Prenatal influences on childhood psychological development

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Prenatal influences on childhood psychological development

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Abstract

The intrauterine period is a sensitive phase for later disease susceptibility, and could affect common expressions of poor psychological development such as internalising and externalising problem behaviours, temperament or social competence.

In this thesis, the associations between birth weight, maternal weight and maternal dietary factors in pregnancy, respectively, and these childhood psychological traits were examined in four European birth cohorts of twins or singletons.

Children of women with pre-pregnancy overweight/obesity who gained 0.5kg/week during gestation had 25-point higher average problem behaviours (on a 0-100 scale) compared to children whose mothers gained 0.2kg/week. Additionally, highest *vs.* lowest maternal Mediterranean-diet score was associated with a 16.3% average reduction in the probability of externalising behaviours, while no association was observed for internalising problems. By contrast, controlling for shared genetic and environmental influences, a greater intrapair birth-weight difference in monozygotic twins was associated with higher internalising symptoms in co-twins with lower compared to larger birth weight. Little-to-no evidence was found for the associations between maternal polyunsaturated fatty acids or pre-pregnancy weight and childhood outcomes. Nonetheless, residual confounding is possible due to the observational study designs. Further research is warranted to strengthen the evidence by addressing the influence of genetics and other parental lifestyle factors.

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List of publications and conference presentations

Papers published or submitted for publication that arose from this thesis (chronological order):

- **Tore EC**, Antoniou EE, Reed K, Southwood TR, Smits L, McCleery JP & Zeegers MP. The Association of Intrapair Birth-Weight Differences with Internalizing and Externalizing Behavior Problems. Twin Res Hum Genet. 2018;21(3):253-262.
- **Tore EC**, Gielen M, Antoniou EE, de Groot RHM, Godschalk R, Southwood TR, Smits L, Stratakis N, van der Wurff ISM & Zeegers MP. The association of maternal polyunsaturated fatty acids during pregnancy with social competence and problem behaviours at 7 years of age: the MEFAB cohort. Prostaglandins, Leukot Essent Fat Acids. 2019;144:1–9.
- **Tore EC**, Antoniou EE, Reed K, Southwood TR, Smits L, McCleery JP & Zeegers MP. Maternal pre-pregnancy weight and twins' temperament. J Dev Orig Health Dis. 2019; 10(5):522-528
- **Tore EC**, Antoniou EE, de Groot RHM, Gielen M, Godschalk R, Roumeliotaki T, Smits L, Southwood TR, Spaanderman MEA, Stratakis N, Vafeiadi M, Chatzi VL & Zeegers MP. Gestational weight gain by maternal pre-pregnancy BMI and childhood problem behaviours in school-age years: a pooled analysis of two European birth cohorts. Matern Child Health J. 2020;24(10):1288–1298.

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WEON, Dutch Epidemiology Conference, Groningen, The Netherlands. Poster presentation: Maternal PUFAs in pregnancy and childhood social competence and behaviour problems.

2020 9th European Conference on Mental Health, online. Oral presentation: Gestational weight gain and child problem behaviours: a pooled analysis.

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Chapter 1

General introduction

Children's psychological development and mental health

Mental illness is the leading cause of disability in young people (1). Anxiety, depression, hyperactivity and conduct problems affect 10%-20% of children and adolescents worldwide, about half of them developing a disorder before the age of 14 years (1–5). Consequences of childhood mental disorders comprise considerable economic burden to the whole society, with significant costs to cover medical expenses, special education needs, parental absence from work and burden to the justice system (4–6). Even more, young psychiatric patients are likely to face a lifetime of mental problems (7–9).

An optimal psychological development is crucial to enable good mental health, by allowing the child to react to experienced emotions in a context-specific and adequate manner. Flexible, adaptive reactions are associated with healthy functioning, while emotion dysregulation is considered at the heart of most mental disorders, including depression, anxiety and conduct problems (10,11). As described below, children can display emotional dysregulations in a large number of ways that could be classified as internalising or externalising expressions of dysfunction, depending on specific genetic and temperamental propensities.

Internalising and externalising problem behaviours

Internalising and externalising problem behaviours are two groupings of common psychopathologies (12,13), widely accepted for research purposes and recently endorsed by the Diagnostic and Statistical Manual of Mental Disorders (DSM) 5th edition. In the DSM, these groupings are defined as an "empirically supported framework" consisting of aetiologically related and highly comorbid syndrome traits (13). As a general description, internalising behaviours in childhood are characterised by anxious/depressive symptoms, social withdrawal or somatic complaints, while childhood externalising problems are characterised by impulsivity, hyperactivity, inattention, aggressiveness, conduct problems or disruptive behaviour (13–15). Nonetheless, the specific traits considered problematic or deviant are highly dependent on the child's age, given the rapid maturation during infancy and childhood (16). For example, separation anxiety is common in infancy but might be concerning if present in older children. Therefore, by grouping together related behavioural problems the general child psychopathological functioning can be examined, instead of focusing on multiple specific traits with limited relevance (17). In addition, due to the proven

stability of dimensions over time (18), understanding the psychopathological functioning of a child during infancy and early childhood consents to make predictions on later development and to design targeted strategies to prevent additional maladjusted behaviours in later years.

It should be considered that the previously mentioned comorbidity is not limited to traits *within* the internalising and externalising groupings but characterises also traits *between* categories (*e.g.*, (19)). In fact, the two groupings are moderately correlated in most populations (20). To account for this association and explain the co-occurrence of virtually all psychopathological traits, a General Psychopathology factor, the *p factor*, has recently been proposed, which represents individuals' propensities to develop any and all psychopathologies (20). The p factor is theorised as a single dimension that unifies all psychopathologies; it can range from low to high, representing an individual's susceptibility to psychopathological severity, persistence and comorbidity (20). The theorisation of the General Psychopathology factor suggests that the same, unspecific factors affect the development of internalising and externalising problems, although a few specific influences for each dimension could be identified. Nonetheless, positive intercorrelations might also result from dynamic processes, such that the presence of a disorder could increase the risk of other disorders (20). Regardless of the actual validity of the p factor, research on the two dimensions of internalising and externalising problems maintains its utility for the identification of specific aetiological factors and for the investigation of the underlying structure of psychopathology and its development over the lifetime (21).

Internalising and externalising problems, as well as each syndrome scale they incorporate and the p factor, are defined as normally distributed and continuous dimensions. Within these dimensions, the healthy and disordered states are separated by infinite qualitatively different traits, rather than representing two distinct categories (21). This novel conceptualisation derives from the realisation that people's uniqueness precludes a standardised manifestation of any mental disorder, and even the health status itself can be qualitatively different between persons. The only possibility to categorise such a continuum would be by arbitrarily deciding when a trait is rare enough for a particular age, gender and culture (22). Consequently, to date there is no univocal definition of disordered state or a gold standard for diagnosis, and prevalence estimates differ depending on the specific tool used to assess children's distress. Nonetheless, most studies have found that internalising or externalising problems can be identified in approximately 15% of individuals aging 18-month to 18-year old (2,12,23).

Social competence

Social competence is considered a central component of an optimal development and healthy functioning (24). It is commonly defined as the ability to behave in a highly adaptive, flexible and effective manner with peers and adults, meeting both personal and others' needs (25). However, there is no full agreement on the specific characteristics of a socially competent child, as they depend on the developmental characteristics of the person being assessed and the specific context in which the child develops and interacts with other people, including the cultural norms, the type of relationship examined and the child's goal underlying the relationship (25–27). Furthermore, being the ability to effectively interact with other people, the working definition of social competence must necessarily consider social interactions, in which the social ability of the index children is affected by the social ability of their peers. It is, therefore, a skill that emerges during social interactions, rather than a characteristic that belongs to the individual (25). Nonetheless, some attributes such as being friendly, helpful or empathetic are largely accepted as important aspects of social competence (25).

The context in which a child lives is, therefore, of particular relevance when examining social competence. The nuclear family represents the centre of social life during infancy and early childhood, and allows the child to learn and improve how to effectively socially interact (28,29). A secure attachment with the mother (or another caregiver), which is the first social relationship for the child, provides a model for children's later social interactions and relationships (30). Conversely, the group of friends becomes the centre of social life from mid-childhood. Therefore, the relative importance of specific social skills changes with age (25). However, the social context is not only age-dependent but is also related to the child's sex. In fact, especially during childhood and pre-adolescence, a high level of social segregation is often present, with boys and girls that tend to socialise in different manners. Gender differences were observed in the broadness of social networks, in the importance attributed to the role played within the group and in the qualities considered essential for friendship (31). This results in the development of different social norms between boys and girls, such that a given behaviour required for efficiently interact within a group of boys could be detrimental for interacting with girls, and *vice versa* (25). For example, while girls focus more on relationship and intimacy issues, physical dominance tends to characterise boys' interactions, especially in preschool-age years. Consequently, antisocial behaviours such as aggression might not be related to poor social competence, if they meet the social requirements in the specific context (25).

Finally, social competence is related to psychological health. In fact, studies have shown that children with externalising problems tend to experience social rejections and isolations due to their excessive reactions or aggressiveness, while children with internalising behaviours are often socially withdrawn and might not develop their social skills appropriately (27,32). Nonetheless, evidence is also available on the importance of high social competence for the prevention of later internalising and externalising behaviours (32). Hence, the relationship between childhood psychopathology and social competence could be bidirectional, with each trait affecting the other reciprocally over time. Alternatively, a third factor, such as emotional and behavioural self-regulation (33), core aspect of temperament (34), might influence both social competence and psychopathology.

Temperament

The concept of temperament traditionally refers to individual differences in behaviour that are evident since birth (35). These characteristics are considered biologically based and in large part stable over time and across situations. Nonetheless, temperament develops as infants acquire new cognitive and physical abilities, so that some temperamental characteristics can only be observed in older infants or children (35). Consequently, temperament is considered the constitutional basis for the development of personality, which matures thanks to the interaction of temperament and experience, with the contribution of higher cognitive and emotional competencies (35,36).

Despite this general agreement, in over 60 years of research no univocal definition, model or measurement tool has been identified. Specifically, a great deal of debate still exists regarding the number and nature of temperamental dimensions, and how these are related to personality (37). Several research traditions can be identified, with the three most important being the "behavioural style" approach of Thomas and Chess (38), the "criterial" approach of Buss and Plomin (39), and the "psychobiological" approach of Rothbart (36).

Thomas and Chess are considered the pioneers in temperament research, thanks to their work on the New York Longitudinal Study (38). In fact, against the prevailing belief at the time, they realised that children are not *tabulae rasae* to be shaped by external forces but are characterised by innate internal forces that differentiate one another. Their observation resulted in the identification of nine temperamental dimensions that focused on the modality (*i.e.*, the *how*) of behaviour rather than the characteristics of the behaviour or the reasons behind it (*i.e.*, the *what* or the *why*). These dimensions were labelled as activity level, adaptability, approach-withdrawal, rhythmicity, threshold, intensity, mood, distractibility, and attention span-persistence. Despite its intrinsic

importance, more recent work on temperament has revealed important limitations of this approach. Firstly, factor analyses in the behavioural style instruments generated based on the New York Longitudinal Study have not supported the originally postulated nine dimensions, yielding only four dimensions of temperament in infancy (40). Furthermore, distinguishing the stylistic components of behaviour from the motivation behind it or its content is often difficult and impracticable (41). Finally, evidence is now available showing that children's tendencies toward positive and negative moods are independent, thus refuting the theorisation of mood as a continuum ranging from positive to negative mood (41).

Buss and Plomin (39) described temperament as a developmental precursor of adult personality. In their view, a dimension should be included in the temperamental model only if it satisfies five criteria: the trait should be 1) inherited, 2) relatively stable during childhood, 3) retained into adulthood, 4) evolutionary adaptive, and 4) present in our phylogenetic relatives. Four dimensions were originally included in Buss and Plomin's model – emotionality, activity, sociability and impulsivity –, although impulsivity was later excluded due to insufficient proof of stability and heritability. A discussion is still open whether the sociability dimension should be rather divided into sociability and shyness (42,43).

Rothbart defines temperament as the constitutionally based individual differences in emotional, motor and attentional reactivity and regulation (36,40,44). The term *reactivity* refers to the latency, intensity, and recovery of response, while *self-regulation* refers to the combination of processes that modulate reactivity. Within reactivity, a distinction is made between emotionality (the reactive tendency to experience and express emotions) and activity (the presence of motor activity). Within self-regulation, attention regulatory processes, which involves the ability to focus or shift attention when required, are differentiated from the ability to inhibit one's behaviour when required (37). In Rothbart's psychobiological model, in infants' temperament is possible to distinguish three higher-order factors that include 14 dimensions: surgency/extraversion (also denoted positive emotionality), negative affectivity and orienting/regulation. Surgency indicates social orientation and includes the traits of approach, vocal reactivity, smiling/laughter, activity level, high-intensity pleasure and perceptual sensitivity. Negative affectivity refers to the tendency to experience negative emotions and includes the traits of sadness, frustration, fear and low levels of falling reactivity. Finally, orienting/regulation refers to the ability of maintain the attention and taking pleasure from low-intensity activities and includes the traits of low-intensity pleasure, cuddliness, duration of orienting and soothability.

Regardless of the specific temperamental model, researchers largely agree upon the importance of temperament in shaping children's psychological development (35–37). A meta-analysis has shown that regulatory problems in infancy, which manifest as excessive and persistent crying, are associated with internalising and externalising behaviours at school age, with medium effect sizes (45). Infants who are extremely shy are more at risk of later internalising problems, while infants who enjoy only high-intensity or novel stimuli might be more at risk of developing externalising problems (35). High levels of fear and anger have been associated with both internalising and externalising problems (35). Furthermore, children who are temperamentally sociable initiate and maintain more positive relationships with peers, while children with a negative or difficult temperament (*e.g.,* negative mood or high inhibition) are more likely to experience peer rejection and social withdrawal (46). Finally, a relationship between specific temperamental characteristics and the General Psychopathology factor has been hypothesised. In this perspective, individuals characterised by low agreeableness, low conscientiousness and high neuroticism, related to emotional/behavioural regulation, score higher in the p factor and thus are more likely to suffer stable and comorbid psychopathologies (20).

Assessment of child psychological development

Children's psychological development can be assessed through direct observations or via rating scales. During a direct observation, the child's behaviour is observed without being filtered by the perception of a parent or teacher (47). The observation can be performed in the child's natural environment (*e.g.,* at home, school or the playground), or in a laboratory setting that might be adapted to reflect the child's environment. Despite being often considered the standard for examining children's behaviour, direct observations are not free from limitations (47). Well-conducted observations are both time-consuming and expensive, and can be subject to bias, especially if children change their behaviour when observed, phenomenon known as reactivity. One way to reduce children's reactivity is to let someone in their environment (*e.g.,* a parent or teacher) observe and assess their behaviours, which however would require them to be trained to appropriately use the chosen coding system, and yet it could lead to unstandardised and biased assessments. Generally, direct observations are mostly preferred in clinical settings, in which few children are carefully and systematically observed for a long period of time. This way, they allow a detailed evaluation of all factors influencing the child's behaviour that can lead to effective interventions (47).

Contrarily to direct observations, the assessment of child psychological traits that relays on rating scales is done using information reported by someone in the child's environment through self-administered questionnaires or face-to-face interviews (47). These methods have several strengths over direct observations: 1) they allow an evaluation of children's overt and rare behaviours, which would be difficult or impossible to assess during a direct observation, 2) are free from reactivity bias, since the child is not aware of being observed, and 3) are less expensive, time-consuming and do not require any previous training. For these reasons, they are often the method of choice in large epidemiological studies. Nonetheless, it is important to consider that ratings always reflect the *perception* that the informant has on the child's behaviour. Consequently, ratings might be subject to report bias, and behavioural assessment performed by multiple raters should be compared in order to obtain a clinical insight on children's traits and their interactions with different informants (47).

Assessment of internalising and externalising problem behaviours

Of the different tools that have been developed to assess childhood problem behaviours, Achenbach's Child Behaviour Checklist (CBCL) (48) is one of the most commonly used and has been employed also in the studies described in **chapters 2, 4, 5 and 6** of this thesis. Translated into over 100 languages and adapted to assess different age-groups (48), the CBCL has shown good psychometric properties, reliability and validity (49,50). All CBCL forms for children and adolescents include items that can be summed up to yield specific syndrome scales and three broadband scales: an internalising behaviour scale, an externalising behaviour scale and a total problem scale, which is the sum of all available syndrome scales. CBCL T-scores, which are age- and sex- standardisations of individual's raw scores with the mean set at 50 and the standard deviation (SD) set at 10, allow comparisons between scales and populations. Apart from the recent addition of DSM-oriented scales (*i.e.,* scales that have been constructed to match the diagnostic criteria enlisted in the DSM 4th edition (51)), the CBCL was empirically derived through factor analysis, and therefore cannot be considered a diagnostic tool. Rather, the scoring system underlying the CBCL refers to the concept of mental state as continuum introduced above, with rare-enough scores set as cut-off points for the identification of children with a borderline or clinical development (*i.e.,* T-scores ≥ 60 or T-scores ≥ 63, respectively).

Assessment of social competence

Given the difficulty in defining the specific characteristics of a socially competent behaviour, the different tools that have been developed to assess children's social competence are hardly comparable and generally limited in their ability to assess the trait in its entirety or examine the child's ability to handle different types of social situations. Common aspects that are investigated include the ability to master specific skills considered important in social connections, the ability to form and maintain successful relationships and the level of popularity or peer appreciation (25). In the present thesis (**chapter 5**), childhood social competence was measured with the school-age CBCL form. It comprises three scales – which examine 1) children's performance in school, 2) their participation in various activities, such as sports and hobbies, and 3) their involvement in social organisations, such as groups of peers – that can be summed into the total competence scale (49). The focus of this checklist is, therefore, on the child's ability to initiate and maintain successful social relationships and to handle academic requirements. Similarly to problem behaviours, scores in the social competence scales can be standardised by age and sex to identify any developmental issue or strength. For total competence, any score greater than 40 is considered within the normal development (49).

Assessment of temperament

One of the most widely used and validated questionnaires for the assessment of infants' temperament is the Infant Behaviour Questionnaire (IBQ) (52), developed by Rothbart and colleagues to overcome some of the previously mentioned limitations of Thomas and Chess's pioneering work. The IBQ was recently revised to reflect the current knowledge on infant temperament. The Revised Infant Behaviour Questionnaire (IBQ-R, employed in **chapter 3** of this thesis) measures temperament as perceived by infants' parents or caregivers, who are asked to rate infants' reactions to specific and realistic situations during the previous week (53). A total of 14 temperamental dimensions are assessed, subdivided into three factors (*i.e.,* Positive Emotionality/Extraversion, Negative affectivity and Orienting/Regulation). Of the 14 scales, three in particular have been linked to childhood internalising and externalising behaviours: activity level (included in the Positive Emotionality/Extraversion factor), distress to limitation (included in the Negative affectivity factor) and duration of orienting (included in the Orienting/Regulation factor). High activity levels (*i.e.,* gross motor activity) in infancy have been associated with higher externalising behaviours both concurrently and longitudinally (54,55). High levels of distress to

limitations (*i.e.,* frustration when confined to a place or position) in infancy have been related to higher internalising and externalising behaviours (55). Finally, duration of orienting (*i.e.*, the ability to maintain the attention) is negatively associated with both internalising and externalising behaviours (14,55).

Genetic and environmental influences on children's psychological development

Problem behaviours, social competence and temperament are all complex traits with numerous genetic and environmental influences. Twin studies have revealed a significant genetic component on internalising and externalising problem behaviours, with heritability (*i.e.,* the proportion of variance that is explained by genetic factors) between 40% and 70% (*e.g.,* (56,57)). Similar estimates of heritability were reported for social competence (*i.e.,* 21% to 78%) (58–60) and temperament (*i.e.,* 20% to 60%) (61).

This observation has two major implications: 1) a large proportion of variance is environmentally influenced, and 2) deviant and dysregulated behaviours might be prevented by tackling modifiable (environmental) risk factors, considering that even a large genetic influence does not imply genetic determination. By employing genetically informed study designs, it is possible to identify the sources of individual variation in a given trait and to examine the relative importance of different environmental influences while accounting for genetic influences.

Genetically informed study designs

Twin studies

Of the different types of genetically informed study designs, the classical twin design is arguably the most widely used to evaluate how much of the variance in a trait is due to genetics, common (or shared) environment and unique (or unshared) environment. Genetics includes both additive influences (*i.e.,* whose combined effects are the sum of each individual effect) and non-additive influences (*i.e.,* responsible for gene-gene interactions such as dominance and epistasis), although the additive component is generally more prominent. The common environment includes all events that affect both twins in the same way. As the name suggests, the unique environment includes all events that happen specifically to one twin, or common events that affect the two twins

differentially. Monozygotic (MZ, or identical) twins share 100% of their genes and of their common environment, while dizygotic (DZ, or fraternal) twins share 100% of their common environment but only 50%, on average, of their genes. Comparing MZ and DZ twins' characteristics, their intrapair similarities and differences, it is therefore possible to calculate the relative importance of genetic, common or shared environmental factors for a given trait (62).

Extensions of the classical twin study design have been developed to examine whether two or more traits are genetically or environmentally related (*i.e.,* bivariate or multivariate models), or to infer causality (62). Specifically, the use of twin data for causal inference takes advantage of the possibility to account for genetic and common environmental influences, so that any residual effect on the outcome is likely to be caused by the exposure of interest (especially after additional sources of unique environment are controlled for) (63). To date, three different statistical methods, all variations of regression analysis, have been proposed for the investigation of exposure-outcome associations and causal inference with twin data (64). The first method employs a mixed-effect regression model to account for the non-independent structure of the data by adding random intercepts for twin pairs to the model. Contrarily to other methods, this one permits the investigation of the effect of a shared environmental factor, for example maternal pre-pregnancy BMI (see **chapter 3** for a more detailed description of this analysis), on an outcome. The second method regresses intrapair twin differences in the outcome over the intrapair twin differences in the exposure. By doing so, in MZ twins, it is possible to completely account for any source of genetic and common environmental influence and thus focus only on sources of the unique environment (see **chapter 2** for a more detailed description of this analysis). The third method employs a mixed-effect regression model to investigate simultaneously any variations in the outcome that is explained by within-pair and between-pair differences in the exposure, which necessarily is a unique environmental factor.

As mentioned before, these methods have been largely employed to investigate causality in situations in which experimental manipulation is not feasible for ethical or practical reasons, by taking advantage of the so-called natural experiment that are twins. Discordant twin study designs (*i.e.,* methods two and three above) are particularly powerful in this regard, as they automatically control for all common influences between co-twins and can test for the temporality of the association if applied to longitudinal data (65). Nonetheless, no twin study design can be considered equivalent to a true experiment, as it is impossible to account for all involved, non-shared environmental factors with an adequate level of accuracy. Furthermore, although internal validity

is generally high in twin studies, the external validity might be modest (63). For example, it is largely accepted that the prenatal environment experienced by twins is not comparable to what singletons experience, with twins being often exposed to pregnancy complications and preterm birth (66). Consequently, some findings might not be applicable to singletons. Twin studies can, therefore, be considered useful methods for gathering information regarding an association, but additional evidences from different populations are required for a final conclusion on causality.

Alternative genetically informed, family study designs

The twin design is certainly the most commonly used study design in behaviour genetics, but other designs are available that use different family structure to examine the influences of genetics and environment on a trait. Among the available options there are 1) the extended-twin study design, which, in addition from MZ and DZ twins, may include data from their non-twin siblings, parents, partners, children, etc., 2) the discordant sibling design, which examines data from non-twin siblings who share 50% of their genes but who experienced different prenatal and postnatal environments, 3) maternal *vs.* paternal exposure design, which uses paternal exposure as negative control, and 4) *in vitro* fertilisation (IVF) design, which uses data from *in-vitro* conceived children and their families, where genetically related and unrelated mother-child pairs are compared. Thanks to the different degrees of genetic relatedness between family members, these study designs are able to investigate research questions that cannot be examined with a classical twin study (63,67). For example, with an extended-twin design it is possible to calculate simultaneously the relative importance of non-additive genetic influences and common environmental factors, which are confounded in the classical twin design. Similarly, it allows to distinguish between different sources of common environment, such as factors shared by all family members and those shared only by siblings. However, the most important advantage of these study designs compared to the classical twin design or non-genetically informed designs is arguably that they allow the disentanglement of purely environmental influences from environmental factors that are genetically influenced, hence allowing to test for the presence of gene-environment correlation (*i.e.,* when the exposure to a particular environment is genetically influenced (68)).

Despite these strengths, these family designs are not free from limitations, including the risk of carry-over effect in the discordant sibling design (*i.e.,* when the exposure and outcome in one child affect the exposure and outcome in their younger siblings), the difficulties related to recruiting a

sufficient number of similar-age children born to MZ-twin mothers, as well as their relatives, for the extended-twin design, and the limited generalisability for the IVF design (67).

Overall, no study design can be considered superior from others in any situations, and all of them rely on important assumptions regarding the relationships between relatives, but the combination of results derived from multiple studies using different designs (even, to some extent, those that lack genetic information) can provide strong evidence for or against a hypothesised association.

