0.03$) at 4 years of age, compared to children of women in the low 25(OH) D tertile (<38.4 nmol/l), after adjustment for several confounders. Similar associations were found with the hyperactivity/inattention score of the SDQ questionnaire. Children of mothers with high 25(OH) D levels had also fewer total behavioral difficulties (beta-coeff: −1.25, 95% CI −2.32, −0.19) and externalizing symptoms (beta-coeff: −0.87, 95% CI −1.58, −0.15) at preschool age. The observed associations were stronger in girls than in boys ($p_{for\ interaction} < 0.1$). No association was observed between maternal 25(OH) D concentrations and cognitive function in preschoolers. Our results suggest that high maternal vitamin D levels in early pregnancy may protect against behavioral difficulties, especially ADHD-like symptoms at preschool age.

Keywords 25-hydroxyvitamin D · Pregnancy · ADHD · Behavior problems · Cognition · Preschool children

Abbreviations

ADHD	Attention deficit hyperactivity disorder
BMI	Body mass index
CI	Confidence interval
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
GAMs	Generalized additive models
IQ	Intelligence quotient
IRR	Incidence rate ratio

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MSCA	McCarthy Scales of Children's Abilities
SDQ	Strengths and Difficulties Questionnaire
SD	Standard deviation
TSH	Thyroid stimulating hormone

Introduction

Early pregnancy is a critical developmental time window for offspring growth and neurodevelopment [1]. Vitamin D has traditionally been viewed as a hormone essential for skeletal growth and calcium metabolism [2], but it has also multiple extraskeletal actions. As vitamin D amounts of the developing fetus are dependent on maternal stores, maternal vitamin D deficiency is of great concern for its consequences in the offspring. Recent data have shown a high prevalence of pregnant populations with vitamin D deficiency [3], even in countries with abundant sunshine [4]. In Europe, the prevalence of maternal vitamin D deficiency during pregnancy is similar or even higher in southern European countries, compared to central or northern countries, a phenomenon known as the vitamin D paradox in the Mediterranean region [5].

Low vitamin D concentrations during pregnancy have been related with fetal growth restriction [6], rickets [7], hypocalcaemia [8], respiratory tract infections [9], and allergic diseases [10]. Animal studies suggest that maternal vitamin D deficiency may impair fetus brain development [11]. Few epidemiological studies have examined so far the association between vitamin D status during pregnancy with offspring cognition [12–19] or behavioral difficulties [13–16, 20–22] with inconclusive results. Studies in the first half of pregnancy support an association of high maternal 25-hydroxyvitamin D [25(OH) D] levels, with improved mental and psychomotor development in infants [12], better receptive language development at 2 years of age [17], less language difficulties at 5 and 10 years of age [13], and a lower risk of developing ADHD-like symptoms in preschoolers [21]. Birth cohorts examining the impact of maternal 25(OH) D status in late pregnancy or cord blood levels on offspring neurodevelopment found very little [15, 18], or no association with offspring IQ [14] and no association with behavioral difficulties [14, 15] or ADHD diagnosis in mid childhood and adolescence [16, 22]. Differences in the sample size, timing of blood collection, and variation in outcome measures may partly explain the heterogeneity between studies. Additionally, most of them examined small sample sizes and had weak statistical power to detect a small to medium effect size.

We aimed to add to the above research more detailed data and investigate the associations of maternal 25(OH) D levels in early pregnancy with multiple neurodevelopmental outcomes, including neurocognitive function and behavioral difficulties at 4 years of age, in a prospective pregnancy cohort in Crete, Greece, after controlling for a wide range of confounders and effect modifiers.

Methods

Study design and population

The present study is part of the "Rhea" study, a prospective pregnancy cohort, at the prefecture of Heraklion, Crete, Greece. Detailed characteristics of the study population have been described elsewhere [23]. In brief, female residents who had become pregnant during the 12-month period starting in February 2007 participated in the study. Maternal inclusion criteria were the following: residents in the study area; pregnant women aged >16 years; no communication handicap. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece) and all participants provided written informed consent after complete description of the study.

In total, 879 singletons participated at the 4-year follow-up, from October 2011 to January 2013, during which neurodevelopmental assessment was performed in 875 children (99.5%). We excluded 26 children with diagnosed neurodevelopmental disorders (i.e., pervasive developmental disorder), other severe medical disorders (i.e. plagiocephalus, microcephaly, hydrocephalus, brain tumor) and/or incomplete examination. Thus, 849 mother–child pairs were available for our analysis. From those, sufficient maternal serum from early pregnancy for 25(OH) D measurement was available for 497 mothers. We further excluded ten mother–child pairs with missing data for possible confounders. Thus, a cohort of 487 mother–child pairs (98% of the children with maternal 25(OH) D data and neurodevelopmental assessment) was available for the present analysis (Fig. 1). We observed no difference between the children included in the analysis and those that were excluded, except breastfeeding duration which was shorter in participants (Table S1).

Measures

Maternal 25(OH) D concentrations in early pregnancy

Maternal non-fasting serum samples during early pregnancy (13 ± 2.4 weeks) were collected in serum gel separator (BD 367958) tubes, centrifuged and stored at −80 °C until assayed. We used chemiluminescent immunoassay (CLIA) test (DiaSorin, Cat. No. 310600) to measure the total amount of 25(OH) D (both serum 25(OH) D_2 and 25(OH) D_3) [24]. The analytical range for the 25(OH) D assay was 10–375 nmol/L. Inter- and intra-assay precision

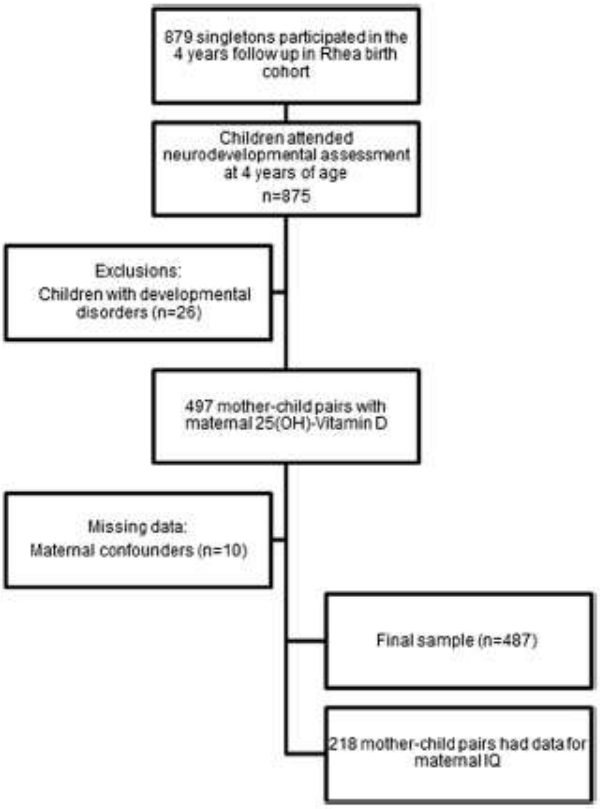

Fig. 1 Flowchart of participants

were <10 and <5%, respectively. We found a weak, but significant correlation between maternal vitamin D serum concentration and dietary intake as measured from a Food Frequency Questionnaire, which included 250 food items and was completed at the end of the first trimester of pregnancy (Spearman's $\rho = 0.12$; $p = 0.006$). FFQ did not take into account vitamin D taken from supplements. We asked about intake of vitamin D supplements in a separate questionnaire; however, none of the study participants were using such supplements. Maternal vitamin D concentration was treated as categorical divided into tertiles: tertile 1: <38.4 nmol/l; tertile 2: 38.4–50.7 nmol/l; tertile 3: >50.7 nmol/l.

Behavioral difficulties at 4 years of age

Information on children's behavior at 4 years of age was obtained via maternal report on two standardized child behavior scales. The parent version of the Strengths and Difficulties Questionnaire (SDQ) [25] is a 25-item behavioral screening instrument designed for children aged 3–16 years. It consists of five subscales generating scores for emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems, and prosocial behavior; all but the last one are summed to generate a total difficulties score, with a high score being less favorable (range 0–40). Two additional scores were calculated: the internalizing problems score by adding up the emotional and peer relationships subscales (range 0–20) and the externalizing problems score by adding up the conduct and hyperactivity subscales (range 0–20). The prosocial behavior scale provides information on protective factors of the child; a low score is less favorable. The SDQ has been translated and adapted for the Greek population [26]. Internal consistency (Cronbach's alpha) varied between 0.38 and 0.70.

The Attention Deficit Hyperactivity Disorder Test (ADHDT) [27] is designed to identify and evaluate ADHD symptoms in ages 3–23 years. It is composed of 36 items in three subscales; (1) hyperactivity, (2) inattention, and (3) impulsivity. All items were rated on a three-point scale (0 = never or rarely, 1 = mild, or 2 = severe).The ADHDT has been translated and adapted for the Greek population [28]. We used the ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) to categorize ADHD-like symptoms. Three quantitative traits were generated for use in our analyses: (1) a count of the number of hyperactive–impulsive symptoms, (2) a count of the number of inattentive symptoms, and (3) a count of the total number of ADHD-like symptoms. In all three cases, a binary measure indicating the presence (severe) or absence (never/rarely or mild) of each symptom was measured and the totals were generated by summing over all symptoms, making the maximum number of symptoms 9, 9, and 18, respectively.

Neurodevelopmental assessment at 4 years of age

Children's cognitive and motor development was assessed by means of the McCarthy Scales of Children's Abilities (MSCA) at 4 years of age [29]. The MSCA is developed for children of ages 2½–8½ years, and includes five conventional subscales (verbal, perceptual performance, quantitative, memory, and motor). A general cognitive score was calculated by combining the verbal, perceptual performance, and quantitative scores [29]. Raw scores were standardized for child's age at test administration using a method for the estimation of age-specific reference intervals based on fractional polynomials. Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales. Higher scores represent better general cognition, language, or psychomotor development. The inter-rater reliability was very high for all scales (intra-class correlation coefficient ≥0.973). MSCA translation and cross-cultural adaptation were conducted according to the internationally recommended methodology. Internal consistency (Cronbach's alpha) varied between 0.76 and 0.89. Confirmatory factor analysis

supported good fit of the model ($\chi 2/df$ = 2, CFI = 0.83, GFI = 0.97, RMSEA = 0.034) [30].

Procedure

Women were invited to provide blood and urine samples and to participate in a face-to-face interview at the first prenatal visit (mean (SD): 12.4 (1.6) weeks). Maternal intelligence quotient (IQ) was measured using the Raven's Standard Progressive Matrices test at the 4-year follow-up [31]. Children's cognitive and motor function at 4 years of age was evaluated by two trained psychologists through the McCarthy Scales of Children Abilities. The administration time ranged from 40 to 60 min. The examiners, also, noted critical comments about the difficulties or special conditions of the neurodevelopmental assessment, so as to evaluate the "quality of assessment" such as: no difficulties, difficulties due to physical problems (e.g., physical illness, tiredness, asleep), difficulties due to behavior problems (e.g., nervousness, shyness). Inter-observer variability was tested in a subsample of 12 children and was <1%. Additional information on children's behavior and ADHD-like symptoms was obtained via maternal report on the SDQ and ADHDT questionnaires. All testing was done at the University Hospital of Heraklion, and Medical Health Centres in the prefecture of Heraklion, Crete, Greece.

Potential covariates

Potential covariates included characteristics that have an established or potential association with the exposure and/or outcomes of interest including: maternal age at delivery (years), maternal education (low: ≤6 years of school; medium: ≤12 years of school; high: university or technical college degree), maternal origin (Greek; non-Greek), smoking during pregnancy (yes; no), parity (multiparous; primiparous), maternal pre-pregnancy BMI (kgr/m^2), maternal IQ measured using the Raven's Standard Progressive Matrices (Raven and Court 1996), first-trimester serum TSH levels, child's sex (male; female), birth weight (kgr), prematurity (preterm; non-preterm), and any breastfeeding duration (months, information on breastfeeding duration was collected during the 9th and the 18th months follow-up).

Statistical analysis

The primary outcomes of interest were SDQ scores, ADHD-like symptoms, and MSCA scores, at 4 years of age. SDQ and MSCA scores were treated as continuous variables, whereas ADHD-like symptoms were treated as quantitative traits. The primary exposure of interest was maternal 25(OH) D in early pregnancy. The distribution of mean 25(OH) D concentration was plotted by calendar month and showed a seasonal variation (Fig S1). As 25(OH) D concentrations followed a sinusoidal pattern, we fitted a cosinor model to the data to predict "deseasonalized" annual mean 25(OH) D concentration for each participant adjusted for month at blood collection.

Descriptive statistics were used to summarize the baseline characteristics of our study population. Bivariate comparisons of normally distributed variables were tested with ANOVA and non-normally distributed variables were tested with non-parametric Kruskal–Wallis test, whereas categorical variables were tested with Pearson's Chi-square test. Generalized additive models (GAMs) were applied to explore the shape of the relationships between 25(OH) D concentration in maternal serum and outcomes under study. These models did not indicate clear linear relationships (p gain defined as the difference in normalized deviance between the GAM model and the linear model for the same predictor < 0.10); thus, maternal 25(OH) D concentration was treated as categorical divided into tertiles. To test the dose–response relationship of 25(OH) D concentrations and outcomes of interest, p for trend was assessed ($p < 0.10$).

We used multivariate linear regression models to assess the association (beta-coefficient, 95% CI) of 25(OH) D levels in early pregnancy on SDQ and standardized MCSA scores at 4 years of age. We also examined the risk [incidence rate ratio (IRR), 95%] of the number of ADHD-like symptoms in association with 25(OH) D levels in early pregnancy using multivariate negative binomial regression models. Covariates were selected if they showed at least marginally significant association ($p < 0.1$) with exposures and outcomes of interest or if they modified the coefficient of maternal 25(OH) D concentration by at least 10% when included in the crude model. Information about child sex and age, the examiner who conducted the developmental testing, and quality of neurodevelopmental assessment were included as a priori confounders. Based on the previous criteria, we created two models: (1) the crude model, minimally adjusted for child sex and age for SDQ scores and ADHD-like symptoms as outcomes, and child sex, examiner, and quality of assessment for MCSA scores; (2) the adjusted model additionally adjusted for maternal age, education, parity, smoking during pregnancy, and pre-pregnancy BMI.

Because relations of 25(OH) D concentration with offspring neurodevelopmental outcomes could be confounded by maternal IQ, we repeated the analysis after adjusting for maternal IQ in a subsample (n = 218) for which information was available. To check for residual confounding, we also adjusted our final models for physical activity, maternal alcohol intake, and total energy intake during pregnancy as well as children's BMI at 4 years of age. We

further examined potential heterogeneity in associations related to maternal pre-pregnancy BMI, maternal TSH levels in early pregnancy, child's sex, birth weight z-score, and breastfeeding duration, by including a multiplicative interaction term in the models (statistically significant effect modification if p value < 0.10) and by stratifying the sample accordingly.

All hypotheses testing were conducted assuming a 0.05 significance level and a two-sided alternative hypothesis. Due to multiple hypotheses testing, Benjamini–Hochberg correction was performed post hoc to control for false discovery rate (FDR = 0.25). We used Stata S.E. version 13 for the statistical analyses (Stata Corp, Texas, USA).

Missing data

In the initial 842 SDQ and ADHDT questionnaires completed by parents at 4 years of age, there were missing values in 1–17 items of the ADHDT questionnaire for 114 subjects and in 1–9 items of the SDQ questionnaire for 57 subjects, respectively. Missing items were imputed to minimize the impact of lack of data and the identification of the number of ADHD-like symptoms based on imputed data. We applied ordinal logistic chained equations to multiply imputed missing values (mi impute procedure in STATA 13.0), and 20 imputed data sets were generated. We have repeated the analysis using cases with complete data and interpretation of results remained unchanged, even though some associations lost statistical significance due to sample size reduction (data not shown).

Results

Sample characteristics

The socio-demographic characteristics of our study population are described in Table 1. Most mothers had a medium education level (52.4%), 46% were primiparous, and 35% were smokers during pregnancy. The mean

Table 1 Maternal and child characteristics by maternal 25(OH) D levels in early pregnancy ($n = 487$)

	Maternal 25(OH) D levels				
	Overall	Tertile 1 < 38.4 nmol/l	Tertile 2 38.4–50.7 nmol/l	Tertile 3 > 50.7 nmol/l	p value[a]
Maternal 25(OH) D (nmol/l), *mean (SD)*	46.3 (15.4)	30.4 (6.3)	45.1 (3.5)	63.4 (10.4)	**<0.01**
Season of maternal blood collection, *n* (%)					0.05
Winter	90 (18.5)	33 (20.4)	29 (17.8)	28 (17.2)	
Spring	142 (29.2)	43 (26.5)	62 (38.1)	37 (22.9)	
Summer	149 (30.6)	50 (30.9)	46 (28.3)	53 (32.8)	
Autumn	106 (21.7)	36 (22.2)	26 (15.8)	44 (27.1)	
Maternal characteristics					
Age at delivery (yr), mean (SD)	29.7 (5.0)	30.1 (5.1)	29.4 (5.2)	29.6 (4.8)	0.66
Education, *n* (%)					0.93
Low	77 (15.8)	23 (14.2)	28 (17.2)	26 (16.1)	
Medium	255 (52.4)	88 (54.3)	85 (52.1)	82 (50.6)	
High	155 (31.8)	51 (31.5)	50 (30.7)	54 (33.3)	
Primiparous, *n* (%)	224 (46.0)	70 (43.2)	85 (52.2)	69 (42.6)	0.15
Smoking during pregnancy, *n* (%)	171 (35.1)	60 (37.1)	62 (38.1)	49 (30.3)	0.27
Pre-pregnancy BMI (kg/m^2), mean (SD)	25 (5.0)	26 (5.8)	25 (4.9)	24 (4.0)	**0.01**
Gestational weight gain (kg), mean (SD)	13.7 (5.6)	13.5 (6.5)	13.8 (5.4)	13.9 (4.8)	0.81
TSH levels(μIU/mL), mean (SD)	1.3 (1.1)	1.4 (1.1)	1.3 (1.0)	1.2 (1.2)	0.27
Child characteristics					
Sex, girl, *n* (%)	230 (47.3)	79 (48.8)	73 (44.8)	78 (48.2)	0.74
Birth weight (kg), mean (SD)	3.2 (0.5)	3.2 (0.4)	3.2 (0.5)	3.2 (0.4)	0.48
Gestational age (weeks), mean (SD)	38.3 (1.6)	38.2 (1.7)	38.2 (1.7)	38.4 (1.3)	0.52
Breastfeeding duration (months), mean (SD)	3.8 (4.1)	3.2 (3.4)	4.0 (4.7)	4.3 (4.0)	**0.03**
BMI (Kg/m^2) at 4 years, mean (SD)	16.5 (1.9)	16.7 (2.0)	16.4 (2.0)	16.4 (1.7)	0.16

BMI body mass index, *TSH* thyroid stimulating hormone

[a] Obtained by Kruskal–Wallis test for more than two independent variables, and $\chi 2$ test or Fisher exact test for categorical variables. Bolds indicate statistically significant differences at $p < 0.05$

(SD) concentration of maternal circulating 25(OH) D was 46.3 (15.4) nmol/l. The second tertile higher threshold (50.7 nmol/l) corresponded well with the recently used definition of vitamin D deficiency as a 25(OH) D concentration <50 nmol/l [31], indicating that two-thirds of pregnant women suffered from vitamin D deficiency. Women in the low 25(OH) D tertile (<38.4 nmol/l) had a higher mean BMI pre-pregnancy and were more likely to breastfeed their children for a shorter interval. We included 257 (52.7%) boys and 230 (47.3%) girls in the present analysis; the mean (SD) age was 4.2 (0.2) years and the mean (SD) birth weight was 3.2(0.5) kg.

Behavioral difficulties

Compared with children of mothers in the low 25(OH) D tertile (<38.4 nmol/l) in early pregnancy children of mothers in the high 25(OH) D tertile (>50.7 nmol/l) had 37% fewer hyperactivity–impulsivity symptoms (IRR 0.63, 95% CI 0.39, 0.99, $p_{trend} = 0.05$) and 40% fewer total ADHD-like symptoms (IRR 0.60, 95% CI 0.37, 0.95, $p_{trend} = 0.03$) at preschool age, after adjustment for several confounders (Fig. 2).

Similar associations were observed between 25(OH) D tertiles in early pregnancy and hyperactivity/inattention subscale score of SDQ questionnaire at preschool age (Table 2). Additionally, children of mothers in the high 25(OH) D tertile in early pregnancy had a significant score reduction in total behavioral difficulties (beta-coeff: −1.25, 95% CI −2.32, −0.19) and more specifically in the scale of externalizing symptoms (beta-coeff: −0.87, 95% CI −1.58, −0.15) at 4 years of age (Table 2). Effect estimates of the crude models for the associations between maternal vitamin D concentrations and behavioral outcomes under study did not differ substantially from the final models adjusted for maternal and child characteristics (Table 2, Table S2).

Neuropsychological outcomes

We did not find a significant association between maternal 25(OH) D tertiles in early pregnancy and offspring cognitive and motor function at preschool age in crude models, although we observed a trend of higher scores in almost all MSCA subscales among children of women in the high 25(OH) D tertile (>50.7 nmol/l) (Table S2). Effect estimates remained to a large extent the same after adjustment for maternal and child characteristics (Table S3).

Effect modification–sensitivity analyses

Further analyses showed that the observed associations were more pronounced in girls than in boys (p for interaction <0.10, Table S4). We saw no evidence for a multiplicative interaction of maternal vitamin D tertiles in early pregnancy with maternal pre-pregnancy BMI, TSH levels, child's birth weight z score and breastfeeding duration.

Further adjustment for maternal intelligence in the subsample ($n = 218$) for which maternal IQ data were available did not change the direction of associations, though confidence intervals were wider, probably due to small sample size (Table S5). The observed associations remained substantially the same, after adjustment for physical activity, alcohol intake during pregnancy, total energy intake during pregnancy, and children's BMI at 4 years of age, although some of our main findings lost statistical significance, probably due to sample size reduction (Table S6). Because the effect of season is close to significant (Table 1) for our analysis, we repeated the analysis by including season in regression models and the associations remained the same (Table S7). To elucidate whether gestational diabetes, gestational hypertension, or prematurity modified the observed results, we performed a sensitivity analysis in which we excluded (a) women diagnosed with gestational diabetes ($n = 41$), (b) women diagnosed with gestational hypertension ($n = 21$), and (c) children born preterm (<37 gestational weeks, $n = 58$). We found no substantial differences in observed estimates (data not shown).

Discussion

In this prospective pregnancy cohort, we examined different domains of child neuropsychological and behavioral development at preschool age affected by vitamin D levels in early pregnancy. To our knowledge, this is the first study to examine the impact of maternal vitamin D status in early pregnancy on both cognitive function and behavioral difficulties in preschoolers. We showed that exposure to high 25(OH) D levels (>50.7 nmol/l) was associated with reduced number of hyperactivity–impulsivity and total ADHD-like symptoms, as well as total behavioral difficulties at preschool age. Our findings suggest that a vitamin D cutoff value of 50 nmol/l may be essential not only for bone health [32], but also for prevention of behavioral difficulties in the offspring. The observed associations persisted after adjustment for several maternal confounders and pre-pregnancy BMI. We also showed for the first time that child sex may modify the impact of vitamin D status in early pregnancy on offspring behavior at 4 years of age. We did not find a strong association between maternal vitamin D levels in early pregnancy and child cognitive and motor function at 4 years of age.