Prenatal influences on children's psychological development

Among all possible environmental factors that might influence child development, those associated with the prenatal period have elicited a great deal of interest for their potential employment in primary prevention. This work gravitates around the idea that pre- and perinatal influences can affect disease risk in later life, concept postulated by the British epidemiologist David Barker in the late 1980s (69–71) and known as Developmental Origin of Health and Disease (DOHaD) hypothesis.

The DOHaD hypothesis is rooted in the concept of *developmental plasticity*, which represents the ability of a genotype to develop into a variety of phenotypes, increasing the individual's chances of adaptation to different environmental conditions. Several sensitive periods in which a system's sensitivity to environmental influences is augmented (72) have been identified in humans, the first matching the intrauterine period (70). In turn, the hypothesis that the intrauterine period might be important for later mental health derives from the fact that during this time the nervous system starts developing and forming its basic structures, and even small perturbations during a critical stage might alter the whole developmental process (73). Although in some cases such alterations are intense and cause severe damage to the developing foetus, such as the neural tube defect caused by a lack of folate in early pregnancy (74), more often they are subtle, with less clearly identifiable consequences to the foetus. Specifically, it has been hypothesised that maternal psychopathology and lifestyle factors induce subtle alterations to the intrauterine environment, influencing infants' birth weight and disease risk in late life (75). In the following paragraphs we will discuss birth weight and maternal lifestyle factors (*i.e.,* weight before and during pregnancy and dietary factors in pregnancy), together with the available evidence of their associations with childhood psychological development.

Birth weight and psychological development

Birth weight is largely used in epidemiological studies as a reliable proxy for foetal growth, since it can be easily and inexpensively obtained with self-reported questionnaires or by accessing clinical data (76). A recent systematic review and meta-analysis reported a small negative association between birth weight and childhood attention deficit/hyperactivity disorder (ADHD) symptoms (meta-analytic effect size Pearson's r: -0.15; 95% confidence interval (C.I.): -0.16, -0.13). However, moderation analyses showed that sample type, geographic region, ethnicity, mean birth weight on the sample, and informant of ADHD symptoms, but not gestational age and maternal smoking during pregnancy, were important contributors to heterogeneity in effect size. Furthermore, the authors highlighted substantial variation in the association that remained unexplained (77). Similarly, another systematic review and meta-analysis comparing children born with an extremely low birth weight (ELBW) to children born with a normal weight reported a greater risk of internalising and externalising behaviours in ELBW children (Hedges' g: 0.42; 95% C.I.: 0.26, 0.58 and g: 0.15; 95% C.I.: 0.02, 0.28 for internalising and externalising behaviours, respectively). However, significant differences in the effect estimates of internalising behaviours were found when examining the moderating effect of geographic region, with significantly larger estimates in Europe than in North America (78).

It should be considered that birth weight is not only environmentally influenced, but it also has a large genetic component (79). As a result, the low-birth weight population is highly heterogeneous, with infants who suffered intrauterine growth restriction and others who are small for genetic reasons (80). Crude birth-weight measurements might, therefore, produce biased results, overestimating the risk for genetically small children and underestimating it for children affected by intrauterine growth restriction.

As explained above, an effective way to control for genetic factors, and thus assess only environmental influences, is by employing a discordant twin study design. Although a small birth-weight discordance between co-twins is physiological and generally due to genetic or small environmental differences, a severe birth-weight discordance might be caused by several different factors, including malformation, lack of intrauterine space and low gestational weight gain (81–84). These factors might randomly affect only one twin in a pair, whose weight would then be lower than that of his or her co-twin (85). Consequently, large birth-weight discordance increases the risk of perinatal mortality and morbidity (84), and might be a risk factor for later developmental delays.

To date, intrapair birth-weight difference has been associated with ADHD symptoms (86–88), and problem behaviours (89–91). Specifically, relative birth-weight discordance was associated with greater total problem score difference in 1,490 Belgian twins aged 6 to 17 years. The difference in total problem scores increased by 0.35% for each percentage point increase in intrapair birth-weight discordance, with similar effects in MZ and DZ twins (90). Increasing birth-weight difference was also associated with a greater difference in total and emotional problems difference in 3,114 Danish twins aged 12 years. Here, a 10%-increase in MZ twins' absolute birth-weight difference was associated with 0.38 SD decrease in the total-problem difference, and with 0.41 SD decrease in emotional-problems difference. Moreover, twins' sex was found to moderate the association, as birth-weight difference in MZ twins was associated with emotional problems in females, and with total problems and hyperactivity in males (91). Conversely, the only study assessing preschool-age twins (n=112 twin pairs, of which only 29 were MZ twin pairs) reported that higher birth-weight twins had more conduct problems compared to their smaller co-twins in 41.1% of pairs, whereas the smaller birth-weight twins had more conduct problems in only 20.5% of pairs. However, although no significant effect of zygosity was found, there was a tendency for greater differences in DZ compared to MZ twins (89), suggesting that uncontrolled genetic influences in DZ twins might underlie the behavioural differences. Consequently, it still needs to be clarified whether birth weight is associated with problem behaviours in younger children and which traits might be mostly affected.

Maternal pre-pregnancy weight, gestational weight gain and child's psychological development

Maternal pre-pregnancy weight

Maternal pre-pregnancy weight is considered a useful proxy for women's health and lifestyle before gestation (92,93). In fact, extreme body weight (too low or too high) at the time of conception have been associated with increased risk of several adverse outcomes – including low birth weight and preterm birth, intrauterine growth restriction, and child and maternal morbidity (94,95) –, which often cannot be reduced by improving diet and physical activity during pregnancy (92). Therefore, entering pregnancy with a healthy weight might significantly improve child outcomes by guaranteeing an optimal prenatal environment since conception.

Numerous studies to date have explored the role of maternal pre-pregnancy weight and investigated its association with child neurocognitive and psychological outcomes. Given the ongoing obesity epidemic (96), a great deal of research has focused on maternal pre-pregnancy overweight or obesity. Results of the associations with childhood mental disorders and neurocognitive outcomes have been summarised in five systematic reviews (97–101), all of whom reported higher risk of poor development in children born to women with overweight or obesity. Specifically, compared to children born to women with a pre-pregnancy normal weight, those born to women with pre-pregnancy obesity had 42% higher odds of childhood internalising or externalising problems (odds ratio [OR]: 1.42; 95% C.I.: 1.26, 1.59) (99). Even higher estimates were reported for childhood ADHD: OR = 1.62 (1.23, 2.14) (99) and relative risk (RR) = 1.64 (1.47, 1.73) (100). Importantly, the evidence points toward a dose-response effect of maternal pre-pregnancy BMI, with lower risk for children born to women with pre-pregnancy overweight (RR: 1.28; 1.17, 1.40) (100). Furthermore, a linear positive association between maternal pre-pregnancy BMI and externalising behaviours in 2-year-old children (n=1,937) was reported (CBCL externalising raw scores increased by 0.13 point, on a 0-22 range, for every unit increase in maternal BMI; 95% C.I.: 0.01, 0.25) (102). In contrast, no association was found with internalising symptoms (102). Focusing on the potential effect of maternal pre-pregnancy underweight, associations were reported with higher total behaviour problems and externalising behaviours in boys aged 9-11 years (total n=5,660). The estimated increase in scores in the underweight group compared to normal maternal BMI was 2.34 points (0.02, 4.66) for total problems and 3.30 points (0.69, 5.91) for externalising behaviours (standardised scores with the mean set at 100 and the SD set at 15 were used) (103). Moreover, compared to maternal normal weight before pregnancy, maternal pre-pregnancy underweight was associated with increased risk of a wide array of behavioural difficulties at 5 years based on teacher but not maternal ratings: concurrent (OR: 2.00; 95% C.I.: 1.09, 3.70), prolonged for at least 6 months (OR: 2.06; 95% C.I.: 1.09, 3.92) and in group settings (OR: 1.92; 95% C.I.: 1.05, 3.50) (total n=1,714) (104). No associations with maternal underweight were however reported when examining psychosocial development at 6 years (total n=1,311) (105) or when assessing affective disorder longitudinally from the age of 5 to 17 years (total n=2,868) (106). Nevertheless, the proportion of women with pre-pregnancy underweight could have been in some cases insufficient to identify an association with childhood outcomes. Hence, an increased risk in children born to women with underweight cannot be ruled out and should be further investigated.

In contrast to the association of maternal pre-pregnancy BMI and child problem behaviours that has been largely investigated, the association with infants' temperament has been mostly neglected. The first study examining this relationship in Australian singletons (n=2,785) at 1 year of age reported no associations (OR for difficult compared to easy temperament: 1.02; 95% C.I.: 0.99, 1.05) (102). However, poorer behavioural regulation was found in infants born to women with pre-pregnancy overweight or obesity (OR for difficulties in multiple behaviour-regulation domains: 1.22; 95% C.I.: 1.05, 1.42; n=2,116) (107) or pre-pregnancy obesity in combination with excessive gestational weight gain (n=159) (108). Maternal pre-pregnancy BMI was also positively associated with negative affectivity in Canadian infants aged 3 months (slope estimate: 0.04 points on a 7-point Likert-type scale; 95% C.I.: 0.01, 0.08; n=16) (109). Finally, pre-pregnancy BMI was associated with negative behaviour at 6 months of age (slope estimate: 0.45; standard error (SE): 0.16; n=62), although the effect was reduced in children of women with high total n-3 polyunsaturated fatty acid (PUFA) concentrations in the third trimester of pregnancy (interaction slope estimate: -0.41; p=0.007) (110). However, in the latter study, maternal pre-pregnancy BMI was associated with negative temperament when assessed through direct observation but not when infants' temperament was reported by parents.

As we will discuss in more details at page 36, a common limitation of all included studies is the lack of control for familial confounding, which might affect these results. In fact, not only child's psychological outcomes are genetically influenced as discussed above, but also maternal BMI has a large genetic component (111). As a result, unadjusted estimates might be inflated and should be interpreted with caution. The strongest evidence of familial confounding on previously reported associations comes from studies investigating child ADHD. Results from 463,474 full biological siblings show that maternal obesity before pregnancy did not increase the risk of childhood ADHD (Hazard ratio [HR]: 1.10; 95% C.I.: 0.94, 1.27) (100). Comparable findings were reported when analysing smaller samples (112,113). However, larger estimates were reported for the association between maternal pre-pregnancy BMI and child problem behaviours after adjusting for the underlying family structure, although the confidence intervals became wider and included the null value (slope for total problems: 10.56; 95% C.I.: -1.81, 22.93; slope for externalising behaviours: 10.74; 95% C.I.: -1.77, 23.25. Estimates obtained from the full sample (not controlling for family structure): slope for total problem: 7.99; 95% C.I.: 3.53, 12.46; slope for externalising behaviours: 5.77; 95% C.I.: 1.50, 10.04. Standardised scores with the mean set at 100 and the SD set at 15 were used) (103). By contrast, no substantial difference in estimates were obtained when using paternal

BMI as negative control (114–116). Finally, a twin study showed a trend for increased risk of externalising behaviours in preschool children exposed to maternal overweight or obesity compared to normal weight (57). Consequently, before accepting the hypothetical association between maternal pre-pregnancy BMI and childhood psychological development, further analyses employing genetically informed study designs should be conducted.

Gestational weight gain

Maternal weight increases physiologically during pregnancy, a biological phenomenon known as gestational weight gain (GWG). GWG is necessary for foetal growth and development, affected by maternal physiology and metabolism and also placental metabolism (93). Three components of weight gain can be identified: 1) maternal fat mass and fat-free mass, including an increase in blood volume and extracellular fluid, and an increase in body fat, and uterine and breast tissues; 2) placental mass, which is created *de novo* shortly after conception and continues to grow throughout gestation; 3) amniotic fluid, which at term can affect GWG by as much as 1 kg, and the foetus (93).

GWG is both genetically and environmentally determined. A twin study estimated that the heritability of GWG is approximately 30%-40%, with somewhat lower estimates for the second pregnancy, in which unique environmental factors might have an even greater role compared to the first pregnancy (117). Molecular genetic analyses showed that common genetic variants associated with BMI might not be independently associated with GWG (118,119). This should not come as a surprise, considering that fat mass accretion represents only a limited portion of GWG. Nonetheless, the sociodemographic determinants of GWG are similar to those of BMI, including the presence or absence of a particularly obesogenic environment, social support and health services, and women's socioeconomic status among others (93).

Adequacy of GWG is generally based on the American Institute of Medicine's (IOM) recommendations, which provide a range of weight gain for each pre-pregnancy BMI category associated with the minimum risk of morbidity and mortality in the perinatal period (93). It has been hypothesised that inadequate GWG (both excessive and insufficient) produces intrauterine alterations able to affect foetal brain development, thus leading to emotional and behavioural dysregulation (93). One of the hypothesised mechanism is the development of the Hypothalamic-Pituitary-Adrenal (HPA), which is considered a putative mechanism also for the association between maternal pre-pregnancy weight and child psychological development (see page 37 for a description of hypothetical mechanisms of action). Sufficient maternal weight during pregnancy is probably

crucial to support an optimal HPA axis development, although an excess of weight might lead to hormonal imbalance and chronic inflammation that would produce an HPA axis dysregulation (120,121). Considering that the evidence shows that over 60% of women with pre-pregnancy overweight or obesity gain weight in excess compared to about 37% of women with a BMI in the normal range (122), it could be hypothesised that children born to overweight or obese women with high GWG could be subject to an even increased risk of developmental problems.

Recent evidence suggests that GWG might influence children's cognitive and psychological development (108,123–130). School-age children (n=12,556, age: 7-12 years) born to women with a high pre-pregnancy BMI and a high GWG had an increased risk of ADHD (OR: 1.24; 95% C.I.: 1.07, 1.44) (128), although a subsequent study (n=511) did not report a greater risks in 10-year-old children born to women with overweight/obesity and excessive GWG (incidence rate ratio (IRR): 1.7; 95% C.I.: 0.9, 2.8), or in children born to normal-weight women with an insufficient GWG (IRR: 1.2; 95% C.I.: 0.9, 1.5) (127). In addition, insufficient GWG was associated with higher hyperactivity/impulsivity symptoms in 331 preschool children, while only a tendency for greater inhibiting behaviours was found in children exposed to excessive compared to adequate GWG (130). Moreover, poorer neonatal neurobehaviour was reported in infants (n=159, of whom 70 in the obesity group) born to women with pre-pregnancy obesity who also gained excess GWG (108). Only one study was found specifically assessing the relationship between GWG and internalising and externalising problem behaviours in children aged 10 years (n=511), reporting null results (127). The latter study, however, examined a high-risk population selected based on first-trimester alcohol or marijuana use, which limits the generalisability of study findings. Consequently, research is needed to evaluate the possible influence of gestational weight gain on childhood problem behaviours in low-risk populations.

Maternal diet during pregnancy and child's psychological development

Among the determinants of GWG, the two that might be directly involved in weight gain are dietary intake and physical activity, as they affect the energy balance. In fact, despite the physiological adaptations that occur in pregnancy (131), significant associations were reported between intakes of total calories, specific dietary components or physical activity and GWG (*e.g.,* (132)). Nonetheless, GWG itself is a physiological phenomenon with a very specific and fundamental role – *i.e.,* to support foetal growth and development –, thus not only the *quantity* but especially the *quality* of food might play a role on GWG and psychological development.

Strong evidence is available on the importance of optimal maternal diet during pregnancy to provide adequate sustainment to foetal development, mostly coming from studies on the effects of severe micronutrient deficiencies or maternal undernutrition on offspring's health (133). On the contrary, evidence of the influence of more subtle variations in maternal diet quality is lacking.

Maternal polyunsaturated fatty acids

PUFAs are fatty-acid molecules (*i.e.,* hydrocarbon chains with a carboxylic acid at one end and a methyl group at the other end) with two or more carbon-carbon double bonds in the hydrocarbon chain. Within this large family of fatty acids, the two clusters of n-3 and n-6 PUFAs can be identified, depending on whether the first double bond is three or six carbons away from the methyl group, respectively (134). Fatty acids of the n-3 and n-6 series are of particular importance, as they cannot be synthesized *de novo* by the human species, which lacks the enzyme required for the insertion of a double bond three or six carbons away from the methyl group of a fatty-acid molecule. For this reason, the α-linolenic acid (ALA, n-3) and the linoleic acid (LA, n–6) are *essential* fatty acids that must be derived from the diet (134). In addition, humans are able to further metabolise ALA and LA into the so-called long-chain PUFAs, which include the eicosapentaenoic acid (EPA, n-3), the docosahexaenoic acid (DHA, n-3) and the arachidonic acid (AA, n-6) (134).

Numerous studies have focused on the association between PUFAs and neurocognitive and behavioural development, given the numerous important functions of PUFAs within brain development and functioning across the lifetime (135–139), as we will discuss at page 38. However, evidence of the influence of pre- and perinatal PUFAs on child emotional and socio-behavioural development is scarce, with several observational studies and a few randomised controlled trials reporting largely inconsistent findings. Assessing maternal food intakes with a food frequency questionnaire (FFQ) twice during pregnancy, a small UK study (n=217) found that eating oily fish (*i.e.,* the most important source of n-3 PUFAs and especially DHA) at least once a week was associated with small improvements in childhood hyperactivity at 9 years (less than once a week in *early* pregnancy compared to never: OR: 0.30; 95% C.I.: 0.12, 0.76; less than once a week in *late* pregnancy compared to never: OR: 0.40; 0.16, 0.98) (140). Similarly, seafood intakes measured with an FFQ in week 32 of gestation was associated with a reduction of childhood prosocial behaviour at 7 years (n=11,875; no seafood intake compared to >340g/week: OR: 1.44; 95% C.I.: 1.05, 1.97) (141). When examining PUFA concentrations directly in maternal blood, every unit increase in maternal DHA concentrations were associated with a small reduction in the risk of emotional symptoms (OR

early pregnancy: 0.75; 95% C.I.: 0.56, 0.99; OR mid-pregnancy: 0.82; 95% C.I.: 0.70, 0.96) in 6-year-old Dutch children from the Generation R cohort (n=2,061 up to 5,307) (142,143). Higher AA concentrations in mid-pregnancy were associated with a marginal increased risk in teacher-assessed problem behaviours (OR: 1.10; 95% C.I.: 1.00, 1.20) (142). In addition, DHA in the umbilical cord was associated with a small reduction in internalising behaviour scores in 7-year-old children fed with formula milk (n=215) but no association was found in breastfed children (n=170) (144). In contrast, umbilical-cord DHA was associated with a small reduction in problem behaviours and hyperactivity/inattention in German children aged 10 years (n=416) (145). In the same cohort, AA alone or in combination with DHA was associated with a reduction in emotional symptoms (145). However, higher concentrations of EPA were associated with an increase in conduct problems, while ALA was predictive of higher peer relation problems (145). Finally, only six, small randomised controlled trials (RCTs) were conducted to investigate the effect of maternal supplementation with PUFAs during pregnancy on child development. As summarised by a systematic review (146), studies examined the effect of n-3 PUFAs (mostly DHA) compared to corn, soybean or olive oil, on infant neurobehaviour, reporting no association. The only RCT that investigated child problem behaviours at 7 years did so in relation to maternal DHA supplementation in the first 4 months of breastfeeding, with no difference in child outcomes between treatment and control groups (147).

Overall, there is a large heterogeneity between these studies, especially concerning the applied statistical methods and the specific PUFAs assessed, which might be the reason for the inconsistency between results. As already mentioned, there is a large variation in the methods of PUFA assessment (*i.e.*, extrapolation from reported dietary intakes, direct measure of PUFA levels in blood or supplementation). Of note is that during pregnancy maternal PUFA levels are affected by dietary intakes in the previous days but also by a process called fat mobilisation, in which free fatty acids stored in the woman's adipose tissue are released in the circulation to support foetal development (148,149). Therefore, assessment of PUFA intakes during pregnancy with an FFQ is not likely to provide a complete representation of prenatal PUFA exposure. Furthermore, given the well-known, non-linear change in relative PUFA levels in maternal blood during pregnancy (151), evaluation of isolated PUFA concentrations without assessing how their levels change over time and in relation to the other PUFAs could have produced biased results. Finally, considering that neurodevelopment starts soon after conception (150), increasing maternal PUFA levels from the second or third trimester, as was done in the RCTs, might not be effective. Additional research is, therefore,

warranted to clarify the association between prenatal PUFA exposure and childhood psychological development.

Maternal diet quality

Although important, maternal PUFAs are certainly not the only dietary components to be investigated in relation to child psychological development. For example, a previous study reported a higher risk of childhood problem behaviours in children whose mothers did not use folic acid supplements during embryogenesis (*i.e.,* the first 10 weeks of gestation; OR: 1.44; 95% C.I.: 1.12, 1.86) (152), although other studies did not support an association with neurocognitive development (153), or reported possible detrimental effects associated with extreme folate intakes (154). Furthermore, minerals such as iron, iodine and zinc might affect neurodevelopment, although the evidence for their effect on child psychopathology is scarce (155).

It is well known that nutrients and food groups are highly correlated within a diet, so that in natural conditions the increase in one food group is associated with a decrease in another and an increase in yet another one. Furthermore, the effect on health outcomes is likely the result of interactions between multiple food groups, so examining only one or a few nutrients or food groups might lead to biased results (156). The whole maternal diet, with different food items and their reciprocal interactions, might be a better predictor of postnatal health (156–159).

Nonetheless, as pointed out by a recently published systematic review and meta-analysis, the large majority of studies to date has focused on single nutrients or food items, with only a few assessing the whole maternal diet during pregnancy (160). Three studies examined the associations of maternal dietary patterns identified with a data-driven method (*i.e.,* principal component analyses, PCA) with child problem behaviours (161–163). Norwegian children (n=23,020) prenatally exposed to a "healthy" diet, characterised by consumption of fruit, vegetables and fish, had lower externalising behaviour scores longitudinally from age 1.5 to 5 years. In contrast, exposure to an "unhealthy" diet prenatally, characterised by consumption of salty and sweet snacks, was associated with higher externalising behaviour scores (161). Similarly, maternal adherence to a dietary pattern that resembled the Mediterranean diet (MD), characterised by consumption of fruit, vegetables, fish, tea and eggs, was associated with a lower risk of externalising behaviours in 3,104 Dutch children aged 1.5 to 6 years (OR: 0.90; 95% C.I.: 0.83, 0.97), while adherence to a dietary pattern that resembled the traditional Dutch one, characterised by consumption of meat and potatoes, was predictive of a higher risk of externalising behaviours (OR: 1.11; 95% C.I.: 1.03, 1.21)

(162). Finally, poor adherence to a "healthy" pattern (characterised by consumption of fruit, vegetables, fish and wholegrains) or high adherence to a "high Western" pattern (characterised by high intake of processed food) were related to higher hyperactivity-inattention symptoms in French children (n=1,242) aged 3 to 8 years (161–163).

Although valuable for hypothesis generation, data-driven methods have been criticised for their excessive dependence on the analysed data and on arbitrary decisions during data analysis (164,165). Besides, diet quality scores or indices, being *a-priori* methods of dietary assessment based on current nutritional knowledge, may provide results that could also be more easily translatable into public health recommendations (165). Together, results obtained with data-driven methods and diet quality scores or indices can provide stronger evidence for an association.

To date, only one study was published that examined the association between maternal adherence to a predefined dietary index and childhood problem behaviours (166). Here, adherence to the Prenatal Diet Quality Index, characterised by high intakes of fruit, vegetables, wholegrains and fish, and low intakes of red meat, sugar and salt, was associated with a small decrease in internalising and externalising scores in Norwegian 27,529 children aged 1.5 to 5 years (Average marginal effects (AMEs) for externalising behaviours: -4%; high density interval (HDI): 2%, 5%; AMEs for internalising behaviours: -3%; HDI: 1%, 5%). In addition, a re-analysis of the data to support findings obtained with PCA found that a higher maternal adherence to the MD score was associated with a small reduction in the odds of child externalising behaviours (0.94; 95% C.I.: 0.90, 0.98; n=3,104) at 1.5 to 6 years (162). Finally, maternal MD score in the highest tertile at the time of conception (*vs.* lowest tertile of MD score) was associated with fewer internalising and externalising behaviour symptoms (OR for atypical behaviours: 0.40; 95% C.I.: 0.20, 0.78; maladaptive behaviours: 0.42; 95% C.I.: 0.18, 0.95; depressive symptoms: 0.28; 95% C.I.: 0.12, 0.64; anxiety: 0.42; 95% C.I.: 0.18, 0.97) and increased odds of social relatedness (2.31; 95% C.I.: 1.04, 5.19) in 325 2-year-old US children (167). These associations related to changes in the methylation patterns in the control regions of several imprinted genes, suggesting a possible mechanism underlying the association between maternal diet quality prenatally and childhood problem behaviours (167). Consequently, results obtained with *a-priori* dietary assessment methods largely confirm associations obtained with data-driven methods. Nonetheless, little is known about the generalisability of these associations to culturally different populations, such as children raised in Southern Europe.

Criticisms to the DOHaD hypothesis

Despite the large number of studies published to date reporting associations between various prenatal environmental factors and childhood outcomes, and thus generally supporting the DOHaD hypothesis, some authors have recently raised doubts about the validity of such associations. Specifically, the main criticism regards the scarce use of genetically informed designs and therefore the lack of control for genetic influences and gene-environment correlation (63,67). Indeed, when familial factors are considered, the strength of widely acknowledged associations is often considerably reduced, suggesting that effect sizes obtained without controlling for genetic and intra-familial environmental factors might be inflated (65). For example, a systematic review reported little to no evidence supporting a causal effect of maternal smoking during pregnancy and child ADHD and conduct problem risks from different types of genetically informed studies (67), while a small residual effect of maternal smoking was observed on externalising but not internalising behaviours in Dutch twins (168). Results from the Norwegian Mother and Child Cohort (MoBa) revealed genetic confounding on the association between maternal prenatal psychopathology, a widely considered important risk factor for child development, and childhood problem behaviours (169–171). Similarly, there was no evidence of an association between maternal pre-pregnancy BMI and child ADHD after controlling for familial confounding with discordant sibling or cousin designs (100,112,113). By contrast, in other studies the strength of the association with problem behaviours in childhood was only modestly reduced after adjusting for the underlying family structure (*i.e.*, singleton or twin siblings) (57,103). Furthermore, the use of paternal BMI as negative control was supportive of a phenotypic effect of maternal pre-pregnancy BMI on child neuropsychological and behavioural development (114–116).

Overall, it seems reasonable to hypothesise that the associations observed within the DOHaD framework might be at least partially inflated by the lack of control for familial confounding, which are likely to be genetic in nature. Observational studies that do not make use of a genetically informed design are therefore likely to find associations that are not necessarily causal. Nonetheless, previous studies did not completely exclude the phenotypic prenatal route, which is also supported by animal models (*e.g.,* (172)) and by studies investigating the plausible biological mechanisms of action (discussed below). Further research is thus needed to shed light on the role of the prenatal environment for childhood psychological development, and a definitive answer can only be obtained with the triangulation of evidence coming from several well-conducted studies with different designs.

Hypothetical mechanisms of action

Several mechanisms of action have been hypothesised to explain the observed or predicted associations between prenatal exposures and childhood psychological development. Given the complexity of psychological development, these mechanisms should not be considered mutually exclusive, but rather they likely represent single facets of a larger biological process.

The Hypothalamic-Pituitary-Adrenal axis

A plausible mechanism concerns the development and functioning of the HPA axis, which regulates the emotional and behavioural response of the organism to stress (*e.g.,* (173)). A prenatal environment characterised by chronic inflammation and hormonal imbalance, such as the one favoured by maternal stress, anxiety/depression, overweight or unhealthy diet, is hypothesised to interfere with the optimal HPA axis development in the foetus, resulting in socio-emotional and behavioural problems (*e.g.,* (121,174–178)). Specifically, chronic systemic inflammation is a feature of both pregnancy and obesity, thus in obese women who are also pregnant inflammatory markers can reach even higher levels. Together with imbalances in insulin, leptin, serotonin and dopamine, chronic inflammation may have significant effects on brain development, affecting neural circuits implicated in behavioural regulation, including the HPA axis (179,180). What is more, growing evidence supports a role of the HPA axis on the intergenerational transmission of physical and psychological susceptibility. Specifically, the high stress reactivity that results from a stress-induced dysregulation of the HPA axis might lead to excessive food intake, especially highly palatable, calorie-dense food (*i.e.,* "comfort food") that could be effective, at least in the short term, in inhibiting the stress response (181–183). A downside of this stress-releasing mechanism is of course the increased risk in visceral fat accumulation and obesity, which in turn leads to metabolic diseases, such as cardiovascular diseases, type 2 diabetes mellitus, (184) and mental disorders (185). According to this hypothesis, women with these metabolic conditions would be more likely to pass on their susceptibilities to the next generation.

Epigenetic modifications

A possible process underlying the HPA axis dysregulation and the intergenerational programming involves epigenetic mechanisms (*i.e.,* functional modifications of the genome that might regulate gene expression without altering the DNA sequence (186)), of which the most widely studied is DNA methylation. Mounting evidence is available linking epigenetic modifications in either candidate

genes involved in brain development or at the genome-wide level to HPA axis dysregulation and stress reactivity (187). Differences in the expression of 205 genes involved in neurodevelopment have been observed in foetuses (14-24 weeks of gestation) of women with obesity compared to foetuses of normal-weight women (188). Similarly, a difference in epigenetic alterations in genes related to neurodevelopment and in white matter integrity was observed in new-borns of obese compared to normal-weight women (189). Differences in the epigenetic modifications were also reported in children prenatally exposed to poor-quality diets or specific food groups or nutrients (159,190–192). However, studies examining the epigenetic modifications in the context of prenatal programming of neurodevelopment are subject to a few limitations, which need to be considered. Firstly, (human) studies need to rely on peripheral, accessible tissues in which the epigenetic pattern might not be comparable to the one present in the tissues involved in neurodevelopment (193). Secondly, it should be noted that an epigenetic modification might not result in an altered gene expression, which could depend on the location of the epigenetic marker relative the genetic sequence (191). Thirdly, most studies employed a cross-sectional or retrospective design, in which epigenetic patterns are examined at the same time of the outcome, limiting the possibility to establish a causal relationship between the exposure and the epigenetic modifications (187). Finally, given that epigenetic modifications are influenced by the DNA sequence and several different environmental factors throughout life, appropriate control for confounding factors and timing of epigenetic assessment is necessary to avoid biased results and infer the causality of the associations (193). However, epigenetic mechanisms might still help explaining various associations between prenatal exposures and child outcomes, and, in the future, better-conceived studies will certainly provide invaluable information about the aetiological mechanisms underlying psychopathology.

Polyunsaturated fatty acids

Another hypothesised mechanism that has been often examined regards the role of PUFAs on brain development and function (135–139). Adequate levels of PUFAs are considered essential to guarantee an optimal development of all neuronal structure and neural circuits, especially considering that 25% to 30% of the brain's dry weight is represented by PUFAs, mostly AA and DHA (138,194–196). Within the nervous system, PUFAs are responsible to maintain the integrity of the neuronal membrane and the functionality of the myelin sheath (*i.e.,* an insulating layer made up of lipids, mostly PUFAs, and proteins that forms around nerves and allows a rapid transmission of electric impulses) (197). Furthermore, in late pregnancy, during the brain's growth spurt, PUFAs are crucial for guaranteeing the formation of synaptic structures and dendritic arborisation (135,194).

Specifically, DHA might influence processes such as synaptogenesis and synaptic activity (135), possibly by reducing the deleterious effects of stress on astrocytes (136). DHA might also mediate the regulation of neuroinflammation, participate in signal transduction and regulate the blood-brain barrier's permeability (198). EPA has anti-inflammatory, neuroprotective and antiapoptotic properties (137), while AA mediates neuronal signalling, limits oxidative stress in the hippocampus and is likely involved in nerve growth and synaptogenesis (199–201). Maternal storage represents the only source of PUFAs for the developing foetus, which are actively transferred through the placenta throughout gestation (148,151). Maternal PUFA levels are therefore important to sustain foetal brain development and might have an effect also on the child's psychopathological development.

The gut microbiota

Finally, recent evidence suggests a possible role of the gut microbiota on brain development and child behaviour. Animal studies demonstrated how shifts in the gut microbiota due to antibiotic use or maternal high-fat diet consumption during pregnancy affected the offspring's HPA axis development and behavioural response to stress (202). In humans, the scarce data available regarding the relation between gut microbiota and psychiatric disorders suggest a difference in microbial composition in children affected by autism spectrum disorder, ADHD or cognitive delays, and in adults affected by major depressive disorder (202,203). Microbial transplant therapy resulted effective in reducing autistic symptoms in 18 children for a minimum of 8 weeks (202). Furthermore, administration of prebiotics or probiotics can reduce symptoms of depression and anxiety (203). Nonetheless, the evidence of the effects of the gut microbiota on neuropsychological disorders is still very preliminary and based on small, cross-sectional studies.

Aims and hypotheses of this thesis

The aim of the present thesis was to examine the associations between prenatal, environmental influences and childhood psychological development.

In **chapter 2**, we examined the association between birth weight, used as a proxy of prenatal environmental factors, and problem behaviours. We used a twin study design to rule out the effect of both genetic and shared environmental factors. The hypothesis was that increasing birth-weight

difference between co-twins would be associated with a rise in problem-behaviour difference, with lower birth-weight twins experiencing higher problem behaviours.

As birth weight is greatly influenced by the prenatal environment (79), we tackled three maternal lifestyle factors that affect birth weight – *i.e.,* weight before pregnancy, gestational weight gain and dietary factors –, hypothesising that they might also be associated with children's psychological development. We hypothesised that higher maternal pre-pregnancy weight would be associated with negative temperament (**chapter 3**) and that gestational weight gain would predict higher problem behaviours (**chapter 4**). In **chapter 5**, we hypothesised an association between changes in maternal PUFA concentrations during pregnancy and childhood problem behaviours and social competence. Finally, we hypothesised that higher maternal diet quality in pregnancy would be associated with lower levels of problem behaviours in childhood (**chapter 6**).

Study populations

The studies presented in this thesis examined data from one twin study, the Twins and Multiple Birth Association Heritability Study (TAMBAHS), and three prospective cohort studies: the Maastricht Essential Fatty Acid Birth cohort (MEFAB) from the Netherlands, the Rhea Mother-Child birth cohort from Greece and the INfancia y Medio Ambiente (INMA) from Spain (figure 1.1).

Figure 1.1: Geographical distribution of the cohorts included in this thesis.

The Twins and Multiple Birth Association Heritability Study (TAMBAHS)

The Twins and Multiple Birth Association Heritability Study (TAMBAHS) aims to 1) establish a new twin cohort for the long-term follow-up of childhood development, 2) determine the associations between maternal BMI, nutritional intrauterine environment, smoking, physical activity and children development, and 3) disentangle the influence of genes, shared and unique environment on infants' temperament and children's behaviour problems. Within the project, two cohorts of

Table 1.1: Characteristics of mothers and twins in TAMBAHS

	Infant twins		Pre-school age twins	
Maternal characteristics	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**
Maternal age (years)	360	34.1 (4.2)	441	36.3 (4.4)
Pre-pregnancy BMI (kg/m2)	366	24.7 (5.3)	447	24.7 (4.8)
Maternal ethnicity, n (%)				
White	388	95.8	478	97.8
Other	17	4.2	11	2.3
Maternal education, n (%)				
Low	80	20	95	20.6
Medium	83	20.7	74	16.1
High	238	59.3	292	63.3
Maternal working status, n (%)				
Working full time	78	19.3	78	15.9
Working part-time	108	26.7	220	44.9
Not working	219	54	192	39.2
Maternal smoking, n (% yes)				
Before pregnancy	63	15.7	94	19.5
During pregnancy	15	4.1	15	3.3
After pregnancy	28	7.2	47	10.2
Twin characteristics[a]	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**
Sex, n (%)				
Male	418	50.1	513	52.2
Female	416	49.9	471	47.9
Zygosity, n (%)				
Monozygotic	376	45.1	360	36.6
Dizygotic	458	54.9	624	63.4
Birth weight (kg)	818	2.5 (0.6)	963	2.4 (0.9)
Gestational age (weeks)	824	36.1 (2.6)	978	35.9 (2.7)
Age (months)	834	8.7 (4.8)	983	35.9 (11.5)
Child's weight (kg)	742	7.8 (2.4)	733	14.3 (7.3)

twins aged 0 to 18 months and 18 months to 5 years, respectively, registered in TAMBA – a UK, volunteer-based association that provides help and advice to families with twins or higher-order multiples – were recruited between July 2008 and May 2010. At recruitment, twins' mothers were asked to retrospectively report about their anthropometric and socioeconomic characteristics during pregnancy, pregnancy outcomes and twins' anthropometric characteristics. Furthermore, mothers of younger twins were required to complete an online questionnaire about their children's temperament (*i.e.,* the IBQ-R), while mothers of older twins filled-in a questionnaire to assess their children's behaviour (*i.e.,* CBCL- 1.5-5). Within the present thesis, data from the cohort of younger twins (n=834) are presented in **chapter 3**, while data from older twins (n=960) are described in **chapter 2**. General population characteristics are reported in table 1.1; children's problem behaviours or temperament scores can be found at pages 68 and 99, respectively.

The Maastricht Essential Fatty Acid Birth cohort (MEFAB)

The Maastricht Essential Fatty Acid Birth cohort (MEFAB, www.mefab.org) is a population-based, prospective cohort established in the Limburg province, the Netherlands in 1989 (204). General aims of this cohort were to 1) study the variations in fatty acid concentrations during pregnancy and how they relate to the fatty acid concentrations in the neonate, and 2) examine the associations of long-chain PUFAs in pregnant women and their infants, with children's characteristics at birth. Between 1989 and 1995, pregnant women attending their first antenatal visit were invited to participate in the study. In total, 1,334 women were initially recruited, of whom 1,203 (90%) were followed up until delivery. When children were 4, 7, 12 and 20-25 years old, specific follow-up studies were organised with the aim of assessing their growth, cognitive and behavioural development, asthma/atopy, and cardiovascular disease risks. The 4- and 7-year follow-up studies were run on two different subpopulations: the cognitive development was assessed in younger children, while the behavioural development was examined in the older group. In this thesis, we focused on the 7-year follow-up, during which data on children's behaviour and social competence, as perceived by their parents, were assessed with the CBCL 4-18. Specifically, data from 378 (**chapter 4**) and 311 (**chapter 5**) mother-child pairs were assessed. Population characteristics are reported in table 1.2; a flow diagram representing the steps undertaken within the MEFAB cohort is shown in the Appendix (figure A1.1). Details on maternal PUFA concentrations, weight during pregnancy, and child problem behaviours and social competence are shown at pages 119, 120, 156 and 158.

Table 1.2: Characteristics of mothers and children in the MEFAB cohort

Maternal characteristics		**N**	**Mean (SD) or %**
Maternal age (years)		1279	29.3 (4.3)
BMI at first trimester (kg/m2)		365	23.6 (3.8)
Maternal ancestry, n (%)			
	Caucasian	1201	97.8
	Other	27	2.2
Parental education, n (%)			
	Low	64	23.2
	Medium	119	43.1
	High	93	33.7
Parental working status, n (%)			
	Both full-time	15	5.9
	One full-time, one part-time	116	45.8
	Both part-time	122	48.3
Maternal smoking during pregnancy, n (% yes)		328	26.0
Parity, n (% primipara)		945	74.3
Type of delivery, n (% cesarean)		152	12.1
Child characteristics		**N**	**Mean (SD) or %**
Sex, n (%)			
	Male	697	54.7
	Female	577	45.3
Birth weight (kg)		1269	3.2 (0.6)
Gestational age (weeks)		1206	39.8 (2.3)
Breastfeeding, n (%)			
	Exclusive breastfeeding	28	9.24
	Mixed breast and formula milk	115	37.95
	Formula milk	160	52.81
Age (years)		305	7.3 (0.3)
Child's BMI (kg/m2)		297	15.6 (1.8)

The Rhea Mother-Child birth cohort

The Rhea Mother-Child birth cohort (www.rhea.gr) was established in Crete, Greece, in 2007 (205). Aims of this cohort were to 1) evaluate maternal health during and after pregnancy, 2) examine the nutritional, environmental and psychosocial determinants of children's growth and obesity, neuropsychological and behavioural development, allergies and asthma, and genotoxicity, and 3) assess the effect of gene-environment interactions on children's growth and development. Between

February 2007 and February 2008, 1610 pregnant women were recruited during their first ultrasound examination, of whom 1363 (84.7%) were followed up until delivery. Data were collected in three occasions during pregnancy (during the first and third trimesters and at delivery), at 8-10 weeks postpartum, and when children aged 9-18 months, 4 years and 6 years. When children were 6 years old, the CBCL 6-18 was used to assess children's behaviour. Data from this cohort are presented in **chapters 4** (n= 413) and **6** (n=293). An overview of the population characteristics is provided in table 1.3, and a flow diagram of the Rhea study is shown in the Appendix (figure A1.2).

Table 1.3: Characteristics of mothers and children in the Rhea cohort

Maternal characteristics		**N**	**Mean (SD) or %**
Maternal age (years)		1470	29.4 (5.0)
Pre-pregnancy BMI (kg/m2)		1373	29.4 (5.0)
Maternal origin, n (%)			
	Greek	1364	91.1
	Non-Greek	133	8.9
Maternal education, n (%)			
	Low	297	21.2
	Medium	709	50.5
	High	397	28.3
Maternal working status, n (%)			
	Employed	657	47.7
	Not working	721	52.3
Maternal smoking during pregnancy, n (% yes)		330	23.8
Parity, n (% primipara)		620	56.7
Type of delivery, n (% cesarean)		766	51.7
Child characteristics		**N**	**Mean (SD) or %**
Sex, n (%)			
	Male	767	50.4
	Female	755	49.6
Birth weight (kg)		1427	3.1 (0.5)
Gestational age (weeks)		1474	38.1 (1.7)
Breastfeeding, n (%)			
	Yes	1113	83.8
	No	215	16.2
Age (years)		626	6.7 (0.3)
Child's BMI (kg/m2)		624	17.0 (2.8)

Details on maternal weight during pregnancy, diet quality and child problem behaviours are shown at pages 119, 120 and 205-208.

The INfancia y Medio Ambiente (INMA)

The INfancia y Medio Ambiente (Childhood and Environment, INMA; www.proyectoinma.org) is a network of prospective birth cohorts consisting of seven Spanish research groups started in 2003,

Table 1.4: Characteristics of mothers and children in the INMA cohorts

	Gipuzkoa		Sabadell		Valencia	
Maternal characteristics	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**
Maternal age (years)	613	32.6 (3.6)	734	31.3 (4.5)	700	31.3 (4.4)
Pre-pregnancy BMI (kg/m2)	638	23.0 (3.7)	750	23.8 (4.5)	825	23.8 (4.7)
Maternal ethnicity, n (%)						
White	622	98.0	685	95.1	773	93.4
Other	13	2.0	35	4.9	55	6.6
Maternal education, n (%)						
Low	86	13.5	201	28.2	288	34.8
Medium	232	36.5	308	43.3	351	42.5
High	318	50.0	204	28.6	188	22.7
Maternal smoking during pregnancy, n (% yes)	143	23.9	214	29.5	322	40.9
Parity, n (% primipara)	345	54.1	365	55.9	458	55.4
Type of delivery, n (% cesarean)	74	12.7	115	15.9	183	23.5
Child characteristics	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**	**N**	**Mean (SD) or %**
Sex, n (%)						
Male	307	50.3	375	50.8	417	53.0
Female	304	49.7	363	49.2	370	47.0
Birth weight (kg)	601	3.3 (0.5)	733	3.3 (0.5)	787	3.2 (0.5)
Gestational age (weeks)	609	39.7 (1.5)	736	39.7 (1.5)	787	39.5 (1.9)
Breastfeeding, n (% yes)	561	87.9	614	79.0	692	80.9
Age (years)	397	7.8 (0.1)	487	9.0 (0.7)	429	9.1 (9.2)
Child's BMI (kg/m2)	391	17.4 (2.3)	489	18.1 (3.0)	410	18.5 (3.4)

when four cohorts (*i.e.,* Asturias, Gipuzkoa, Sabadell and Valencia) were established based on the experience acquired by three existing cohorts (*i.e.,* Granada, Menorca and Ribera d'Ebre). The aim shared by these cohorts was to assess the protective or detrimental effect of the exposure to different environmental factors, including pollutants, metals and diet, during pregnancy and early childhood on children's health, although in some cases their protocols differed (206–208). The three cohorts of Gipuzkoa, Sabadell and Valencia collected data regarding maternal diet during pregnancy and child problem behaviours, which were analysed in **chapter 6** of this thesis. A total of 2,270 pregnant women were recruited within these three cohorts during the first antenatal visit (*i.e.,* n=638 in Gipuzkoa, n=777 in Sabadell and n=855 in Valencia), of whom 2,141 (94.32%) were followed up until delivery and 1,255 (58.62%) provided complete information on maternal diet during pregnancy and childhood problem behaviours (assessed with the CBCL 6-18). An overview of the population characteristics is provided in table 1.4; a flow diagram of the recruitment process is shown in the Appendix (figure A1.3). Details on maternal diet quality and child problem behaviours are shown at pages 205-208.

Thesis outline

In **chapter 2**, we present the analysis of the association between intrapair birth-weight difference and later problem behaviours in the TAMBAHS twins.

Chapters 3 and 4 focus on the influence of maternal weight before pregnancy or GWG on childhood psychological development. Specifically, in **chapter 3**, we examined data of the TAMBAHS study to assess the association between maternal pre-pregnancy BMI on twins' temperament during infancy. Then, in **chapter 4**, we harmonised and pooled individual data of the MEFAB and Rhea cohorts to examine the association between gestational weight gain and problem behaviours in school-age singletons, as well as the effect modification of maternal pre-pregnancy BMI.

Chapters 5 and 6 presents maternal dietary factors as possible exposures in childhood development. In **chapter 5**, we analysed data of the MEFAB cohort to investigate the associations between changes in PUFA concentrations during pregnancy and childhood social competence and problem behaviours. In **chapter 6**, we harmonised and pooled individual data of the Rhea and INMA cohorts to assess the relationship between maternal diet quality during pregnancy and childhood problem behaviours.

Finally, **chapter 7** brings together all studies presented in this thesis by critically discussing the research findings in light of the main limitations. Furthermore, it considers the main implications of these findings and suggests future research directions.

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Appendix to chapter 1

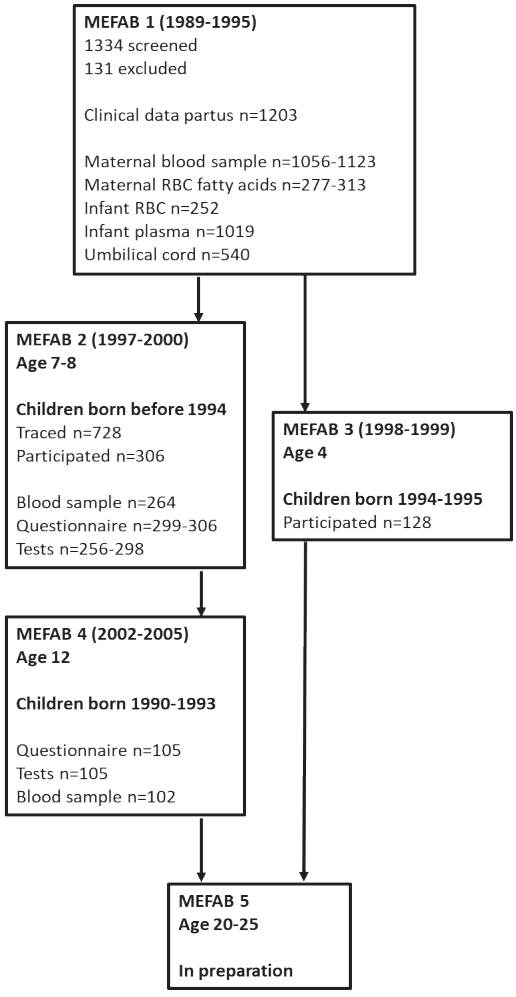

Figure A1.1: Flow diagram of the MEFAB cohort. From van der Wurff *et al.* 2015 (199)

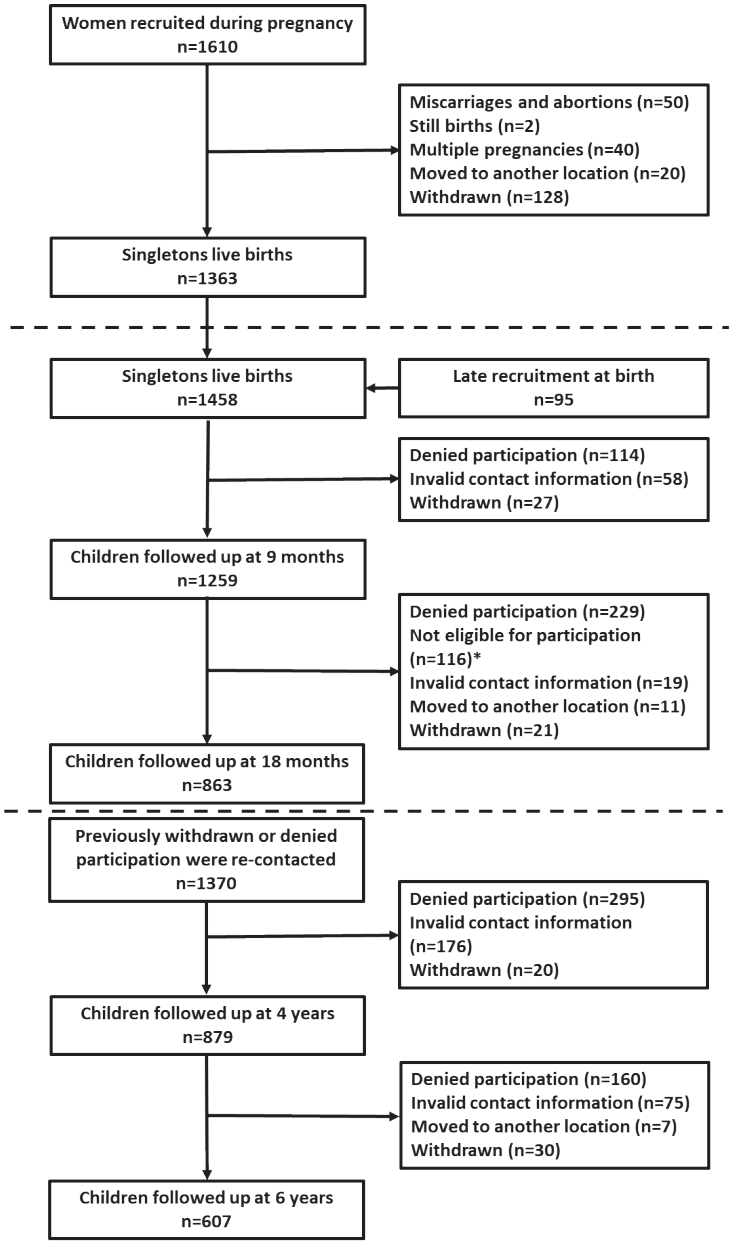

Figure A1.2: Flow diagram of the Rhea study. From Chatzi *et al.*, 2017 (200)

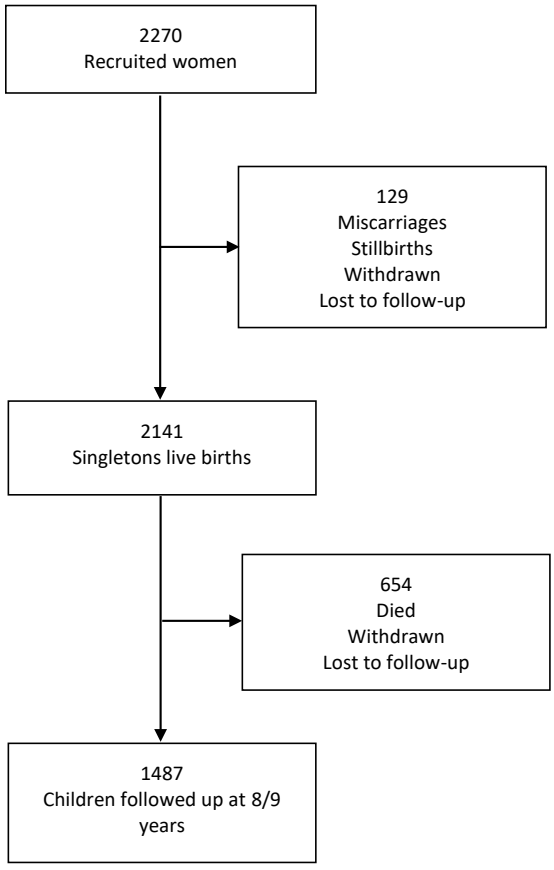

Figure A1.3: Flow diagram of the INMA study. Pooled data of the three cohorts examined in chapter 6 (*i.e.*, Gipuzkoa, Sabadell and Valencia) are shown.