Although maternal vitamin D status has an important role in early brain development, data on the association between child neurodevelopment and vitamin D status in

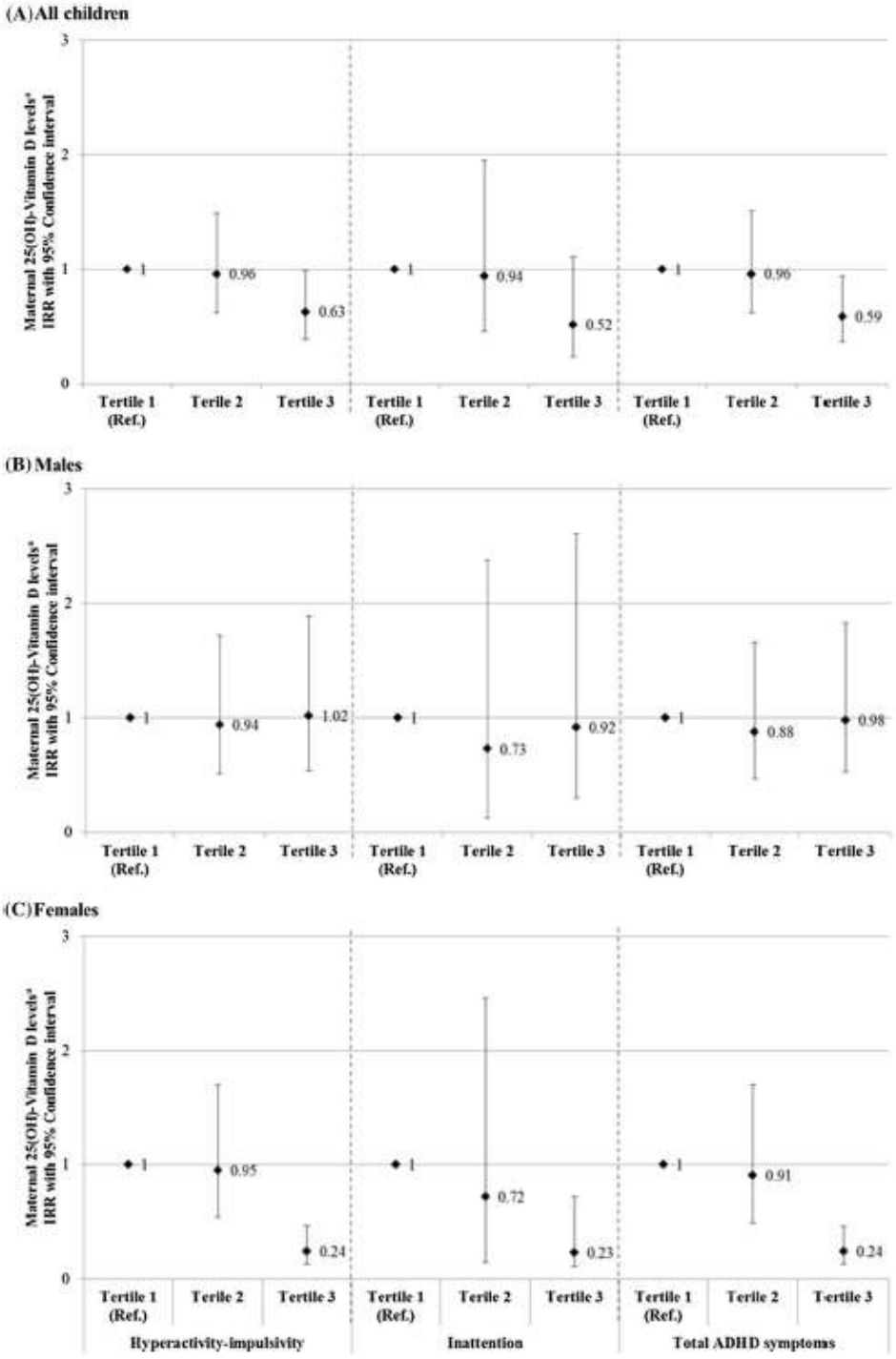

Fig. 2 Association [incidence rate ratio (IRR) with 95% confidence interval] of maternal 25(OH) D concentrations with ADHD-like symptoms in **a** all children, **b** males, and **c** females at 4 years of age. All models were adjusted for child age at assessment, maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy. The models including all children were additionally adjusted for child sex

early pregnancy are limited. Previous studies support that high maternal 25(OH) D levels in the first half of pregnancy were associated with improved mental and psychomotor development in infants [12], better receptive language development at 2 years of age [17], and less language difficulties at 5 and 10 years of age [13]. We examined a wide broad of cognitive abilities at 4 years of age and also found a trend of higher scores among children of women in the high 25(OH) D tertile. However, our results did not reach statistical significance probably due to the small sample size. We also found an inverse relationship between maternal vitamin D status in early pregnancy and behavioral difficulties, including ADHD-like symptoms at 4 years of age. Our results are consistent with the findings of Morales et al.

Table 2 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties at 4 years of age ($n = 445$)

SDQ	Maternal 25(OH) D levels					
	Tertile 1 < 38.4 nmol/l		Tertile 2 38.4–50.7 nmol/l		Tertile 3 > 50.7 nmol/l	
	Ref.	β-coeff	95% CI	β-coeff	95% CI	$P_{\text{for trend}}$
Crude model						
Emotional symptoms	0	−0.04	−0.42, 0.33	−0.20	−0.58, 0.17	0.289
Conduct problems	0	0.15	−0.21, o.50	−0.14	−0.49, 0.21	0.426
Hyperactivity/inattention	0	−0.24	−0.73, 0.24	**−0.73**	**−1.21, −0.24**	**0.003**
Peer relationship problems	0	0.25	−0.06, 0.57	−0.26	−0.58, 0.05	0.093
Prosocial behavior	0	0.04	−0.37, 0.45	−0.05	−0.46, 0.35	0.793
Internalizing symptoms	0	0.21	−0.35, 0.77	−0.46	−1.02, 0.09	0.096
Externalizing symptoms	0	−0.09	−0.82, 0.64	**−0.87**	**−1.59, −0.14**	**0.018**
Total difficulties score	0	0.12	−0.98, 1.21	**−1.33**	**−2.42, −0.24**	**0.016**
Adjusted model						
Emotional symptoms	0	−0.07	−0.44, 0.29	−0.15	−0.52, 0.22	0.421
Conduct problems	0	0.11	−0.25, 0.46	−0.15	−0.50, 0.21	0.410
Hyperactivity/inattention	0	−0.35	−0.82, 0.13	**−0.72**	**−1.19, −0.25**	**0.003**
Peer relationship problems	0	0.23	−0.07, 0.54	−0.23	−0.54, 0.07	0.129
Prosocial behavior	0	0.07	−0.34, 0.48	−0.04	−0.44, 0.37	0.859
Internalizing symptoms	0	0.16	−0.38, 0.70	−0.38	−0.93, 0.16	0.157
Externalizing symptoms	0	−0.24	−0.95, 0.47	**−0.87**	**−1.58, −0.15**	**0.017**
Total difficulties score	0	−0.08	−1.14, 0.98	**−1.25**	**−2.32, −0.19**	**0.019**

Beta-coefficients (β-coeff) and 95% confidence intervals retained from linear regression models

Crude model: minimally adjusted for child sex and child age of assessment

Adjusted model: crude model further adjusted for maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy

SDQ Strengths and Difficulties Questionnaire

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model. Bolds indicate statistically significant differences at $p < 0.05$, after Benjamini–Hochberg procedure for multiple testing correction

[21], who found a significant association between high 25(OH) D levels in early pregnancy and reduced number of ADHD-like symptoms in preschool children. However, we investigated more aspects of child behavior in our study and additionally found that high vitamin D levels in early pregnancy were associated with a reduced number of total behavioral problems, including externalizing symptoms at 4 years of age. Further adjustment for maternal IQ did not change the direction of the associations, suggesting a limited role of maternal genetic confounding.

It is well known that early pregnancy is a time window of particular vulnerability, as cortical structures critical to cognitive function and behavioral regulation are first formed. Maternal vitamin D performs a number of biological functions that are fundamental to early brain development [11], including proliferation and differentiation of brain cells [33], regulation of axonal growth [34], calcium signaling within the brain, and neurotrophic and neuroprotective actions [34]. In animal models, prenatal vitamin D deficiency has been associated with morphological changes [33] that may persist despite a postnatal return to normal vitamin D levels, resulting in abnormal behaviors in adulthood [35]. However, studies in humans are limited and plausible biological mechanisms are not clear yet.

In our study, we showed for the first time that child sex may modify the impact of maternal vitamin D status on offspring neurodevelopment. Higher levels of 25(OH) D in early pregnancy had a stronger protective effect on behavioral difficulties in females compared to males, especially on hyperactivity/inattention, externalizing symptoms and total ADHD-like symptoms. Limited data in adults have shown that immunomodulatory effects of vitamin D are significantly stronger in females than in males in multiple sclerosis patients supporting estrogen-promoted differences on vitamin D metabolism and action [36]. Whether there is also a functional synergy between estradiol and vitamin D action on prenatal brain development remains to be investigated.

With the vitamin D deficiency epidemic among pregnant women, the present results have important public health implications, which may be more profound in countries with higher prevalence of vitamin D deficiency.

Despite a hypothetical excess of sunshine hours in the Mediterranean region, maternal hypovitaminosis D remains common in pregnant populations of these countries [5]. In our study, we found that almost two-thirds of pregnant women ($n = 313$) had vitamin D deficiency [25(OH) D levels < 50 nmol/l] in early pregnancy [31]. Possible reasons for this paradox could be maternal darker skin pigmentation, poor dietary vitamin D intake, veiled clothing reducing sunshine exposure, and increased prevalence of obesity [5]. In addition, preventive strategies for maternal vitamin D deficiency in the Mediterranean region are lacking so far, as hypovitaminosis D is largely unrecognized and underrated in several South European countries [5].

The strengths of our study include its prospective population-based study design, well-established neurodevelopmental outcome measures, and control for several maternal and child characteristics. The inclusion of maternal intelligence, although available for a subsample of the total population, should be considered as an additional strength. We estimated maternal vitamin D status in early pregnancy by measuring circulating 25(OH) D concentration, a reliable indicator of vitamin D synthesis and intake. We minimized the potential effect of season in our results by using the deseasonalized variable of 25(OH) D in our analysis. Neurodevelopment assessment at preschool age was performed with the use of MCSA [29], a valid, standardized psychometric test which provides both a general level of child's intellectual functioning and an assessment of separate neurodevelopmental domains. In the present analysis, we used standardized neurodevelopmental scales (mean of 100 points with a 15 SD). There is extensive literature on the public health impact of a 1-point loss of a neuropsychological scale, but most are based on the effects of lead exposure on IQ [37]. Although a seemingly small change of a 1-point decrease in IQ score might not be relevant at the individual level, at the population level it is possible to shift the distribution of IQ to the left and increase the number of persons below the normal range [38].

A limitation of our study is the assessment of children's ADHD symptoms and behavioral difficulties by parent-reported measures, which could be different from assessments made by a health-care professional. However, both ADHDT and SDQ questionnaire are well established and widely used screening tools with high specificity and sensitivity. Although we incorporated extensive information on potential social and environmental factors that are associated with child neurodevelopment, we acknowledge that residual confounding because of other unmeasured confounders such as social class and child's vitamin D status may still occur.

In conclusion, our findings support a possible inverse relationship between vitamin D levels in early pregnancy and behavioral problems, especially hyperactivity/inattention, externalizing symptoms, and total ADHD-like symptoms in early childhood. These associations are more pronounced in females and may have important implications from a public health perspective. Whether these findings translate into long-term increased risk of abnormal behavior for the offspring of vitamin D-deficient women is unknown. However, unlike other causes of neurodevelopmental disorders, maternal vitamin D deficiency may be prevented. We speculate that appropriate supplementation during pregnancy may reduce the incidence of behavioral difficulties and ADHD-like symptoms later in life. Further investigation is needed to assess the long-term effects of vitamin D supplements in pregnancy on neuropsychological and behavioral development in offspring.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

1. Kubota T, Miyake K, Hariya N, Mochizuki K (2015) Understanding the epigenetics of neurodevelopmental disorders and DOHaD. J Dev Orig Health Dis 6(2):96–104. doi:10.1017/S2040174415000057
2. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357(3):266–281. doi:10.1056/NEJMra070553
3. Urrutia RP, Thorp JM (2012) Vitamin D in pregnancy: current concepts. Curr Opin Obstet Gynecol 24(2):57–64. doi:10.1097/GCO.0b013e3283505ab3
4. Hamilton SA, McNeil R, Hollis BW, Davis DJ, Winkler J, Cook C, Warner G, Bivens B, McShane P, Wagner CL (2010) Profound vitamin D deficiency in a diverse group of women during pregnancy living in a sun-rich environment at latitude 32°N. Int J Endocrinol 2010:917428. doi:10.1155/2010/917428
5. Karras SN, Anagnostis P, Annweiler C, Naughton DP, Petroczi A, Bili E, Harizopoulou V, Tarlatzis BC, Persinaki A, Papadopoulou F, Goulis DG (2014) Maternal vitamin D status during pregnancy: the Mediterranean reality. Eur J Clin Nutr 68(8):864–869. doi:10.1038/ejcn.2014.80
6. Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O'Beirne M, Rabi DM (2013) Association between maternal serum

25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. BMJ 346:f1169. doi:10.1136/bmj.f1169
7. Paterson CR, Ayoub D (2015) Congenital rickets due to vitamin D deficiency in the mothers. Clin Nutr 34(5):793–798. doi:10.1016/j.clnu.2014.12.006
8. Camadoo L, Tibbott R, Isaza F (2007) Maternal vitamin D deficiency associated with neonatal hypocalcaemic convulsions. Nutr J 6:23. doi:10.1186/1475-2891-6-23
9. Morales E, Romieu I, Guerra S, Ballester F, Rebagliato M, Vioque J, Tardon A, Rodriguez Delhi C, Arranz L, Torrent M, Espada M, Basterrechea M, Sunyer J (2012) Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. Epidemiology 23(1):64–71. doi:10.1097/EDE.0b013e31823a44d3
10. Sharief S, Jariwala S, Kumar J, Muntner P, Melamed ML (2011) Vitamin D levels and food and environmental allergies in the United States: results from the National Health and Nutrition Examination Survey 2005–2006. J Allergy Clin Immunol 127(5):1195–1202. doi:10.1016/j.jaci.2011.01.017
11. Groves NJ, McGrath JJ, Burne TH (2014) Vitamin D as a neurosteroid affecting the developing and adult brain. Annu Rev Nutr 34:117–141. doi:10.1146/annurev-nutr-071813-105557
12. Morales E, Guxens M, Llop S, Rodriguez-Bernal CL, Tardon A, Riano I, Ibarluzea J, Lertxundi N, Espada M, Rodriguez A, Sunyer J (2012) Circulating 25-hydroxyvitamin D3 in pregnancy and infant neuropsychological development. Pediatrics 130(4):e913–e920. doi:10.1542/peds.2011-3289
13. Whitehouse AJ, Holt BJ, Serralha M, Holt PG, Kusel MM, Hart PH (2012) Maternal serum vitamin D levels during pregnancy and offspring neurocognitive development. Pediatrics 129(3):485–493. doi:10.1542/peds.2011-2644
14. Gale CR, Robinson SM, Harvey NC, Javaid MK, Jiang B, Martyn CN, Godfrey KM, Cooper C (2008) Maternal vitamin D status during pregnancy and child outcomes. Eur J Clin Nutr 62(1):68–77. doi:10.1038/sj.ejcn.1602680
15. Keim SA, Bodnar LM, Klebanoff MA (2014) Maternal and cord blood 25(OH)-vitamin D concentrations in relation to child development and behaviour. Paediatr Perinat Epidemiol 28(5):434–444. doi:10.1111/ppe.12135
16. Strom M, Halldorsson TI, Hansen S, Granstrom C, Maslova E, Petersen SB, Cohen AS, Olsen SF (2014) Vitamin D measured in maternal serum and offspring neurodevelopmental outcomes: a prospective study with long-term follow-up. Ann Nutr Metab 64(3–4):254–261. doi:10.1159/000365030
17. Tylavsky FA, Kocak M, Murphy LE, Graff JC, Palmer FB, Volgyi E, Diaz-Thomas AM, Ferry RJ (2015) Gestational vitamin 25(OH) D status as a risk factor for receptive language development: a 24-month, longitudinal, observational study. Nutrients 7(12):9918–9930. doi:10.3390/nu7125499
18. Zhu P, Tong SL, Hao JH, Tao RX, Huang K, Hu WB, Zhou QF, Jiang XM, Tao FB (2015) Cord blood vitamin D and neurocognitive development are nonlinearly related in toddlers. J Nutr 145(6):1232–1238. doi:10.3945/jn.114.208801
19. Gould JF, Anderson AJ, Yelland LN, Smithers LG, Skeaff CM, Zhou SJ, Gibson RA, Makrides M (2016) Association of cord blood vitamin D with early childhood growth and neurodevelopment. J Paediatr Child Health. doi:10.1111/jpc.13308
20. Whitehouse AJ, Holt BJ, Serralha M, Holt PG, Hart PH, Kusel MM (2013) Maternal vitamin D levels and the autism phenotype among offspring. J Autism Dev Disord 43(7):1495–1504. doi:10.1007/s10803-012-1676-8
21. Morales E, Julvez J, Torrent M, Ballester F, Rodriguez-Bernal CL, Andiarena A, Vegas O, Castilla AM, Rodriguez-Dehli C, Tardon A, Sunyer J (2015) Vitamin D in pregnancy and attention deficit hyperactivity disorder-like symptoms in childhood. Epidemiology 26(4):458–465. doi:10.1097/EDE.0000000000000292
22. Gustafsson P, Rylander L, Lindh CH, Jonsson BA, Ode A, Olofsson P, Ivarsson SA, Rignell-Hydbom A, Haglund N, Kallen K (2015) Vitamin D status at birth and future risk of attention deficit/hyperactivity disorder (ADHD). PLoS ONE 10(10):e0140164. doi:10.1371/journal.pone.0140164
23. Chatzi L, Plana E, Daraki V, Karakosta P, Alegkakis D, Tsatsanis C, Kafatos A, Koutis A, Kogevinas M (2009) Metabolic syndrome in early pregnancy and risk of preterm birth. Am J Epidemiol 170(7):829–836. doi:10.1093/aje/kwp211
24. Katrinaki M, Kampa M, Margioris A, Castanas E, Malliaraki N (2016) Vitamin D levels in a large Mediterranean cohort: reconsidering normal cut-off values. Hormones (Athens) 15(2):205–223. doi:10.14310/horm.2002.1674
25. Goodman R (1997) The strengths and difficulties questionnaire: a research note. J Child Psychol Psychiatry 38(5):581–586
26. Mpipou-Nakou I, Stogiannidou A, Kisseoglou G (2001) Strengths and difficulties of school-age children in the family and school context. Psychologia 8(4):506–525
27. Gilliam JE (1995) Examiners manual for the attention-deficit/hyperactivity disorder test: a method for identifying individuals with ADHD. pro-Ed, Austin
28. Maniadaki K, Kakouros E (2002) Translation and adaptation of the attention deficit hyperactivity disorder test (ADHDT; Giliam, 1995). In: Stalikas A, Triliva S, Roussi P (eds) The psychometric Instruments in Greece. Ellinika Grammata, Athens, pp 102–103
29. McCarthy D (1972) Manual for the McCarthy scales of children's abilities. Psychological Corp, New York
30. Kampouri M, Kyriklaki A, Roumeliotaki T, Koutra K, Anousaki D, Sarri K, Vassilaki M, Kogevinas M, Chatzi L (2017) Patterns of early-life social and environmental exposures and child cognitive development, Rhea birth cohort, Crete, Greece. Child Dev. doi:10.1111/cdev.12782
31. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96(7):1911–1930. doi:10.1210/jc.2011-0385
32. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96(1):53–58. doi:10.1210/jc.2010-2704
33. Eyles D, Brown J, Mackay-Sim A, McGrath J, Feron F (2003) Vitamin D3 and brain development. Neuroscience 118(3):641–653
34. Eyles DW, Burne TH, McGrath JJ (2013) Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. Front Neuroendocrinol 34(1):47–64. doi:10.1016/j.yfrne.2012.07.001
35. O'Loan J, Eyles DW, Kesby J, Ko P, McGrath JJ, Burne TH (2007) Vitamin D deficiency during various stages of pregnancy in the rat; its impact on development and behaviour in adult offspring. Psychoneuroendocrinology 32(3):227–234. doi:10.1016/j.psyneuen.2006.12.006
36. Correale J, Ysrraelit MC, Gaitan MI (2010) Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. J Immunol 185(8):4948–4958. doi:10.4049/jimmunol.1000588
37. Grandjean P, Landrigan PJ (2006) Developmental neurotoxicity of industrial chemicals. The Lancet 368(9553):2167–2178
38. Bellinger DC (2012) A strategy for comparing the contributions of environmental chemicals and other risk factors to neurodevelopment of children. Environ Health Perspect 120(4):501

Online Supplemental Material

Figure S1 The distribution of mean 25(OH) D concentration plotted by calendar month in our study population

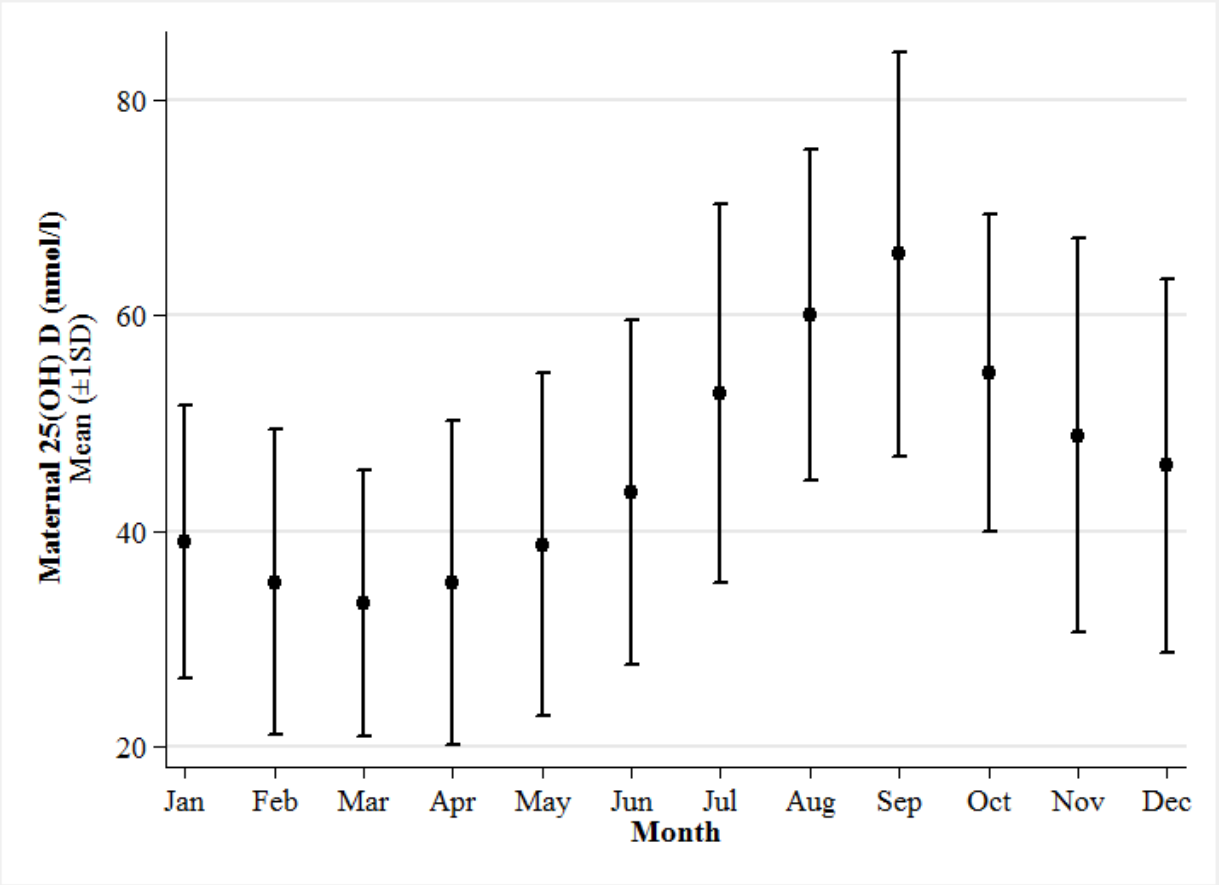

Table S1 Maternal and child characteristics of participants and non-participants

	N	% or Mean	(SD)	N	% or Mean	(SD)	p-value[a]
Maternal characteristics							
Age at delivery (yr), *mean (SD)*	354	29.8	(4.9)	487	29.7	(5.0)	0.45
Education, *n (%)*							0.75
Low	53	15.8		77	15.8		
Medium	168	50.0		255	52.4		
High	115	34.2		155	31.8		
Primiparous, *n (%)*	165	54.2		224	46.0		0.93
Smoking during pregnancy, *n (%)*	140	46.9		171	35.1		0.08
Pre-pregnancy BMI (kg/m^2), *mean (SD)*	316	24.3	(4.7)	487	25.0	(5.0)	0.06
Gestational weight gain (kg), *mean (SD)*	279	14.0	(5.9)	397	13.7	(5.6)	0.66
Child characteristics							
Sex, girl, *n (%)*	191	52.7		230	47.3		0.11
Birth weight (kg), *mean (SD)*	331	3.2	(0.4)	487	3.2	(0.5)	0.25
Gestational age (weeks), *mean (SD)*	337	38.2	(1.4)	487	38.3	(1.6)	0.29
Breastfeeding duration (months), *mean (SD)*	326	4.6	(4.6)	470	3.8	(4.1)	**0.01**
BMI (Kg/m^2) at 4 years, *mean (SD)*	361	16.4	(1.9)	485	16.5	(1.9)	0.65

BMI body mass index

[a] Obtained by Kruskal–Wallis test for more than two independent variables, and $\chi 2$ test for categorical variables.