Chapter 2

The association of intrapair birth-weight differences with internalising and externalising behaviour problems

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Abstract

Background: Mounting evidence is available suggesting an association between low birth weight and problem behaviours in children. However, given the substantial genetic and environmental influences on children's birth weight and problem behaviours, a discordant twin study is useful to isolate the unique environmental influences.

Aims: To examine the association between intrapair birth-weight difference and problem-behaviour difference in pre-school twins.

Methods: The Twins and Multiple Births Association Heritability Study (TAMBAHS), a volunteer-based study from the UK, recruited mothers of twins aged 18 months to 5 years (n=480 twin pairs). At recruitment, twins' mothers reported about maternal and twins' characteristics and problem behaviours by completing the Child Behaviour Checklist (CBCL) 1.5-5. We calculated the absolute intrapair difference in birth weight and in each CBCL scale by subtracting the weight (or the score) of the smaller twin from the weight (or the score) of the larger twin. We used multivariate linear regressions to examine the associations between the absolute birth-weight difference and each CBCL scale's score difference and calculated the expected mean CBCL score differences for ease of interpretation.

Results: In monozygotic twins, we found relatively large estimates for the associations between birth-weight difference on one hand and differences in total problems (β= -5.95; 95% C.I.: -11.08, -0.82), internalising problems (β= -4.17; 95% C.I.: -7.65, -0.69), and emotional reactiveness (β= -2.70; 95% C.I.: -5.23, -0.17) on the other. No associations were observed in dizygotic twins when analysed as a separate group, nor in monozygotic and dizygotic twins combined. However, none of the associations were significant after controlling for multiple testing.

Conclusions: Despite the lack of statistical significance, results suggest that an increase in absolute birth-weight difference might be associated with an intrapair difference in total problems, internalising behaviours and emotionality in young twins. Findings are supported by previous studies in older twin populations, but additional research is warranted.

Background

Problem behaviours, such as internalising (*i.e.*, anxiety, depression, emotional problems, somatic complaints, withdrawnness) and externalising (*i.e.*, aggression and attention problems), may manifest during the pre-school years, potentially extending over childhood and adolescence (1). Early identification and deeper understanding of the aetiology of these behaviours are important mainly because children with behavioural difficulties are at greater risk of adverse developmental outcomes, including poor academic performance, conflictual relationships with peers and family members, delinquency, and even early death (2–4).

Twin studies have revealed significant genetic influences on internalising and externalising behaviours, with common and non-shared environmental effects generally being considered more modest (1,5). Among the diverse mechanisms via which the environment can influence twins' behaviour, the prenatal environment is receiving increasing interest (6). In this context, birth-weight discordance is considered a good proxy of prenatal non-shared environment, especially in MZ twins were genetic influences are completely controlled for. In fact, as twins are characterised by the same gestational age, a difference in MZ twins' birth weight must be the result of any factor affecting the growth of each individual twin (7,8). In DZ twins, birth weight discordance results from differences in both genetic and environmental influences between co-twins (8). Several specific risk factors have been described for intertwin birth-weight discordance, including genetic or structural abnormalities, adverse intrauterine factors (*e.g.*, small placental weight, single umbilical artery, excessive velamentous cord insertions) and various placental abnormalities (9,10). Additional risk factors include advanced maternal age, low gestational weight gain, nulliparity, smoking, diabetes, hypertensive disorders, and low gestational age, which may randomly affect the growth of only one of the twins by affecting placental function (11).

Growth discordance has been associated with symptoms of attention deficit/hyperactivity disorder (ADHD) (7,12,13), and problem behaviours (14–16). Particularly, lower birth weight was associated with higher total problem behaviour scores in 1,490 Belgian twins aged 6 to 17 years (15), and higher total problem and emotional problems in 3,114 Danish twins aged 12 years (16). Conversely, in a study examining 112 Jewish twin pairs, higher birth-weight twins were more likely to have conduct problems compared to their co-twins (14).

The aim of the current study was to replicate the previously reported associations between the intrapair birth-weight difference and the intrapair difference in problem behaviours, measured by

the Child Behaviour Checklist (CBCL) questionnaire. Our primary interest was to examine whether and to what extent the difference in birth weight may predict problematic behaviours in twins. Specifically, we focused on each CBCL subscale and presented absolute birth-weight differences, in order to help parents and clinicians to easily identify the more challenging areas of development. Furthermore, we examined young, pre-school-age twins to reduce as much as possible external, uncontrolled influences.

Materials and methods

The data collection process has been described previously (17). Briefly, the Twins and Multiple Births Association Heritability Study (TAMBAHS) is a UK, volunteer-based study investigating the psychological development of twins from birth until 5 years of age. For this study, the older cohort of twins, aged 18 months to 5 years, was selected. Between July 2008 and May 2010, an invitation to complete an online questionnaire about maternal and twins' demographic and anthropometric characteristics and twins' problem behaviours was sent to families registered in the Twins and Multiple Births Association (TAMBA). Participants' geographical spread was representative of the twin families' spread across the UK. This study was approved by the University of Birmingham Ethical Review Committee. Written informed consent was obtained from all participating families.

Birth weight

At recruitment, mothers reported each twin's birth weight in pounds or kg. In addition, information regarding twins' sex (male or female), age (months), gestational age (weeks), weight at the time of survey (pounds or kg), maternal height (feet or meter), pre-pregnancy weight (pounds or kg), maternal age at delivery (years), and smoking status before, during and after pregnancy (ever/never). Weight and height measure reported on the imperial system were converted into metric measures during data cleaning.

Zygosity determination

The adapted version of the Goldsmith's zygosity questionnaire (18) was used to assess the zygosity of the twins included in the TAMBAHS dataset. This questionnaire has been validated against determination by identity of polymorphic DNA markers, reaching an accuracy of verifying zygosity in 95% of cases.

Behaviour problems

The Child Behaviour Checklist 1.5-5 (19) is a questionnaire developed to obtain a standardised report of children's problem behaviours as perceived by their parents. Numerous versions of this questionnaire have been developed to target different age groups. The CBCL 1.5-5 was developed to assess children from 18 months to 5 years of age. It contains 99 problem items, split into 7 subscales: emotional reactiveness, anxiety/depression, somatic complaints, withdrawnness, sleep problems, attention problems, and aggressive behaviour, originally derived by factor analyses. The broadband scale internalising is the sum score of the first four syndrome scales, whereas externalising is the sum score of attention problems and aggressive behaviour. Total problems is the sum score of all ninety-nine problem items. Each item is scored 0-2 ("not true", "somewhat or sometimes true", and "very true or often true"), based on the preceding 2 months. Good reliability and validity criteria have been reported for this checklist (19).

Statistical analysis

Twins' absolute birth-weight difference in grams (*i.e.*, birth weight of the larger twin minus birth weight of the smaller twin) and CBCL score discordance (*i.e.,* CBCL score of the larger twin minus CBCL score of the smaller twin) were calculated. The association between birth-weight difference and CBCL score difference was investigated by means of multivariate linear regression analyses. The hypothesis under test was that an increase in birth-weight difference between co-twins would be associated with a CBCL score difference. The expected mean CBCL difference was calculated using the LINCOM command in Stata to determine how the score is expected to change with increasing birth-weight difference per 100 g difference while keeping constant all covariates at their mean values. Twins' gender (male-male, female-female, opposite-sex), age, gestational age, weight difference at the time of survey, zygosity, and maternal pre-pregnancy BMI, age, and smoking status before, during and after pregnancy were controlled for. An interaction term was added to the regressions in order to test the possible interaction of zygosity with birth-weight difference.

The Benjamini-Hochberg procedure (20) with a false discovery rate of 5% was used to correct for multiple testing. Statistical analysis was performed in Stata v.13 (21).

Results

Summary statistics

A total of 960 twins was included in the analysis, of which 202 were monozygotic (MZ) male twins, 152 MZ female twins, 162 dizygotic (DZ) male twins, 166 DZ female twins and 278 opposite-sex twins. Mean twins' birth weight, gestational age and age at survey are presented in table 2.1, subdivided by zygosity. Monozygotic twins' mean gestational age was significantly shorter than that of dizygotic twins (35.38±2.48 weeks of MZ twins, compared to 36.26±2.69 weeks of DZ twins; p<0.001). Furthermore, MZ twins' mean birth weight was significantly lower than that of dizygotic twins (2326.6±575.9 g, compared to 2487.0±578.8 g, for MZ and DZ twins, respectively; p<0.001),

Table 2.1: Phenotypic characteristics of twins according to their zygosity

		MZ		DZ		
Twins' characteristics		**Mean or n**	**SD or %**	**Mean or n**	**SD or %**	**p-value**
Gender						
	Male	202	57.06%	301	49.67%	0.027
	Female	152	42.94%	305	50.33%	
	Same-sex pairs	177	100%	164	54.13%	
	Different-sex pairs	NA	NA	139	45.87%	<0.001
Birth weight (g)		2326.64	575.86	2487.02	578.78	<0.001
Gestational age (weeks)		35.38	2.48	36.26	2.69	<0.001
Children age (months)		37.50	11.63	34.67	11.35	<0.001
Twins' weight (Kg)		11.25	11.74	10.51	6.59	0.235
Maternal characteristics		**Mean or n**	**SD or %**	**Mean or n**	**SD or %**	**p-value**
Age		35.53	4.69	36.69	4.17	<0.001
Pre-pregnancy BMI		24.52	4.32	24.76	5.11	0.570
Ethnicity	White	346	97.19%	610	98.07%	0.057
	Other	10	2.81%	12	1.82%	
Smoking status (% yes)						
	Before	78	22.94%	108	17.94%	0.190
	During	8	2.50%	20	3.56%	0.542
	After	34	10.69%	58	9.63%	0.799

Note: SD: standard deviation; NA: not applicable

while MZ twins' age at the time of survey was significantly higher than DZ twins' mean age (37.50±11.63 months and 34.67±11.35 months for MZ and DZ twins, respectively; p<0.001). Dizygotic twins' mothers were significantly older than mothers of monozygotic twins (36.69±4.17 years compared to 35.53±4.69 years; p<0.001), while no difference was observed in maternal pre-pregnancy BMI (24.52±4.32 kg/m2 of MZ twins' mothers compared to 24.76±5.11 kg/m2 of DZ twins' mothers; p=0.570). Mothers of monozygotic and dizygotic twins did not differ much regarding their ethnicity, with the vast majority of them being white Caucasian (97.19% and 98.07% of mothers of MZ and DZ twins, respectively).

Table 2.2: Distribution of twin pairs in each birth-weight difference category by zygosity

	MZ twin pairs		DZ twin pairs	
Birth-weight difference categories	**N**	**%**	**N**	**%**
0 g	18	5%	46	7.37%
0-100 g	96	26.67%	120	19.23%
100-200 g	84	23.33%	118	18.91%
200-300 g	44	12.22%	76	12.18%
300-400 g	48	13.33%	84	13.46%
400-500 g	24	6.67%	60	9.62%
500-600 g	6	1.67%	40	6.41%
600-700 g	4	1.11%	16	2.56%
700-800 g	22	6.11%	10	1.60%
800-900 g	4	1.11%	10	1.60%
900-1000 g	2	0.56%	6	0.96%
1000-1100 g	0	0%	8	1.28%
>1100 g	8	2.22%	30	4.81%

The mean intrapair birth-weight difference between MZ twins was lower than DZ twins (246.1±228.5 g compared to 295.7±271.7 g; p= 0.004). The distribution of twin pairs for each birth-weight difference category and by zygosity is presented in table 2.2. As expected, given the genetic component of birth weight, a larger variation was found between DZ co-twin compared to MZ co-twins.

Table 2.3 shows mean CBCL scores for each zygosity group, subdivided by gender and adjusted for age at survey to account for the rapid development that occurs in the age period considered. As can be seen, mean scores were higher in female monozygotic twins as compared to female dizygotic twins in total problems, internalising problems, anxiety/depression and withdrawnness. No differences in mean scores were observed among male twins.

Table 2.3: Mean CBCL scores, by zygosity and twin's sex

		MZ		DZ		
CBCL scales		**Mean**	**SE**	**Mean**	**SE**	**p-value**
Externalising						
Attention problems	males	2.46	0.13	2.45	0.11	0.931
	females	2.22	0.17	2.26	0.12	0.844
Aggressive behaviour	males	11.08	0.42	10.35	0.35	0.190
	females	10.13	0.51	10.18	0.37	0.935
Total Externalising	males	13.58	0.50	12.83	0.42	0.255
	females	12.40	0.61	12.46	0.44	0.941
Internalising						
Emotional reactiveness	males	1.44	0.15	1.31	0.12	0.519
	females	1.80	0.20	1.35	0.14	0.065
Anxiety/Depression	males	2.15	0.15	2.34	0.13	0.356
	females	3.17	0.20	2.20	0.14	<0.001
Somatic complaints	males	1.75	0.14	1.90	0.12	0.403
	females	2.03	0.17	1.95	0.12	0.685
Withdrawnness	males	1.20	0.10	1.37	0.09	0.215
	females	1.82	0.12	1.27	0.09	<0.001
Total Internalising	males	6.56	0.37	6.96	0.31	0.422
	females	8.90	0.51	6.80	0.36	0.001
Sleep problems	males	2.53	0.16	2.24	0.14	0.173
	females	2.58	0.20	2.41	0.14	0.503
Total problems	males	32.71	1.11	32.14	0.95	0.699
	females	34.69	1.43	31.11	1.02	0.042

Note: Means are corrected for twins' age at survey; SE: standard error.

Table 2.4 shows mean differences in CBCL scores in MZ and DZ twins, subdivided by gender and adjusted by age at survey. Among female-female twin pairs, larger differences were found in MZ twin pairs in the emotional reactiveness scale (1.40 (0.51) and -0.40 (0.49) for females, MZ and DZ twin pairs, respectively). On the other hand, DZ twins showed larger differences in somatic complaints (-0.48 (0.28) for female MZ twin pairs, and 0.67 (0.28) for female DZ twin pairs) and

withdrawnness (Males: 0.19 (0.13) and -0.34 (0.16) for MZ and DZ twins, respectively. Females: 0.13 (0.15) and -0.38 (0.15) for MZ and DZ twin pairs, respectively). Finally, a difference in mean score was observed in male twin pairs in the sleep problems scale (-0.14 (0.16) and 0.33 (0.18) for MZ and DZ twins, respectively). Note that negative values indicate that twins with a lower birth weight scored higher on average than their larger co-twins, and vice versa.

Table 1.4: CBCL mean difference, by zygosity and sex of twin pairs

		MZ		DZ	
CBCL scales		**Mean difference**	**SE**	**Mean difference**	**SE**
Attention problems	male	0.06	0.17	-0.12	0.20
	female	0.13	0.26	-0.00	0.25
	opposite sex			0.24	0.18
Aggressive behaviour	male	0.10	0.43	-0.31	0.51
	female	0.10	0.56	-0.45	0.54
	opposite sex			-0.15	0.37
Total Externalising	male	0.16	0.50	-0.42	0.58
	female	0.19	0.65	-0.45	0.63
	opposite sex			-0.08	0.47
Emotional reactiveness	male	-0.11	0.36	0.56	0.43
	female	1.40	0.51	-0.40	0.49
	opposite sex			-0.19	0.25
Anxiety/Depression	male	0.07	0.21	-0.12	0.24
	female	-0.12	0.25	0.20	0.24
	opposite sex			-0.15	0.16
Somatic complaints	male	-0.01	0.21	0.37	0.25
	female	-0.48	0.28	0.67	0.28
	opposite sex			0.15	0.19
Withdrawnness	male	0.19	0.13	-0.34	0.16
	female	0.13	0.15	-0.38	0.15
	opposite sex			0.08	0.11
Total Internalising	male	0.15	0.53	0.47	0.62
	female	0.96	0.79	0.09	0.77
	opposite sex			-0.14	0.43
Sleep problems	male	-0.14	0.16	0.33	0.18
	female	-0.46	0.25	-0.00	0.24
	opposite sex			0.17	0.17
Total problems	male	0.73	1.02	0.94	1.20
	female	0.93	1.34	-0.14	1.30
	opposite sex			0.10	0.84

Note: Means are corrected for twins' age at survey; SE: standard error.

We computed intrapair correlations by using Pearson's correlation coefficients between the standardised residuals of birth-weight difference, adjusted for gestational age and sex of the pair (*i.e.*, male-male, female-female or opposite-sex), and CBCL score differences, adjusted for age at survey and sex of the pair (table 2.5). All residuals were standardised to a mean of 0 and a standard deviation of 1. MZ correlations were higher than DZ correlations only in four out of ten scales (*i.e.*, externalising and internalising problems, attention problems and emotional reactiveness), while in three scales monozygotic twins standardised correlations were below the mean value of zero (*i.e.*, sleep problems, somatic complaints and withdrawnness). However, when we computed intrapair twin correlations of raw CBCL scores, monozygotic twins' correlations were always higher than those of dizygotic twins (table 2.5). Specifically, MZ twins' correlations ranged from 0.136 to 0.921, with most of them being higher than 0.8, while DZ twins' correlations ranged from -0.093 to 0.639. In both cases, the lowest correlations were associated with emotional reactiveness.

Table 2.5: Intrapair correlations for each CBCL scale, subdivided into zygosity group

	MZ		DZ	
	r^a	r^b	r^a	r^b
Attention problems	0.801	0.128	0.274	-0.038
Aggressive behaviour	0.865	0.044	0.639	0.070
Total externalising	0.888	0.083	0.623	0.042
Emotional reactiveness	0.136	0.122	-0.093	0.052
Anxiety/Depression	0.820	0.017	0.469	0.023
Somatic complaints	0.600	-0.084	0.251	0.023
Withdrawnness	0.856	-0.094	0.404	-0.004
Total internalising	0.807	0.055	0.559	0.048
Sleep problems	0.863	-0.114	0.540	-0.019
Total problems	0.921	0.034	0.750	0.075

Note: r: Pearson's correlation coefficient; a: intrapair correlations of raw CBCL scores; b: correlations of standardized residuals. Birth weight difference was adjusted for gestational age and sex, while CBCL scales score differences were adjusted for age at survey and sex.

Multiple linear regression

Multivariate linear regressions were performed firstly with absolute birth-weight difference as a continuous variable and secondly after subdividing it into categories of 100g difference (total number of twin pairs per category can be found in table 2.2). No associations were found when treating birth-weight difference as a continuous variable. However, when subdividing it into birth-weight difference categories, associations were found in total problems (β= -5.95; 95% CI: -11.08, -0.82), internalising behaviour (β= -4.17; 95% CI: -7.65, -0.69) and emotional reactiveness scales (β=

Table 2.6: Expected mean difference score for increasing birth weight difference

BWD	Total Problems			Internalising			Externalising		
	M	SE	p	M	SE	p	M	SE	p
0 g	-4.04	6.04		0.98	3.38		-6.07	2.93	
100 g	-5.15	5.89		0.76	3.30		-6.29	2.86	
200 g	-6.26	5.83		0.54	3.26		-6.52	2.83	
300 g	-7.37	5.86		0.32	3.28		-6.74	2.84	
400 g	-8.49	5.98		0.10	3.35		-6.97	2.90	
500 g	-9.60	6.18		-0.12	3.46		-7.20	3.00	
600 g	-10.71	6.47	0.13	-0.33	3.62	0.59	-7.42	3.14	0.53
700 g	-11.82	6.82		-0.55	3.82		-7.65	3.31	
800 g	-12.93	7.22		-0.77	4.05		-7.86	3.50	
900 g	-14.05	7.68		-0.99	4.30		-8.10	3.72	
1000 g	-15.16	8.17		-1.21	4.58		-8.33	3.97	
1100 g	-16.27	8.70		-1.43	4.88		-8.55	4.22	
>1100 g	-17.38	9.26		-1.65	5.19		-8.78	4.49	

BWD	Anxiety/Depression			Emotional reactiveness			Somatic complaints			Withdrawnness		
	M	SE	p	M	SE	p	M	SE	p	M	SE	p
0 g	1.42	1.11		-2.09	2.13		2.87	1.28		-1.23	0.72	
100 g	1.45	1.09		-2.09	2.08		2.69	1.25		-1.31	0.70	
200 g	1.48	1.07		-2.09	2.06		2.52	1.24		-1.39	0.69	
300 g	1.51	1.08		-2.08	2.07		2.35	1.25		-1.46	0.70	
400 g	1.54	1.10		-2.08	2.11		2.17	1.27		-1.54	0.71	
500 g	1.58	1.14		-2.08	2.19		2.00	1.32		-1.62	0.74	
600 g	1.61	1.19	0.82	-2.08	2.27	0.99	1.82	1.38	0.26	-1.70	0.77	0.37
700 g	1.64	1.26		-2.08	2.41		1.65	1.45		-1.78	0.81	
800 g	1.67	1.33		-2.07	2.55		1.48	1.54		-1.86	0.86	
900 g	1.70	1.42		-2.07	2.71		1.30	1.63		-1.94	0.91	
1000 g	1.73	1.51		-2.07	2.89		1.13	1.74		-2.01	1.04	
1100 g	1.76	1.60		-2.07	3.08		0.95	1.85		-2.09	1.04	
>1100 g	1.80	1.71		-2.06	3.27		0.78	1.97		-2.17	1.10	

BWD	Aggressive behaviour			Attention problems			Sleep problems		
	M	SE	p	M	SE	p	M	SE	p
0 g	-4.29	2.43		-1.89	1.11		0.20	1.13	
100 g	-4.57	2.37		-1.83	1.08		-0.03	1.10	
200 g	-4.85	2.34		-1.77	1.07		-0.26	1.09	
300 g	-5.13	2.35		-1.71	1.07		-0.49	1.10	
400 g	-5.40	2.40		-1.64	1.10		-0.72	1.16	
500 g	-5.68	2.48		-1.58	1.33		-0.95	1.16	
600 g	-5.96	2.60	0.35	-1.52	1.19	0. 64	-0.18	1.21	0.10
700 g	-6.24	2.74		-1.46	1.25		-1.41	1.26	
800 g	-6.52	2.90		-1.39	1.32		-1.64	1.35	
900 g	-6.79	3.09		-1.33	1.41		-1.87	1.44	
1000 g	-7.07	3.29		-1.27	1.50		-2.10	1.53	
1100 g	-7.35	3.50		-1.21	1.60		-2.33	1.63	
>1100 g	-7.63	3.72		-1.14	1.70		-2.55	1.73	

Note: BWD: birth-weight difference; M: mean; SE: standard error; Total number of twin pairs per category can be found in table 2.2.