Bolds indicate statistically significant differences at $p < 0.05$

Table S2 Crude association of maternal 25(OH) D[a] levels with offspring ADHD-like symptoms at 4 years of age (n = 445)

	Maternal 25(OH) D levels					
	Tertile 1	Tertile 2		Tertile 3		
ADHD–like symptoms	<38.4 nmol/l	38.4-50.7 nmol/l		>50.7 nmol/l		
	Ref[b]	*IRR*	95%CI	*IRR*	95%CI	$P_{for\ trend}$
Hyperactivity-impulsivity	1	0.95	0.61, 1.51	**0.57**	**0.35, 0.93**	**0.031**
Inattention	1	1.04	0.51, 2.15	0.46	0.21, 1.02	0.073
Total ADHD symptoms	1	0.96	0.61, 1.54	**0.54**	**0.33, 0.88**	**0.019**

ADHD Attention Deficit Hyperactivity Disorder;

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model.β -Coefficients and 95% confidence intervals are retained from linear regression models

All models were minimally adjusted for child sex and child age of assessment

Bolds indicate statistically significant differences at p <0.05, after Benjamini-Hochberg procedure for multiple testing correction

Table S3 Association of maternal 25(OH) D[a] levels with offspring cognitive and motor function at 4 years of age (*n* = 487)

	Maternal 25(OH) D levels					
	Tertile 1	Tertile 2		Tertile 3		
MSCA scales	<38.4 nmol/l	38.4-50.7 nmol/l		>50.7 nmol/l		
Crude Model	Ref[b]	*β-coeff*	95%CI	*β-coeff*	95%CI	$P_{for\ trend}$
Verbal scale	0	1.17	-2.00, 4.33	1.82	-1.36, 5.00	0.260
Perceptual scale	0	0.28	-2.95, 3.51	2.32	-0.92, 5.56	0.160
Quantitative scale	0	3.36	0.02, 6.69	1.76	-1.58, 5.11	0.300
General Cognitive scale	0	1.32	-1.93, 4.57	2.23	-1.04, 5.49	0.180
Memory scale	0	-0.08	-3.34, 3.18	0.69	-2.59, 3.96	0.680
Motor scale	0	2.14	-1.20, 5.49	3.85	0.49, 7.21	0.025
Adjusted Model	Ref[b]	*β-coeff*	95%CI	*β-coeff*	95%CI	$P_{for\ trend}$
Verbal scale	0	1.13	-1.89, 4.16	1.77	-1.28, 4.82	0.255
Perceptual scale	0	0.21	-2.84, 3.26	1.50	-1.57, 4.57	0.338
Quantitative scale	0	3.59	0.36, 6.81	1.58	-1.66, 4.84	0.343
General Cognitive scale	0	1.32	-1.71, 4.36	1.80	-1.26, 4.86	0.248
Memory scale	0	-0.09	-3.25, 3.06	0.29	-2.88, 3.47	0.855
Motor scale	0	2.10	-1.24, 5.44	3.29	-0.07, 6.66	0.055

MSCA McCarthy Scales of Children's Abilities;

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model. β-Coefficients and 95% CI are retained from linear regression models

Crude model: minimally adjusted for child sex, examiner and quality of assessment

Adjusted model: crude model further adjusted for maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy.

Bolds indicate statistically significant differences at p <0.05, after Benjamini-Hochberg procedure for multiple testing correction

Table S4 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties at 4 years of age, stratified by child sex

	Maternal 25(OH) D levels								
	Boys				Girls				
	Tertile 2[b]		Tertile 3[b]		Tertile 2[b]		Tertile 3[b]		
SDQ	38.4-50.7 nmol/l		>50.7 nmol/l		38.4-50.7 nmol/l		>50.7 nmol/l		
	β-coeff	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI	*P for interaction*
Emotional symptoms	-0.11	-0.62, 0.39	0.01	-0.49, 0.51	-0.06	-0.63, 0.51	-0.29	-0.86, 0.28	0.58
Conduct problems	0.37	-0.14, 0.88	0.28	-0.22, 0.79	-0.22	-0.72, 0.27	**-0.58**	**-1.08, -0.08**	0.05
Hyperactivity/ inattention	0.01	-0.68, 0.68	-0.09	-0.77, 0.59	**-0.78**	**-1.44, -0.14**	**-1.39**	**-2.04, -0.75**	0.03
Peer relationship problems	0.22	-0.22, 0.66	-0.05	-0.49, 0.38	0.33	-0.11, 0.77	-0.37	-0.80, 0.07	0.27
Prosocial behavior	0.37	-0.22, 0.96	0.11	-0.49, 0.69	-0.13	-0.71, 0.44	-0.14	-0.71, 0.43	0.55
Internalizing symptoms score	0.11	-0.64, 0.86	-0.05	-0.79, 0.69	0.27	-0.54, 1.08	-0.66	-1.47, 0.15	0.27
Externalizing symptoms score	0.37	-0.66, 1.41	0.19	-0.84, 1.22	**-1.01**	**-1.99, -0.02**	**-1.98**	**-2.96, -0.99**	0.01
Total difficulties score	0.48	-1.04, 2.01	0.14	-1.38, 1.65	-0.74	-2.24, 0.76	**-2.64**	**-4.14, -1.14**	0.03

SDQ Strengths and Difficulties Questionnaire

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

β-Coefficients and 95% confidence intervals are retained from linear regression models / IRR (Incidence rate ratio) and 95% confidence interval are retained from negative binomial regression models; All models were adjusted for child age of assessment, maternal age, maternal education, parity, smoking during pregnancy, and maternal body

mass index pre-pregnancy

Bolds indicate statistically significant differences at p <0.05, after Benjamini-Hochberg procedure for multiple testing correction

[b]Reference category: maternal 25(OH) D levels <38.4 nmol/l

Table S5 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties and ADHD-like symptoms at 4 years of age, in the subsample in which maternal IQ was available (*n* = 218)

	Maternal 25(OH) D levels								
		Without adjustment for maternal IQ				Adjusted for maternal IQ			
	Tertile 1	Tertile 2		Tertile 3		Tertile 2		Tertile 3	
	<38.4 nmol/l	38.4-50.7 nmol/l		>50.7 nmol/l		38.4-50.7 nmol/l		>50.7 nmol/l	
SDQ	Ref	*β-coeff*	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI
Emotional symptoms	0	-0.33	-0.86, 0.19	-0.23	-0.72, 0.25	-0.35	-0.88, 0.17	-0.25	-0.73, 0.23
Conduct problems	0	0.13	-0.43, 0.69	-0.21	-0.72, 0.31	0.15	-0.41, 0.72	-0.19	-0.72, 0.32
Hyperactivity/inattention	0	-0.27	-1.02, 0.46	**-0.87**	**-1.55, -0.18**	-0.17	-0.91, 0.56	**-0.80**	**-1.48, -0.13**
Peer relationship problems	0	0.11	-0.34, 0.56	-0.26	-0.68, 0.15	0.16	-0.28, 0.62	-0.23	-0.64, 0.18
Prosocial behavior	0	-0.17	-0.82, 0.47	-0.18	-0.77, 0.41	-0.19	-0.85, 0.45	-0.19	-0.79, 0.39
Internalizing symptoms score	0	-0.22	-1.01, 0.55	-0.51	-1.22, 0.22	-0.19	-0.97, 0.59	-0.48	-1.20, 0.24
Externalizing symptoms score	0	-0.14	-1.32, 1.03	-1.08	-2.16, 0.01	-0.02	-1.19, 1.16	-1.01	-2.08, 0.07
Total score	0	-0.36	-2.10, 1.37	-1.58	-3.17, 0.01	-0.21	-1.94, 1.52	-1.48	-3.06, 0.11
ADHD–like symptoms	Ref[b]	*IRR*	95%CI	*IRR*	95%CI	*IRR*	95%CI	*IRR*	95%CI
Hyperactivity-impulsivity	1	1.06	0.54, 2.10	0.69	0.36, 1.31	1.17	0.59, 2.32	0.74	0.39, 1.40
Inattention	1	1.16	0.38, 3.54	0.44	0.14, 1.34	1.44	0.48, 4.32	0.52	0.17, 1.58
Total ADHD symptoms	1	1.08	0.54, 2.17	0.62	0.33, 1.18	1.23	0.62, 2.45	0.68	0.36, 1.30

SDQ Strengths and Difficulties Questionnaire; *ADHD* Attention Deficit Hyperactivity Disorder; *IQ* Intelligence Quotient;

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

β-Coefficients and 95% confidence intervals are retained from linear regression models / IRR (Incidence rate ratio) and 95% confidence interval are retained from negative binomial regression models; All models were adjusted for child sex, child age of assessment, maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy

Bolds indicate statistically significant differences at $p < 0.05$, after Benjamini-Hochberg procedure for multiple testing correction

Table S6 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties and ADHD-like symptoms at 4 years of age, in the subsample in which additional covariates[b] were available (n = 344)

	Maternal 25(OH) D levels								
		Without adjustment for additional covariates				Adjusted for additional covariates			
	Tertile 1	Tertile 2		Tertile 3		Tertile 2		Tertile 3	
	<38.4 nmol/l	38.4-50.7 nmol/l		>50.7 nmol/l		38.4-50.7 nmol/l		>50.7 nmol/l	
SDQ	Ref	*β-coeff*	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI	*β-coeff*	95%CI
Emotional symptoms	0	-0.04	-0.48, 0.41	0.02	-0.44, 0.47	0.01	-0.44, 0.46	0.05	-0.40, 0.52
Conduct problems	0	0.19	-0.22, 0.61	0.02	-0.40, 0.43	0.19	-0.22, 0.62	0.02	-0.41, 0.45
Hyperactivity/inattention	0	-0.21	-0.77, 0.35	**-0.62**	**-1.19, -0.06**	-0.21	-0.77, 0.35	**-0.63**	**-1.21, -0.05**
Peer relationship problems	0	0.04	-0.32, 0.39	-0.25	-0.61, 0.11	0.03	-0.33, 0.39	-0.26	-0.63, 0.11
Prosocial behavior	0	0.16	-0.32, 0.63	-0.03	-0.51, 0.44	0.21	-0.27, 0.68	0.02	-0.46, 0.51
Internalizing symptoms score	0	0.01	-0.65, 0.66	-0.23	-0.89, 0.43	0.04	-0.62, 0.70	-0.20	-0.87, 0.47
Externalizing symptoms score	0	-0.02	-0.85, 0.82	-0.61	-1.46, 0.23	-0.01	-0.86, 0.83	-0.61	-1.47, 0.25
Total score	0	-0.02	-1.21, 1.24	-0.84	-2.11, 0.43	0.02	-1.24, 1.29	-0.81	-2.10, 0.48
ADHD–like symptoms	Ref[b]	*IRR*	95%CI	*IRR*	95%CI	*IRR*	95%CI	*IRR*	95%CI
Hyperactivity-impulsivity	1	1.05	0.64, 1.71	0.71	0.42, 1.18	1.06	0.65, 1.73	0.74	0.44, 1.25
Inattention	1	1.00	0.43, 2.32	0.44	0.17, 1.15	1.00	0.43, 2.34	0.47	0.18, 1.22
Total ADHD symptoms	1	1.05	0.64, 1.74	0.66	0.39, 1.12	1.05	0.64, 1.73	0.69	0.40, 1.18

SDQ Strengths and Difficulties Questionnaire; *ADHD* Attention Deficit Hyperactivity Disorder;

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

[b]Additional covariates added in the final models were physical activity, alcohol intake during pregnancy, total energy intake during pregnancy, and children´s BMI at 4 years of age.

β-Coefficients and 95% confidence intervals are retained from linear regression models / IRR (Incidence rate ratio) and 95% confidence interval are retained from negative binomial regression models; All models were adjusted for child sex, child age of assessment, maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy

Bolds indicate statistically significant differences at $p < 0.05$, after Benjamini-Hochberg procedure for multiple testing correction

Table S7 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties or ADHD-like symptoms at 4 years of age, after adjustment for season

	Maternal 25(OH) D levels					
	Tertile 1	Tertile 2		Tertile 3		
	<38.4 nmol/l	38.4-50.7 nmol/l		>50.7 nmol/l		
SDQ	Ref.	*β-coeff*	95%CI	*β-coeff*	95%CI	$P_{for\ trend}$
Emotional symptoms	0	-0.07	-0.44, 0.31	-0.16	-0.53, 0.21	0.391
Conduct problems	0	0.12	-0.23, 0.48	-0.16	-0.51, 0.19	0.370
Hyperactivity/inattention	0	-0.36	-0.83, 0.11-	**-0.74**	**-1.22, -0.28**	**0.002**
Peer relationship problems	0	0.24	-0.08, 0.54	-0.27	-0.53, 0.08	0.143
Prosocial behaviour	0	0.02	-0.39, 0.43	-0.02	-0.43, 0.38	0.906
Internalizing symptoms	0	0.17	-0.38, 0.72	-0.39	-0.93, 0.16	0.156
Externalizing symptoms	0	-0.23	-0.95, 0.48	**-0.91**	**-1.62, -0.19**	**0.012**
Total difficulties score	0	-0.07	-1.13, 1.01	**-1.29**	**-2.36, -0.24**	**0.016**
ADHD-like symptoms	Ref	*IRR*	*95%CI*	*IRR*	*95%CI*	$P_{for\ trend}$
Hyperactivity-impulsivity	1	0.97	0.62, 1.51	**0.59**	**0.37, 0.93**	**0.028**
Inattention	1	0.89	0.42, 1.88	0.49	0.23, 1.06	0.077

Total symptoms	1	0.98	0.63, 1.54	**0.57**	**0.36, 0.89**	**0.019**

SDQ Strengths and Difficulties Questionnaire; *ADHD* Attention Deficity and Hyperactivity Disorder;

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

All models were adjusted for child sex, child age of assessment, maternal age, maternal education, parity, smoking during pregnancy, maternal body mass index pre-pregnancy and season.

Bolds indicate statistically significant differences at $p < 0.05$, after Benjamini-Hochberg procedure for multiple testing correction

4.4 Paper 4. Effect of very low vitamin D levels in pregnancy on offspring obesity indices and cardiometabolic traits in childhood

Main Findings:

1) Low maternal 25(OH)D levels in the first half of pregnancy were associated with increased offspring BMI SD score, central adiposity and body fat percentage at pre-school age.

2) Maternal obesity pre-pregnancy may modify effect estimates of 25(OH)D levels on offspring adiposity measures.

3) Maternal 25(OH) levels in the first half of pregnancy had no effect on child blood pressure or lipid levels at preschool age.

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TITLE PAGE

Title: Effect of very low vitamin D levels in pregnancy on offspring obesity indices and cardiometabolic traits in childhood

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Keywords: Vitamin D, pregnancy, child obesity, child blood pressure, child lipids, preschool age

Running Title: Maternal 25(OH)D and child obesity outcomes

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‘What is already known’

Low maternal 25(OH)D levels have been associated with increased fat mass in offspring.

‘What this study adds’

Very low maternal 25(OH)D levels in the first half of pregnancy were associated with increased offspring adiposity measures, predominantly in girls.

Maternal obesity pre-pregnancy may modify the association of maternal 25(OH)D levels during pregnancy and offspring adiposity outcomes.

Very low maternal 25(OH)D levels were not associated with offspring blood pressure or lipid levels in childhood.

Abstract

Background: Vitamin D may modulate adipogenesis. However, limited studies have investigated the effect of maternal vitamin D during pregnancy on offspring adiposity or cardiometabolic parameters with inconclusive results.

Objectives: To examine the associations of maternal 25(OH)-vitamin D [25(OH)D] levels with offspring obesity and cardiometabolic characteristics in 532 mother-child pairs from the prospective pregnancy cohort Rhea in Crete, Greece.

Methods: Maternal 25(OH)D levels were measured at the first prenatal visit (mean: 14weeks, SD: 4). Child outcomes included BMI SD score, waist circumference, skin-fold thickness, body fat percentage (%BF), blood pressure, and serum lipids at ages 4 and 6 years. BMI growth trajectories from birth to 6 years were estimated by mixed effects models with fractional polynomials of age. Adjusted associations were obtained via multivariable linear regression analyses.

Results: About two-thirds of participating mothers had 25(OH)D levels <50nmol/l. Offspring of women in the low 25(OH)D tertile (<37.7nmol/l) had higher BMI SD score (β 0.20, 95% CI: 0.03, 0.37), waist circumference (β 0.87 95% CI: 0.12, 1.63) and %BF (β 1.48, 95% CI: 0.46, 2.49) at preschool age, compared to the offspring of women with higher 25(OH)D measurements (≥37.7nmol/l), on covariate-adjusted analyses. The observed relationships persisted at age 6 years. We found no association between maternal 25(OH)D concentrations and offspring blood pressure or serum lipids at both time points.

Conclusions: Exposure to very low 25(OH)D levels in utero may increase childhood adiposity indices. Given that vitamin D is a modifiable risk factor, our findings may have important public health implications.

Abbreviations

BIA: Bioelectric Impedance Analysis

BF: Body Fat

BMI: Body Mass Index

CI: Confidence Interval

FM: Fat Mass

FFM: Free Fat Mass

GAMs: Generalized additive models

HDL: High-Density Lipoprotein Cholesterol

IOM: Institute of Medicine

LDL: Low-Density Lipoprotein Cholesterol

VitD: Vitamin D

25(OH)D: 25(OH)-vitamin D

Introduction

Maternal vitamin D (VitD) is an essential biological factor for intrauterine skeletal growth and muscle development[104]. However, recent data suggest multiple effects beyond mineral metabolism. VitD deficiency among pregnant women has increased dramatically [124] even in countries with abundant sunlight [181]. Observational studies across Europe have shown a similar or even higher prevalence of maternal hypovitaminosis D in southern countries, compared to countries of Mediterranean region, a phenomenon known as the Mediterranean VitD paradox[114].

Given that VitD levels of the developing fetus depend exclusively on maternal supply, low VitD levels during pregnancy is of great concern for its possible pleiotropic consequences on the offspring later in life. Maternal VitD deficiency has been linked to low muscle mass [147], poorer bone mineral accrual [148], fetal growth restriction [140] and increased prevalence of respiratory tract infections [184] and allergic diseases [185]. However, the effect of maternal VitD status on fetal adipogenesis is unclear. Experimental studies suggest that VitD may modulate inflammation, and adipocyte formation and secretion[197]. Additionally, human studies support an association of low maternal 25(OH)-vitamin D [25(OH)D] status with offspring lower fat mass at birth [149], but increased odds of overweight at the first year [153], greater fat mass at ages 4 and 6 years [149,154], and higher fat percentage at 9.5 years [147]. However, other studies have not observed these relationships[150-152]. Differences in sample size, timing of blood collection and variation in outcome measures may partly explain the above heterogeneity. To our knowledge the impact of very low maternal VitD levels on child obesity indices and cardiovascular traits has not been investigated so far.

We aimed to investigate the effect of very low maternal 25(OH)D levels in the first half of pregnancy with multiple offspring metabolic outcomes, including BMI growth trajectories, adiposity outcomes, lipids and blood pressure levels at 4 and 6 years of age, in a prospective pregnancy cohort in Crete, Greece, after controlling for a wide range of confounders.

Methods

Study design and population

The present study is part of the "Rhea" study, a prospective pregnancy cohort, at the prefecture of Heraklion, Crete, Greece. Detailed characteristics of the study population have been described elsewhere [198]. In brief, female residents (Greek and immigrants) who became pregnant within a 12-month period starting in February 2007 were asked to participate in the study. The first contact was made at the time of the first major ultrasound. Maternal inclusion criteria were: residents in the study area; pregnant women aged >16 years; no communication handicap. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece), and all participants provided written informed consent after complete description of the study.

For the present study we used measures of 25(OH)D concentrations from maternal blood samples collected during pregnancy (n=1226), and offspring obesity indices and cardiovascular traits measured at 4 (n=766) and 6 years (n=522) follow-up assessments. Mother-child pairs at 4 (n = 211) and 6 (n = 152) years follow up with missing data on maternal VitD concentrations due to inadequate quantity of serum or incomplete covariate information were excluded from our analysis. The final study sample with complete data on maternal 25(OH)D measures, covariates and offspring outcomes included 532, and 370 mother-child pairs at 4 and 6 years follow up, respectively (Figure S1). We observed no difference in sociodemographic characteristics (p for all 0.19–0.86) or outcome scores (p for all 0.09–0.89) between participating and non-participating mothers (Table S1), except the sum of skinfolds at 4 years that was higher in the participants' group (p=0.03).

Measures

Details of 25(OH)D assaying, and measurements of outcomes and covariates, are included in the online supplementary material.

Statistical analysis

The distribution of mean 25(OH)D concentration was plotted by calendar month and showed a seasonal variation (Figure S2). As 25(OH)D concentrations followed a sinusoidal pattern, we fitted a cosinor model to the data to predict "deseasonalized" annual mean 25(OH)D concentration for each participant adjusted for month at blood collection. Because of the lack of a universally accepted

definition of VitD deficiency during pregnancy, we treated maternal 25(OH)D concentration as categorical by dividing it into tertiles: tertile 1: <37.7nmol/l; tertile 2: 37.7-51.2nmol/l; tertile 3: >51.2nmol/l. For our analysis, we have grouped together tertile 2 and 3 in order to examine the impact of very low maternal 25(OH)D levels (<37.7nmol/l) on offspring adiposity indices and cardiometabolic traits in childhood.

Differences in distributions of normally distributed variables were tested with t-test, non-normally distributed continuous variables were tested by using non parametric tests (i.e., Mann-Whitney, Kruskal-Wallis, and Spearman'rho), whereas categorical variables were tested with Pearson's Chi square test. Generalized additive models (GAMs) were applied to explore the shape of the relationships between maternal 25(OH)D concentration and outcomes under study indicating linear relationships for all exposure-outcomes associations.

The time points of interest in childhood were the ages of 4 years, and 6 years to reflect different developmental ages (preschooler, and school-aged child, respectively). We used multivariate linear regression models to assess the association (beta-coefficient, 95% CI) of 25(OH)D levels during pregnancy on offspring adiposity indices and cardiometabolic traits. We also examined the risk [relative risk (RR), 95% CI] of rapid growth at 24 months in association with 25(OH)D levels during pregnancy using log binomial regression models. Covariates were selected if they modified the coefficient of maternal 25(OH)D concentration by at least 10% when included in the crude models. Based on the previous criteria we created 2 models: (1) the crude model, minimally adjusted for child sex and age (except models using child rapid growth, and child BMI and blood pressure SD scores as an outcome; models using waist circumference, sum of skinfolds, FFM, FM, and %BF as an outcome were further adjusted for child height). (2) The full model additionally adjusted for maternal age, education, parity and smoking during pregnancy. Models using child lipid levels as an outcome were further adjusted for child BMI SD score.

We used a two-step approach to assess differences in BMI trajectories from birth to 6 years of age for low and high maternal 25(OH)D groups. First, we identified the best fitting fractional polynomials of age and constructed sex-and age-specific BMI growth curves[199,200]. Then we used mixed-effects linear

regression models with the previously identified fractional polynomials of age, including an interaction term of 25(OH)D levels with age along with previously described covariates, a random intercept for child and a random age slope.

Additional analysis

In order to address the missing data on maternal 25(OH)D concentrations and covariates at 4 and 6 years follow-up we applied chained equations to multiply impute missing values (the mi impute procedure in STATA 13.0) and 20 imputed data sets were generated. We used imputation models that were more general than the analyses models and included all covariates, maternal 25(OH)D concentrations and offspring outcomes. Results of the models based on multiple imputation (n = 766) compared with those generated by using the complete data set (n = 532) revealed no differences in interpretation (data not shown). In order to have compliance with the analysis of BMI growth trajectories, which is based only on actual data, we only present results from the complete data set for all outcomes.

We examined potential heterogeneity in associations related to maternal overweight/obesity, gestational age at sampling, child sex, birth weight SD score, and breastfeeding duration, by including a multiplicative interaction term in the models. Statistically significant effect modification if p value <0.10 or by comparing the model using likelihood ratio test. If significant effects were detected we stratified the sample accordingly. To elucidate whether gestational diabetes, or preterm birth modified the observed results due to residual confounding, we repeated our analysis excluding a) women diagnosed with gestational diabetes (n = 45) and b) children born preterm (<37 gestational weeks, n=61).

All hypotheses testing were conducted assuming a 0.05 significance level and a 2-sided alternative hypothesis. We used Stata S.E. version 13 for the statistical analyses (Stata Corp, Texas, USA).

Results

Participants' characteristics

Sociodemographic characteristics of our study population are presented in Table 1. Participating mothers had a mean (±SD) age of 29.7 ±4.9 years, 51% had a medium education level, 46% were primiparous and 17% were smokers during pregnancy. A total of 122 (23%) women were overweight and 76 (14%) were obese pre-pregnancy, while 45 (9%) were diagnosed with gestational diabetes. The mean (±SD) concentration of maternal circulating 25(OH)D was 46.3 (±15.7) nmol/l. Mothers in the low VitD tertile (25(OH)D levels <37.7nmol/l) constituted almost one third of our study population and had a higher mean BMI pre-pregnancy compared to women with higher 25(OH)D levels. The mean age of the children at 4 and 6 years follow up was 4.2 (±0.2) and 6.5 (±0.3) years respectively and 47 % were girls (Table 1). About 11% (n=61) of the children were born preterm (<37 week of gestation) and 13% (n=37) were rapid growers from birth to 24 months.

Adiposity outcomes

A 10nmol/l decrement of maternal 25(OH)D concentrations in the first half of pregnancy was inversely associated with BMI SD score (*β coef* 0.06, 95% CI: 0.01, 0.11), waist circumference (*β coef* 0.24, 95% CI: 0.02, 0.46) and %BF (*β coef* 0.32, 95% CI:0.03, 0.62) at 4 years of age, after adjustment for multiple confounders (Table 2). Moreover, women in the low 25(OH)D tertile (<37.7nmol/l) gave birth to children with higher BMI SD score (*β coef* 0.20, 95% CI: 0.03, 0.37,), larger waist circumference by 0.87cm (95% CI: 0.12, 1.63,), and higher %BF (*β coef* 1.48, 95% CI: 0.46, 2.49,) at preschool age, compared to women with 25(OH)D levels ≥37.7nmol/l, in the fully adjusted models (Table 2). The observed associations persisted at age 6 years (Table 2). We found no association between maternal 25(OH)D levels and rapid infant growth from birth to 24 months of life (Table 2). Effect estimates of the crude models did not differ substantially from the final models adjusted for maternal and child characteristics (Table 2).

Figure 1 depicts the modeled BMI SD score trajectories of children up to 6 years according to different 25(OH) D levels during pregnancy. Children of mothers with very low 25(OH)D levels (<37.7nmol/l) exhibited consistently higher values than did those of mothers with higher 25(OH)D levels (≥37.7nmol/l), and at 4.5 years onwards, the difference became more pronounced.

Cardiometabolic traits

We found no significant effect of 25(OH)D status during pregnancy on offspring blood pressure measurements and lipid levels at pre-school or school age, even in offspring of women with 25(OH)D levels during pregnancy <37.7nmol/l (Table S2).

VitD and maternal obesity

Maternal obesity modified 25(OH)D effect estimates on offspring BMI SD score and waist circumference. Children of women with normal weight or overweight and 25(OH)D levels<37.7nmol/l had higher BMI SD scores and waist circumference, whereas children of mothers with obesity and low 25(OH)D levels tended to have decreased BMI SD scores and waist circumference, at 4 and 6 years follow up (all p for interaction<0.1, Figure S3). Similar findings were observed in the analysis of BMI SD score trajectories stratified by pre-pregnancy BMI categories (Figure 1).

Effect modification-Sensitivity analyses

Examining potential heterogeneity in associations related to child sex we found that the inverse relationship between 25(OH)D levels <37.7nmol/l and waist circumference or fat mass at 6 years follow up were more pronounced in girls than in boys (p for interaction 0.08 and 0.09 respectively, Table S3). We found no evidence for a multiplicative interaction of very low maternal 25(OH)D levels during pregnancy with gestational age at sampling, child's birthweight SD score or breastfeeding duration. Excluding gestational diabetes or preterm birth did not change the observed estimates (data not shown).

Discussion

To our knowledge, this is the first study examining the association of very low 25(OH)D concentrations in the first half of pregnancy on offspring obesity indices and cardiometabolic traits. We found that maternal 25(OH)D levels <37nmol/l were associated with increased offspring BMI SD score, central adiposity and body fat percentage at pre-school and school age. The observed associations persisted after adjustment for several maternal and child characteristics. We also, showed

for the first time that maternal obesity pre-pregnancy may modify effect estimates on offspring adiposity measures, as children of mothers with obesity and low 25(OH)D levels had decreased BMI SD scores and central adiposity, at 4 and 6 years follow up. We did not find an association between maternal 25(OH)D levels and child blood pressure or lipid levels at 4 or 6 years of age.

Two birth cohorts in the first half of pregnancy found also a significant inverse association of maternal 25(OH)D concentrations with offspring BMI z-score and increased odds of overweight at age 1 year [153] and increased fat percentage at 5-6 years [154]. In contrast studies focusing on the effect of maternal 25(OH)D status in late pregnancy support no significant effect of maternal vitamin D status with offspring adiposity measures [147,150,152]. Only one birth cohort examining the potential role of maternal 25(OH)D status in late pregnancy suggested an association between lower 25(OH)D levels with greater offspring fat mass in childhood [149]. Differences in sample size, timing of sampling, severity of vitamin D deficiency, and outcomes studied may explain this discrepancy. Previous studies support that fetal exposure to 25(OH)D levels is unlikely to influence offspring most cardiovascular traits later in life [147,151,154], except insulin resistance [147,154]. In our study we also showed no association of 25(OH)D status with offspring blood pressure or lipid levels, but we were not able to estimate child glucose metabolism, as child glucose and insulin measurements were not available.

Accumulating evidence supports the view that VitD influences adipocyte differentiation but the exact mechanisms are largely unknown[197]. Animal studies have shown that VitD suppresses differentiation of preadipocytes to mature adipocytes by inhibiting the expression of key adipogenesis regulators, like PPARγ[201]. In vitro studies have also shown that exposure of human preadipocytes to VitD metabolites reduces lipid accumulation early in developmental process and may inhibit the initiation of human preadipocyte differentiation [202]. In the human fetus, white fat lobules appear at 14 weeks of gestation, while after the 23rd week, their total number remains approximately constant [203]. After birth, expansion of adipose tissue occurs mainly through increase in adipocyte size. Our findings support that exposure to very low VitD levels in the first half of pregnancy-the critical time point of adipogenesis- may increase fetal adipocyte number resulting in increased fat mass later in life. However, further research is needed to understand the plausible biological mechanisms.

An unexpected finding in our study was the different direction of maternal 25(OH)D associations with offspring adiposity indices of mothers with normal weight/overweight compared to mothers with obesity. A previous study investigating the associations of maternal 25(OH)D in early pregnancy with offspring adiposity measures at ages 5-6 years found no interaction with pre-pregnancy BMI [154]. Differences in outcomes under study may explain this inconsistency. Women with normal weight have a higher rate of weight increment and an increased preperitoneal fat thickness across gestation compared to women with overweight/obesity [204]. As circulating levels of 25(OH)D depend on its storage in visceral adipose tissue [205] variations in fat distribution during pregnancy in women with different BMI levels may partly explain our findings.

Given the alarming increase of childhood obesity and subsequent cardiovascular risk, the present results may have important public health implications, which may be more profound in countries with higher prevalence of hypovitaminosis D. In our study, almost two-thirds of participating women (*n* = 346) suffered from VitD deficiency [25(OH)D levels <50nmol/l] [113], while 172 women had 25(OH)D levels <37.7nmol/l. Our findings are in accordance with the high prevalence of maternal hypovitaminosis D in the Mediterranean region, despite a hypothetical excess of sunshine hours[114]. Possible reasons for this paradox could be maternal darker skin pigmentation, poor dietary VitD intake, veiled clothing, reduced sunshine exposure, and increased prevalence of obesity, while preventive strategies in these countries are lacking so far[114].

Strengths of our study include its prospective population-based study design, the repeated measures of offspring BMI, the detailed cardiometabolic measures, and the control for several maternal and child characteristics. We estimated maternal vitamin D status by measuring circulating 25(OH)D concentration, a reliable indicator of vitamin D synthesis and intake. We minimized the potential effect of season in our results by using the deseasonalised variable of 25(OH)D in our analysis. Unlike previous epidemiologic studies, blood pressure measurements and lipids concentrations were not collected from medical records but measured during the study follow up according to validated protocols.

Selection bias due to loss to follow-up is always of concern in cohort studies, even though no significant differences in baseline characteristics were revealed between participants and no-participants. Loss to follow up may limit the generalizability of our results. Chance findings are always of concern when multiple comparisons are performed, but because the outcomes were highly correlated and our analysis was considered exploratory, correction for multiple testing would be inappropriate and very restrictive [206]. A limitation of our study could be that we were not able to measure fasting lipid levels, however, it has been shown that fasting lipid levels in children have small differences with non-fasting levels [207]. Although we incorporated extensive information on potential social and environmental factors, that are associated with childhood adiposity and cardiometabolic characteristics, we acknowledge that residual confounding because of other unmeasured confounders, such as social class, and child's vitamin D status may still occur.

In conclusion, our findings support that very low 25(OH)D levels in the first half of pregnancy may increase offspring adiposity indices at preschool and school age. Unlike other causes of childhood obesity maternal hypovitaminosis D is a preventable factor. Further studies and clinical trials are needed to explore the role of vitamin D supplementation during pregnancy in childhood obesity prevention.

Conflicts of interest

The authors have no conflicts of interest to declare.

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VD conducted the data analysis and wrote the first draft of the manuscript, TR supervised the statistical analyses and helped with manuscript preparation, GC, MK, MKar, VL, MV, KS, and MVas participated in data collection, data cleansing, result interpretation and manuscript preparation, SP and MKog provided feedback and critical revision of the manuscript and helped with data interpretation, LC conceived the study, supervised the data collection, and provided feedback and critical revision of the manuscript. All authors have approved the manuscript for submission.

References

1. Holick MF. Vitamin D deficiency. *N Engl J Med.* 2007;357(3):266-281.

2. Urrutia RP, Thorp JM. Vitamin D in pregnancy: current concepts. *Curr Opin Obstet Gynecol.* 2012;24(2):57-64.

3. Hamilton SA, McNeil R, Hollis BW, et al. Profound Vitamin D Deficiency in a Diverse Group of Women during Pregnancy Living in a Sun-Rich Environment at Latitude 32 degrees N. *Int J Endocrinol.* 2010;2010:917428.

4. Karras SN, Anagnostis P, Annweiler C, et al. Maternal vitamin D status during pregnancy: the Mediterranean reality. *Eur J Clin Nutr.* 2014;68(8):864-869.

5. Krishnaveni GV, Veena SR, Winder NR, et al. Maternal vitamin D status during pregnancy and body composition and cardiovascular risk markers in Indian children: the Mysore Parthenon Study. *Am J Clin Nutr.* 2011;93(3):628-635.

6. Javaid MK, Crozier SR, Harvey NC, et al. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. *Lancet.* 2006;367(9504):36-43.

7. Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O'Beirne M, Rabi DM. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. *BMJ.* 2013;346:f1169.

8. Morales E, Romieu I, Guerra S, et al. Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. *Epidemiology.* 2012;23(1):64-71.

9. Sharief S, Jariwala S, Kumar J, Muntner P, Melamed ML. Vitamin D levels and food and environmental allergies in the United States: results from the National Health and Nutrition Examination Survey 2005-2006. *J Allergy Clin Immunol.* 2011;127(5):1195-1202.

10. Mutt SJ, Hypponen E, Saarnio J, Jarvelin MR, Herzig KH. Vitamin D and adipose tissue-more than storage. *Front Physiol.* 2014;5:228.

11. Crozier SR, Harvey NC, Inskip HM, Godfrey KM, Cooper C, Robinson SM. Maternal vitamin D status in pregnancy is associated with adiposity in the offspring: findings from the Southampton Women's Survey. *Am J Clin Nutr.* 2012;96(1):57-63.

12. Morales E, Rodriguez A, Valvi D, et al. Deficit of vitamin D in pregnancy and growth and overweight in the offspring. *Int J Obes (Lond).* 2015;39(1):61-68.

13. Hrudey EJ, Reynolds RM, Oostvogels AJ, Brouwer IA, Vrijkotte TG. The Association between Maternal 25-Hydroxyvitamin D Concentration during Gestation and Early Childhood Cardio-metabolic Outcomes: Is There Interaction with Pre-Pregnancy BMI? *PLoS One.* 2015;10(8):e0133313.

14. Gale CR, Robinson SM, Harvey NC, et al. Maternal vitamin D status during pregnancy and child outcomes. *Eur J Clin Nutr.* 2008;62(1):68-77.

15. Williams DM, Fraser A, Fraser WD, et al. Associations of maternal 25-hydroxyvitamin D in pregnancy with offspring cardiovascular risk factors in childhood and adolescence: findings from the Avon Longitudinal Study of Parents and Children. *Heart.* 2013;99(24):1849-1856.

16. Rytter D, Bech BH, Halldorsson TI, et al. Maternal Vitamin D Status at Week 30 of Gestation and Offspring Cardio-Metabolic Health at 20 Years: A Prospective Cohort Study over Two Decades. *PLoS One.* 2016;11(10):e0164758.

17. Chatzi L, Leventakou V, Vafeiadi M, et al. Cohort Profile: The Mother-Child Cohort in Crete, Greece (Rhea Study). *Int J Epidemiol.* 2017;In press.

18. Royston P, Wright E. A method for estimating age-specific reference intervals (`normal ranges') based on fractional polynomials and exponential transformation. *Journal of the Royal Statistical Society, Series A.* 1998(161):79-101.

19. Long J, Ryoo J. Using fractional polynomials to model non-linear trends in longitudinal data. *Br J Math Stat Psychol.* 2010;63(Pt 1):177-203.

20. Kong J, Li YC. Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. *Am J Physiol Endocrinol Metab.* 2006;290(5):E916-924.

21. Ding C, Gao D, Wilding J, Trayhurn P, Bing C. Vitamin D signalling in adipose tissue. *Br J Nutr.* 2012;108(11):1915-1923.

22. Poissonnet CM, Burdi AR, Garn SM. The chronology of adipose tissue appearance and distribution in the human fetus. *Early Hum Dev.* 1984;10(1-2):1-11.

23. Straughen JK, Trudeau S, Misra VK. Changes in adipose tissue distribution during pregnancy in overweight and obese compared with normal weight women. *Nutr Diabetes.* 2013;3:e84.

24. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr.* 2000;72(3):690-693.

25. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2011;96(7):1911-1930.

26. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology.* 1990;1(1):43-46.

27. Steiner MJ, Skinner AC, Perrin EM. Fasting might not be necessary before lipid screening: a nationally representative cross-sectional study. *Pediatrics.* 2011;128(3):463-470.

Table and figure legends

Table 1 Maternal-child characteristics by maternal 25(OH)-vitamin D levels, Rhea pregnancy cohort, Crete, Greece

Table 2 Associations of maternal 25(OH)-vitamin D levels and offspring obesity outcomes at 4 and 6 years of age

Figure 1 BMI SD score trajectories from birth to 6 years of age for each stratum of high (≥37.7nmol/l) or low (<37.7nmol/l) 25(OH)D levels in (A) all children (b) children with maternal BMI pre-pregnancy≤30 (C) children with maternal BMI pre-pregnancy>30. All models were adjusted for maternal age, maternal education, parity, smoking during pregnancy, and gestational age at sampling.

Table 1 Maternal-child characteristics by maternal 25(OH)-vitamin D[a] levels, Rhea pregnancy cohort, Crete, Greece

		Maternal 25(OH)-vitamin D levels		
	Overall	<37.7nmol/l	≥37.7nmol/l	[b]*P*- value
Season in which maternal blood was collected, *n (%)*				0.16
Winter	107 (20.1)	44 (25.6)	63 (17.5)	
Spring	153 (28.8)	46 (26.7)	107 (29.7)	
Summer	154 (28.9)	44 (25.6)	110 (30.6)	
Autumn	118 (22.2)	38 (22.1)	80 (22.2)	
Maternal characteristics				
Maternal age at delivery (yr), *mean (SD)*	29.7 (4.9)	30.3 (5.0)	29.5 (4.9)	0.22
Maternal Education, *n (%)*				0.72
Low	85 (15.9)	25 (14.5)	60 (16.7)	
Medium	272 (51.2)	87 (50.6)	185 (51.4)	
High	175 (32.9)	60 (34.9)	115 (31.9)	
Primiparous, *n (%)*	243 (45.7)	75 (43.6)	168 (46.7)	0.51
Smoking status during pregnancy, *n (%)*	91 (17.1)	35 (20.4)	56 (15.6)	0.17
Pre-pregnancy maternal BMI, *mean (SD)*	24.9 (4.5)	26.1 (5.8)	24.5 (4.5)	**<0.01**
Maternal pre-pregnancy BMI categories, *n (%)*				**<0.01**
Normal (< 25 kg/m^2)	333 (62.7)	94 (54.9)	239 (66.4)	
Overweight (25 to <30 kg/m^2)	122 (22.9)	41 (24.0)	81 (22.5)	
Obese (≥ 30 kg/m^2)	76 (14.4)	36 (21.1)	40 (11.1)	
Gestational weight gain, *mean (SD)*	13.9 (5.8)	13.6 (6.6)	14.1 (5.5)	0.33
Gestational diabetes, *n (%)*	45 (9.3)	20 (7.6)	25 (12.6)	0.07
Child characteristics in infancy (n=532)				
Sex, girl, *n (%)*	251 (47.2)	81 (47.1)	170 (47.2)	0.97
Birth weight (kg), *mean (SD)*	3.2 (0.4)	3.2 (0.5)	3.2 (0.4)	0.14
Gestational age (weeks), *mean (SD)*	38.3 (1.5)	38.2 (1.7)	38.3 (1.5)	0.80
Breastfeeding duration (months), *mean (SD)*	4.1 (5.4)	4.0 (6.8)	4.2 (4.6)	0.09
Preterm birth, *n (%)*	61 (11.5)	28 (16.3)	33 (9.2)	**0.01**

Rapid growth 0-24 months, *n (%)*	37 (13.4)	12 (13.9)	25 (13.1)	0.84
Child characteristics at 4 years of age (n=532)				
BMI (Kg/m^2), *mean (SD)*	16.5 (1.8)	16.8 (2.0)	16.3 (1.8)	**0.02**
Waist circumference (cm), *mean (SD)*	53.7 (4.7)	54.2 (5.1)	53.5 (4.6)	**0.04**
Sum of skinfolds (mm), *mean (SD)*	41.2 (13.7)	42.9 (14.7)	40.4 (13.1)	0.09
%BF as estimated by skinfolds, *mean (SD)*	19.5 (5.9)	20.4 (6.6)	19.1 (5.6)	**0.03**
Child characteristics at 6 years of age (n=370)				
BMI (Kg/m^2), *mean (SD)*	17.1 (2.6)	17.4 (2.7)	16.8 (2.5)	**0.02**
Waist circumference (cm), *mean (SD)*	58.9 (6.9)	59.8 (6.9)	58.5 (7.0)	**0.04**
Sum of skinfolds (mm), *mean (SD)*	42.7 (17.6)	45.5 (18.1)	41.5 (17.2)	**0.02**
FFM as estimated by BIA, *mean (SD)*	18.6 (2.9)	18.7 (3.1)	18.5 (2.8)	0.96
FM as estimated by BIA, *mean (SD)*	6.3 (2.9)	6.8 (3.0)	6.1 (2.9)	**0.02**
%BF as estimated by BIA, *mean (SD)*	24.5 (6.8)	25.8 (6.9)	23.9 (6.7)	**0.02**

BMI, Body Mass Index; %BF, Body Fat Percentage; FFM, Free Fat Mass; FM, Fat Mass; BIA, Bioelectric Impedance Analysis; *P*-values obtained by Mann-Whitney U test for two independent samples, and χ^2 test or Fisher exact test

[a]Deseasonalized maternal 25(OHD)-vitamin D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

[b]Bolds indicate statistically significant differences at p <0 .05

Table 2 Associations of maternal 25(OH)-vitamin D[a] levels and offspring adiposity outcomes at 4 and 6 years of age

	Maternal 25(OH)-vitamin D per 10nmol/l decrease				Maternal 25(OH)-vitamin D levels[b] <37.7nmol/l			
Adiposity outcomes	Crude Model		Adjusted model		Crude Model		Adjusted model	
Infancy (n = 277)	*RR*	95%CI	*RR*	95%CI	*RR*	95%CI	*RR*	95%CI
Rapid growth 0-24 months	0.99	0.82, 1.21	0.96	0.79, 1.17	0.97	0.53, 1.78	0.91	0.49, 1.71
4 years of age (n = 532)	*β*	95%CI	*β*	95%CI	*β*	95%CI	*β*	95%CI
BMI SD score	**0.05**	**0.10, 0.01**	**0.06**	**0.01, 0.11**	**0.17**	**0.01, 0.34**	**0.20**	**0.03, 0.37**
Waist circumference (cm)	**0.23**	**0.45, 0.01**	**0.24**	**0.02, 0.46**	**0.80**	**0.06,1.55**	**0.87**	**0.12, 1.63**
Sum of skinfolds (mm)	0.61	-0.09, 1.31	0.69	-0.03, 1.40	2.19	-0.21, 4.58	**2.57**	**0.13, 5.01**
%BF as estimated by skinfolds	0.27	-0.03, 0.56	**0.32**	**0.03, 0.62**	**1.21**	**0.21, 2.22**	**1.48**	**0.46, 2.49**
6 years of age (n = 370)	*β*	95%CI	*β*	95%CI	*β*	95%CI	*β*	95%CI
BMI SD score	0.05	-0.01, 0.11	0.05	-0.01, 0.11	0.19	-0.01, 0.39	**0.22**	**0.01, 0.42**
Waist circumference (cm)	**0.39**	**0.01, 0.76**	0.35	-0.02, 0.72	1.24	-0.04, 2.52	**1.39**	**0.13, 2.65**
Sum of skinfolds (mm)	0.82	-0.32, 1.96	0.85	-0.31, 2.01	3.45	-0.43, 7.32	3.64	-0.32, 7.61
FFM as estimated by BIA	0.05	-0.05, 0.15	0.06	-0.04, 0.15	0.20	-0.14, 0.53	0.24	-0.36, 0.84
FM as estimated by BIA	0.17	-0.01, 0.34	0.14	-0.02, 0.32	0.56	-0.04, 1.17	0.60	-0.04, 1.24
%BF as estimated by BIA	**0.46**	**0.03, 0.88**	0.39	-0.02, 0.82	**1.65**	**0.20, 3.09**	**1.59**	**0.13, 3.05**

BMI, Body Mass Index; %BF, Body Fat Percentage; FFM, Free Fat Mass; FM, Fat Mass; BIA, Bioelectric Impedance Analysis;

[a]Deseasonalized maternal 25(OH)-vitamin D concentrations based on month at blood collection for each subject derived from the sinusoidal model. Crude model: minimally adjusted for child sex, age, and height, except models using BMI SD score and rapid growth as an outcome. Adjusted model: crude model further adjusted for maternal age, maternal education, parity, smoking during pregnancy, and gestational age at sampling.