Table 2.7: Expected mean difference score for increasing birth weight difference in monozygotic twins

BWD	Total Problems			Internalising			Externalising		
	M	SE	p	M	SE	p	M	SE	p
0 g	15.11	12.03		11.27	8.16		6.06	5.35	
100 g	9.16	10.56		7.10	7.16		5.39	4.70	
200 g	3.21	9.58		2.92	6.49		4.74	4.26	
300 g	-2.74	9.24		-1.25	6.27		4.08	4.11	
400 g	-8.69	9.61		-5.42	6.52		3.42	4.28	
500 g	-14.64	10.63		-9.59	7.21		2.76	4.73	
600 g	-20.59	12.12	0.02	-13.76	8.22	0.02	2.11	5.39	0.57
700 g	-26.54	13.93		-17.94	9.45		1.45	6.20	
800 g	-33.49	15.97		-22.11	10.83		0.79	7.10	
900 g	-38.44	18.14		-26.28	12.30		0.13	8.07	
1000 g	-44.39	20.41		-30.45	13.84		-0.53	9.08	
1100 g	-50.34	22.75		-34.63	15.43		-1.18	10.12	
>1100 g	-56.29	25.14		-38.80	17.05		-1.84	11.18	

BWD	Anxiety/ Depression			Emotional reactiveness			Somatic complaints			Withdrawnness		
	M	SE	p	M	SE	p	M	SE	p	M	SE	p
0 g	0.65	2.11		7.31	5.92		1.95	2.80		1.41	0.99	
100 g	0.41	1.86		4.60	5.20		0.91	2.46		1.19	0.87	
200 g	0.17	1.68		1.90	4.72		-0.14	2.23		0.97	0.79	
300 g	-0.06	1.62		-0.81	4.55		-1.18	2.15		0.75	0.76	
400 g	-0.30	1.69		-3.51	4.74		-2.22	2.24		0.53	0.79	
500 g	-0.54	1.87		-6.21	5.24		-3.26	2.48		0.30	0.88	
600 g	-0.78	2.13	0.6	-8.92	5.97	0.04	-4.31	2.83	0.09	0.08	1.00	0.3
700 g	-1.01	2.45		-11.62	6.86		-5.35	3.25		-0.14	1.15	
800 g	-1.25	2.81		-14.33	7.86		-6.39	3.72		-0.36	1.32	
900 g	-1.49	3.19		-17.03	8.94		-7.43	4.23		-0.58	1.50	
1000 g	-1.73	3.59		-19.74	10.05		-8.48	4.76		-0.81	1.68	
1100 g	-1.96	4.00		-22.44	11.21		-9.52	5.30		-1.03	1.88	
>1100 g	-2.20	4.42		-25.15	12.38		-10.56	5.86		-1.25	2.07	

BWD	Aggressive behaviour			Attention problems			Sleep problems		
	M	SE	p	M	SE	p	M	SE	p
0 g	5.39	5.10		0.57	1.78		-0.45	1.99	
100 g	4.89	4.48		0.46	1.57		-1.29	1.75	
200 g	4.39	4.07		0.35	1.42		-2.13	1.59	
300 g	3.90	3.92		0.24	1.37		-2.97	1.53	
400 g	3.40	4.08		0.13	1.43		-3.81	1.59	
500 g	2.90	4.51		0.02	1.58		-4.65	1.76	
600 g	2.41	5.14	0.65	-0.09	1.80	0.78	-5.49	2.01	0.05
700 g	1.91	5.91		-0.20	2.07		-6.33	2.31	
800 g	1.41	6.78		-0.30	2.37		-7.17	2.64	
900 g	0.92	7.70		-0.41	2.69		-8.01	3.01	
1000 g	0.42	8.66		-0.52	3.03		-8.86	3.38	
1100 g	-0.08	9.66		-0.63	3.37		-9.70	3.77	
>1100 g	-0.57	10.67		-0.74	3.73		-10.54	4.16	

Note: BWD: birth-weight difference; M: mean; SE: standard error; Total number of twin pairs per category can be found in table 2.2.

Table 2.8: Expected mean difference score for increasing birth weight difference in dizygotic twins

BWD	Total Problems			Internalising			Externalising		
	M	SE	p	M	SE	p	M	SE	p
0 g	0.32	9.84		0.11	5.20		-6.29	4.85	
100 g	-1.77	8.78		0.35	4.64		-6.93	4.33	
200 g	-3.85	7.98		0.58	4.21		-7.57	3.93	
300 g	-5.94	7.51		0.82	3.97		-8.21	3.70	
400 g	-8.02	7.44		1.06	3.93		-8.85	3.67	
500 g	-10.11	7.78		1.29	4.11		-9.49	3.83	
600 g	-12.19	8.48	0.24	1.53	4.48	0.80	-10.13	4.18	0.46
700 g	-14.28	9.46		1.77	5.00		-10.78	4.66	
800 g	-16.36	10.64		2.00	5.62		-11.42	5.24	
900 g	-18.45	11.97		2.24	6.32		-12.06	5.90	
1000 g	-20.53	13.39		2.48	7.07		-12.70	6.60	
1100 g	-22.68	14.89		2.71	7.87		-13.34	7.34	
>1100 g	-24.70	16.44		2.95	8.69		-13.98	8.10	

BWD	Anxiety/ Depression			Emotional reactiveness			Somatic complaints			Withdrawnness		
	M	SE	p	M	SE	p	M	SE	p	M	SE	p
0 g	1.69	1.85		-3.41	2.98		2.96	2.06		-1.17	1.28	
100 g	1.94	1.65		-3.25	2.66		2.88	1.84		-1.25	1.14	
200 g	2.18	1.50		-3.08	2.41		2.80	1.67		-1.33	1.03	
300 g	2.43	1.41		-2.91	2.27		2.72	1.57		-1.41	0.97	
400 g	2.67	1.40		-2.75	2.25		2.63	1.56		-1.49	0.96	
500 g	2.92	1.46		-2.58	2.35		2.55	1.63		-1.57	1.01	
600 g	3.16	1.59	0.46	-2.41	2.56	0.76	2.47	1.77	0.83	-1.66	1.10	0.72
700 g	3.41	1.78		-2.25	2.86		2.39	1.98		-1.74	1.23	
800 g	3.66	2.00		-2.08	3.22		2.31	2.23		-1.82	1.38	
900 g	3.90	2.25		-1.92	3.62		2.23	2.50		-1.90	1.55	
1000 g	4.15	2.52		-1.75	4.05		2.15	2.80		-1.98	1.74	
1100 g	4.39	2.80		-1.58	4.50		2.07	3.12		-2.06	1.93	
>1100 g	4.64	3.09		-1.42	4.97		1.99	3.44		-2.14	2.13	

BWD	Aggressive behaviour			Attention problems			Sleep problems		
	M	SE	p	M	SE	p	M	SE	p
0 g	-4.26	3.88		-2.22	1.90		1.27	1.91	
100 g	-4.83	3.47		-2.27	1.69		0.98	1.71	
200 g	-5.41	3.15		-2.33	1.54		0.69	1.46	
300 g	-5.98	2.96		-2.39	1.45		0.41	1.46	
400 g	-6.55	2.94		-2.44	1.44		0.12	1.44	
500 g	-7.12	3.07		-2.50	1.50		-0.16	1.51	
600 g	-7.70	3.35	0.41	-2.56	1.64	0.87	-0.45	1.65	0.41
700 g	-8.27	3.73		-2.61	1.82		-0.73	1.84	
800 g	-8.84	4.20		-2.67	2.05		-1.02	2.07	
900 g	-9.42	4.72		-2.73	2.31		-1.30	2.32	
1000 g	-9.99	5.29		-2.78	2.58		-1.59	2.60	
1100 g	-10.56	5.88		-2.84	2.87		-1.87	2.89	
>1100 g	-11.13	6.49		-2.90	3.17		-2.16	3.19	

Note: BWD: birth-weight difference; M: mean; SE: standard error; Total number of twin pairs per category can be found in table 2.2.

-2.70; 95% CI: -5.23, -0.17), in MZ twins analysed as a separate group. Moreover, we found a tendency for sleep problem to be associated with birth-weight difference in MZ twins (β= -0.84; 95% CI: -1.69, 0.01). No associations were found in MZ and DZ twins combined, nor in DZ twins analysed separately (full-model estimates are reported in the Appendix, tables A2.1-A2.9).

We then computed expected mean differences for every birth-weight category, subdivided by CBCL scale, in monozygotic and dizygotic twins combined (table 2.6), monozygotic twins (table 2.7) and dizygotic twins (table 2.8) analysed separately.

In monozygotic twins analysed separately, the expected means for the intrapair difference in total problems score ranged from 15.11 (12.03) to -59.29 (25.14). Furthermore, the expected means for internalising score ranged from 11.27 (8.16) to -38.80 (17.05), while this figure ranged from 7.31 (5.92) to -25.15 (12.38) for emotional reactiveness.

None of the reported associations could be considered significant after controlling for multiple testing.

Post-hoc and sensitivity analysis

Among the independent variables included in the multivariate regressions, gestational age was given special attention because of its possible association with childhood neurodevelopment. For this reason, all multiple regressions were repeated without controlling for gestational age. The results were not significantly affected by the exclusion of gestational age (Appendix, table A1.10).

Furthermore, we repeated all the analysis after removing all twin pairs (n=2 and n=9, for MZ and DZ twins respectively) with at least one extremely low birth weight (ELBW) twin (*i.e.*, with a birth weight ≤1000g, according to the World Health Organisation's definition) from the sample. Again, no difference in the results was observed (Appendix, table A1.11).

Discussion

The aim of the current study was to replicate the previously reported association between the intrapair birth-weight difference and the intrapair difference in behaviour problems, measured with the CBCL. We found negative estimates for the associations between birth-weight difference and total problems, internalising behaviours and emotional reactiveness differences in MZ twins when examining categorical, but not continuous, birth-weight difference. Due to the computation

method, negative estimates imply a rise in CBCL scores in smaller twins (or a reduction in the larger ones) with increasing birth-weight difference.

Despite the fact that none of the identified associations could be considered statistically significant after controlling for multiple testing, a few aspects suggest our results might not be completely due to chance. Firstly, the scales associated with birth weight are related to each other, in that emotional reactiveness is a subscale of internalising behaviours, which is in turn a subscale of total problems, suggesting that results might not be spurious. In accordance with our study, Møllegaard (16) has reported significant associations only with emotional problems and total problems in MZ twins. Secondly, larger intrapair differences have been previously reported for internalising behaviours and especially emotional problems compared to externalising problems (*e.g.,* (5)), similarly as the present population, as shown in tables 2.4 and 2.5. Hence, it could be hypothesised that genetic and common environmental factors are more important in externalising behaviours and that birth weight affects externalising behaviours in a minor extent compared to emotionality. This hypothesis is supported by the study by Møllegaard (16), the only study so far to have examined both internalising and externalising behaviours and the included subscales in relation to birth weight, in which smaller estimates are reported for hyperactivity/inattention compared to emotional problems in MZ twins. Moreover, two studies examined the relative importance of genetic and environmental factors on problem behaviours in infant twins and reported a greater influence of shared genetic and environmental factors on externalising compared to internalising behaviours (5,17). Thirdly, the effect of birth weight might vary depending on the adequacy of foetal growth, so that moderately large effects might be observed only in case of low birth weight (*i.e.*, below 2500g) or sufficient birth weight discordance (16). In the present study, 50.5% (n=497) of twins had a low birth weight, of whom 42.3% (n=210) were MZ twins, but 61.2% of twin pairs (n=301, of which 40.2% were MZ twin pairs) had a birth weight difference of less than 300g. This distribution of birth weight difference might have been insufficient to show an effect on externalising problems. Finally, the lack of associations in the group of DZ twins is an indication of unadjusted genetic confounding, which might be either increasing or decreasing the phenotypic difference depending on the specific twin pair considered. For this reason, most studies with a discordant twin design restrict their analyses to MZ twins only. We decided to include DZ twins in our analyses to identify any trait in which variations are mainly driven by genetics (8).

These results are in line with previews studies, which have explored the effect of birth-weight discordance on problem behaviours and psychopathology. In fact, compared to their larger co-

twins, smaller twins in discordant twin pairs showed higher ADHD symptoms in two populations of twins aged between 2 and 16 years (7,12). Van Os and colleagues showed that birth-weight discordance was associated with a discordance in CBCL scores in 6- to 17-year-old Belgian twins (15). Despite the similar result, two aspects differentiate the present study from van Os' and colleagues' study (15), the first being that they analysed only the influence of birth weight on the total CBCL score, while our aim was to determine which specific subscale(s) might be influenced the most by birth weight. The second aspect is that van Os' research group analysed the linear regressions of relative differences, as opposed to the present study, in which the absolute CBCL score differences were regressed over the absolute birth-weight difference. Although this differential approach limits the comparability between the two studies, our choice of presenting expected mean differences for 100g birth-weight difference was led by the will of presenting more interpretable results for clinicians and parents. Moreover, Møllegaard (16) recently reported a negative association between birth weight and behaviour problems in 12-year-old Danish twins. Specifically, birth weight difference was associated with total problems and hyperactive/inattentive behaviour in male twin pairs, and with emotional problems in female twin pairs, suggesting a differential effect of the prenatal environment on male and female foetuses. Furthermore, similar results have been reported in studies of non-twin populations. As Drvaric and colleagues discussed in their review (22), ELBW infants are at increased risk of emotional regulation issues, internalising and externalising behaviour problems compared to normal-weight infants. In the present study, we did not observe any influence of ELBW on the results of the linear regression analyses, probably due to the low number of twin pairs with at least one ELBW twin (*i.e.*, 11 out of 480). Similarly, Breslau and colleagues (23) reported a higher risk of internalising, externalising, and attention problems in children born with a low birth weight. Contrasting results were reported only in two small twin studies. Compared to their smaller co-twins, at birth, larger twins (n=70 pairs) were more active while awake and during sleep, more irritable and difficult to soothe (24). Similarly, larger at-birth twins (n=112) were more likely to have more conduct problems in preschool years compared to their co-twins (14).

Previous heritability analyses showed that internalising and externalising problem behaviours in young twins are influenced by genetic and environmental factors, with the majority of the observed variance explained by genetic influences (5,25). The analysis of discordances in MZ twins allows one to focus on unique environmental factors only, which, according to a meta-analysis on child and adolescent psychopathology, are thought to explain 26% (95% CI: 24.8%, 26.9%) and 33% (31.8%,

34.3%) of the observed variance in externalising and internalising problems, respectively (25). A previous heritability analysis done in this population of twins reported that unique environmental factors explain 13% (10%, 16%) and 49% (42%, 56%) of the variance in externalising behaviours and internalising behaviours, respectively (17), suggesting that the relative importance of non-shared environmental factors on internalising behaviours might be greater in younger compared to older children. However, when we analysed MZ twins alone, the R^2 values indicated that each of the association considered accounted only for about 8%-11% of the variance. At the same time, the expected mean differences in CBCL scores at 0g birth-weight difference strongly diverged from the expected value of zero. These results could mean that other factors are likely to influence twins' problem behaviours. For example, it is possible that unreported intrauterine growth restriction (IUGR) affected the smaller twins' psychological development independently from birth weight, although removing all pairs with ELBW twins in sensitivity analyses did not affect the results. Alternatively, other risk factors able to affect the individuals' behaviour independently from their co-twins (*i.e.*, increasing the difference between the two) could influence the association between birth-weight difference and problem behaviours.

The present study comes with some limitations. Data were retrospectively collected by means of an online questionnaire. This has precluded the collection of any data regarding chorionicity and intrauterine growth and might have affected the precision of maternal anthropometric data before and during pregnancy. Nonetheless, previous studies have shown that maternal report of children's birth weight is reliable even after a long follow-up time (26). Furthermore, because of the volunteer-based nature of the study, it was susceptible to selection bias, in that mothers of twins with dysfunctional behaviours might have been more interested in participating in the research. Moreover, parental ratings are subject to several types of limitations, including report bias. However, they are still considered a critically important source of information in epidemiological research. In fact, even though psychologists and psychiatrics would provide more detailed and less biased description of children's behaviour, their observations would involve stressful, standardised situations and a laboratory setting, in which observed responses and reactions may not reflect children's usual behaviour. Conversely, parents' ratings, while not perfect, are thought to better summarise children's normal reactions to everyday stimuli, especially during the preschool years (27). Even so, we assessed children's problem behaviour with a widely used and validated questionnaire to minimise report bias (19). Furthermore, we had no data regarding parenting behaviours. It is possible that differential parenting practices towards co-twins might naturally

derive from birth-weight discordance, as parents might tend to provide more attention to their smaller, weaker child, which might, in turn, differentially influence twins' behaviour (28,29). Other risk factors for externalising and internalising behaviours (*i.e.*, familial socio-economic status, maternal education levels, substance abuse, and psychopathology) reported by Carneiro and colleagues (30) would probably not have affected the results in the current research design, as they are factors common to the co-twins and, therefore, likely to increase their similarities instead of their differences. Finally, even though we had over 80% power to detect medium effect sizes, the statistical power to detect small effect sizes (*i.e.*, $f^2<0.15$) was <76%. This might have limited our ability to detect small associations.

In conclusion, this study suggests that the absolute intrapair birth-weight difference might be associated with total problems, internalising behaviours and emotional reactiveness score differences, respectively, in preschool-age MZ twins, in which all genetic and environmental factors shared between co-twins are controlled for. No associations were observed in DZ twins or in MZ and DZ twins analysed together. These results indicate that unique environmental factors (*i.e.*, those causing the different birth weight) might be involved in young twins' psychological and behavioural development. However, no association was significant after controlling for multiple testing and the role of other non-shared environmental influences, such as chorionicity and differential parental treatment between co-twins cannot be excluded. Future studies are therefore warranted to investigate further the role of intrapair birth-weight difference on problem-behaviour development in young twins.

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Appendix to chapter 2

Full-model-estimate tables

Table A2.1: Full-model estimates from the regressions of total problems on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-1.11	-2.55, 0.33	-5.95	-11.08, -0.82	-2.09	-5.56, 1.39
Gestational age	-0.09	-0.51, 0.33	-0.17	-0.77, 0.43	0.00	-0.58, 0.57
Mother's age	0.06	-0.22, 0.35	-0.25	-0.60, 0.10	0.34	-0.08, 0.75
Twins' age	0.04	-0.06, 0.14	0.13	-0.00, 0.26	0.00	-0.15, 0.14
Pre-pregnancy BMI	0.19	-0.08, 0.46	-0.05	-0.41, 0.31	0.30	-0.08, 0.67
Smoking before pregnancy (yes)	0.98	-2.85, 4.81	1.14	-4.10, 6.38	2.18	-3.11, 7.48
Smoking during pregnancy (yes)	-2.53	-10.15, 5.10	2.61	-10.43, 15.65	-5.26	-15.11, 4.58
Smoking after pregnancy (yes)	-1.14	-6.42, 4.14	-6.15	-13.31, 1.01	1.28	-5.97, 8.54
Twins' weight difference	0.03	-0.08, 0.14	0.01	-0.08, 0.10	0.21	-0.22, 0.63
Zygosity	-3.02	-6.63, 0.59	NA	NA	NA	NA
Sex of the twin pair (both females)	-0.73	-3.50, 2.04	0.38	-2.67, 3.42	-2.24	-6.77, 2.29
Sex of the twin pair (male-female)	0.24	-3.03, 3.50	NA	NA	-0.46	-4.48, 3.56
Constant	-2.84	-21.10, 15.42	15.49	-8.20, 39.19	-19.18	-46.07, 7.70

Note: NA: not applicable.

Table A2.2: Full-model estimates from the regressions of internalising behaviours on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-0.22	-1.03, 0.59	-4.17	-7.65, -0.69	0.24	-1.60, 2.07
Gestational age	-0.07	-0.30, 0.17	-0.02	-0.43, 0.39	-0.12	-0.43, 0.18
Mother's age	-0.02	-0.18, 0.14	-0.12	-0.35, 0.12	0.07	-0.15, 0.29
Twins' age	-0.01	-0.07, 0.05	0.02	-0.07, 0.11	-0.02	-0.10, 0.06
Pre-pregnancy BMI	0.06	-0.09, 0.21	-0.06	-0.30, 0.19	0.11	-0.09, 0.30
Smoking before pregnancy (yes)	-0.61	-2.75, 1.53	-0.06	-3.61, 3.49	-0.36	-3.15, 2.44
Smoking during pregnancy (yes)	1.40	-2.87, 5.67	1.40	-7.44, 10.24	1.44	-3.76, 6.63
Smoking after pregnancy (yes)	-1.04	-3.99, 1.92	-2.57	-7.43, 2.29	-0.62	-4.45, 3.21
Twins' weight difference	0.01	-0.05, 0.07	0.01	-0.05, 0.07	-0.07	-0.29, 0.15
Zygosity	-0.38	-2.40, 1.65	NA	NA	NA	NA
Sex of the twin pair (both females)	0.22	-1.33, 1.78	0.36	-1.70, 2.43	-0.28	-2.67, 2.12
Sex of the twin pair (male-female)	-0.53	-2.35, 1.30	NA	NA	-0.78	-2.90, 1.35
Constant	2.42	-7.81, 12.64	7.91	-8.16, 23.98	0.27	-13.93, 14.47

Note: NA: not applicable.

Table A2.3: Full-model estimates from the regressions of externalising behaviours on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-0.23	-0.92, 0.47	-0.66	-2.94, 1.62	-0.64	-2.35, 1.07
Gestational age	0.05	-0.16, 0.25	0.01	-0.25, 0.28	0.15	-0.14, 0.43
Mother's age	0.09	-0.05, 0.23	-0.05	-0.20, 0.10	0.21	0.00, 0.41
Twins' age	0.03	-0.02, 0.08	0.08	0.02, 0.14	0.01	-0.07, 0.08
Pre-pregnancy BMI	0.03	-0.10, 0.17	0.00	-0.17, 0.16	0.06	-0.12, 0.25
Smoking before pregnancy (yes)	1.59	-0.26, 3.45	1.04	-1.29, 3.37	2.28	-0.33, 4.89
Smoking during pregnancy (yes)	-4.34	-8.04, -0.64	3.36	-2.44, 9.16	-7.17	-12.02, -2.32
Smoking after pregnancy (yes)	-0.18	-2.74, 2.38	-2.41	-5.60, 0.77	1.04	-2.53, 4.62
Twins' weight difference	0.02	-0.04, 0.07	0.00	-0.04, 0.04	0.20	-0.01, 0.41
Zygosity	-1.88	-3.64, -0.13	NA	NA	NA	NA
Sex of the twin pair (both females)	-0.14	-1.49, 1.20	0.70	-0.65, 2.06	-0.93	-3.16, 1.30
Sex of the twin pair (male-female)	0.94	-0.64, 2.53	NA	NA	0.63	-1.35, 2.61
Constant	-6.27	-15.13, 2.59	-1.16	-11.70, 9.38	-15.26	-28.51, -2.01

Note: NA: not applicable.

Table A2.4: Full-model estimates from the regressions of anxiety/depression on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	0.03	-0.23, 0.30	-0.24	-1.14, 0.66	0.25	-0.41, 0.90
Gestational age	0.04	-0.04, 0.11	0.08	-0.02, 0.19	0.02	-0.09, 0.12
Mother's age	0.05	-0.00, 0.10	-0.01	-0.07, 0.06	0.08	0.00, 0.16
Twins' age	-0.01	-0.03, 0.01	0.00	-0.03, 0.02	-0.01	-0.04, 0.02
Pre-pregnancy BMI	0.03	-0.02, 0.08	-0.03	-0.09, 0.03	0.07	-0.01, 0.14
Smoking before pregnancy (yes)	-0.18	-0.88, 0.53	-0.25	-1.17, 0.67	0.02	-0.98, 1.01
Smoking during pregnancy (yes)	1.30	-0.11, 2.70	0.72	-1.57, 3.02	1.31	-0.54, 3.16
Smoking after pregnancy (yes)	-0.17	-1.14, 0.80	-0.34	-1.60, 0.92	-0.10	-1.47, 1.26
Twins' weight difference	0.00	-0.02, 0.02	0.00	-0.02, 0.01	-0.05	-0.12, 0.03
Zygosity	-0.12	-0.78, 0.55	NA	NA	NA	NA
Sex of the twin pair (both females)	0.11	-0.41, 0.62	-0.29	-0.83, 0.25	0.45	-0.40, 1.31
Sex of the twin pair (male-female)	-0.10	-0.70, 0.50	NA	NA	0.11	-0.65, 0.86
Constant	-3.61	-6.98, -0.25	-1.61	-5.78, 2.55	-5.34	-10.40, -0.29

Note: NA: not applicable.