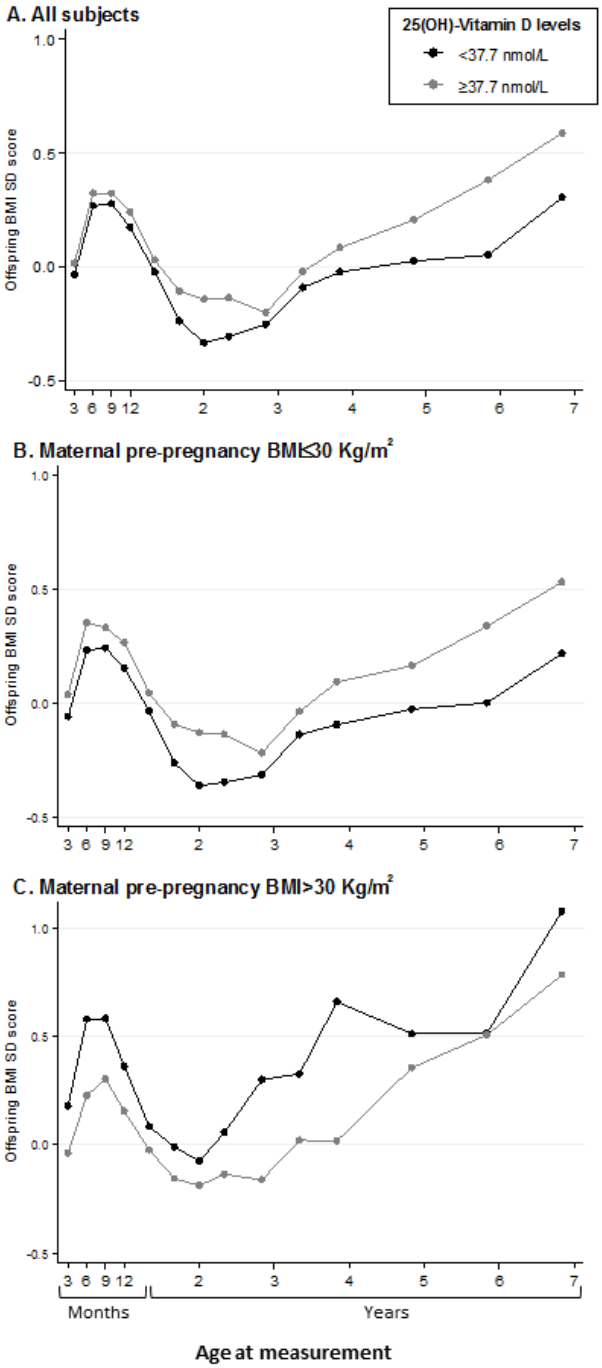

Figure 1 BMI SD score trajectories from birth to 6 years of age for each stratum of high (≥37.7nmol/l) or low (<37.7nmol/l) 25(OH)D levels in (A) all children (b) children with maternal BMI pre-pregnancy≤30 (C) children with maternal BMI pre-pregnancy>30. All models were adjusted for maternal age, maternal education, parity, smoking during pregnancy, and gestational age at sampling**.**

Online Supporting Information

Title: Effect of very low vitamin D levels in pregnancy on offspring obesity indices and cardiometabolic traits in childhood

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Maternal serum 25(OH)D measurements during pregnancy

Maternal non-fasting serum samples at the first trimester of pregnancy (mean: 14weeks, SD: 4) were collected in serum gel separator (BD 367958) tubes, centrifuged and stored at -80ºC until assayed. We used chemiluminescent immunoassay (CLIA) test (DiaSorin, Cat. No. 310600) to measure the total amount of 25(OH)D (both serum $25(OH)D_2$ and $25(OH)D_3$) [166]. The analytical range for the 25(OH)D assay was 10 nmol/l to 375 nmol/l. Inter- and intra-assay precision were <10% and <5% respectively.

Child adiposity indices

Information on weight and length at birth was obtained from the hospital delivery logs and medical records. At follow-up visits (0-24 months, 4 years and 6 years) weight and length/height were measured using validated scales (Seca 354 baby scale, Seca Bellisima 841) and stadiometers (Seca 210 measuring mat, Seca 213), by specially trained research assistants, according to standard operating procedures. Repeated measures of weight and length/height were also abstracted from the children's health cards. We calculated BMI and converted raw values into sex and age specific standard deviation scores (SD scores) using internally generated growth reference curves from multi-level (mixed) linear models fitted with fractional polynomials and random effects for child. We defined rapid infant growth from birth to 24 months as a SD score change in weight of greater than 0.67 [208]. We analyzed child BMI SD score as a continuous outcome at 4 years and 6 years of age.

Waist circumference was measured at 4 and 6 years of age in duplicate to the nearest 0.1 cm in a standing position, at the high point of the iliac crest at the end of a gentle expiration, using a measuring tape (Seca 201). Skinfold thickness was measured at four anatomical sites (triceps, subscapular, suprailiac and thigh) on the right side of the body in triplicate to the nearest 0.1 mm, using a calibrated caliper (Harpenden HSK- BI, CE-0120). Intra- and inter-observer reliability was above 0.98 and 0.82 for all anthropometric measurements respectively.

Body composition was estimated at 6 years follow up by a bioelectric impedance analysis (BIA) performed using a tetra-polar device (Bodystat 1500). All children had not eaten or participated in physical activity a minimum of 120 mins prior to being measured. We used pediatric specific BIA equation [169] to obtain free fat mass (FFM), fat mass (FM) and body fat percentage (%BF) at 6 years of

age. As BIA was not available at the 4 years follow up, we estimated child %BF at 4 years using the Slaughter skinfold-thickness equations [170].

Child cardiometabolic traits

At age four, trained research assistants measured systolic and diastolic blood pressure after 5 minutes rest in the seated position, at the child's right arm, with a cuff of appropriate size for arm circumference using a Dinamap Pro Care 400 (Critikon Inc, Tampa, FL). At age six, children were measured using an OMRON device. We used the average of five consecutive measurements –at age four- or three consecutive measurements –at age six- that were taken at 1-minute intervals. We then calculated blood pressure age, sex, and height specific SD scores [209].

Non-fasting blood samples were collected from the children at both time points. Blood samples were processed within 2 hours, with serum stored at −80°C until analysis. We measured lipids [total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL)] using standard enzymatic methods (Medicon, Greece) on an automatic analyzer (AU5400 high-volume chemistry analyzer; Olympus America, Inc., Melville, New York). Low density lipoprotein cholesterol (LDL) concentration was estimated by using the formula: LDL-C=TC-[(TG/5) + HDL-C].

Potential covariates

Potential confounders included characteristics that have an established or potential association with maternal VitD status and outcomes of interest including: maternal age (years); maternal education (low level: ≤6 yrs. of school; medium level: ≤12 yrs. of school; high level: university or technical college degree); maternal origin (Greek/other); marital status (married/other); parity (primiparous/multiparous); smoking during pregnancy (yes/no); maternal pre-pregnancy BMI (kgr/m^2)), and as normal, overweight, obese; gestational weight gain according to 2009 Institute of Medicine (IOM) guidelines [210]; gestational diabetes defined according to the criteria proposed by Carpenter and Coustan [211]; child sex (male/female); birth weight (g); gestational age, preterm birth (<37 weeks of gestation; yes/no); duration of breastfeeding (months).**e S1.** Maternal and child

characteristics of participants and non-participants in the childhood 4 and 6 years follow up of the Rhea pregnancy cohort Crete, Greece

	4 years					6 years				
	Non participants (n=211)		Participants (n=532)			Non participants (n=152)		Participants (n=370)		
	n	% or Mean (SD)	n	% or Mean (SD)	*P*-value[a]	n	% or Mean (SD)	n	% or Mean (SD)	*P*-value[a]
Maternal characteristics										
Maternal age (years), *mean(SD)*	209	29.9 (5.1)	532	29.8 (4.9)	0.63	151	29.9 (5.1)	370	30.0 (4.8)	0.82
Maternal education, *n (%)*	209		532		0.59	149		370		0.68
Low		40 (19.2)		85 (16.0)			26 (17.4)		54 (14.6)	
Medium		103 (49.3)		272 (51.1)			74 (49.7)		185 (50.0)	
High		66 (31.5)		175 (32.9)			49 (32.9)		131 (35.4)	
Smoking during pregnancy, *n (%)*	192		532		0.76	138		370		0.62
Smoker		31 (16.2)		91 (17.1)			22 (15.9)		66 (17.8)	
Non-smoker		161 (83.8)		441 (82.9)			116 (84.1)		304 (82.2)	
Parity, *n (%)*	210		532		0.41	152		370		0.86
Primiparous		89 (42.4)		243 (45.7)			69 (45.4)		165 (44.6)	
Multiparous		121 (57.6)		289 (54.3)			83 (54.6)		205 (55.4)	
Maternal pre-pregnancy BMI (kg/m^2), *mean(SD)*	211	24.2 (4.4)	531	25.0 (5.0)	0.11	144	24.3 (4.7)	367	25.0 (5.1)	0.27
Maternal pre-pregnancy BMI categories, *n (%)*	200		531		0.43	144		367		0.81
Normal (< 25 kg/m^2)		134 (67.0)		333 (62.7)			97 (67.4)		237 (64.6)	
Overweight (25 to <30 kg/m^2)		44 (22.0)		122 (22.9)			29 (20.1)		83 (22.6)	

Obese (≥ 30 kg/m^2)		22 (11.0)		76 (14.4)			18 (12.5)		47 (12.8)	
Child characteristics										
Child sex, *n (%)*	211		532		0.76	152		370		0.23
Boy		114 (54.1)		281 (52.8)			78 (51.3)		211 (57.1)	
Girl		97 (45.9)		251 (47.2)			74 (48.7)		159 (42.9)	
Gestational age (months), *mean(SD)*	211	38.1 (1.5)	532	38.3 (1.5)	0.19	152	38.0 (1.6)	370	38.2 (1.6)	0.06
Birth weight (kg), *mean(SD)*	201	3.2 (0.4)	532	3.2 (0.5)	0.58	143	3.2 (0.4)	370	3.2 (0.4)	0.73
Breastfeeding duration (months), *mean (SD)*	205	4.7 (5.5)	524	4.1 (5.4)	0.29	152	4.5 (5.7)	370	4.3 (5.9)	0.80
BMI (Kg/m^2), *mean (SD)*	205	16.4 (53.5)	532	16.5 (53.7)	0.19	152	17.0 (3.1)	369	17.0 (2.6)	0.32
Waist circumference (cm), *mean (SD)*	203	53.5 (5.4)	527	53.7 (4.8)	0.15	150	58.4 (7.6)	368	59.0 (7.0)	0.20
Sum of skinfolds (mm), *mean (SD)*	182	39.6 (14.6)	480	41.2 (13.7)	**0.03**	135	42.1 (16.6)	311	42.8 (17.6)	0.79
%BF as estimated by skinfolds, *mean (SD)*	192	18.7 (6.2)	509	19.5 (6.0)	0.05	NA	NA	NA	NA	
FFM as estimated by BIA, *mean (SD)*	NA	NA	NA	NA		147	18.3 (2.9)	360	18.6 (2.9)	0.19
FM as estimated by BIA, *mean (SD)*	NA	NA	NA	NA		147	6.4 (3.7)	360	6.4 (3.2)	0.60
%BF as estimated by BIA, *mean (SD)*	NA	NA	NA	NA		147	24.8 (7.6)	360	24.5 (6.8)	0.86

BMI, Body Mass Index; %BF, Body Fat Percentage; FFM, Free Fat Mass; FM, Fat Mass; BIA, Bioelectric Impedance Analysis; NA, Not Applicable;

[a] Statistically significant differences ($p<0.05$), based on Mann-Whitney U test for two independent samples and Pearson's $\chi 2$ test for independence

Table S2 Associations of maternal 25(OH)-vitamin D[a] levels and offspring cardiometabolic traits at 4 and 6 years of age

	Maternal 25(OH)-vitamin D per 10nmol/l decrease				Maternal 25(OH)-vitamin D[b] levels <37.7nmol/l			
Cardiometabolic traits	Crude Model		Adjusted model		Crude Model		Adjusted model	
4 years of age	*β*	95%CI	*β*	95%CI	*β*	95%CI	*β*	95%CI
Offspring lipid levels (n = 457)								
Total cholesterol (mg/dl)	0.30	-1.39, 1.99	0.28	-1.44, 2.00	0.65	-4.87, 6.17	0.49	-5.15, 6.14
LDL cholesterol (mg/dl)	0.24	-1.21, 1.68	0.31	-1.17, 1.78	1.03	-3.69, 5.76	1.30	-3.54, 6.15
HDL cholesterol (mg/dl)	0.17	-0.47, 0.82	0.11	-0.55, 0.76	0.28	-1.82, 2.39	0.12	-2.04, 2.27
Triglycerides (mg/dl)	0.41	-2.03, 1.21	-0.52	-2.17, 1.14	-2.81	-8.10, 2.47	-4.14	-9.56, 1.28
Blood pressure levels (n = 432)								
SBP SD score	0.05	-0.08, 0.18	0.05	-0.08, 0.18	-0.01	-0.45, 0.44	0.05	-0.40, 0.50
DBP SD score	-0.05	-0.17, 0.08	-0.05	-0.18, 0.08	-0.06	-0.49, 0.37	-0.11	-0.55, 0.33
6 years of age	*β*	95%CI	*β*	95%CI	*β*	95%CI	*β*	95%CI
Offspring lipid levels (n = 345)								
Total cholesterol (mg/dl)	0.63	-0.91, 2.17	0.70	-0.88, 2.29	-0.35	-5.65, 4.95	-1.00	-6.49, 4.50

LDL cholesterol (mg/dl)	0.64	-0.70, 1.98	0.80	-0.58, 2.18	0.51	-4.10, 5.13	0.54	-4.25, 5.33
HDL cholesterol (mg/dl)	0.01	-0.76, 0.77	0.02	-0.77, 0.82	-0.90	-3.53, 1.73	-0.87	-3.62, 1.89
Triglycerides (mg/dl)	0.92	-1.37, 3.21	0.67	-1.67, 3.00	3.91	-3.96, 11.77	1.36	-6.74, 9.45
Blood pressure levels (n = 361)								
SBP SD score	-0.02	-0.07, 0.03	-0.02	-0.07, 0.03	-0.04	-0.21, 0.13	-0.03	-0.20, 0.15
DBP SD score	0.01	-0.03, 0.05	0.01	-0.02, 0.05	-0.01	-0.13, 0.11	0.00	-0.12, 0.13

LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure;

[a]Deseasonalized maternal 25(OH)-vitamin D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

Crude model: minimally adjusted for child sex and age, except models using blood pressure SD score as an outcome.

Adjusted model: crude model further adjusted for maternal age, maternal education, parity, smoking during pregnancy, and gestational age at sampling. Models using offspring lipid levels as an outcome were additionally adjusted for child BMI SD score.

[b]Reference category: maternal 25(OH)-vitamin D levels ≥37.7nmol/l

Table S3 Associations of maternal 25(OH)-vitamin D[a] levels and offspring adiposity outcomes at 4 and 6 years of age stratified by child sex

Adiposity outcomes	Maternal 25(OH)-vitamin D per 10nmol/l decrease					Maternal 25(OH)-vitamin D[b] levels <37.7nmol/l				
	Male		Female		*P for interaction*	Male		Female		*P for interaction*
Infancy (n = 277)	*RR*	95%CI	*RR*	95%CI		*RR*	95%CI	*RR*	95%CI	
Rapid growth 0-24 months	0.96	0.77, 1.20	1.01	0.69, 1.48	0.879	0.82	0.38, 1.74	1.22	0.41, 3.72	0.671
4 years of age (n = 532)	*β*	95%CI	*β*	95%CI		*β*	95%CI	*β*	95%CI	
BMI SD score	0.05	-0.02, 0.12	0.05	-0.02, 0.12	0.985	0.22	-0.02, 0.44	0.19	-0.06, 0.45	0.952
Waist circumference (cm)	0.12	-0.19, 0.44	0.33	0.01, 0.65	0.399	0.64	-0.36, 1.65	1.13	-0.04, 2.29	0.553
Sum of skinfolds (mm)	0.03	-0.91, 0.96	**1.42**	**0.32, 2.52**	**0.069**	1.05	-1.96, 4.07	4.82	0.81, 8.84	0.156
%BF as estimated by skinfolds	0.10	-0.27, 0.48	0.58	0.10, 1.05	0.161	0.78	-0.43, 2.01	2.48	0.77, 4.19	0.136
6 years of age (n = 370)	*β*	95%CI	*β*	95%CI		*β*	95%CI	*β*	95%CI	
BMI SD score	0.03	-0.05, 0.11	0.07	-0.02, 0.16	0.520	0.14	-0.13, 0.40	0.37	0.06, 0.68	0.339
Waist circumference (cm)	0.11	-0.41, 0.63	0.54	0.01, 1.07	0.251	0.37	-1.33, 2.06	**2.56**	**0.65, 4.48**	**0.088**
Sum of skinfolds (mm)	0.59	-1.15, 2.33	1.13	-0.39, 2.65	0.638	2.75	-2.91, 8.40	5.42	-0.14, 10.98	0.564
FFM as estimated by BIA	0.05	-0.09, 0.18	0.07	-0.09, 0.23	0.837	0.09	-0.34, 0.52	0.55	-0.01, 1.11	0.215

FM as estimated by BIA	-0.01	-0.25, 0.24	0.28	0.04, 0.52	0.119	0.16	-0.63, 0.96	**1.19**	**0.34, 2.04**	**0.094**
%BF as estimated by BIA	0.01	-0.59, 0.60	0.73	0.13, 1.33	0.115	0.52	-1.41, 2.45	2.86	0.69, 5.03	0.125

BMI, Body Mass Index; %BF, Body Fat Percentage; FFM, Free Fat Mass; FM, Fat Mass; BIA, Bioelectric Impedance Analysis;

[a]Deseasonalized maternal 25(OH)-vitamin D concentrations based on month at blood collection for each subject derived from the sinusoidal model.

All models were adjusted for child age and height, maternal age, maternal education, parity, and smoking during pregnancy. Models using BMI SD score and rapid growth and BMI SD score as an outcome were not adjusted for child age and height.

[b]Reference category: maternal 25(OH)-vitamin D levels ≥37.7nmol/l

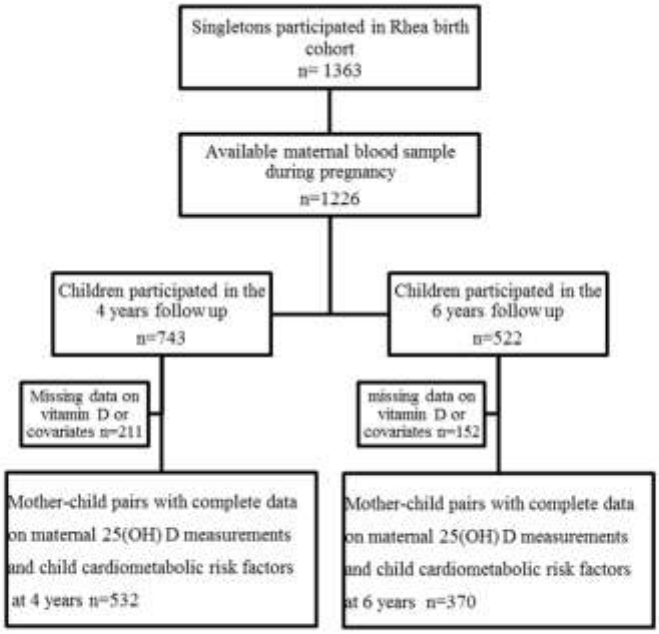

Figure S1 Flowchart of our study population

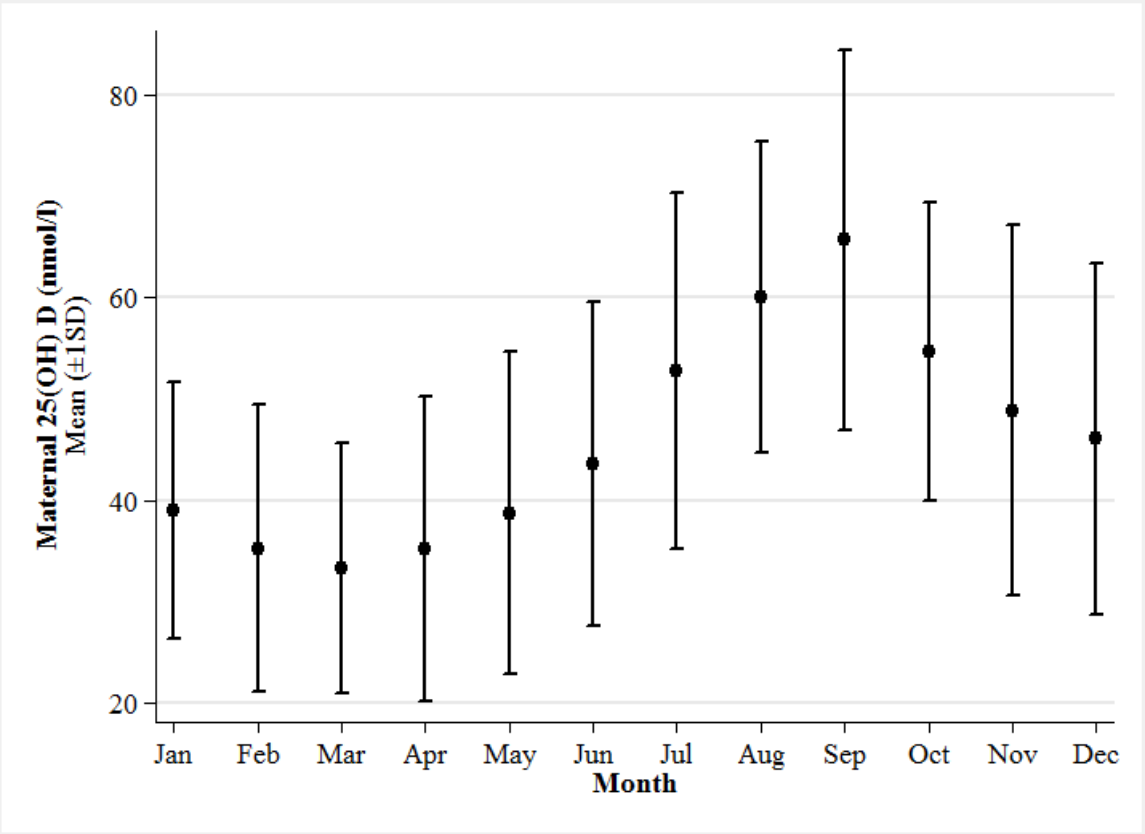

Figure S2 The distribution of mean 25(OH)-vitamin D concentration plotted by calendar month in our study population

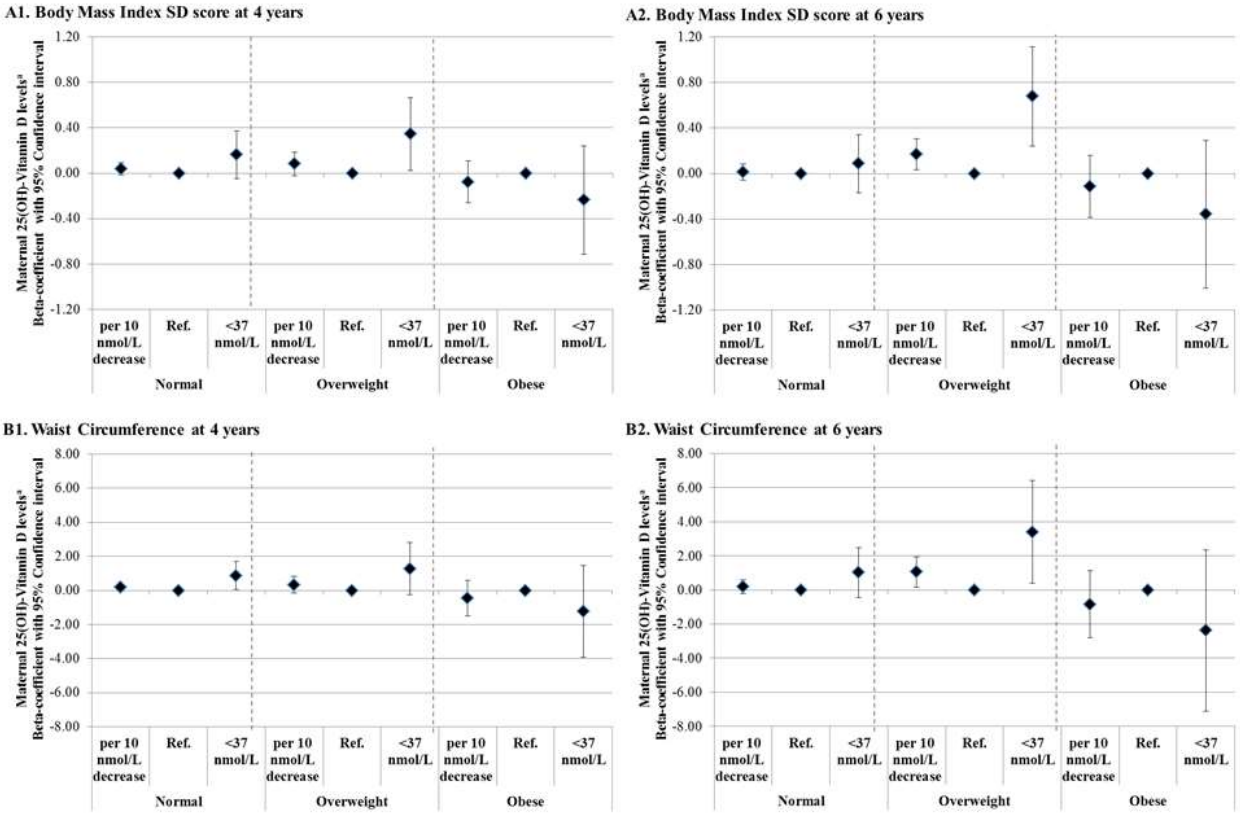

Figure S3 Associations of maternal 25(OH)-vitamin D levels with child BMI SD score (A1) at 4 years (A2) at 6 years and child waist circumference (B1) at 4 years (B2) at 6 years, stratified by maternal BMI pre-pregnancy categories. All models were adjusted for maternal age, maternal education, parity, smoking during pregnancy, and gestational age at sampling. Models with waist circumference were further adjusted with child sex, age, and height.