Table A2.5: Full-model estimates from the regressions of emotional reactiveness on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	0.00	-0.51, 0.51	-2.70	-5.23, -0.18	0.17	-0.88, 1.22
Gestational age	-0.01	-0.16, 0.14	-0.10	-0.40, 0.19	0.01	-0.16, 0.18
Mother's age	-0.04	-0.14, 0.06	-0.07	-0.24, 0.10	0.01	-0.12, 0.13
Twins' age	-0.01	-0.04, 0.03	0.03	-0.04, 0.09	-0.03	-0.07, 0.02
Pre-pregnancy BMI	0.02	-0.08, 0.11	-0.02	-0.20, 0.16	0.02	-0.09, 0.13
Smoking before pregnancy (yes)	-0.74	-2.09, 0.61	0.27	-2.31, 2.85	-0.91	-2.51, 0.69
Smoking during pregnancy (yes)	-1.27	-3.97, 1.43	-0.36	-6.78, 6.06	-1.49	-4.47, 1.48
Smoking after pregnancy (yes)	0.35	-1.51, 2.22	-1.61	-5.14, 1.91	1.06	-1.13, 3.26
Twins' weight difference	0.02	-0.02, 0.06	0.02	-0.03, 0.06	0.02	-0.11, 0.14
Zygosity	-0.56	-1.83, 0.72	NA	NA	NA	NA
Sex of the twin pair (both females)	0.30	-0.68, 1.28	1.24	-0.26, 2.74	-0.76	-2.13, 0.61
Sex of the twin pair (male-female)	-0.17	-1.32, 0.99	NA	NA	-0.76	-1.98, 0.45
Constant	1.96	-4.50, 8.41	6.63	-5.04, 18.31	0.19	-7.94, 8.32

Note: NA: not applicable.

Table A2.6: Full-model estimates from the regressions of somatic complaints on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-0.17	-0.48, 0.13	-1.04	-2.24, 0.15	-0.08	-0.81, 0.65
Gestational age	-0.07	-0.16, 0.02	0.00	-0.14, 0.14	-0.1	-0.24, 0.00
Mother's age	-0.03	-0.09, 0.03	-0.04	-0.12, 0.04	-0.03	-0.12, 0.05
Twins' age	0.00	-0.02, 0.03	-0.01	-0.04, 0.02	0.01	-0.02, 0.04
Pre-pregnancy BMI	0.00	-0.06, 0.05	0.01	-0.08, 0.09	-0.01	-0.09, 0.07
Smoking before pregnancy (yes)	-0.11	-0.92, 0.70	-0.31	-1.53, 0.91	-0.07	-1.18, 1.04
Smoking during pregnancy (yes)	1.92	0.30, 3.54	0.21	-2.83, 3.25	2.62	0.56, 4.68
Smoking after pregnancy (yes)	-0.74	-1.87, 0.38	-0.02	-1.69, 1.65	-1.14	-2.66, 0.38
Twins' weight difference	0.00	-0.03, 0.02	-0.01	-0.03, 0.01	-0.03	-0.12, 0.06
Zygosity	0.84	0.07, 1.61	NA	NA	NA	NA
Sex of the twin pair (both females)	-0.25	-0.84, 0.34	-0.62	-1.33, 0.09	-0.10	-1.05, 0.85
Sex of the twin pair (male-female)	-0.68	-1.38, 0.01	NA	NA	-0.62	-1.46, 0.22
Constant	3.56	-0.33, 7.44	2.29	-3.23, 7.82	6.08	0.46, 11.71

Note: NA: not applicable.

Table A2.7: Full-model estimates from the regressions of withdrawnness on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-0.08	-0.25, 0.09	-0.22	-0.65, 0.20	-0.08	-0.53, 0.37
Gestational age	-0.02	-0.07, 0.03	0.00	-0.05, 0.05	-0.03	-0.10, 0.05
Mother's age	0.01	-0.03, 0.04	0.00	-0.03, 0.02	0.01	-0.04, 0.07
Twins' age	0.00	-0.01, 0.01	0.01	-0.00, 0.02	0.00	-0.02, 0.02
Pre-pregnancy BMI	0.01	-0.02, 0.05	-0.02	-0.05, 0.01	0.03	-0.02, 0.08
Smoking before pregnancy (yes)	0.42	-0.04, 0.87	0.23	-0.20, 0.66	0.60	-0.08, 1.29
Smoking during pregnancy (yes)	-0.52	-1.43, 0.39	0.86	-0.22, 1.94	-0.99	-2.27, 0.28
Smoking after pregnancy (yes)	-0.48	-1.11, 0.15	-0.63	-1.22, -0.04	-0.42	-1.36, 0.52
Twins' weight difference	0.00	-0.01, 0.01	0.00	-0.01, 0.01	-0.01	-0.07, 0.04
Zygosity	-0.53	-0.96, -0.10	NA	NA	NA	NA
Sex of the twin pair (both females)	0.02	-0.31, 0.35	-0.02	-0.27, 0.23	0.10	-0.49, 0.69
Sex of the twin pair (male-female)	0.42	0.03, 0.81	NA	NA	0.51	-0.02, 1.03
Constant	0.38	-1.79, 2.56	0.51	-1.45, 2.47	-0.80	-4.29, 2.68

Note: NA: not applicable.

Table A2.8: Full-model estimates from the regressions of attention problems on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	0.06	-0.20, 0.33	-0.11	-0.87, 0.65	-0.06	-0.73, 0.61
Gestational age	-0.02	-0.09, 0.06	-0.12	-0.21, -0.03	0.05	-0.06, 0.16
Mother's age	0.00	-0.05, 0.06	-0.03	-0.08, 0.02	0.04	-0.04, 0.12
Twins' age	0.01	-0.01, 0.03	0.02	0.00, 0.04	0.00	-0.03, 0.03
Pre-pregnancy BMI	0.01	-0.04, 0.06	0.03	-0.02, 0.09	0.00	-0.07, 0.07
Smoking before pregnancy (yes)	-0.03	-0.73, 0.67	0.12	-0.66, 0.89	-0.02	-1.05, 1.00
Smoking during pregnancy (yes)	-1.31	-2.71, 0.09	-0.09	-2.02, 1.85	-1.81	-3.71, 0.09
Smoking after pregnancy (yes)	0.38	-0.58, 1.35	-0.04	-1.10, 1.03	0.61	-0.79, 2.01
Twins' weight difference	0.00	-0.02, 0.02	0.00	-0.01, 0.02	0.07	-0.02, 0.15
Zygosity	-0.15	-0.81, 0.51	NA	NA	NA	NA
Sex of the twin pair (both females)	-0.03	-0.54, 0.48	0.21	-0.24, 0.66	-0.26	-1.14, 0.61
Sex of the twin pair (male-female)	0.45	-0.15, 1.05	NA	NA	0.31	-0.46, 1.09
Constant	-0.18	-3.52, 3.17	3.71	0.20, 7.22	-3.17	-8.36, 2.01

Note: NA: not applicable.

Table A2.9: Full-model estimates from the regressions of sleep problems on intrapair birth-weight difference

	MZ + DZ twins		MZ twins		DZ twins	
Variables	**β**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Birth weight difference (trend)	-0.23	-0.50, 0.04	-0.84	-1.69, 0.01	-0.29	-0.96, 0.39
Gestational age	0.01	-0.07, 0.08	-0.05	-0.15, 0.05	0.03	-0.08, 0.15
Mother's age	-0.01	-0.06, 0.04	-0.04	-0.09, 0.02	0.01	-0.07, 0.09
Twins' age	0.00	-0.02, 0.02	0.01	-0.01, 0.03	-0.01	-0.04, 0.02
Pre-pregnancy BMI	0.04	-0.02, 0.09	0.02	-0.04, 0.08	0.04	-0.04, 0.11
Smoking before pregnancy (yes)	-0.06	-0.78, 0.65	0.63	-0.23, 1.50	-0.39	-1.42, 0.64
Smoking during pregnancy (yes)	-0.15	-1.57, 1.28	-1.18	-3.34, 0.98	-0.02	-1.93, 1.89
Smoking after pregnancy (yes)	0.17	-0.82, 1.15	-0.90	-2.08, 0.29	0.77	-0.64, 2.18
Twins' weight difference	0.00	-0.02, 0.02	0.00	-0.02, 0.01	0.07	-0.01, 0.15
Zygosity	0.08	-0.59, 0.76	NA	NA	NA	NA
Sex of the twin pair (both females)	-0.31	-0.83, 0.21	-0.38	-0.88, 0.13	-0.21	-1.09, 0.67
Sex of the twin pair (male-female)	-0.06	-0.67, 0.55	NA	NA	-0.05	-0.83, 0.73
Constant	-0.54	-3.96, 2.87	2.50	-1.42, 6.43	-1.91	-7.13, 3.31

Note: NA: not applicable.

Post-hoc and sensitivity analyses

Table A2.10: Results of the multiple linear regression analyses excluding gestational age

	MZ + DZ twins	MZ twins	DZ twins
Problem behaviour scale		**β (95% C.I.)**	
Total problems	-1.21 (-2.65, 0.24)	-5.78 (-10.86, -0.70)	-2.76 (-6.19, 0.66)
Internalizing behaviours			
Total internalizing	-0.26 (-1.06, 0.55)	-4.15 (-7.59, -0.71)	-0.05 (-1.86, 1.75)
Anxiety/depression	0.04 (-0.22, 0.31)	-0.32 (-1.22, 0.58)	0.27 (-0.36, 0.91)
Emotional reactiveness	-0.02 (-0.53, 0.49)	-2.60 (-5.11, -0.10)	-0.02 (-1.06, 1.01)
Somatic complaints	-0.19 (-0.50, 0.12)	-1.04 (-2.23, 0.14)	-0.16 (-0.88, 0.56)
Withdrawnness	-0.08 (-0.26, 0.09)	-0.22 (-0.64, 0.19)	-0.13 (-0.57, 0.31)
Externalizing behaviours			
Total externalizing	-0.26 (-0.96, 0.44)	-0.67 (-2.93, 1.59)	-0.91 (-2.60, 0.77)
Aggressive behaviour	-0.30 (-0.88, 0.28)	-0.62 (-2.79, 1.54)	-0.79 (-2.14, 0.56)
Attention problems	0.05 (-0.21, 0.31)	0.01 (-0.77, 0.78)	-0.10 (-0.76, 0.55)
Sleep problems	-0.24 (-0.51, 0.03)	-0.79 (-1.64, 0.05)	-0.35 (-1.01, 0.31)

Table A2.11: Results of the multiple linear regression analyses excluding pairs with extremely low birth-weight twins

	MZ + DZ twins	MZ twins	DZ twins
Problem behaviour scale		**β (95% C.I.)**	
Total problems	-1.11 (-2.56, 0.34)	-5.70 (-10.82, -0.59)	-2.11 (-5.64, 1.42)
Internalizing behaviours			
Total internalizing	-0.20 (-1.01, 0.61)	-4.06 (-7.55, -0.57)	0.28 (-1.57, 2.13)
Anxiety/depression	0.03 (-0.24, 0.29)	-0.22 (-1.13, 0.69)	0.23 (-0.43, 0.90)
Emotional reactiveness	0.01 (-0.51, 0.52)	-2.70 (-5.24, -0.15)	0.20 (-0.88, 1.26)
Somatic complaints	-0.16 (-0.46, 0.14)	-0.97 (-2.16, 0.22)	-0.05 (-0.77, 0.67)
Withdrawnness	-0.07 (-0.24, 0.10)	-0.20 (-0.62, 0.22)	-0.08 (-0.53, 0.38)
Externalizing behaviours			
Total externalizing	-0.24 (-0.95, 0.47)	-0.66 (-2.96, 1.64)	-0.71 (-2.46, 1.04)
Aggressive behaviour	-0.28 (-0.87, 0.30)	-0.59 (-2.77, 1.58)	-0.63 (-2.03, 0.77)
Attention problems	0.06 (-0.20, 0.32)	-0.01 (-0.72, 0.70)	-0.07 (-0.75, 0.61)
Sleep problems	-0.24 (-0.51, 0.03)	-0.81 (-1.66, 0.04)	-0.30 (-0.99, 0.39)

Chapter 3

Maternal pre-pregnancy weight and twins' temperament

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Re-analysis of the chapter "Maternal pre-pregnancy weight and infant temperament" included in the PhD thesis: The influence of genetic, environmental and intrauterine factors on child development: The east Flanders prospective twin survey (EFPTS) & the twins and multiple births association heritability study (TAMBAHS) by Evangelia Antoniou (2012).

Abstract

Maternal pre-pregnancy weight has been related with young singletons' behavioural development, but it is not clear if it has an effect on temperament. We used a twin cohort to evaluate the association between maternal pre-pregnancy body mass index (BMI) and infants' temperament. The mothers of 834 twins answered questions regarding their pre-pregnancy BMI and their 0- to 18-month-old children's temperament using the Infant Behaviour Questionnaire- Revised. Three temperamental dimensions were examined: activity level, distress to limitation and duration of orienting. The relationship between maternal pre-pregnancy BMI and each temperamental component was investigated by means of multilevel mixed-effects linear regression analyses. We found no evidence of an association of maternal pre-pregnancy BMI with twins' temperament, which might be influenced by a different set of factors from those influencing children's problem behaviours.

Background

Following Rothbart's definition, temperament can be considered the combination of individual differences in reactivity and self-regulation, observed in the emotional, motor and attentional domains (1,2). The term *reactivity* refers to the latency, intensity, and recovery of response, while *self-regulation* refers to the processes that modulate reactivity. These characteristics are generally evident since birth and remain relatively stable over the lifetime, although personal experiences and acquired physical skills contribute to their development (3). In total, 14 temperamental dimensions have been identified, grouped into three higher-order factors: 1) surgency/extraversion, which includes the traits of approach, vocal reactivity, smiling/laughter, activity level, high-intensity pleasure and perceptual sensitivity; 2) negative affectivity, which includes the traits of sadness, frustration (or distress to limitation), fear and low levels of falling reactivity; and 3) orienting/regulation, which includes low-intensity pleasure, cuddliness, duration of orienting and soothability (1).

An increasing body of literature has suggested a link between temperamental dimensions and child psychopathology (3–5). A meta-analysis has shown that regulatory problems in infancy, which manifest as excessive and persistent crying, are associated with internalising and externalising behaviours at school age, with medium effect sizes (6). Persistent problems with behavioural control, such as the inability to regulate feeding and sleeping behaviour in infancy are precursors of behaviour control difficulties such as hyperactivity or conduct problems in childhood (7–9). Specifically, three temperamental dimensions have been linked with development of psychopathology later in life (10–12): activity level, distress to limitations, and duration of orienting. Activity level refers to gross motor activity, including squirming and locomotor activity while doing daily activities; distress to limitations refers to fussing, crying or showing distress when confined in a place/position or when unable to perform a desired action; duration of orienting refers to the child's ability to sustain the attention on and/or interact with an object or task. High activity levels in infancy have been associated with higher externalising behaviours both concurrently and longitudinally (13,14). High levels of distress in infancy have been related to higher internalising and externalising behaviours (14), and especially aggression (15). Finally, duration of orienting is negatively associated with both internalising and externalising behaviours (14,15).

Since both pregnancy and obesity are associated with massive metabolic alterations (16,17), their combination might substantially affect the environment experienced by the foetus. It has been

hypothesised that maternal adiposity at the time of conception and during pregnancy might influence child mental health through oxidative stress, chronic inflammation and hormonal dysregulation (18). Higher maternal pre-pregnancy body mass index (BMI) has been associated with a higher risk of child problem behaviours and attention deficit/hyperactivity disorder (19–22). Nonetheless, the possible influence of maternal weight before pregnancy, as a proxy for the prenatal environment, on infant temperament has only recently been studied. Van Lieshout and colleagues (23) were the first to analyse the association of maternal pre-pregnancy BMI with offspring temperament in children aged 1 year. In their analysis, maternal BMI was not found to be related with any temperamental component, measured using the Toddler Temperament Scale (23). However, in a very small study (n=16) maternal pre-pregnancy BMI was reported to be positively associated with negative affectivity in Canadian infants aged 3 months (24). Similarly, in two studies examining infants' regulation, infants born to women with pre-pregnancy overweight or obesity (25) or pre-pregnancy obesity in combination with excessive gestational weight gain (26) had higher regulatory behavioural problems. Finally, another small, preliminary study (n=68, including 6 sibling pairs) reported that pre-pregnancy BMI was associated with negative behaviour at 6 months of age. However, the observed effects of pre-pregnancy BMI was reduced in children of women with high total n-3 polyunsaturated fatty acid (PUFA) concentrations in the third trimester of pregnancy (27).

Twin studies have provided strong evidence of genetic influence on temperament including activity level (28), distress to limitations (29) and duration of orienting (30,31). Estimates of heritability suggest that genetic factors account for 20% to 60% of the variability of temperament within a population with no substantial difference across dimensions (32). Uncontrolled genetic influences might therefore have affected previously reported associations. In fact, although no studies were published examining the association between maternal pre-pregnancy BMI and infants' temperament with a genetically informed study design, analyses of siblings have questioned the association between maternal pre-pregnancy BMI and child psychopathology (22,33,34). Therefore, we aimed to investigate the association between maternal pre-pregnancy BMI and infants' temperament in monozygotic (MZ) and dizygotic (DZ) twins. A deeper understanding of this association could help preventing the development of difficult temperament in children, as well as problem behaviours later in life, by informing prospective mothers on the possible effects of overweight and obesity on children's psychopathology.

Method

Study Population

The Twins and Multiple Births Association Heritability Study (TAMBAHS) is a volunteer-based study focusing on the development of twins from birth until the age of 5 years (35). For the present study, mothers of twins aged 0 to 18 months at the time of the survey were invited to participate. Between July 2008 and May 2010, 417 women completed a questionnaire on their twins' temperament and reported information about maternal and twins' demographic and anthropometric characteristics. For the determination of the twins' zygosity, the adapted version of Goldsmith's zygosity questionnaire was used (36). This adapted questionnaire, as a method of assigning zygosity, has been validated against determination by identity of polymorphic DNA markers and has reached accuracy in verifying zygosity in 95% of cases (37). In total, 834 twins were included in the analyses: 188 MZ male twins, 188 MZ female twins, 120 DZ male twins, 118 DZ female twins and 220 DZ opposite-sex twins. This study was approved by the University of Birmingham Ethical Review Committee. Written informed consent was obtained from all participating families.

Maternal and twins' characteristics

Pre-pregnancy BMI (expressed as kg/m^2) was based on maternal self-report of weight and height and introduced as a continuous variable in the mixed-effect linear regressions. Gestational age (measured in completed weeks of gestation), level of education (high school diploma or less, college/professional education, and university degree), employment status (housekeeper or unemployed, working part-time, working full-time, other) and smoking (before, during, after pregnancy; yes/no) were noted for mothers; age (in months), sex and birthweight (in grams) were noted for all twins.

Temperament

Infants' temperament was assessed using the revised Infant Behaviour Questionnaire (IBQ-R) (38). The parents were asked to report on a 7-point Likert-type scale the relative frequency of occurrence of specified infants' reactions to concrete situations during the previous seven days. The scale ranged from 1 to 7 (never, very rarely, less than half the times, about half the times, more than half the times, almost always, always, does not apply). The IBQ-R consists of 14 scales. For the purposes of this study we used only three dimensions of temperament; 1) Activity level, which consists of items examining the twins' movement of arms and legs, squirming, and locomotor activity; 2)

Distress to Limitations, which consists of items looking into twins' fussing, crying or showing distress while a) in a confining place or position; b) involved in caretaking activities; c) unable to perform a desired action; 3) Duration of Orienting, which consists of items on the twins' attention to and/or interaction with a single object for extended periods of time. Reliability, convergent validity, and relative stability have been demonstrated for the IBQ-R (39,40). The internal consistency for the IBQ-R items was high with the Cronbach's alpha, ranging from 0.81 to 0.90. Consistency between parent report on the IBQ-R and indicators of temperament based on home and laboratory observations has been demonstrated (41).

Statistical analysis

Mean and standard deviation (SD) or median and interquartile range (IQR) for each continuous variable were calculated as appropriate, stratified by zygosity group. Intrapair twin correlations for each variable, subdivided into MZ and DZ twins, were calculated by using Pearson's (r) and Spearman's (ρ) coefficient statistics where appropriate.

The association between maternal pre-pregnancy BMI and each temperamental dimension was analysed using mixed-effect linear regressions, in which a random-effects model was added to the default fixed effect. The intercept of each twin pair was modelled as a function of the population intercept plus a unique contribution of the twin pair, as thoroughly explained by Carlin and colleagues (42) and summarised by the following equation:

$$Y_{ij} = \beta_0 + \beta_C X_{i(j)} + \alpha_i + \epsilon_{ij}$$

in which Y_{ij} represents the outcome (*i.e.,* the specific temperamental dimension's score) of the j^{th} twin in the i^{th} pair. In addition to the linear regression model, a twin pair-specific error term (α_i) allows a random shift of the intercept. Regression coefficients (βs) were employed as a measure of change on the temperamental dimensions' scores by a unit change in the mother's BMI.

Twins' age, gender, birth weight and gestational age, and maternal age, smoking (before, during and after pregnancy), level of education and employment status were adjusted for in the analysis.

All analyses were performed using Stata v.14 (43).

Results

Descriptive statistics as well as Pearson's and Spearman's correlations between each covariate and the three temperament dimensions stratified by zygosity are shown in Table 3.1. Women's mean age at delivery was 33.5 years (4.2) for mothers of MZ and 34.7 years (4.1) for mothers of DZ twins. Median (IQR) for pre-pregnancy BMI was 23.4 (21.6, 26.2) for mothers of MZ and 23.5 (21.5, 26.9) for mothers of DZ, respectively. For MZ and DZ twins, respectively, median (IQR) gestational age was 36 weeks (34.6, 37.5) and 37 weeks (35.7, 38); for birthweight it was 2438g (1984, 2778) and 2590g (2211, 2934). Monozygotic and dizygotic twins were significantly different with respect to gestational age (p=0.0001), birthweight (p<0.0001) and maternal age (p=0.0001).

Means of temperamental dimensions and intra-pair twin correlations for MZ and DZ twins are shown in Table 3.2. For activity level, MZ correlation was r=0.75 and the DZ correlation was r=0.45. For distress to limitations, MZ and DZ correlations were r=0.83 and r=0.56, respectively. For duration of orienting, MZ and DZ correlations were r=0.94 and r=0.85, respectively.

Linear Regressions

Table 3.3 presents the results of the linear regressions. In the unadjusted analyses, we found a small decrease in the dizygotic twins' distress to limitations score for every unit increase of mother's pre-pregnancy BMI (β for the fixed effect= -0.03; 95% confidence intervals (CI): -0.050, -0.001; p=0.042). This association was not substantially affected after important confounders were adjusted for (β for the fixed effect = -0.04; 95% CI: -0.065, -0.013; p=0.003). Similar results were obtained when analysing the MZ and DZ twins combined (Appendix, table A3.1). No association remained statistically significant after controlling for multiple testing, setting the cut-off for statistical significance at α=(0.05/18)=0.0028. The normality of residuals was checked through normal quantile plots and confirmed for all regression analyses (Appendix, figures A3.1-A3.3). Full-model-estimate tables are shown in the Appendix (tables A3.2-A3.4).