References

1. Katrinaki M, Kampa M, Margioris A, Castanas E, Malliaraki N. Vitamin D levels in a large Mediterranean cohort: reconsidering normal cut-off values. *Hormones (Athens).* 2016;15(2):205-223.

2. Monteiro PO, Victora CG. Rapid growth in infancy and childhood and obesity in later life--a systematic review. *Obes Rev.* 2005;6(2):143-154.

3. Clasey JL, Bradley KD, Bradley JW, Long DE, Griffith JR. A new BIA equation estimating the body composition of young children. *Obesity (Silver Spring).* 2011;19(9):1813-1817.

4. Slaughter MH, Lohman TG, Boileau RA, et al. Skinfold equations for estimation of body fatness in children and youth. *Hum Biol.* 1988;60(5):709-723.

5. Falkner B, Daniels SR. Summary of the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents. *Hypertension.* 2004;44(4):387-388.

6. Rasmussen KM, & Yaktine, A. L.,. *Institute of medicine (committee to reexamine iom pregnancy weight guidelines, food and nutritions board and board on children, youth, and families) weight gain during pregnancy : Reexamining the guidelines.* Washington D.C.2009.

7. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* 2008;31 Suppl 1:S55-60.

5. Discussion

Findings from the present thesis support an important effect of maternal metabolic diseases and vitamin D status in early pregnancy on children's adiposity indices, cardiometabolic traits and neurodevelopmental outcomes at preschool age. To our knowledge, this is the first study that assesses the impact of metabolic dysregulation and low vitamin D levels on obesity indices, cardiometabolic traits and neurodevelopmental difficulties in the Greek children. This section is meant to be a global discussion and provides a broader and more integrated interpretation of the entire research study. It summarizes the detailed discussions of the individual articles presented in the results section of this thesis.

5.1 General discussion of the present findings

Metabolic profile in early pregnancy and offspring adiposity and cardiometabolic traits at preschool age

The association of metabolic dysregulation in early pregnancy with offspring adiposity measures and cardiometabolic traits, is described in detail in paper 1. To our knowledge this is the first study examining the potential role of multiple components of metabolic syndrome in early pregnancy, in association with offspring cardiometabolic outcomes at preschool age.

In accordance with previous epidemiological data we found that pre-pregnancy overweight/obesity was associated with increased BMI measures[33,34,212], central adiposity, and fat mass[36,40,64,213,214] at 4 years of age. It has been hypothesized, that shared environmental, life-style and genetic characteristics may explain the aforementioned associations[215]. In our study effect estimates remained the same after adjustment for paternal BMI and several family and child characteristics, implying potential intrauterine mechanisms in the observed relationships. Animal studies, also, support a potential role of maternal obesity in fetal metabolic programming, by modulating the expression of several genes, such as genes encoding hormones (e.g., leptin), nuclear receptors (adipogenic and lipogenic transcription factors such as PPARγ and PPARα, respectively), gluconeogenic enzymes and transmembrane proteins[216]. Additionally, maternal obesity may impact placental morphology,

blood flow, feto-maternal exchanges and endocrine function, resulting in increased susceptibility to metabolic disorders later in life[217].

Our findings were more pronounced in girls whose mothers were overweight/obese prior to gestation. However, evidence on offspring sex-specific responses to maternal weight status are inconsistent, while sex specificity in response to maternal anthropometry has previously been shown only in fetal growth measures[218]. Timing of exposure to maternal weight changes and subsequent fetal over nutrition may explain in part the observed heterogeneity between studies. Female fetuses seem to respond more than males to the mother's nutrition and metabolism prior to conception, while male fetuses are more vulnerable to metabolic changes after the first trimester of pregnancy[219,220], through sex-specific differences in the regulation and expression of placental genes, proteins, steroids and structure[221,222].

We showed for the first time, that high diastolic blood pressure levels in early pregnancy were associated with increased risk of offspring overweight/obesity and increased fat mass at 4 years of age, independently of pre-pregnancy BMI, gestational weight gain, or pre-eclampsia. Potential mechanisms for the observed associations remain unclear. In normal pregnancies the first trimester is characterized by a decrease in maternal blood pressure levels due to vasodilatation[223]. However, when the typical physiological changes in the spiral arteries of the decidua and myometrium are disrupted, women may exhibit higher blood pressure levels than expected, resulting in poor placental perfusion, early placental hypoxia and oxidative stress[223], which in turn may alter fetal metabolic programing, independently of the effect of pre-eclampsia in late pregnancy.

Finally, in this study, we examined for the first time the potential role of fasting glucose and maternal lipids in early pregnancy on multiple cardiometabolic traits in preschoolers. We did not found an association between maternal glucose intolerance in early pregnancy and cardiometabolic outcomes examined in offspring. However, in accordance with a previous study[64], we found that high fasting cholesterol levels in early pregnancy were associated with increased risk of offspring overweight/obesity, and greater fat mass at 4 years of age. Our findings were not attenuated after adjustment for maternal BMI pre-pregnancy, gestational diabetes status, birthweight, and child BMI at age of outcome assessment. Potential mechanisms for the observed associations could be genetic, environmental and shared life style characteristics, but also increased oxidative stress in the fetus due to maternal dyslipidemia. Animal studies have shown that intrauterine exposure to high fat levels results

in decreased activity of mitochondrial enzymes and increased expression of several genes involved in the oxidative stress and inflammatory pathways in the liver of offspring[224], causing permanent metabolic alterations and predisposing to metabolic syndrome later in life[7]. Hypercholesterolemia in early pregnancy has also been associated with increased offspring atherosclerotic lesions both in animal models[225], and in human tissues[226]; thus may further increase offspring cardiometabolic risk probably due to the induction of a constitutional state of overexpression of "atherogenes" in the fetal vascular wall[226].

Overall, the findings of the present study indicate that among components of metabolic syndrome in early pregnancy, maternal overweight/obesity, diastolic blood pressure levels, and hypercholesterolemia may, independently, determine offspring adiposity at preschool age. The complex underlying mechanisms that explain these findings are not clear, but modulation of gene expression and increased oxidative stress may have a profound role. We found no association between maternal metabolic diseases in early pregnancy and blood pressure or lipid levels in preschoolers.

Parental obesity, glucose intolerance in early pregnancy, and gestational diabetes on child neuropsychological and behavioral development at preschool age

The impact of metabolic dysregulation during pregnancy on offspring neuropsychological and behavioral development, is described in detail in paper 2. Our findings support an association between maternal obesity pre-pregnancy with decreased cognitive scores and increased behavioral problems and ADHD-like symptoms at preschool age. Adjustment for paternal BMI, maternal intelligence, and several other family and child characteristics did not change the direction of the associations, suggesting a limited role of socioeconomic and family parameters or maternal genetic confounding, on the observed estimates. We did not find evidence of an association between paternal overweight/obesity with child neurodevelopmental outcomes in preschoolers.

Few previous studies have controlled for family background by comparing the associations of maternal and paternal BMI on child neurodevelopmental outcomes, with inconclusive results[45-48], probably due to different study designs, and neurodevelopmental outcomes examined, by each study. In addition, most of these studies have examined only one neurodevelopmental outcome, making it difficult to determine whether offspring are at risk

for cognitive dysfunction or abnormal behavior. In this analysis, we examined a greater board of neurodevelopmental outcomes, including cognitive abilities, behavioral problems and ADHD-like symptoms in preschoolers, and we found an important impact of maternal, but not paternal obesity on almost all outcomes examined.

Maternal obesity pre-pregnancy has been linked to an inflammatory in utero environment, resulting in dysregulation of hormonal or immune system, placental transport of excess nutrients and increased oxidative stress[79,227] which may impair fetal neurodevelopment[228]. Evidence from animal studies support that maternal obesity in mice can affect oxidative status and progenitor cell division in offspring brain, resulting in decreased hippocampal neurogenesis[42], and consequently impaired hippocampus-dependent cognitive functions in offspring. Moreover, analysis of the amniotic fluid transcriptome in human fetuses of obese women revealed gene expression patterns suggestive of decreased brain apoptosis; lipid, insulin and appetite dysregulation; and increased estrogen and inflammatory signalling, which may predispose to mental disorders later in life[43]. However, plausible biological mechanisms are not clear yet.

We also showed for the first time that the effect of maternal obesity pre-pregnancy on offspring neurodevelopmental outcomes, was more pronounced in children born large for gestational age (LGA). Previous studies have already demonstrated the metabolic impact of over nutrition on the growing fetus[229], but little is known for its impact on brain formation and function. Plausible mechanisms could be developmental adaptations to mild maternal hyperglycaemia, changes in hormonal status and increased pre-inflammatory cytokines[33]. However, further studies are needed to replicate the above results.

In this analysis we also investigated for the first time the association between maternal fasting glucose and insulin levels in early pregnancy and offspring neurodevelopment at preschool age, and we found no significant impact. In contrast we observed that exposure to GDM, resulted in a positive trend in almost all the examined cognitive scores at 4 years of age. The observed estimates remained after adjustment for several sociodemographic characteristics. The lack of significant differences in offspring metabolic outcomes, including LGA and prematurity between women exposed to GDM and women with normal glucose metabolism supported a good diabetic control in our study population, which may explain our positive

results. A recent review also supported that offspring cognitive performance could be within normal limits in well-controlled GDM women[80].

Maternal vitamin D levels in early pregnancy and offspring cognitive function and behavioral difficulties at preschool age

Despite abundant sunshine, Greece is highly recognized among the countries with increasing prevalence of vitamin D deficiency. Paper 3 is the first study to examine the association of 25(OH)D concentrations during pregnancy on multiple offspring neurodevelopmental outcomes, in a Greek population. In line with previous studies[158], we found that exposure to high 25(OH)D levels (>50.7 nmol/l) was associated with reduced number of hyperactivity-impulsivity and total ADHD-like symptoms, compared to lower 25(OH)D levels, at preschool children. However we investigated more aspects of child behavior in our study and we additionally found a strong association between high 25(OH)D levels in early pregnancy and reduced number of total behavioral problems, including externalizing symptoms in preschoolers. We also observed a trend of higher scores in a wide broad of cognitive abilities at 4 years of age, among children of women in the high 25(OH)D tertile, compared to children of women in the low 25(OH)D tertile. Effect estimates remained the same after adjustment for maternal IQ, suggesting a limited role of maternal genetic confounding. Our findings support that a vitamin D cut off value of 50 nmol/l during pregnancy, may be essential beyond skeletal development, for prevention of behavioral difficulties in childhood.

Maternal vitamin D performs a number of biological functions that are fundamental in early brain development[186], including proliferation and differentiation of brain cells[191], regulation of axonal growth[192], calcium signalling within the brain, and neurotrophic and neuroprotective actions[192]. Animal studies have shown an association between prenatal vitamin D deficiency and brain morphological changes[191] that may persist postnatally, despite restoring vitamin D levels to normal values, resulting in abnormal behaviors in adulthood[193]. However, human studies are limited so far and plausible biological mechanisms are not clear yet.

In this study, we showed for the first time that high levels of 25(OH)D in early pregnancy had a stronger protective effect on behavioral difficulties and total ADHD-like symptoms in females compared to males. It has been previously shown that immunomodulatory effects of vitamin D are significantly stronger in females than in males suffering from multiple

sclerosis, supporting estrogen-promoted differences on vitamin D metabolism and action[194]. Whether there is also a functional synergy between estradiol and vitamin D action on prenatal brain development remains to be investigated.

Maternal vitamin D levels during pregnancy and offspring obesity indices and cardiometabolic traits in childhood

The impact of very low maternal 25(OH)D levels in the first half of pregnancy on offspring obesity indices and cardiometabolic traits, at preschool age, is examined for the first time in a Greek population in paper 4 (currently under revision). Interestingly, about two-thirds of pregnant women, participating in our study, had vitamin D deficiency [25(OH)D levels <50 nmol/l] during pregnancy, while one third of women had 25(OH)D levels<37.7nmol/l . In accordance with previous studies investigating the impact of maternal 25(OH)D levels in the first half of pregnancy on offspring adiposity[154], we found an inverse relationship with body fat percentage at 4 and 6 years of age. However we investigated a greater board of obesity outcomes and we showed that maternal 25(OH)D levels <37.7 nmol/l were, additionally, associated with increased offspring BMI SD score and central adiposity at preschool and school age.

Underlying mechanisms affecting the aforementioned associations remain unclear. Animal studies support a key modulating role of vitamin D in adipocyte differentiation, by inhibiting the expression of significant adipogenesis regulators, like PPARγ[201]. In vitro studies have, also, shown that exposure of human pre-adipocytes to vitamin D metabolites reduces lipid accumulation early in developmental process and may inhibit the initiation of human pre-adipocyte differentiation[202]. During human embryogenesis, white fat lobules appear at 14 weeks of gestation, and their total number remains approximately constant after the 23rd gestational week[203]. After birth, expansion of adipose tissue occurs mainly through increase in adipocyte size. Our findings support that exposure to very low vitamin D levels in the first half of pregnancy-the critical time point of adipogenesis- may increase fetal adipocyte number, which in turn may result to increased fat mass and central adiposity later in life.

In our study, we showed for the first time that maternal obesity may modify the direction of maternal 25(OH)D associations with adiposity indices in childhood, as in contrast with our main findings, offspring of mothers with very low 25(OH)D levels and obesity had decreased BMI SD scores and central adiposity, at 4 and 6 years of age. Because circulating levels of

25(OH)D depend on its storage in visceral adipose tissue[205] variations in fat distribution during pregnancy in women with different BMI levels may partly explain our findings. Recently has been shown that women with normal weight have a higher rate of weight increment and an increased pre-peritoneal fat thickness across gestation compared to women with overweight/obesity[204], probably resulting in a higher 25(OH)D accumulation in visceral fat.

Finally in line with previous data we did not find an effect of 25(OH)D status on offspring blood pressure or lipid levels in childhood. However we were not able to estimate a possible 25(OH)D impact on offspring glucose metabolism, as child glucose and insulin measurements were not available in our study.

5.2 Methodological issues

This thesis includes studies that aimed to investigate the impact of components of metabolic syndrome and vitamin D status in early pregnancy on children's adiposity, cardiometabolic traits, and neurodevelopment. The data of these studies were mainly derived from the Rhea pregnancy cohort, a prospective population based birth cohort study in Greece. Several methodological issues are acknowledged in the following section which provides a general description of selection and information bias.

Selection and Information bias

Selection bias are a major concern in epidemiological studies and occur, when the subjects studied, are not representative of the target population, due to the procedures followed to select the study participants from the source population at the stage of the recruitment and/or during the procedure of retaining them in the study (follow ups)[230]. It is generally accepted that the enrollment of subjects will not bias prospective cohort studies, because the outcome has not yet occurred. However, retention of subjects during long follow-up periods may be differentially related to exposure and outcome, while poor response is usually associated with a lower educational level or socioeconomic status and a less favorable lifestyle[231]. Overall, attrition in the Rhea pregnancy cohort was inevitable and may limit the generalizability of our results. However, no significant differences were revealed, between participants and no-participants' baseline characteristics, in almost all the studies including in the present thesis.

Therefore, this type of selection bias may not have biased our observations in either direction (i.e. overestimation or underestimation).

The other major class of bias in epidemiological studies arises from errors in measuring exposure or disease, during the data collection, known as information bias[232]. In the present thesis, information on maternal and paternal pre-pregnancy weight were self-reported, which might have led to misclassification of maternal/paternal BMI pre-pregnancy in papers 1 and 2. However, we have performed a validation study comparing self-reported maternal pre-pregnancy weight with clinically measured weight in the first prenatal visit, which showed high correlation (r 0.93) and a fairly good agreement between self-reported and objectively measured maternal BMI. Because clinical examination of fathers was not included in our study protocol, paternal misreporting due to maternal providing information on fathers anthropometry cannot be discounted. However, we did not found a significant association between paternal BMI categories and offspring outcomes examined; therefore data on paternal BMI could not have biased our observations. Other source of information bias in the present thesis could be assessment of children's ADHD-like symptoms and behavioral difficulties, using parent-reported measures, which could be different from assessments made by a health-care professional. In order to overcome these issues, we used well-established and widely used questionnaires, with high specificity and sensitivity. Parents were also blind to the main hypotheses of the research.

5.3 Strengths and limitations

A general description of strengths and limitations of the present thesis that have been extensively discussed in the papers, is summarized in the following section. Studies included in this thesis are part of a population-based prospective pregnancy cohort the 'Rhea' study which provided us the opportunity to account for the effect of exposures prospectively within the cohort and for a number of potential predictors of child adiposity, cardiometabolic traits and neurodevelopmental outcomes. Strengths of this thesis, also, include the use of fasting serum samples and the detailed cardiometabolic serum measurements in early pregnancy according to validated protocols. In addition, we estimated maternal vitamin D status during pregnancy by measuring 25(OH)D concentration, a reliable indicator of vitamin D synthesis and intake, whereas we minimized the potential effect of season in our results, by using the deseasonalised variable of 25(OH)D in our analysis.

Unlike previous epidemiological studies, offspring cardiometabolic traits at preschool age, were measured during the study follow ups, by special trained assistants, according to standardized procedures. In addition, offspring neurodevelopmental assessments at 4 years of age, were conducted by two trained psychologists via a strict protocol, by using McCarthy Scales of Children's Abilities (MSCA) a valid, standardized psychometric test which provides both a general level of child's intellectual functioning, as well as an assessment of specific neurodevelopmental domains (cognition, language and motor abilities) and thus, detect which domain is mostly affected. In order to assess behavioral problems at preschool age, we also used the ADHD test and the Strengths and Difficulties (SDQ) questionnaire, which are well-established and widely used child behavior scales, with high specificity and sensitivity. The SDQ questionnaire assess several child behavioral aspects including, emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems, and prosocial behavior, whereas the ADHD test estimates different ADHD-like symptoms, such as impulsivity, inattention, and hyperactivity symptoms. Both tests enable a more detailed evaluation of child behavior, but they also provide a total score of behavioral difficulties in childhood.

We acknowledge that there are also some limitations in the present thesis. Although, we incorporated extensive information on potential social and environmental factors, that were associated with exposures and outcomes of interest in our multivariate models, residual confounding of other unmeasured confounders may still occur, due to the observational study design. Another limitation of papers 1 and 3, was that we were not able to measure fasting lipid levels in offspring, however, it has been shown that fasting lipid levels in children have small differences with non-fasting levels[207].

5.4 Public health implications and future research

Studies on the developmental origins of health and disease have mainly been focused on nutrition during pregnancy. However, the identification of other potential adverse influences in utero, such as maternal metabolic diseases and maternal vitamin D status, on offspring development and long term consequences remains challenging. Data exist so far only for maternal exposures in late pregnancy, such as the potential impact of gestational diabetes or pre-eclampsia on offspring outcomes. However, the first months of gestation, are recently

recognized as a period of particular metabolic and epigenetic plasticity, as several fetal structures and organs are first formed. Therefore, the investigation of the impact of maternal exposures in early pregnancy provides a potential window for developing preventive strategies and therapeutic interventions, for a better metabolic and neurodevelopmental programming in offspring.

Maternal dysregulation in early pregnancy and child health outcomes

Our findings highlight the importance of maternal BMI pre-pregnancy on offspring adiposity measures and neurodevelopmental outcomes. We observed a positive linear association of maternal pre-pregnancy overweight/obesity with the probability of overweight/obesity, increased central adiposity and increased fat mass at preschool age (paper 1). We also found an inverse relationship between maternal weight status prior to gestation, with both cognitive function and behavioral difficulties in preschoolers (paper 2). These findings support the need for national and international strategies to increase awareness in pregnancy-care professionals to promote preconceptional normalization of weight among young women, during family planning.

We also found that maternal hypercholesterolemia and increased diastolic blood pressure in early pregnancy were positively associated with increased risk of overweight/obesity and fat mass at 4 years of age (paper 1). Our results provide evidence that components of metabolic syndrome in early pregnancy may contribute to obesity epidemic in childhood, and indicate the need of developing strategies for early identification and appropriate treatment of maternal metabolic disorders, even in the first months of pregnancy.

Maternal 25(OH)D status in early pregnancy and child health outcomes

In this thesis we also highlight the profound role of sufficient maternal vitamin D status during pregnancy on offspring metabolism and mental health. We found for the first time that high maternal 25(OH)D levels in early pregnancy may protect from several behavioral difficulties, including hyperactivity/inattention, and total ADHD-like symptoms at 4 years of age (paper 3), whereas very low maternal 25(OH)D levels in the first half of pregnancy may increase BMI SD score, central adiposity and fat mass percentage at preschool and school age (paper 4). Additionally, in line with previous data, on countries of Mediterranean region, we

found a high prevalence of vitamin D deficiency in our study population, a phenomenon known as the Mediterranean paradox. Because of the increased sunshine hours, hypovitaminosis D is unrecognized and underrated in several South European countries including Greece, with severe consequences in both the mother and the child. Our findings support the need of developing national and international preventive strategies, for maternal vitamin D deficiency, such as screening in high-risk future mothers and fortification of dairy products with vitamin D. Furthermore, appropriately designed randomized controlled clinical trials for vitamin D supplements during pregnancy are emergently needed to provide definite clinical recommendations, regarding safety and efficiency of vitamin D supplementation in pregnant women.

Future Research

In this thesis, we support an important association between maternal exposures in early pregnancy and offspring anthropometric measures, cardiometabolic traits and neurodevelopmental outcomes at preschool age, but we could not provide data on plausible pathogenetic mechanisms. In vitro and animal studies have shown that maternal obesity[233] and maternal vitamin D status[234], investigated in our study, may cause epigenetic regulation of gene expression, such as DNA methylation, that alter gene expression independent of gene sequence. Advances in genomic technologies have opened up the possibility of population-based assessment of epigenetic patterns and their influence on disease, mainly throughout birth cohorts, that start in early life[235]. In RHEA cohort we have hold DNA, collected at multiple time points, and we may be able to investigate, in the future, epigenetic mechanisms underlying the observed associations in early pregnancy, a period when the epigenome is believed to be particularly plastic.

In addition, it is increasingly acknowledged that the precise mode of action and the full spectrum of activities of vitamin D on human health depend not only on its levels, but also on genetically modified expression of its receptor VDR or other genes involved in vitamin D metabolism. Recent data support an association of VDR polymorphisms with increased susceptibility to multiple human diseases, including osteoporosis[236], autoimmune diseases[237] and cancer[238]. Further investigation of VDR polymorphisms in mother-child pairs of our cohort may contribute to a better understanding of the role of vitamin D-VDR system in utero and reveal individuals with increased susceptibility to metabolic and neurodevelopmental disorders later in life.

Almost all maternal exposures investigated in our study had an important impact on offspring adiposity indices, including BMI SD score, waist circumference and fat mass estimated by skinfolds measures. It is well known that increased anthropometric measures are associated with cardiometabolic risk later in life. However, this relationship is not straightforward; as for any given BMI level, there is a significant variability, with some lean individuals developing cardiovascular disease and others remaining healthy, despite severe obesity, the so-called metabolically healthy obese. This phenomenon may be due to variation in fat distribution and depot differences in adipose tissue function. Visceral adipose tissue and smaller fat depots in close proximity to the heart (epicardial adipose tissue) are considered most accurate indicators of the subsequent risk, severity and progression of atherosclerosis[239]. Future research in our cohort could deepen in plausible pathophysiologic mechanisms, by examining body fat distribution in late childhood and adolescence, using imaging techniques, such as CT or MRI of the abdomen. These tools provide quantitative measurement of body fat and estimate with higher accuracy than anthropometric measurements, visceral fat and smaller fat depots in close proximity to vital organs. In addition, measurement of essential adipokines, such as adiponectin and resistin in children and the investigation of their association with maternal metabolic environment or vitamin D levels in early pregnancy may provide additional data of offspring susceptibility to later cardiometabolic disease.