Discussion

The aim of this study was to examine the relationship between maternal pre-pregnancy weight and infants' temperament in twins, measured with three dimensions of the Infant Behaviour Questionnaire- Revised: activity level, distress to limitation and duration of orienting. A statistically

Table 3.1: Means/frequencies, standard deviations/percentages and Pearson correlations with the three temperament scales for each covariate, stratified by zygosity

	MZ					DZ				
Maternal Characteristics	**Mean, median or n**	**SD, IQR or %**	r_a	r_b	r_c	**Mean, median or n**	**SD, IQR or %**	r_a	r_b	r_c
Pre-pregnancy BMI	23.4	21.6, 26.2	-0.04	-0.01	0.04	23.5	21.5, 26.9	-0.03	-0.13*	0.00
Age (years)	33.5	4.2	-0.12*	-0.05	-0.10	34.7	4.1	-0.19**	-0.14***	-0.09
Gestational age (weeks)	36	34.6, 37.5	0.04	-0.06	0.06	37	35.7, 38	0.03	0.03	-0.04
Smoking (% yes)										
Before	28	16%	-0.03	-0.02	0.03	35	16%	-0.03	0.09	0.04
During	4	2%	0.00	0.07	0.03	11	5%	0.02	0.07	-0.02
After	11	6%	-0.04	0.02	0.16***	17	8%	-0.03	0.09	0.9***
Level of education										
High School diploma or less	33	18.6%				47	21.1%			
College/professional education	31	17.4%	-0.15	-0.11	-0.07	52	23.3%	-0.06	-0.13	-0.05
University education	114	64.0%				124	55.6%			
Employment status										
Housekeeper or unemployed	192	53.6%				216	47.8%			
Working part-time	80	22.3%	0.01	-0.19**	0.02	136	30.1%	-0.04	0.04	-0.04
Working full-time	68	19%				88	19.5%			
Other	18	5.1%				12	2.6%			
Twin Characteristics										
Birth weight (grams)	2438	1984, 2778	0.08	-0.03	0.05	2595	2211, 2934	-0.03	-0.03	-0.02
Age (months)	8	4, 12	0.35**	0.11*	0.10*	9	5, 13	0.16**	0.04	0.20**
Sex										
Male	188	50%				120	26.2%			
Female	188	50%	-0.14**	-0.16**	-0.04	118	25.8%	-0.03	-0.11*	0.07
Opposite sex	-	-				220	48%			

Note: r_a Pearson/Spearman correlation with activity level; r_b Pearson/Spearman correlation with distress to limitations; r_c Pearson/Spearman correlation with duration of orienting; *p<0.05; **p<0.001; ***p<0.0001

Table 3.2: Descriptive statistics of temperament scales for monozygotic and dizygotic twin pairs

MZ twin pairs		Twin 1		Twin 2		
Temperament scales	**N**	**Mean**	**SD**	**Mean**	**SD**	**r**
Activity level	185	4.12	1.01	4.00	0.93	0.75**
Distress to Limitations	185	3.48	1.02	3.55	0.98	0.83**
Duration of Orienting	171	3.72	1.20	3.69	1.21	0.95**
DZ twin pairs		**Twin 1**		**Twin 2**		
Temperament scales	**N**	**Mean**	**SD**	**Mean**	**SD**	**r**
Activity level	215	4.13	0.98	3.99	0.95	0.45**
Distress to Limitations	215	3.57	0.99	3.58	0.97	0.56**
Duration of Orienting	202	3.57	1.13	3.53	1.18	0.85**

Note: N: number of twin pairs; SD: standard deviation; r: intra-pair correlations; twin 1 and twin 2 denote the first and the second born, respectively; **p<0.001

Table 3.3: Results of the associations between maternal pre-pregnancy BMI and each temperamental scale stratified by zygosity

				MZ twins		
Temperament scales	**β**	**95% CI**	**p**	**β[a]**	**95% CI[a]**	**p[a]**
Activity Level	-0.01	-0.04, 0.02	0.57	-0.01	-0.04, 0.03	0.71
Distress to Limitations	-0.00	-0.04, 0.03	0.94	0.00	-0.03, 0.04	0.88
Duration of Orienting	0.01	-0.03, 0.06	0.61	0.02	-0.03, 0.07	0.37
				DZ twins		
Temperament scales	**β**	**95% CI**	**p**	**β[a]**	**95% CI[a]**	**p[a]**
Activity Level	-0.01	-0.03, 0.02	0.61	-0.01	-0.04, 0.01	0.33
Distress to Limitations	-0.03	-0.05, -0.00	0.04	-0.04	-0.07, -0.01	0.003
Duration of Orienting	0.00	-0.03, 0.04	0.98	0.02	-0.01, 0.06	0.25

Note: β coefficients and 95% confidence intervals of the fixed effects of maternal pre-pregnancy BMI on temperamental scales are shown; a: adjusted for twins' age, sex, birth weight, gestational age, mother's age, level of education, employment status and smoking (before, during, after pregnancy).

significant, negative association between maternal pre-pregnancy weight and distress to limitation in dizygotic twins initially emerged from the present analysis. However, the relative p-value failed to reach the critical level of significance after controlling for multiple testing. Although the Bonferroni method for multiple testing adjustment has been criticised and could be considered excessively conservative in this case (44), the true value of beta is likely close to null, as the 95% confidence interval ranges between -0.065 and -0.013. This means that for every unit increase in maternal pre-pregnancy BMI, scores in distress to limitation would decrease on average by 0.04

points, on a scale from 1 to 7. Consequently, the effect of a high maternal BMI on children's temperament we observed would certainly not be enough to suggest that a higher maternal pre-pregnancy weight could be beneficial, especially in light of the many negative effects overweight or obesity have on maternal and offspring's health (45).

Research on the relationship between maternal pre-pregnancy BMI and infants' temperament has been conducted mainly in virtue of the evidence of an association between pre-pregnancy BMI and childhood psychopathology (19–22), and given the relationship between temperament and psychopathology, where temperament is often considered a sub-clinical manifestation of psychopathology (46). Nonetheless, the evidence supporting an effect of maternal BMI on infants' temperament is less strong. Poorer regulatory behaviours were reported in children born to women who were overweight or obese prior to pregnancy (25), while another study reported an association between maternal pre-pregnancy obesity and infants' regulation only in combination with an excessive gestational weight gain (26). Maternal pre-pregnancy BMI was associated with increased negative affectivity measured with the IBQ-R (24), or assessed through direct observation (27). However, in the latter study no associations were reported when infants' negative affectivity was measured with the IBQ-R (27). In addition, the latter two studies utilised very small populations, hence findings should be considered preliminary (24,27). Finally, no associations were found between pre-pregnancy BMI and temperamental dimensions in 1-year-old children, despite an association was reported with child externalising (but not internalising) behaviours in the same children at 2 years of age (23).

An important limitation of most previous studies investigating the relationship between maternal weight and child's temperament and psychopathology is the lack of adjustment for genetics. Consequently, it could be hypothesised that reported associations were confounded by uncontrolled genetic influences. In fact, it has been demonstrated that temperament, as well as problem behaviours, is substantially genetically and environmentally influenced (47). A lack of adequate control for genetics could, therefore, have confounded previously reported association. In fact, we used a twin design to control for genetic and common environmental influences and found no evidence of an association between maternal pre-pregnancy BMI and infants' temperament. Similarly, previous studies employing genetically informed designs (*i.e.*, sibling design) reported no associations with child problem behaviours and ADHD risk (22,33,34), although with some exceptions (e.g., (48)). Only a tendency for aggressiveness and externalising behaviours was observed in preschool-age twins born to women with a pre-pregnancy overweight or obesity

(35). In this scenario, the genes transmitted by the mother (and the father) to the child would influence childhood temperament and psychopathology to a greater extent than the prenatal environment created by maternal weight.

However, there is some evidence suggesting a role for maternal BMI on offspring's psychological development independent from genetic factors, although mainly coming from animal studies (18). In humans, maternal pre-pregnancy obesity was associated with white matter development in new-borns, as well as a differential methylation pattern in genes related to brain development (49). Excessive body weight at the time of conception have been associated with increased risk of several adverse outcomes – including low birth weight and preterm birth, intrauterine growth restriction, and child and maternal morbidity (45) –, which often cannot be reduced by improving diet and physical activity during pregnancy (50). Therefore, entering pregnancy with a healthy weight might significantly improve child outcomes by guaranteeing an optimal prenatal environment since conception. Nonetheless, the strongest evidence from epidemiological studies of an association between pre-pregnancy BMI and child development is available for psychological traits measured in older children as opposed to infants and preschool-age children. A possible reason for this pattern of findings might be that the effect of maternal pre-pregnancy BMI may manifest later in children's development. In this respect, pre-pregnancy BMI might be a proxy for maternal lifestyle, which is fairly stable over time (52). Maternal lifestyle would influence children's dietary intake and physical activity (53–55), which in turn affect their risk of psychopathology (56–59). Temperament in infancy is arguably assessed too early to be affected by childhood lifestyle. An alternative explanation might be that environmental factors important for problem behaviours might not affect temperament. In this regard, behavioural genetic studies suggest that genetic factors might be completely responsible for the phenotypic correlation between temperament and problem behaviours (e.g., (60)). Consequently, temperament in infancy would be a moderator of the association between maternal pre-pregnancy BMI and child psychopathology, rather than mediating it (46,61). In this perspective, a "difficult" temperament might lead to psychopathology only in a high-risk environment, such as the one created by a high maternal BMI, and/or in the absence of protective factors. Investigating the association between maternal pre-pregnancy BMI and temperament in preschool- or school-age children might provide some evidence for or against this hypothesis.

The results of this study should be interpreted in light of some limitations. There were no data available to assess maternal psychopathology, a risk factor for obesity and internalising/externalising problem behaviours that may also affect maternal perception of

offspring's temperament (62–66). Similarly, it was not possible to assess parents' soothing practices towards their twins, which is related to psychological development in infancy and childhood (67). We had no data regarding breastfeeding and maternal Type II Diabetes Mellitus, which have been associated with children's temperament and behaviour (68,69). Full control for the familiar socioeconomic status (SES) was not possible, since we lacked the data regarding parental income. Nonetheless, maternal level of education and employment status are considered the other two core components of SES (70) and can provide a reliable indication of the familiar situation. Additionally, we were not able to account for the presence of other children in the family, which might have affected maternal ratings. Although previous studies did not observe an association between chorionicity and children's psychopathology (71), we cannot exclude an uncontrolled effect of chorion type on our results. Chorionicity might affect nutrient and space scarcity in multiple pregnancies, which may lead to discordant growth or pathological complications such as the twin-to-twin transfusion syndrome (71). This implies that twins, and especially MZ twins, might be more susceptible to challenging intrauterine environment that might influence their future temperament. Finally, although self-reported height and weight are widely used in epidemiological studies, overweight and obese women are more likely to under-report their pre-pregnancy weight (72). Consequently, we cannot rule out an effect of biased reports on results.

Conclusions

In sum, the results of this study do not support an association between maternal pre-pregnancy BMI and infants' temperament. These findings suggest that genetic and environmental influences shared between the family might confound the association between maternal pre-pregnancy BMI and infants' temperament. Additional studies are warranted to investigate the developmental paths of temperament, accounting for markers of maternal pre- and post-pregnancy metabolic and mental health, as well as infants' genetic susceptibility.

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Appendix to chapter 3

Linear regression analyses in MZ and DZ twins combined

Table A3.1: Linear regressions for the temperamental scales in MZ and DZ twins combined

	MZ and DZ twins					
	β	**95% CI**	**p**	**β[a]**	**95% CI[a]**	**p[a]**
Activity Level	-0.01	-0.03, 0.01	0.46	-0.01	-0.03, 0.01	0.41
Distress to Limitations	-0.02	-0.04, 0.01	0.14	-0.02	-0.05, -0.00	0.03§
Duration of Orienting	0.00	-0.02, 0.03	0.80	0.02	-0.01, 0.05	0.15

Note: a: Adjusted for twins' age, sex, birth weight, gestational age, mother's age, level of education, employment status and smoking (before, during, after pregnancy); § p≤ 0.05; * p≤ 0.0028

Normal quantile plots of linear regressions' residuals

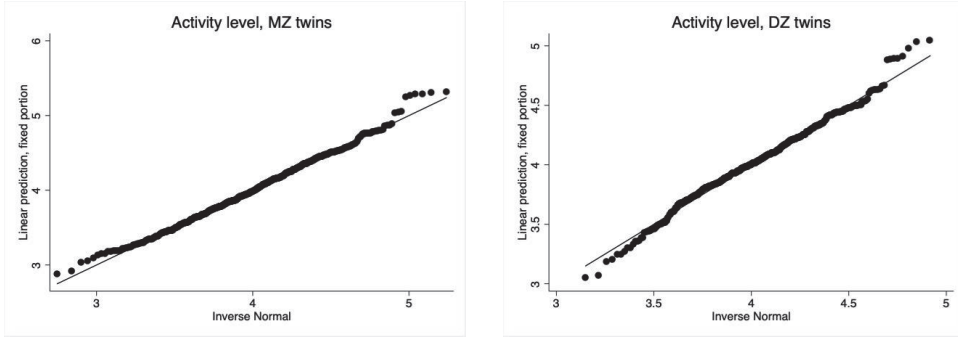

Figure A3.1: Normal quantile plot in MZ and DZ twins, respectively (outcome: activity level)

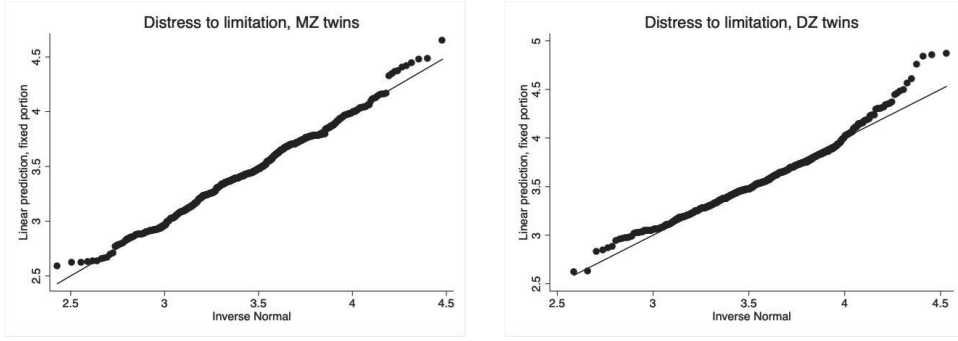

Figure A3.2: Normal quantile plot in MZ and DZ twins, respectively (outcome: distress to limitation)

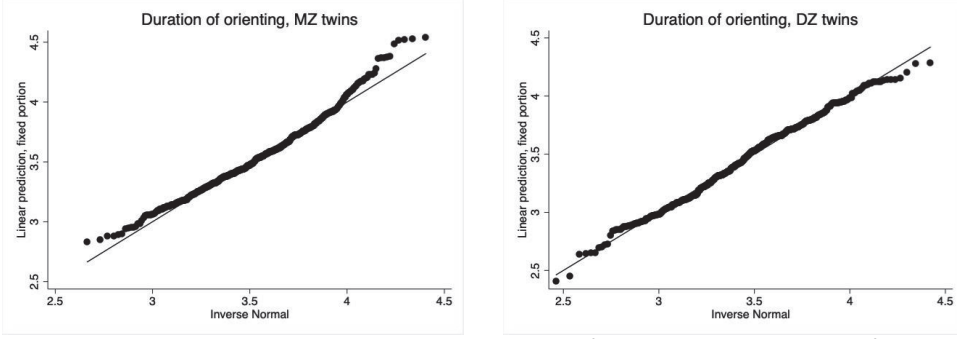

Figure A3.3: Normal quantile plot in MZ and DZ twins, respectively (outcome: duration of orienting)

Full-model-estimate tables

Table A3.2: Full-model estimates for the association between maternal pre-pregnancy BMI and twins' activity level

Activity level	MZ twins		DZ twins	
	β	95% C.I.	β	95% C.I.
Maternal BMI	-0.01	-0.04, 0.03	-0.01	-0.04, 0.01
Gestational age	-0.01	-0.08, 0.05	0.00	0.00, 0.00
Birth weight	0.00	0.00, 0.00	0.02	-0.01, 0.05
Twins' age	0.05	0.02, 0.08	-0.06	-0.25, 0.13
Twin's sex (female)	-0.26	-0.55, 0.03	-0.05	-0.08, -0.01
Mother's age	-0.04	-0.08, -0.01	-0.21	-0.63, 0.21
Smoking before pregnancy (yes)	-0.05	-0.54, 0.43	0.17	-0.77, 1.11
Smoking during pregnancy (yes)	0.27	-0.71, 1.25	-0.41	-1.17, 0.35
Smoking after pregnancy (yes)	-0.48	-1.20, 0.24	-0.30	-0.69, 0.09
Maternal education (College)	-0.24	-0.73, 0.25	-0.31	-0.64, 0.02
Maternal education (University)	-0.46	-0.82, -0.09	0.12	-0.17, 0.40
Maternal employment (part-time)	0.26	-0.10, 0.62	-0.02	-0.36, 0.31
Maternal employment (fulltime)	0.19	-0.18, 0.55	-0.04	-0.72, 0.64
Maternal employment (other)	-0.27	-1.09, 0.55	6.66	4.33, 8.99
Constant	5.66	3.29, 8.04	-0.02	-0.08, 0.05

Table A3.3: Full-model estimates for the association between maternal pre-pregnancy BMI and twins' distress to limitation

Distress to limitation	MZ twins		DZ twins	
	β	95% C.I.	β	95% C.I.
Maternal BMI	0.00	-0.03, 0.04	-0.04	-0.07, -0.01
Gestational age	0.03	-0.03, 0.10	0.02	-0.04, 0.09
Birth weight	0.00	0.00, 0.00	0.00	0.00, 0.00
Twins' age	0.00	-0.03, 0.03	0.00	-0.03, 0.02
Twin's sex (female)	-0.31	-0.61, -0.01	-0.26	-0.43, -0.08
Mother's age	-0.01	-0.04, 0.03	-0.02	-0.06, 0.01
Smoking before pregnancy (yes)	0.04	-0.48, 0.55	0.22	-0.22, 0.66
Smoking during pregnancy (yes)	0.82	-0.21, 1.85	0.56	-0.43, 1.54
Smoking after pregnancy (yes)	-0.30	-1.06, 0.46	0.04	-0.76, 0.84
Maternal education (College)	0.15	-0.37, 0.66	-0.04	-0.44, 0.37
Maternal education (University)	-0.23	-0.62, 0.15	-0.32	-0.66, 0.03
Maternal employment (part-time)	-0.12	-0.49, 0.26	0.11	-0.19, 0.41
Maternal employment (fulltime)	-0.44	-0.83, -0.06	0.20	-0.15, 0.55
Maternal employment (other)	-1.23	-2.09, -0.36	0.91	0.20, 1.62
Constant	2.93	0.42, 5.44	4.92	2.52, 7.31

Table A3.4: Full-model estimates for the association between maternal pre-pregnancy BMI and twins' duration of orienting

Duration of orienting	MZ twins		DZ twins	
	β	95% C.I.	β	95% C.I.
Maternal BMI	0.02	-0.03, 0.07	0.02	-0.01, 0.06
Gestational age	0.01	-0.06, 0.08	0.02	-0.06, 0.09
Birth weight	0.00	0.00, 0.00	0.00	0.00, 0.00
Twins' age	0.02	-0.02, 0.06	0.04	0.00, 0.08
Twin's sex (female)	-0.02	-0.40, 0.35	0.04	-0.10, 0.19
Mother's age	-0.03	-0.08, 0.01	-0.02	-0.07, 0.02
Smoking before pregnancy (yes)	-0.13	-0.78, 0.51	0.19	-0.39, 0.76
Smoking during pregnancy (yes)	-0.50	-1.91, 0.91	-0.71	-2.07, 0.64
Smoking after pregnancy (yes)	1.55	0.56, 2.54	0.19	-0.94, 1.32
Maternal education (College)	-0.64	-1.28, 0.00	0.14	-0.40, 0.68
Maternal education (University)	-0.45	-0.93, 0.03	0.22	-0.23, 0.68
Maternal employment (part-time)	-0.51	-0.98, -0.04	0.36	-0.05, 0.77
Maternal employment (fulltime)	0.18	-0.32, 0.68	-0.37	-0.84, 0.10
Maternal employment (other)	-0.88	-1.93, 0.17	0.02	-0.90, 0.95
Constant	3.91	0.97, 6.86	2.67	-0.37, 5.71

Chapter 4

Gestational weight gain by maternal pre-pregnancy BMI and childhood problem behaviours in school-age years: a pooled analysis of two European birth cohorts

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Abstract

Background: Maternal pre-pregnancy weight is known to affect foetal development. However, it has not been clarified yet whether gestational weight gain is associated with childhood behavioural development.

Methods: We performed a pooled analysis of two prospective birth cohorts to investigate the association between gestational weight gain and childhood problem behaviours, and the effect modification of maternal pre-pregnancy BMI. In total, 378 mother-child pairs from the Maastricht Essential Fatty Acids Birth cohort (MEFAB) and 414 pairs from the Rhea Mother-Child cohort were followed up from early-pregnancy to 6-7 years post-partum. At follow up, parents assessed their children's behaviour, measured as total problems, internalising and externalising behaviours, with the Child Behaviour Checklist. We computed cohort- and subject-specific gestational weight gain trajectories using mixed-effect linear regression models. Fractional polynomial regressions, stratified by maternal pre-pregnancy BMI status, were then used to examine the association between gestational weight gain and childhood problem behaviours.

Results: In the pre-pregnancy overweight/obese group, greater gestational weight gain was associated with higher problem behaviours. On average, children of women with overweight/obesity who gained 0.5 kg/week scored 25 points higher (on a 0-100 scale) in total problems and internalising behaviours, and about 18 points higher in externalising behaviours than children whose mothers gained 0.2 kg/week. Inconsistent results were found in the pre-pregnancy normal-weight group.

Conclusions: Excessive gestational weight gain in women with pre-pregnancy overweight/obesity might increase problem behaviours in school-age children. Attention should be granted to avoid excessive weight gain in women who enter pregnancy while being overweight or obese.

Background

In Western countries, an increasing proportion of pregnant women does not follow the recommendations regarding gestational weight gain (GWG) (1,2). Excessive GWG is characterised by a disproportionate increase in maternal fat mass (1) and has been hypothesised to affect maternal inflammation and hormonal regulation (3–5). In turn, these metabolic alterations might affect the development of foetal hypothalamic-pituitary-adrenal (HPA) axis, resulting in an impaired behavioural response of the offspring to stress (6). By contrast, insufficient GWG, especially when combined with low pre-pregnancy BMI, might not provide adequate sustainment for the foetal brain and HPA axis development (7).

In the attempt to test this hypothesis, previous studies have examined the association between maternal GWG and childhood problem behaviours, a group of psychopathological disorders that affects stress reactivity. Internalising problems are typified by anxious and depressive traits, whereas aggressiveness, attention problems and hyperactivity characterise externalising behaviours (8,9). However, to date the evidence of an association is scarce. Only a tendency for increased impulsivity was found in 10-year-old children (n=511) born to women with a normal pre-pregnancy BMI who gained insufficient weight in pregnancy, as well as in children of women with pre-pregnancy overweight who gained excessive gestational weight (10). No associations were reported when examining parent- or teacher-rated internalising or externalising behaviours in the same children (10). In contrast, high maternal pre-pregnancy BMI was associated with greater problem behaviours and attention problems (10). Higher hyperactivity/impulsivity symptoms were also observed in children of women with insufficient compared to adequate GWG, while only a tendency for greater inhibiting behaviours was found in children exposed to excessive compared to adequate GWG (11). By contrast, no associations were observed with continuous GWG (adjusting for maternal pre-pregnancy BMI) (11). Furthermore, increasing weekly weight gain combined with high pre-pregnancy BMI or weight loss in lean women were associated with greater odds of attention deficit/hyperactivity disorder (ADHD) in 12,556 children aged 7 to 12 years (12). Finally, for women with pre-pregnancy obesity (n=70), those who gained excessive weight during pregnancy were more likely to have children with poorer neonatal neurobehaviour (13).

With the present study we, therefore, aimed to further examine the association between GWG and problem behaviours in school-age children. To increase the generalisability of our results, we pooled together individual data from two European prospective birth cohorts, the Maastricht Essential

Fatty Acid Birth (MEFAB) from the Netherlands and the Rhea Mother-Child Cohort from Greece. We leveraged the repeated gestational weight measurements obtained by both cohorts to investigate the trajectory of GWG. Compared to the standard calculation of total GWG, computed as the difference between pre-pregnancy and delivery weight, this method does not assume a steady increase in weight throughout gestation, which over-simplifies the common GWG trajectory (14–16). The possible effect modification of maternal pre-pregnancy BMI status was assessed, based on previous evidence of the correlation between GWG and pre-pregnancy weight on child psychological traits (1,12,13).

Methods

Study participants

The MEFAB cohort is a prospective birth cohort established in the South of Netherlands. Details of the study population and the data collection have been previously reported (17). Briefly, between 1989 and 1995, 1,203 pregnant women free of any cardiovascular, neurological, renal or metabolic condition were recruited during their first antenatal visit. Of these, 750 were eligible for the 7-year follow-up evaluation.

The Rhea Cohort recruited pregnant women during their first-trimester ultrasound examination in Crete, Greece in 2007–2008. Eligible women were resident in the wider Heraklion region and did not present any communication disability. A total of 1,363 singleton pregnancies were followed-up until delivery, as previously described (18).

To meet inclusion criteria for the present study, participants had to attend a minimum of two prenatal visits during which weight was measured at least once. Additionally, complete information on child behaviour at age 6/7 years had to be available. Consequently, this analysis included a total of 378 mother-child pairs from MEFAB (50.4% of the eligible participants for the follow up) and 414 from Rhea (30.4% of the pregnancies followed-up until delivery).

The MEFAB study was approved by the Medical Ethics Committee, University Hospital, Maastricht/ University of Maastricht, while the Rhea study was approved by the Ethics Committee of the University Hospital in Heraklion. Written informed consent was obtained from all participants included in the study.

Results of the non-response analyses are presented in the Appendix (table A4.1A and A4.1B). Children with follow-up data had mothers who were less likely to be overweight/obese in MEFAB (27.25% *vs.* 34.20%) and more likely to be highly educated (40.58% *vs.* 30.78%) in Rhea. Other differences were smaller and not expected to affect participation rate; no differences were observed in important maternal and child characteristics such as maternal age at delivery and child gestational age.