Given the increased prevalence of behavioral difficulties and ADHD disorders in children the last decades, it would also be important to investigate in our cohort, whether metabolic dysregulation and maternal vitamin D status in early pregnancy are associated with offspring different patterns of brain function and response to several stimulus, predisposing to neurodevelopmental disorders. Neuroimaging methods, such as positron emission tomography and functional magnetic resonance imaging may help to have a more comprehensive picture of complexity of networks involved in ADHD[240] or other behavioral and cognitive problems and deepen in the mechanisms of the impact of adverse intrauterine environment in these disorders.

The interaction of clinical and epidemiological science is essential in evaluation of study outcomes and in defining future directions. Multiple groups need to work together in large national and international level to better understand biological mechanisms and design appropriate preventive strategies. Researches in the field of epidemiology, endocrinology,

pediatrics, psychology, psychiatry and genetics should collaborate in order to improve the current knowledge of how maternal metabolic diseases and hormone status in utero affect offspring metabolism and mental health in later life.

6. Conclusions

In summary, findings in the present thesis support that maternal metabolic diseases and vitamin D levels, in the first months of gestation, may have a significant impact in the development of metabolic and neuropsychological disorders later in life. More specifically, overweight/obese women prior to gestation gave birth to overweight/obese offspring with increased central adiposity and fat mass, whereas maternal hypercholesterolemia and increased diastolic blood pressure in early pregnancy had also a positive relationship with increased adiposity measures at preschool age. From the maternal metabolic diseases examined, only maternal pre-pregnancy obesity was associated with decreased cognitive scores and increased behavioral problems at preschool age. In addition, maternal vitamin D levels in early pregnancy had a profound role in both metabolic and mental programming in offspring. Sufficient maternal vitamin D status in the first half of pregnancy seemed to improve cognitive abilities and have a protective role on behavioral difficulties and ADHD-like symptoms at 4 years of age. In contrast, very low vitamin D levels were associated with increased offspring obesity measures and particularly central adiposity and increased fat percentage in early childhood, predisposing to metabolic syndrome later in life.

7. References

1. Lau C, Rogers JM, Desai M, Ross MG. Fetal programming of adult disease: implications for prenatal care. *Obstet Gynecol.* Apr 2011;117(4):978-985.

2. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. *Am J Clin Nutr.* May 2000;71(5 Suppl):1344S-1352S.

3. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med.* Jul 3 2008;359(1):61-73.

4. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia.* Jul 1992;35(7):595-601.

5. Gluckman PD, Hanson MA, Morton SM, Pinal CS. Life-long echoes--a critical analysis of the developmental origins of adult disease model. *Biol Neonate.* 2005;87(2):127-139.

6. Kieling C, Baker-Henningham H, Belfer M, et al. Child and adolescent mental health worldwide: evidence for action. *Lancet.* Oct 22 2011;378(9801):1515-1525.

7. Barnes SK, Ozanne SE. Pathways linking the early environment to long-term health and lifespan. *Prog Biophys Mol Biol.* Jul 2011;106(1):323-336.

8. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. *Science.* Sep 17 2004;305(5691):1733-1736.

9. Gaillard R. Maternal obesity during pregnancy and cardiovascular development and disease in the offspring. *Eur J Epidemiol.* Nov 2015;30(11):1141-1152.

10. Van Lieshout RJ, Taylor VH, Boyle MH. Pre-pregnancy and pregnancy obesity and neurodevelopmental outcomes in offspring: a systematic review. *Obes Rev.* May 2011;12(5):e548-559.

11. Van Lieshout RJ. Role of maternal adiposity prior to and during pregnancy in cognitive and psychiatric problems in offspring. *Nutr Rev.* Oct 2013;71 Suppl 1:S95-101.

12. Bechet D. Control of gene expression by steroid hormones. *Reprod Nutr Dev.* 1986;26(5A):1025-1055.

13. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr.* Apr 2008;87(4):1080S-1086S.

14. Hossein-nezhad A, Holick MF. Optimize dietary intake of vitamin D: an epigenetic perspective. *Curr Opin Clin Nutr Metab Care.* Nov 2012;15(6):567-579.

15. Al-Garawi A, Carey VJ, Chhabra D, et al. The Role of Vitamin D in the Transcriptional Program of Human Pregnancy. *PLoS One.* 2016;11(10):e0163832.

16. Schroth RJ, Lavelle CL, Moffatt ME. Review of vitamin D deficiency during pregnancy: who is affected? *Int J Circumpolar Health.* Apr 2005;64(2):112-120.

17. Amegah AK, Klevor MK, Wagner CL. Maternal vitamin D insufficiency and risk of adverse pregnancy and birth outcomes: A systematic review and meta-analysis of longitudinal studies. *PLoS One.* 2017;12(3):e0173605.

18. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA.* May 16 2001;285(19):2486-2497.

19. Kassi E, Pervanidou P, Kaltsas G, Chrousos G. Metabolic syndrome: definitions and controversies. *BMC Med.* May 05 2011;9:48.

20. Parikh RM, Mohan V. Changing definitions of metabolic syndrome. *Indian J Endocrinol Metab.* Jan 2012;16(1):7-12.

21. Ervin RB. Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003-2006. *Natl Health Stat Report.* May 05 2009(13):1-7.

22. Huda SS, Brodie LE, Sattar N. Obesity in pregnancy: prevalence and metabolic consequences. *Semin Fetal Neonatal Med.* Apr 2010;15(2):70-76.

23. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. *JAMA.* Jan 20 2010;303(3):235-241.

24. Grammatikopoulou MG, Pritsa AA, Badeka S, et al. A pilot study on the prevalence of maternal obesity in selected Greek counties. *Endocrinol Nutr.* Nov 2013;60(9):507-512.

25. Chu SY, Callaghan WM, Kim SY, et al. Maternal obesity and risk of gestational diabetes mellitus. *Diabetes Care.* Aug 2007;30(8):2070-2076.

26. Catalano PM, Ehrenberg HM. The short- and long-term implications of maternal obesity on the mother and her offspring. *BJOG.* Oct 2006;113(10):1126-1133.

27. Weiss JL, Malone FD, Emig D, et al. Obesity, obstetric complications and cesarean delivery rate--a population-based screening study. *Am J Obstet Gynecol.* Apr 2004;190(4):1091-1097.

28. Stothard KJ, Tennant PW, Bell R, Rankin J. Maternal overweight and obesity and the risk of congenital anomalies: a systematic review and meta-analysis. *JAMA.* Feb 11 2009;301(6):636-650.

29. Gaudet L, Ferraro ZM, Wen SW, Walker M. Maternal obesity and occurrence of fetal macrosomia: a systematic review and meta-analysis. *Biomed Res Int.* 2014;2014:640291.

30. Chu SY, Kim SY, Lau J, et al. Maternal obesity and risk of stillbirth: a metaanalysis. *Am J Obstet Gynecol.* Sep 2007;197(3):223-228.

31. Johansson S, Villamor E, Altman M, Bonamy AK, Granath F, Cnattingius S. Maternal overweight and obesity in early pregnancy and risk of infant mortality: a population based cohort study in Sweden. *BMJ.* Dec 02 2014;349:g6572.

32. Poston L, Harthoorn LF, Van Der Beek EM. Obesity in pregnancy: implications for the mother and lifelong health of the child. A consensus statement. *Pediatr Res.* Feb 2011;69(2):175-180.

33. Yu Z, Han S, Zhu J, Sun X, Ji C, Guo X. Pre-pregnancy body mass index in relation to infant birth weight and offspring overweight/obesity: a systematic review and meta-analysis. *PLoS One.* 2013;8(4):e61627.

34. Patro B, Liber A, Zalewski B, Poston L, Szajewska H, Koletzko B. Maternal and paternal body mass index and offspring obesity: a systematic review. *Ann Nutr Metab.* 2013;63(1-2):32-41.

35. Catalano PM, Farrell K, Thomas A, et al. Perinatal risk factors for childhood obesity and metabolic dysregulation. *Am J Clin Nutr.* Nov 2009;90(5):1303-1313.

36. Gaillard R, Steegers EA, Duijts L, et al. Childhood cardiometabolic outcomes of maternal obesity during pregnancy: the Generation R Study. *Hypertension.* Apr 2014;63(4):683-691.

37. Lawlor DA, Timpson NJ, Harbord RM, et al. Exploring the developmental overnutrition hypothesis using parental-offspring associations and FTO as an instrumental variable. *PLoS Med.* Mar 11 2008;5(3):e33.

38. Bekkers MB, Brunekreef B, Smit HA, et al. Early-life determinants of total and HDL cholesterol concentrations in 8-year-old children; the PIAMA birth cohort study. *PLoS One.* 2011;6(9):e25533.

39. Oostvogels AJ, Stronks K, Roseboom TJ, van der Post JA, van Eijsden M, Vrijkotte TG. Maternal pre-pregnancy BMI, offspring's early postnatal growth and metabolic profile at age 5-6 years: the ABCD-study. *J Clin Endocrinol Metab.* Jun 23 2014:jc20141561.

40. Perng W, Gillman MW, Mantzoros CS, Oken E. A prospective study of maternal prenatal weight and offspring cardiometabolic health in midchildhood. *Ann Epidemiol.* Sep 3 2014.

41. Derraik JG, Ayyavoo A, Hofman PL, Biggs JB, Cutfield WS. Increasing maternal prepregnancy body mass index is associated with reduced insulin sensitivity and increased blood pressure in their children. *Clin Endocrinol (Oxf).* Nov 11 2014.

42. Tozuka Y, Wada E, Wada K. Diet-induced obesity in female mice leads to peroxidized lipid accumulations and impairment of hippocampal neurogenesis during the early life of their offspring. *FASEB J.* Jun 2009;23(6):1920-1934.

43. Edlow AG, Vora NL, Hui L, Wick HC, Cowan JM, Bianchi DW. Maternal obesity affects fetal neurodevelopmental and metabolic gene expression: a pilot study. *PLoS One.* 2014;9(2):e88661.

44. Smith GD. Assessing intrauterine influences on offspring health outcomes: can epidemiological studies yield robust findings? *Basic Clin Pharmacol Toxicol.* Feb 2008;102(2):245-256.

45. Casas M, Chatzi L, Carsin AE, et al. Maternal pre-pregnancy overweight and obesity, and child neuropsychological development: two Southern European birth cohort studies. *Int J Epidemiol.* Apr 2013;42(2):506-517.

46. Bliddal M, Olsen J, Stovring H, et al. Maternal pre-pregnancy BMI and intelligence quotient (IQ) in 5-year-old children: a cohort based study. *PLoS One.* 2014;9(4):e94498.

47. Brion MJ, Zeegers M, Jaddoe V, et al. Intrauterine effects of maternal prepregnancy overweight on child cognition and behavior in 2 cohorts. *Pediatrics.* Jan 2011;127(1):e202-211.

48. Suren P, Gunnes N, Roth C, et al. Parental obesity and risk of autism spectrum disorder. *Pediatrics.* May 2014;133(5):e1128-1138.

49. Herrera E. Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus. *Eur J Clin Nutr.* Mar 2000;54 Suppl 1:S47-51.

50. Herrera E. Lipid metabolism in pregnancy and its consequences in the fetus and newborn. *Endocrine.* Oct 2002;19(1):43-55.

51. Mazurkiewicz JC, Watts GF, Warburton FG, Slavin BM, Lowy C, Koukkou E. Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. *J Clin Pathol.* Aug 1994;47(8):728-731.

52. Woollett LA. Review: Transport of maternal cholesterol to the fetal circulation. *Placenta.* Mar 2011;32 Suppl 2:S218-221.

53. Hollingsworth DR, Grundy SM. Pregnancy-associated hypertriglyceridemia in normal and diabetic women. Differences in insulin-dependent, non-insulin-dependent, and gestational diabetes. *Diabetes.* Dec 1982;31(12):1092-1097.

54. Ziaei S, Bonab KM, Kazemnejad A. Serum lipid levels at 28-32 weeks gestation and hypertensive disorders. *Hypertens Pregnancy.* 2006;25(1):3-10.

55. Wiznitzer A, Mayer A, Novack V, et al. Association of lipid levels during gestation with preeclampsia and gestational diabetes mellitus: a population-based study. *Am J Obstet Gynecol.* Nov 2009;201(5):482 e481-488.

56. Baker AM, Klein RL, Moss KL, Haeri S, Boggess K. Maternal serum dyslipidemia occurs early in pregnancy in women with mild but not severe preeclampsia. *Am J Obstet Gynecol.* Sep 2009;201(3):293 e291-294.

57. Amundsen AL, Khoury J, Iversen PO, et al. Marked changes in plasma lipids and lipoproteins during pregnancy in women with familial hypercholesterolemia. *Atherosclerosis.* Dec 2006;189(2):451-457.

58. Catov JM, Bodnar LM, Kip KE, et al. Early pregnancy lipid concentrations and spontaneous preterm birth. *Am J Obstet Gynecol.* Dec 2007;197(6):610 e611-617.

59. Edison RJ, Berg K, Remaley A, et al. Adverse birth outcome among mothers with low serum cholesterol. *Pediatrics.* Oct 2007;120(4):723-733.

60. Di Cianni G, Miccoli R, Volpe L, et al. Maternal triglyceride levels and newborn weight in pregnant women with normal glucose tolerance. *Diabet Med.* Jan 2005;22(1):21-25.

61. Vrijkotte TG, Krukziener N, Hutten BA, Vollebregt KC, van Eijsden M, Twickler MB. Maternal lipid profile during early pregnancy and pregnancy complications and outcomes: the ABCD study. *J Clin Endocrinol Metab.* Nov 2012;97(11):3917-3925.

62. Sattar N, Greer IA, Galloway PJ, et al. Lipid and lipoprotein concentrations in pregnancies complicated by intrauterine growth restriction. *J Clin Endocrinol Metab.* Jan 1999;84(1):128-130.

63. Vrijkotte TG, Algera SJ, Brouwer IA, van Eijsden M, Twickler MB. Maternal triglyceride levels during early pregnancy are associated with birth weight and postnatal growth. *J Pediatr.* Nov 2011;159(5):736-742 e731.

64. Gademan MG, Vermeulen M, Oostvogels AJ, et al. Maternal prepregancy BMI and lipid profile during early pregnancy are independently associated with offspring's body composition at age 5-6 years: the ABCD study. *PLoS One.* 2014;9(4):e94594.

65. Chang GQ, Gaysinskaya V, Karatayev O, Leibowitz SF. Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J Neurosci.* Nov 12 2008;28(46):12107-12119.

66. Di Cianni G, Miccoli R, Volpe L, Lencioni C, Del Prato S. Intermediate metabolism in normal pregnancy and in gestational diabetes. *Diabetes Metab Res Rev.* Jul-Aug 2003;19(4):259-270.

67. Butte NF. Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am J Clin Nutr.* May 2000;71(5 Suppl):1256S-1261S.

68. Buchanan TA. Pancreatic B-cell defects in gestational diabetes: implications for the pathogenesis and prevention of type 2 diabetes. *J Clin Endocrinol Metab.* Mar 2001;86(3):989-993.

69. Galtier-Dereure F, Boegner C, Bringer J. Obesity and pregnancy: complications and cost. *Am J Clin Nutr.* May 2000;71(5 Suppl):1242S-1248S.

70. Yogev Y, Visser GH. Obesity, gestational diabetes and pregnancy outcome. *Semin Fetal Neonatal Med.* Apr 2009;14(2):77-84.

71. Pettitt DJ, Aleck KA, Baird HR, Carraher MJ, Bennett PH, Knowler WC. Congenital susceptibility to NIDDM. Role of intrauterine environment. *Diabetes.* May 1988;37(5):622-628.

72. Pettitt DJ, Baird HR, Aleck KA, Bennett PH, Knowler WC. Excessive obesity in offspring of Pima Indian women with diabetes during pregnancy. *N Engl J Med.* Feb 03 1983;308(5):242-245.

73. Kim SY, England JL, Sharma JA, Njoroge T. Gestational diabetes mellitus and risk of childhood overweight and obesity in offspring: a systematic review. *Exp Diabetes Res.* 2011;2011:541308.

74. Krishnaveni GV, Veena SR, Hill JC, Kehoe S, Karat SC, Fall CH. Intrauterine exposure to maternal diabetes is associated with higher adiposity and insulin resistance and clustering of cardiovascular risk markers in Indian children. *Diabetes Care.* Feb 2010;33(2):402-404.

75. Tam WH, Ma RC, Yang X, et al. Glucose intolerance and cardiometabolic risk in children exposed to maternal gestational diabetes mellitus in utero. *Pediatrics.* Dec 2008;122(6):1229-1234.

76. Tam WH, Ma RC, Yang X, et al. Glucose intolerance and cardiometabolic risk in adolescents exposed to maternal gestational diabetes: a 15-year follow-up study. *Diabetes Care.* Jun 2010;33(6):1382-1384.

77. Clausen TD, Mathiesen ER, Hansen T, et al. High prevalence of type 2 diabetes and pre-diabetes in adult offspring of women with gestational diabetes mellitus or type 1 diabetes: the role of intrauterine hyperglycemia. *Diabetes Care.* Feb 2008;31(2):340-346.

78. Ornoy A, Reece EA, Pavlinkova G, Kappen C, Miller RK. Effect of maternal diabetes on the embryo, fetus, and children: congenital anomalies, genetic and epigenetic changes and developmental outcomes. *Birth Defects Res C Embryo Today.* Mar 2015;105(1):53-72.

79. Georgieff MK. The effect of maternal diabetes during pregnancy on the neurodevelopment of offspring. *Minn Med.* Mar 2006;89(3):44-47.

80. Ornoy A. Growth and neurodevelopmental outcome of children born to mothers with pregestational and gestational diabetes. *Pediatr Endocrinol Rev.* Dec 2005;3(2):104-113.

81. Veena SR, Krishnaveni GV, Srinivasan K, et al. Childhood cognitive ability: relationship to gestational diabetes mellitus in India. *Diabetologia.* Oct 2010;53(10):2134-2138.

82. Dahlquist G, Kallen B. School marks for Swedish children whose mothers had diabetes during pregnancy: a population-based study. *Diabetologia.* Sep 2007;50(9):1826-1831.

83. Fraser A, Nelson SM, Macdonald-Wallis C, Lawlor DA. Associations of existing diabetes, gestational diabetes, and glycosuria with offspring IQ and educational attainment: the Avon Longitudinal Study of Parents and Children. *Exp Diabetes Res.* 2012;2012:963735.

84. Nielsen GL, Andersen E, Lundbye-Christensen S. Maternal blood glucose in diabetic pregnancies and cognitive performance in offspring in young adulthood: a Danish cohort study. *Diabet Med.* Jul 2010;27(7):786-790.

85. Nielsen GL, Dethlefsen C, Sorensen HT, Pedersen JF, Molsted-Pedersen L. Cognitive function and army rejection rate in young adult male offspring of women with diabetes: a Danish population-based cohort study. *Diabetes Care.* Nov 2007;30(11):2827-2831.

86. Fraser A, Almqvist C, Larsson H, Langstrom N, Lawlor DA. Maternal diabetes in pregnancy and offspring cognitive ability: sibling study with 723,775 men from 579,857 families. *Diabetologia.* Jan 2014;57(1):102-109.

87. Bonilla C, Lawlor DA, Ben-Shlomo Y, et al. Maternal and offspring fasting glucose and type 2 diabetes-associated genetic variants and cognitive function at age 8: a Mendelian randomization study in the Avon Longitudinal Study of Parents and Children. *BMC Med Genet.* 2012;13:90.

88. Camprubi Robles M, Campoy C, Garcia Fernandez L, Lopez-Pedrosa JM, Rueda R, Martin MJ. Maternal Diabetes and Cognitive Performance in the Offspring: A Systematic Review and Meta-Analysis. *PLoS One.* 2015;10(11):e0142583.

89. Mustafa R, Ahmed S, Gupta A, Venuto RC. A comprehensive review of hypertension in pregnancy. *J Pregnancy.* 2012;2012:105918.

90. Magee LA, Helewa M, Moutquin JM, von Dadelszen P. Diagnosis, evaluation, and management of the hypertensive disorders of pregnancy. *J Obstet Gynaecol Can.* Mar 2008;30(3 Suppl):S1-48.

91. Barton JR, O'Brien J M, Bergauer NK, Jacques DL, Sibai BM. Mild gestational hypertension remote from term: progression and outcome. *Am J Obstet Gynecol.* Apr 2001;184(5):979-983.

92. MacKay AP, Berg CJ, Atrash HK. Pregnancy-related mortality from preeclampsia and eclampsia. *Obstet Gynecol.* Apr 2001;97(4):533-538.

93. Hauth JC, Ewell MG, Levine RJ, et al. Pregnancy outcomes in healthy nulliparas who developed hypertension. Calcium for Preeclampsia Prevention Study Group. *Obstet Gynecol.* Jan 2000;95(1):24-28.

94. Davis EF, Lazdam M, Lewandowski AJ, et al. Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. *Pediatrics.* Jun 2012;129(6):e1552-1561.

95. Seed PT, Chappell LC, Black MA, et al. Prediction of preeclampsia and delivery of small for gestational age babies based on a combination of clinical risk factors in high-risk women. *Hypertens Pregnancy.* 2011;30(1):58-73.

96. McCowan L, Horgan RP. Risk factors for small for gestational age infants. *Best Pract Res Clin Obstet Gynaecol.* Dec 2009;23(6):779-793.

97. Rosen CJ, Adams JS, Bikle DD, et al. The nonskeletal effects of vitamin D: an Endocrine Society scientific statement. *Endocr Rev.* Jun 2012;33(3):456-492.

98. Holick MF. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. *Rev Endocr Metab Disord.* Jun 2017;18(2):153-165.

99. Wang H, Chen W, Li D, et al. Vitamin D and Chronic Diseases. *Aging Dis.* May 2017;8(3):346-353.

100. van Schoor NM, Lips P. Worldwide vitamin D status. *Best Pract Res Clin Endocrinol Metab.* Aug 2011;25(4):671-680.

101. Vandevijvere S, Amsalkhir S, Van Oyen H, Moreno-Reyes R. High prevalence of vitamin D deficiency in pregnant women: a national cross-sectional survey. *PLoS One.* 2012;7(8):e43868.

102. Dawodu A, Wagner CL. Mother-child vitamin D deficiency: an international perspective. *Arch Dis Child.* Sep 2007;92(9):737-740.

103. Gray TK, Lowe W, Lester GE. Vitamin D and pregnancy: the maternal-fetal metabolism of vitamin D. *Endocr Rev.* Summer 1981;2(3):264-274.

104. Holick MF. Vitamin D deficiency. *N Engl J Med.* Jul 19 2007;357(3):266-281.

105. Holick MF. Resurrection of vitamin D deficiency and rickets. *J Clin Invest.* Aug 2006;116(8):2062-2072.

106. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev.* Jan 2016;96(1):365-408.

107. Wagner CL, Taylor SN, Dawodu A, Johnson DD, Hollis BW. Vitamin D and its role during pregnancy in attaining optimal health of mother and fetus. *Nutrients.* Mar 2012;4(3):208-230.

108. Calvo MS, Whiting SJ, Barton CN. Vitamin D intake: a global perspective of current status. *J Nutr.* Feb 2005;135(2):310-316.

109. Salle BL, Delvin EE, Lapillonne A, Bishop NJ, Glorieux FH. Perinatal metabolism of vitamin D. *Am J Clin Nutr.* May 2000;71(5 Suppl):1317S-1324S.

110. DeLuca HF. Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr.* Dec 2004;80(6 Suppl):1689S-1696S.

111. Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev.* Aug 2005;26(5):662-687.

112. Margolis RN, Christakos S. The nuclear receptor superfamily of steroid hormones and vitamin D gene regulation. An update. *Ann N Y Acad Sci.* Mar 2010;1192:208-214.

113. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* Jul 2011;96(7):1911-1930.

114. Karras SN, Anagnostis P, Annweiler C, et al. Maternal vitamin D status during pregnancy: the Mediterranean reality. *Eur J Clin Nutr.* Aug 2014;68(8):864-869.

115. Steichen JJ, Tsang RC, Gratton TL, Hamstra A, DeLuca HF. Vitamin D homeostasis in the perinatal period: 1,25-dihydroxyvitamin D in maternal, cord, and neonatal blood. *N Engl J Med.* Feb 07 1980;302(6):315-319.

116. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res.* Oct 2011;26(10):2341-2357.

117. Stevenson JC, Hillyard CJ, MacIntyre I, Cooper H, Whitehead MI. A physiological role for calcitonin: protection of the maternal skeleton. *Lancet.* Oct 13 1979;2(8146):769-770.

118. Zhong Y, Armbrecht HJ, Christakos S. Calcitonin, a regulator of the 25-hydroxyvitamin D3 1alpha-hydroxylase gene. *J Biol Chem.* Apr 24 2009;284(17):11059-11069.

119. Ajibade DV, Dhawan P, Fechner AJ, Meyer MB, Pike JW, Christakos S. Evidence for a role of prolactin in calcium homeostasis: regulation of intestinal transient receptor potential vanilloid type 6, intestinal calcium absorption, and the 25-hydroxyvitamin D(3) 1alpha hydroxylase gene by prolactin. *Endocrinology.* Jul 2010;151(7):2974-2984.

120. Bikle DD, Gee E, Halloran B, Haddad JG. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *J Clin Invest.* Dec 1984;74(6):1966-1971.

121. Wagner CL, Taylor SN, Johnson DD, Hollis BW. The role of vitamin D in pregnancy and lactation: emerging concepts. *Womens Health (Lond).* May 2012;8(3):323-340.

122. Hollis BW, Pittard WB, 3rd. Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences. *J Clin Endocrinol Metab.* Oct 1984;59(4):652-657.

123. Delvin EE, Glorieux FH, Salle BL, David L, Varenne JP. Control of vitamin D metabolism in preterm infants: feto-maternal relationships. *Arch Dis Child.* Oct 1982;57(10):754-757.

124. Urrutia RP, Thorp JM. Vitamin D in pregnancy: current concepts. *Curr Opin Obstet Gynecol.* Mar 2012;24(2):57-64.

125. Morales E, Guxens M, Llop S, et al. Circulating 25-hydroxyvitamin D3 in pregnancy and infant neuropsychological development. *Pediatrics.* Oct 2012;130(4):e913-920.

126. Cadario F, Savastio S, Pozzi E, et al. Vitamin D status in cord blood and newborns: ethnic differences. *Ital J Pediatr.* Jun 04 2013;39:35.

127. Nicolaidou P, Hatzistamatiou Z, Papadopoulou A, et al. Low vitamin D status in mother-newborn pairs in Greece. *Calcif Tissue Int.* Jun 2006;78(6):337-342.

128. Shahbazi M, Jeddi-Tehrani M, Zareie M, et al. Expression profiling of vitamin D receptor in placenta, decidua and ovary of pregnant mice. *Placenta.* Sep 2011;32(9):657-664.

129. Enquobahrie DA, Williams MA, Qiu C, Siscovick DS, Sorensen TK. Global maternal early pregnancy peripheral blood mRNA and miRNA expression profiles according to plasma 25-hydroxyvitamin D concentrations. *J Matern Fetal Neonatal Med.* Aug 2011;24(8):1002-1012.

130. Kovacs CS. Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies. *Am J Clin Nutr.* Aug 2008;88(2):520S-528S.

131. Anagnostis P, Karras S, Goulis DG. Vitamin D in human reproduction: a narrative review. *Int J Clin Pract.* Mar 2013;67(3):225-235.

132. Hypponen E, Cavadino A, Williams D, et al. Vitamin D and pre-eclampsia: original data, systematic review and meta-analysis. *Ann Nutr Metab.* 2013;63(4):331-340.

133. De-Regil LM, Palacios C, Lombardo LK, Pena-Rosas JP. Vitamin D supplementation for women during pregnancy. *Sao Paulo Med J.* May-Jun 2016;134(3):274-275.

134. Zhou SS, Tao YH, Huang K, Zhu BB, Tao FB. Vitamin D and risk of preterm birth: Up-to-date meta-analysis of randomized controlled trials and observational studies. *J Obstet Gynaecol Res.* Feb 2017;43(2):247-256.

135. Anatoliotaki M, Tsilimigaki A, Tsekoura T, Schinaki A, Stefanaki S, Nicolaidou P. Congenital rickets due to maternal vitamin D deficiency in a sunny island of Greece. *Acta Paediatr.* 2003;92(3):389-391.

136. Orbak Z, Karacan M, Doneray H, Karakelleoglu C. Congenital rickets presenting with hypocalcaemic seizures. *West Indian Med J.* Sep 2007;56(4):364-367.

137. Brooke OG, Brown IR, Bone CD, et al. Vitamin D supplements in pregnant Asian women: effects on calcium status and fetal growth. *Br Med J.* Mar 15 1980;280(6216):751-754.

138. Ioannou C, Javaid MK, Mahon P, et al. The effect of maternal vitamin D concentration on fetal bone. *J Clin Endocrinol Metab.* Nov 2012;97(11):E2070-2077.

139. Mahon P, Harvey N, Crozier S, et al. Low maternal vitamin D status and fetal bone development: cohort study. *J Bone Miner Res.* Jan 2010;25(1):14-19.

140. Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O'Beirne M, Rabi DM. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. *BMJ.* Mar 26 2013;346:f1169.

141. Wei SQ, Qi HP, Luo ZC, Fraser WD. Maternal vitamin D status and adverse pregnancy outcomes: a systematic review and meta-analysis. *J Matern Fetal Neonatal Med.* Jun 2013;26(9):889-899.

142. Thorne-Lyman A, Fawzi WW. Vitamin D during pregnancy and maternal, neonatal and infant health outcomes: a systematic review and meta-analysis. *Paediatr Perinat Epidemiol.* Jul 2012;26 Suppl 1:75-90.

143. Karras SN, Anagnostis P, Petroczi A, Annweiler C, Naughton DP, Goulis DP. Maternal vitamin D status in pregnancy: a critical appraisal of current analytical data on maternal and neonatal outcomes. *Hormones (Athens).* Apr-Jun 2015;14(2):224-231.

144. Belderbos ME, Houben ML, Wilbrink B, et al. Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis. *Pediatrics.* Jun 2011;127(6):e1513-1520.

145. Hansdottir S, Monick MM, Lovan N, Powers L, Gerke A, Hunninghake GW. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and

cytokines in airway epithelium while maintaining the antiviral state. *J Immunol.* Jan 15 2010;184(2):965-974.

146. Christesen HT, Elvander C, Lamont RF, Jorgensen JS. The impact of vitamin D in pregnancy on extraskeletal health in children: a systematic review. *Acta Obstet Gynecol Scand.* Dec 2012;91(12):1368-1380.

147. Krishnaveni GV, Veena SR, Winder NR, et al. Maternal vitamin D status during pregnancy and body composition and cardiovascular risk markers in Indian children: the Mysore Parthenon Study. *Am J Clin Nutr.* Mar 2011;93(3):628-635.

148. Javaid MK, Crozier SR, Harvey NC, et al. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. *Lancet.* Jan 07 2006;367(9504):36-43.

149. Crozier SR, Harvey NC, Inskip HM, Godfrey KM, Cooper C, Robinson SM. Maternal vitamin D status in pregnancy is associated with adiposity in the offspring: findings from the Southampton Women's Survey. *Am J Clin Nutr.* Jul 2012;96(1):57-63.

150. Gale CR, Robinson SM, Harvey NC, et al. Maternal vitamin D status during pregnancy and child outcomes. *Eur J Clin Nutr.* Jan 2008;62(1):68-77.

151. Williams DM, Fraser A, Fraser WD, et al. Associations of maternal 25-hydroxyvitamin D in pregnancy with offspring cardiovascular risk factors in childhood and adolescence: findings from the Avon Longitudinal Study of Parents and Children. *Heart.* Dec 2013;99(24):1849-1856.

152. Rytter D, Bech BH, Halldorsson TI, et al. Maternal Vitamin D Status at Week 30 of Gestation and Offspring Cardio-Metabolic Health at 20 Years: A Prospective Cohort Study over Two Decades. *PLoS One.* 2016;11(10):e0164758.

153. Morales E, Rodriguez A, Valvi D, et al. Deficit of vitamin D in pregnancy and growth and overweight in the offspring. *Int J Obes (Lond).* Jan 2015;39(1):61-68.

154. Hrudey EJ, Reynolds RM, Oostvogels AJ, Brouwer IA, Vrijkotte TG. The Association between Maternal 25-Hydroxyvitamin D Concentration during Gestation and Early Childhood Cardio-metabolic Outcomes: Is There Interaction with Pre-Pregnancy BMI? *PLoS One.* 2015;10(8):e0133313.

155. Eyles D, Burne T, McGrath J. Vitamin D in fetal brain development. *Semin Cell Dev Biol.* Aug 2011;22(6):629-636.

156. Tylavsky FA, Kocak M, Murphy LE, et al. Gestational Vitamin 25(OH)D Status as a Risk Factor for Receptive Language Development: A 24-Month, Longitudinal, Observational Study. *Nutrients.* 2015;7(12):9918-9930.

157. Whitehouse AJ, Holt BJ, Serralha M, Holt PG, Kusel MM, Hart PH. Maternal serum vitamin D levels during pregnancy and offspring neurocognitive development. *Pediatrics.* Mar 2012;129(3):485-493.

158. Morales E, Julvez J, Torrent M, et al. Vitamin D in Pregnancy and Attention Deficit Hyperactivity Disorder-like Symptoms in Childhood. *Epidemiology.* Jul 2015;26(4):458-465.

159. Zhu P, Tong SL, Hao JH, et al. Cord blood vitamin D and neurocognitive development are nonlinearly related in toddlers. *J Nutr.* Jun 2015;145(6):1232-1238.

160. Keim SA, Bodnar LM, Klebanoff MA. Maternal and cord blood 25(OH)-vitamin D concentrations in relation to child development and behaviour. *Paediatr Perinat Epidemiol.* Sep 2014;28(5):434-444.

161. Strom M, Halldorsson TI, Hansen S, et al. Vitamin D measured in maternal serum and offspring neurodevelopmental outcomes: a prospective study with long-term follow-up. *Ann Nutr Metab.* 2014;64(3-4):254-261.

162. Gustafsson P, Rylander L, Lindh CH, et al. Vitamin D Status at Birth and Future Risk of Attention Deficit/Hyperactivity Disorder (ADHD). *PLoS One.* 2015;10(10):e0140164.

163. Metzger BE, Gabbe SG, Persson B, et al. International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. *Diabetes Care.* Mar 2010;33(3):676-682.

164. Jellinger PS, Smith DA, Mehta AE, et al. American Association of Clinical Endocrinologists' Guidelines for Management of Dyslipidemia and Prevention of Atherosclerosis: executive summary. *Endocr Pract.* Mar-Apr 2012;18(2):269-293.

165. Chatzi L, Plana E, Daraki V, et al. Metabolic syndrome in early pregnancy and risk of preterm birth. *Am J Epidemiol.* Oct 1 2009;170(7):829-836.

166. Katrinaki M, Kampa M, Margioris A, Castanas E, Malliaraki N. Vitamin D levels in a large Mediterranean cohort: reconsidering normal cut-off values. *Hormones (Athens).* Apr 2016;15(2):205-223.

167. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ.* May 6 2000;320(7244):1240-1243.

168. Linardakis M, Sarri K, Bertsias G, Papadaki A, Kafatos A. Waist circumference and waist-to-height percentiles for the youth of Crete, Greece. *International Journal of Chld Health and Human Development.* 2011;3:329-342.

169. Clasey JL, Bradley KD, Bradley JW, Long DE, Griffith JR. A new BIA equation estimating the body composition of young children. *Obesity (Silver Spring).* Sep 2011;19(9):1813-1817.

170. Slaughter MH, Lohman TG, Boileau RA, et al. Skinfold equations for estimation of body fatness in children and youth. *Hum Biol.* Oct 1988;60(5):709-723.

171. Steinberger J, Daniels SR, Eckel RH, et al. Progress and challenges in metabolic syndrome in children and adolescents: a scientific statement from the American Heart Association Atherosclerosis, Hypertension, and Obesity in the Young Committee of the Council on Cardiovascular Disease in the Young; Council on Cardiovascular Nursing; and Council on Nutrition, Physical Activity, and Metabolism. *Circulation.* Feb 3 2009;119(4):628-647.

172. Karachaliou M, Georgiou V, Roumeliotaki T, et al. Association of trimester-specific gestational weight gain with fetal growth, offspring obesity, and cardiometabolic traits in early childhood. *Am J Obstet Gynecol.* Dec 31 2014.

173. McCarthy D. *Manual for the McCarthy scales of children's abilities*: Psychological Corp.; 1972.

174. Kampouri M, Kyriklaki A, Roumeliotaki T, et al. Patterns of Early-Life Social and Environmental Exposures and Child Cognitive Development, Rhea Birth Cohort, Crete, Greece. *Child Dev.* Mar 28 2017.

175. Goodman R. The Strengths and Difficulties Questionnaire: a research note. *Journal of child psychology and psychiatry.* 1997;38(5):581-586.

176. Mpipou - Nakou I, Stogiannidou A, Kisseoglou G. Strengths and difficulties of school-age children in the family

and school context. *Psychologia.* 2001;8(4):506-525.

177. R. GJ. *Examiners manual for the Attention-Deficit/Hyperactivity Disorder Test: A method for identifying individuals with ADHD.* . Austin, TX: pro-Ed; 1995.

178. Maniadaki K, Kakouros E. Translation and Adaptation of the Attention Deficit Hyperactivity Disorder Test (ADHDT; Giliam, 1995). In: A. Stalikas, S. Triliva, & P. Roussi (Eds.) The psychometric Instruments in Greece (pp. 102-103). *Athens: Ellinika Grammata.* 2002.

179. McNutt LA, Wu C, Xue X, Hafner JP. Estimating the relative risk in cohort studies and clinical trials of common outcomes. *Am J Epidemiol.* May 15 2003;157(10):940-943.

180. Kubota T, Miyake K, Hariya N, Mochizuki K. Understanding the epigenetics of neurodevelopmental disorders and DOHaD. *J Dev Orig Health Dis.* Apr 2015;6(2):96-104.

181. Hamilton SA, McNeil R, Hollis BW, et al. Profound Vitamin D Deficiency in a Diverse Group of Women during Pregnancy Living in a Sun-Rich Environment at Latitude 32 degrees N. *Int J Endocrinol.* 2010;2010:917428.

182. Paterson CR, Ayoub D. Congenital rickets due to vitamin D deficiency in the mothers. *Clin Nutr.* Oct 2015;34(5):793-798.

183. Camadoo L, Tibbott R, Isaza F. Maternal vitamin D deficiency associated with neonatal hypocalcaemic convulsions. *Nutr J.* 2007;6:23.

184. Morales E, Romieu I, Guerra S, et al. Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. *Epidemiology.* Jan 2012;23(1):64-71.

185. Sharief S, Jariwala S, Kumar J, Muntner P, Melamed ML. Vitamin D levels and food and environmental allergies in the United States: results from the National Health and Nutrition Examination Survey 2005-2006. *J Allergy Clin Immunol.* May 2011;127(5):1195-1202.

186. Groves NJ, McGrath JJ, Burne TH. Vitamin D as a neurosteroid affecting the developing and adult brain. *Annu Rev Nutr.* 2014;34:117-141.

187. Gould JF, Anderson AJ, Yelland LN, et al. Association of cord blood vitamin D with early childhood growth and neurodevelopment. *J Paediatr Child Health.* Aug 27 2016.

188. Whitehouse AJ, Holt BJ, Serralha M, Holt PG, Hart PH, Kusel MM. Maternal vitamin D levels and the autism phenotype among offspring. *J Autism Dev Disord.* Jul 2013;43(7):1495-1504.

189. Gilliam JE. *Examiners manual for the Attention-Deficit/Hyperactivity Disorder Test: A method for identifying individuals with ADHD.* Austin, TX: pro-Ed; 1995.

190. Ross AC, Manson JE, Abrams SA, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab.* Jan 2011;96(1):53-58.

191. Eyles D, Brown J, Mackay-Sim A, McGrath J, Feron F. Vitamin D3 and brain development. *Neuroscience.* 2003;118(3):641-653.

192. Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front Neuroendocrinol.* Jan 2013;34(1):47-64.

193. O'Loan J, Eyles DW, Kesby J, Ko P, McGrath JJ, Burne TH. Vitamin D deficiency during various stages of pregnancy in the rat; its impact on development and behaviour in adult offspring. *Psychoneuroendocrinology.* Apr 2007;32(3):227-234.

194. Correale J, Ysrraelit MC, Gaitan MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. *J Immunol.* Oct 15 2010;185(8):4948-4958.

195. Grandjean P, Landrigan PJ. Developmental neurotoxicity of industrial chemicals. *The Lancet.* 2006;368(9553):2167-2178.

196. Bellinger DC. A strategy for comparing the contributions of environmental chemicals and other risk factors to neurodevelopment of children. *Environmental health perspectives.* 2012;120(4):501.

197. Mutt SJ, Hypponen E, Saarnio J, Jarvelin MR, Herzig KH. Vitamin D and adipose tissue-more than storage. *Front Physiol.* 2014;5:228.

198. Chatzi L, Leventakou V, Vafeiadi M, et al. Cohort Profile: The Mother-Child Cohort in Crete, Greece (Rhea Study). *Int J Epidemiol.* 2017;In press.

199. Royston P, Wright E. A method for estimating age-specific reference intervals (`normal ranges') based on fractional polynomials and exponential transformation. *Journal of the Royal Statistical Society, Series A.* 1998 1998(161):79-101.

200. Long J, Ryoo J. Using fractional polynomials to model non-linear trends in longitudinal data. *Br J Math Stat Psychol.* Feb 2010;63(Pt 1):177-203.

201. Kong J, Li YC. Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. *Am J Physiol Endocrinol Metab.* May 2006;290(5):E916-924.

202. Ding C, Gao D, Wilding J, Trayhurn P, Bing C. Vitamin D signalling in adipose tissue. *Br J Nutr.* Dec 14 2012;108(11):1915-1923.

203. Poissonnet CM, Burdi AR, Garn SM. The chronology of adipose tissue appearance and distribution in the human fetus. *Early Hum Dev.* Sep 1984;10(1-2):1-11.

204. Straughen JK, Trudeau S, Misra VK. Changes in adipose tissue distribution during pregnancy in overweight and obese compared with normal weight women. *Nutr Diabetes.* Aug 26 2013;3:e84.

205. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr.* Sep 2000;72(3):690-693.

206. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology.* Jan 1990;1(1):43-46.

207. Steiner MJ, Skinner AC, Perrin EM. Fasting might not be necessary before lipid screening: a nationally representative cross-sectional study. *Pediatrics.* Sep 2011;128(3):463-470.

208. Monteiro PO, Victora CG. Rapid growth in infancy and childhood and obesity in later life--a systematic review. *Obes Rev.* May 2005;6(2):143-154.

209. Falkner B, Daniels SR. Summary of the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents. *Hypertension.* Oct 2004;44(4):387-388.

210. Rasmussen KM, & Yaktine, A. L.,. *Institute of medicine (committee to reexamine iom pregnancy weight guidelines, food and nutritions board and board on children, youth, and families) weight gain during pregnancy : Reexamining the guidelines.* Washington D.C.2009.

211. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* Jan 2008;31 Suppl 1:S55-60.

212. Drake AJ, Reynolds RM. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. *Reproduction.* Sep 2010;140(3):387-398.

213. Gale CR, Javaid MK, Robinson SM, Law CM, Godfrey KM, Cooper C. Maternal size in pregnancy and body composition in children. *J Clin Endocrinol Metab.* Oct 2007;92(10):3904-3911.

214. Blair NJ, Thompson JM, Black PN, et al. Risk factors for obesity in 7-year-old European children: the Auckland Birthweight Collaborative Study. *Arch Dis Child.* Oct 2007;92(10):866-871.

215. Lawlor DA, Relton C, Sattar N, Nelson SM. Maternal adiposity--a determinant of perinatal and offspring outcomes? *Nat Rev Endocrinol.* Nov 2012;8(11):679-688.

216. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. *Int J Obes (Lond).* Feb 2 2015.

217. Tarrade A, Panchenko P, Junien C, Gabory A. Placental contribution to nutritional programming of health and diseases: epigenetics and sexual dimorphism. *J Exp Biol.* Jan 1 2015;218(Pt 1):50-58.

218. Lampl M, Gotsch F, Kusanovic JP, et al. Sex differences in fetal growth responses to maternal height and weight. *Am J Hum Biol.* Jul-Aug 2010;22(4):431-443.

219. Eriksson JG, Kajantie E, Osmond C, Thornburg K, Barker DJ. Boys live dangerously in the womb. *Am J Hum Biol.* May-Jun 2010;22(3):330-335.

220. Regnault N, Gillman MW, Rifas-Shiman SL, Eggleston E, Oken E. Sex-specific associations of gestational glucose tolerance with childhood body composition. *Diabetes Care.* Oct 2013;36(10):3045-3053.

221. Clifton VL. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta.* Mar 2010;31 Suppl:S33-39.

222. Mao J, Zhang X, Sieli PT, Falduto MT, Torres KE, Rosenfeld CS. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proc Natl Acad Sci U S A.* Mar 23 2010;107(12):5557-5562.

223. Suzannah Bennett M, Cheryllee Bourgeois R, Andrea Cassidy R, et al. Clinical Practice Guideline No.15 Hypertensive Disorders of Pregnancy. *Association of Ontario Midwives.* 2012.

224. Bruce KD, Cagampang FR, Argenton M, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology.* Dec 2009;50(6):1796-1808.

225. Napoli C, de Nigris F, Welch JS, et al. Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray. *Circulation.* Mar 19 2002;105(11):1360-1367.

226. Napoli C, Glass CK, Witztum JL, Deutsch R, D'Armiento FP, Palinski W. Influence of maternal hypercholesterolaemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of Early Lesions in Children (FELIC) study. *Lancet.* Oct 9 1999;354(9186):1234-1241.

227. Bolton JL, Bilbo SD. Developmental programming of brain and behavior by perinatal diet: focus on inflammatory mechanisms. *Dialogues Clin Neurosci.* Sep 2014;16(3):307-320.

228. van der Burg JW, Sen S, Chomitz VR, Seidell JC, Leviton A, Dammann O. The role of systemic inflammation linking maternal BMI to neurodevelopment in children. *Pediatr Res.* Jan 2016;79(1-1):3-12.

229. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics.* Mar 2005;115(3):e290-296.

230. Ellenberg JH. Selection bias in observational and experimental studies. *Stat Med.* Mar 15-Apr 15 1994;13(5-7):557-567.

231. Sheikh K. Predicting risk among non-respondents in prospective studies. *Eur J Epidemiol.* Mar 1986;2(1):39-43.

232. Song JW, Chung KC. Observational studies: cohort and case-control studies. *Plast Reconstr Surg.* Dec 2010;126(6):2234-2242.

233. Heerwagen MJ, Miller MR, Barbour LA, Friedman JE. Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. *Am J Physiol Regul Integr Comp Physiol.* Sep 2010;299(3):R711-722.

234. Xue J, Schoenrock SA, Valdar W, Tarantino LM, Ideraabdullah FY. Maternal vitamin D depletion alters DNA methylation at imprinted loci in multiple generations. *Clin Epigenetics.* 2016;8:107.

235. Ng JW, Barrett LM, Wong A, Kuh D, Smith GD, Relton CL. The role of longitudinal cohort studies in epigenetic epidemiology: challenges and opportunities. *Genome Biol.* Jun 29 2012;13(6):246.

236. Wood RJ, Fleet JC. The genetics of osteoporosis: vitamin D receptor polymorphisms. *Annu Rev Nutr.* 1998;18:233-258.

237. Cantorna MT, Mahon BD. Mounting evidence for vitamin D as an environmental factor affecting autoimmune disease prevalence. *Exp Biol Med (Maywood).* Dec 2004;229(11):1136-1142.

238. Ahn J, Albanes D, Berndt SI, et al. Vitamin D-related genes, serum vitamin D concentrations and prostate cancer risk. *Carcinogenesis.* May 2009;30(5):769-776.

239. Shen W, Wang Z, Punyanita M, et al. Adipose tissue quantification by imaging methods: a proposed classification. *Obes Res.* Jan 2003;11(1):5-16.

240. Cortese S, Castellanos FX. Neuroimaging of attention-deficit/hyperactivity disorder: current neuroscience-informed perspectives for clinicians. *Curr Psychiatry Rep.* Oct 2012;14(5):568-578.