Maternal weight in pregnancy

In MEFAB, hospital staff measured women's weight in four occasions during pregnancy: at study entry (median; interquartile range (IQR): week 10.14; 8.29, 12.29), during the second (week 21.86; 21.00, 22.86) and third study visits (week 32; 31.43, 32.57), and at delivery (39.43 weeks; 38.29, 40.43). In Rhea, women's weight was measured by trained midwives during clinical visits in the first (week 12; 11, 13) and third (week 32; 30, 35) trimesters, while data on women's weight at delivery was collected by means of phone interviews 8-10 weeks after giving birth (final gestational age: week 38; 38, 39).

Maternal pre-pregnancy BMI

In MEFAB, pre-pregnancy BMI (kg/m^2) was calculated using the measured first-trimester weight as an estimator of weight before conception (19), since no information was recorded regarding pre-pregnancy weight. In Rhea, given the relatively late recruitment (median: week 12), information on self-reported pre-pregnancy weight, collected at study entry, was used to compute pre-pregnancy BMI.

Due to a limited number of women falling in the underweight (BMI <18.5; n=25, 3.05%) and obese (BMI ≥30; n=81, 9.88%) pre-pregnancy BMI categories, pre-pregnancy BMI status was computed as normal (BMI <25 kg/m^2) *vs*. overweight/obese (BMI ≥25 kg/m^2).

Child problem behaviour

The Child Behaviour Checklist (CBCL) 4/18 and its revised version, the CBCL 6/18, were used in MEFAB and Rhea, respectively, to assess children's problem behaviours as perceived by their parents (9,20). The CBCL has demonstrated good psychometric properties and reliability (21). Validation of the two CBCL forms were reported for both the Dutch and Greek populations (22,23). This study assessed the three CBCL broadband scales: total problems, internalising and externalising behaviours. To allow comparability between studies, age-standardized T-scores (with a mean of 50

and a standard deviation of 10) were used. T-scores range from 0 to 100; high values (*i.e.,* above 63) indicate clinical levels of symptomatology.

Statistical analysis

Computation of gestational weight gain trajectories (analyses stratified by cohort)

To increase modelling precision, this step of the analyses included all women with available information on at least one measure of gestational weight and at least two measures of gestational age at which weight (or other data) was collected (n=1,227 in MEFAB and n=1,353 in Rhea; median (IQR) number of measurements per woman: 4 (4, 4) in MEFAB, 3 (2, 3) in Rhea; percent of women with one weight measurement: 0.24% in MEFAB, 6.95% in Rhea). The linearity of the association was explored in each cohort separately; no evidence of deviation from linearity was found. Mixed-effect linear regression models with two levels (*i.e.,* random intercepts for participants and random slopes for measurement occasion) were, then, used to model maternal weights during pregnancy against gestational age. The best linear unbiased predicted slope was obtained for each woman and used as exposure in the subsequent step of the analyses (24). The main exposure of interest was the predicted slope of gestational weight, and it represents the average weekly increment in weight during pregnancy.

Multivariate regressions (pooled analyses)

The associations between GWG and childhood problem behaviours were assessed with multivariate regression analyses with the best-fitting fractional polynomials of GWG, since these associations did not follow a linear pattern. Interaction between maternal pre-pregnancy BMI status and GWG were tested for all outcomes. Furthermore, given the role of sex on prenatal brain development (*e.g.*, (25)), the interaction between GWG and children's sex was evaluated. Finally, we assessed the interaction between GWG and cohort to test for heterogeneity in the associations across cohorts. Subsequent analyses were stratified based on the effect modifier's categories in case of statistically significant interactions ($p<0.05$).

We used a Directed Acyclic Graph (DAG, Appendix figure A4.1 (26)) to identify the covariates to control for, which comprised maternal age at delivery (years), smoking and alcohol consumption in pregnancy (ever/never), parental education (low/medium/high, according to the highest completed education level of either parent) and parity at the index pregnancy (no children/one child/two or more children). Moreover, maternal first-trimester (MEFAB) or pre-pregnancy (Rhea) weight (kg), children's age at assessment (years) and a cohort indicator variable were additionally adjusted for,

while children's sex (male/female) was controlled for in the non-stratified analyses (*i.e.*, those for which a significant interaction with children's sex was not found).

We treated GWG as a continuous variable in all analyses. However, for ease of interpretation, we used the MIMRGNS command (27) in Stata to predict problem behaviour scores at the 5th, 25th, 50th, 75th and 95th percentiles of GWG, while keeping constant at their mean values all other variables included in the model.

To increase the sample size and reduce the bias due to missing values, multiple imputation of missing covariate data was performed using chained equations where 50 completed datasets were generated, separately for the two cohorts (28). An imputation model including all exposures, outcomes, covariates and additional auxiliary variables was constructed. Auxiliary variables comprised maternal height, subject-specific mean weight in pregnancy, birth weight, gestational age, pregnancy outcomes, children's BMI at follow-up, breastfeeding status and day-care attendance.

Several sensitivity analyses were performed to assess the robustness of our results. First, we excluded women who gave birth before week 37 of pregnancy, since a preterm birth might influence both GWG and child development. Second, we included only women with complete information on weight during pregnancy, to rule out the possibility that the group of women with fewer weight measurements differs from the group with complete data. Third, we repeated the analyses in each cohort separately to evaluate potential heterogeneity. Fourth, we additionally controlled for breastfeeding and day-care attendance, since they might independently influence the outcomes. Fifth, we repeated the analyses excluding pre-pregnancy underweight and obese women. Sixth, we additionally controlled for maternal Mediterranean diet score, calculated based on women's early-pregnancy dietary intakes (29). For these analyses, data was restricted to the Rhea cohort, as no information on dietary intake during pregnancy was available for women included in MEFAB. Finally, complete-case data analyses were performed by including only participants without missing covariate data.

All statistical analyses were conducted with either Stata version 14.2 (30) or R version 3.5.1 (31).

Results

Table 4.1 presents maternal and children's characteristics subdivided by cohort and maternal pre-pregnancy BMI status. A total of 255 (32.2%) women had a pre-pregnancy BMI in the overweight or obesity ranges, of whom 103 (40.4%) were included in MEFAB and 152 (59.6%) in Rhea. In both cohorts, a higher percentage of women with a pre-pregnancy BMI in the overweight/obese range had a low level of education compared to normal-weight women. A tendency for children of overweight/obese women of having higher problem behaviours compared to children of normal weight women can be observed.

Mean GWG was 0.40 (SD=0.11) kg/week in MEFAB, and 0.41 (SD=0.05) kg/week in Rhea (p=0.407). The mean intercept of the linear regression between gestational weight and gestational age was 60.99 kg (SD=10.71) in MEFAB and 63.26 kg (SD=12.99) in Rhea (p=0.008). This value can be compared with the reported pre-pregnancy weight in Rhea (mean=65.79 kg; SD=14.21; p<0.0001).

The interaction between GWG and pre-pregnancy BMI was statistically significant on all three outcomes. Furthermore, statistically significant interactions with children's sex were found on total problems and internalising behaviours in the normal pre-pregnancy BMI group. Besides, interactions between GWG and children's sex were not statistically significant on externalising behaviours in the pre-pregnancy normal weight group and on any outcomes in the pre-pregnancy overweight/obese group. No statistically significant effect modifications by cohort were observed. The analyses were, therefore, stratified to account for the two effect modifiers – *i.e.,* pre-pregnancy BMI and children's sex; results are presented in figures 4.1 and 4.2A-C, and in the Appendix (text A4.2-A4.5). Percentiles of GWG were calculated in each group separately; 5^{th} and 95^{th} percentiles of GWG corresponded to about 0.25 kg/week and 0.55 kg/week, respectively, in all groups (exact estimates are reported in the Appendix, text A4.4). Higher scores in all three problem-behaviour scales were observed in children born to women with pre-pregnancy overweight/obesity who gained the most weight during pregnancy. Total problems and internalising behaviour scores were on average 25 points higher, respectively, in children born to overweight/obese women who gained the most weight during their pregnancy, compared to children of women with the lowest weight gain. Average scores at 5^{th} and 95^{th} percentiles of GWG (95% confidence interval) were 40.95 (30.35, 51.55) and 66.13 (53.69, 78.57) for total problems, and 40.49 (30.43, 50.54) and 66.08 (54.28, 77.87) for internalising behaviours. A smaller difference (*i.e.,* 18 points) was found in externalising behaviour scores in these children (45.73 (35.34, 56.12) and 63.77 (51.58, 75.97), for the 5^{th} and

Table 4.1: Population's characteristics

	MEFAB				Rhea			
	n	Normal weight[a]	Overweight/obese[a]	p-value	n	Normal weight[a]	Overweight/obese[a]	p-value
Maternal characteristics								
Age at delivery (years)	378	29.8 (3.9)	28.9 (4.1)	0.047	413	29.6 (4.5)	30.7 (4.9)	0.026
Ancestry (% Caucasian)	377	272 (98.9%)	101 (99 %)	0.926	410	261 (100%)	149 (100%)	1.000
Pre-pregnancy BMI (kg/m^2)[a]	378	21.9 (1.77)	27.3 (25.8, 29.3)	<0.001	414	22 (1.9)	28.4 (26.2, 32.7)	<0.001
Smoking during pregnancy (% ever smokers)	376	63 (23%)	31 (30.4%)	0.141	371	40 (16.7%)	25 (18.9%)	0.593
Alcohol during pregnancy (% ever drinkers)	376	8 (2.9%)	5 (4.9%)	0.350	361	65 (29%)	36 (26.3%)	0.574
Parity	378			0.314	408			0.041
No children		200 (72.7%)	81 (78.6%)			127 (49.2%)	58 (38.7%)	
One child		62 (22.6%)	16 (15.5%)			97 (37.6%)	60 (40%)	
Two or more children		13 (4.7%)	6 (5.8%)			34 (13.2%)	32 (21.3%)	
Level of education	263			0.001	414			0.009
Low		40 (20.9%)	27 (37.5%)			13 (5%)	20 (13.2%)	
Middle		72 (37.7%)	32 (44.4%)			143 (54.6%)	70 (46.1%)	
High		79 (41.4%)	13 (18.1%)			106 (40.5%)	62 (40.8%)	
Weight in pregnancy (kg)								
First trimester	378	61 (6.6)	77.52 (11.2)	<0.001	342	60.77 (6.7)	80.59 (14.1)	<0.001
Second trimester	377	65.2 (6.9)	80.85 (11.1)	<0.001	-	-	-	-
Third trimester	376	69.6 (7.2)	84.71 (11.2)	<0.001	362	70.91 (8.1)	88.68 (13.9)	<0.001
At delivery	375	72.73 (7.7)	87.88 (11.9)	<0.001	377	73.24 (8.7)	87.04 (14.5)	<0.001

Table 4.1 (continued)

	MEFAB				Rhea			
	n	Normal weight[a]	Overweight/obese[a]	p-value	n	Normal weight[a]	Overweight/obese[a]	p-value
Children's characteristics								
Gestational age (weeks)	378	39.8 (1.5)	39.9 (1.8)	0.680	411	38.3 (1.5)	37.9 (1.7)	0.035
Birth weight (g)	377	3304 (510)	3305 (553)	0.978	409	3221 (437)	3200 (481)	0.647
Sex (% male)	378	150 (54.6%)	55 (53.4%)	0.842	414	139 (53.1%)	93 (61.2%)	0.108
Breastfeeding (% ever breastfed)	268	98 (50%)	27 (37.5%)	0.069	401	223 (88.1%)	121 (81.8%)	0.077
Age at survey (years)	268	7.3 (0.3)	7.3 (0.3)	0.902	413	6.6 (0.3)	6.9 (0.3)	0.616
Total problems	378	50.3 (11.3)	52.8 (10.9)	0.054	414	51.3 (9.8)	52.3 (8.4)	0.275
Internalising behaviours	378	51.7 (10.9)	52.5 (9.4)	0.506	414	51.3 (9)	52 (8.7)	0.391
Externalising behaviours	378	50.3 (10.6)	53.3 (10.5)	0.013	414	53.4 (9.7)	54.6 (8.1)	0.198

Note: a: Maternal BMI was computed using first-trimester weight in MEFAB and pre-pregnancy BMI in Rhea; values are expressed as mean (SD), median (IQR) or number (%) as appropriate; p-values are calculated using Student's T-test for continuous variables or chi square test for categorical variables.

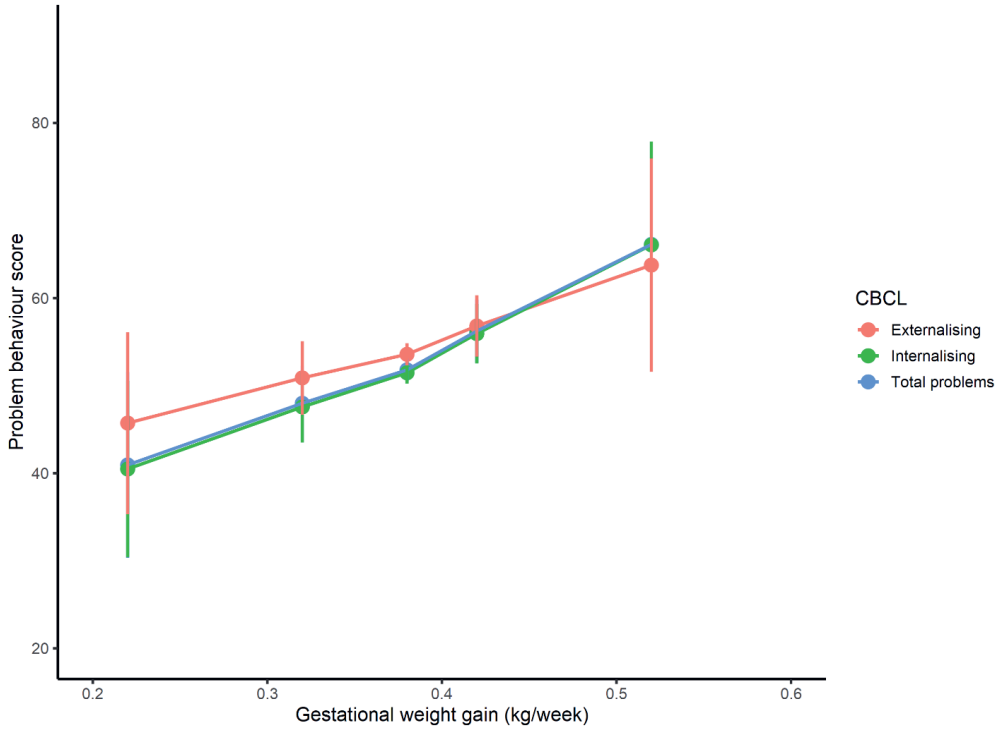

Figure 4.1. Predicted problem behaviour scores by GWG in children of pre-pregnancy overweight or obese women

Note: n= 255; models were adjusted for maternal first-trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's sex and children's age at assessment; 95% confidence intervals are shown.

95th percentiles of GWG, respectively). Of note is that average predicted problem-behaviour scores for children of women with overweight/obesity who gained about 0.5 kg/week fell within the clinical level of symptomatology, as defined by the CBCL (*i.e.,* above 63).

Besides, in children whose mothers had a normal pre-pregnancy BMI, internalising behaviour scores decreased by 23 points for increasing GWG in males (63.90 (47.95, 79.84) and 40.83 (23.26, 58.41)), while increasing slightly (*i.e.,* about 10 points) in females (44.53 (35.39, 53.67) and 55.11 (44.16, 66.05)). No association was evident in children of normal-weight women for what concerns total problems (males: 49.53 (46.50, 52.56) and 51.96 (49.45, 54.47); females: 49.83 (47.05, 52.60) and 49.00 (46.24, 51.76)), and only a small reduction (*i.e.,* about 10 points) was found in externalising behaviour scores for increasing GWG (56.56 (51.66, 61.46) and 45.71 (39.38, 52.03)).

The results of the sensitivity analyses are presented in the Appendix, text A4.6. Overall, with the exception of the association between GWG and internalising behaviours in the analyses restricted

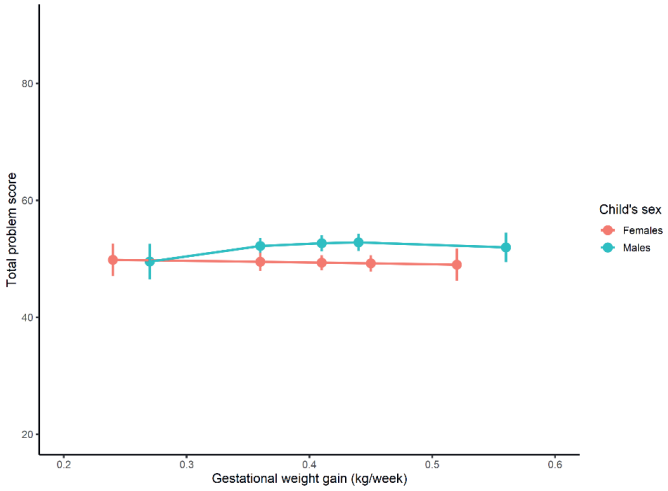

Figure 4.2A. Predicted total problem scores by GWG in children of pre-pregnancy underweight or normal weight women, stratified by children's sex

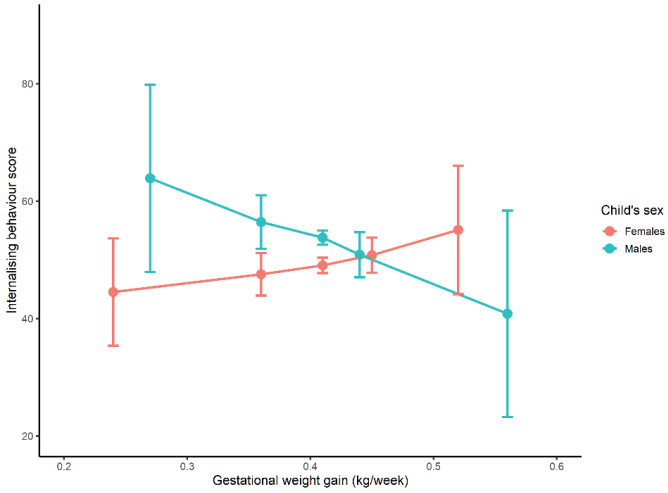

Figure 4.2B. Predicted internalising behaviour scores by GWG in children of pre-pregnancy underweight or

Note (fig 4.2A and B): n= 289 (males) and 248 (females); models were adjusted for maternal first-trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education,

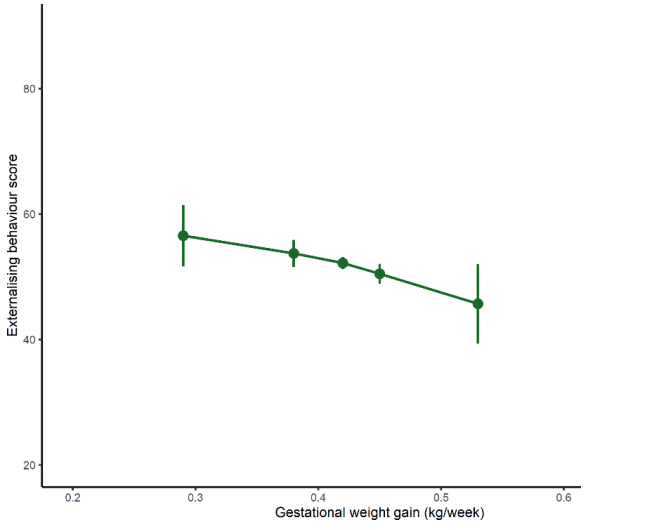

Figure 4.2C. Predicted externalising behaviour scores by GWG in children of pre-pregnancy underweight or normal weight women

Note: n= 537; models were adjusted for maternal first-trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy,

to the Rhea cohort, all regressions in the pre-pregnancy overweight/obese group showed similar estimates and a clear increase in the outcome's predicted score. Besides, the associations between GWG and problem behaviours in the normal-weight group were inconsistent.

Discussion

The aim of the present study was to examine the association between GWG and childhood problem behaviours in school-age years by pooling together individual data from two prospective European birth cohorts, MEFAB and Rhea. Children born to women with pre-pregnancy overweight/obesity exhibited a rise in all problem behaviours for increasing GWG. In contrast, in children of pre-pregnancy normal-BMI women, the associations were inconsistent. We observed a sex-specific trend of change for increasing GWG in internalising behaviours, with scores decreasing in males and slightly increasing in females. A reduction in externalising behaviours for increasing GWG was also evident in males and females combined, while no association was observed with total problems.

Taken as a whole, these results provide evidence for the association between maternal weight in pregnancy and problem behaviours in school-age children. The role of maternal pre-pregnancy BMI on childhood problem behaviours is generally recognised (32–36), although recent analyses on siblings suggested that uncontrolled genetic and familial confounding might be responsible for an inflation of the estimates (35,37,38). In contrast, observational studies using paternal BMI as negative control (39–41) have been published supporting the hypothesised role of maternal weight on child development. Consequently, although the strength of the association is yet to be ascertained, it is still reasonable to assume a phenotypic effect of maternal pre-pregnancy BMI on child problem behaviours. Furthermore, we showed that excessive GWG, associated with pre-pregnancy overweight/obesity, might have important consequences on childhood problem behaviours. In the overweight/obesity group, we observed a 25-point difference (on a 0-100 scale) in the average scores of the total problem and internalising behaviour scales between children of women in the lower-end of the GWG range and children of women in the higher-end of this range. Furthermore, our results showed that offspring of women who excessively gain weight during pregnancy (*i.e.,* about 0.5 kg/week) may attain mean problem behaviour scores in the clinical range of symptomatology (*i.e.*, over 63, as defined by the CBCL). These results are likely to be of clinical relevance, considering that children with behaviour in the clinical range are at increased risk of poor developmental outcomes, with higher odds for each unit increase in CBCL scores (42,43).

Previous studies examined the association between GWG and infants' neurobehaviour (13) and childhood ADHD risk (12), which are strongly related to problem behaviours in mid-childhood (44,45). Poor outcomes are reported in children of obese women who gained excessive weight during pregnancy (12,13), supporting the present study's findings. In contrast, Pugh and colleagues have investigated the relationship between GWG and childhood problem behaviours, reporting no statistically significant association (10). However, their results cannot be directly compared with findings reported in the present study due to a few methodological differences. Firstly, the study population was enriched with low-income, high-risk women enrolled based on first-trimester alcohol or marijuana use, while we analysed a population-based sample, which mostly included highly educated, low-risk families. Secondly, in their study, all gestational weights were self-reported, which might have introduced bias in their exposure and moderator variables. Furthermore, only the pre-pregnancy and the delivery weights were available, precluding the examination of maternal GWG trajectory, which has been shown to be non-linear (*e.g.*, (16)). Although in the present study gestational weight trajectories were better described by linear patterns, comparing the intercept of maternal weight's trajectory with the self-reported pre-pregnancy weight in Rhea, a different pattern of weight gain could be hypothesised for the first weeks of pregnancy, with lower rates of weight gain in this period. The frequency of weight measurements during pregnancy might not have been sufficient to capture the full complexity of the weight gain trajectory. Consequently, the trajectory we described might resemble more the characteristic pattern of the second and third trimesters, overestimating slightly the weight gain in the first trimester.

If replicated, the results of the present study may have public health relevance, given the constantly rising number of overweight and obese women entering pregnancy (1). In line with the recommendations of the American Institute of Medicine guidelines (1), which are also adopted by several European countries (2), – *i.e.,* an average increase of 0.22 kg/week in the second and third trimesters is recommended for women with pre-pregnancy obesity, while in the first trimester a total increase of 0.5-2kg is assumed –, we showed that for overweight/obese women a lower weight gain is associated with the lowest childhood problem behaviours. However, given that 50%-60% of overweight or obese women gain weight in excess during their pregnancy (1), a careful monitoring of overweight and obese women should be planned to prevent an excessive weight gain.

Maternal weight in pregnancy might influence the development of children's behaviour, and especially the likelihood of anxious and depressive traits, via increased glucose levels and

consequent rise in insulin secretion by the foetus or through elevated levels of inflammatory cytokines (3,4). Additionally, obesity might result in leptin resistance, with consequent excessive leptin levels and disproportionate release of cortisol (5). In fact, a previous study found that evening cortisol levels were elevated in pre-pregnancy obese women during the third trimester of pregnancy, with an even greater increase in case of excessive GWG (46). Nonetheless, additional research is needed to clarify the possible pathway(s) underlying the relationship between pre-pregnancy overweight/obesity, GWG and child problem behaviours.

Strengths of this study include the pooling of individual data from two European prospective birth cohorts, MEFAB and Rhea, which has led to greater generalisability of the results. In fact, both MEFAB and Rhea are population-based cohorts that targeted any women living in the designated regions who were free from specific disabilities or disorders. The included study populations were varied in terms of maternal age, education level, parity, smoking and alcohol consumption. Furthermore, pooling together individual data from these two cohorts allowed us to examine two geographically and culturally different populations, which however did not show significant variability regarding the studied associations. Of note is, however, that both cohorts suffered uneven loss to follow up, so that the included sample had a smaller proportion of families with a low socioeconomic status. Additional strengths include a centralised statistical-analysis approach with harmonised exposure, confounder and outcome variables and the assessment of children's behaviour using similar versions of the CBCL. Furthermore, women's weight in pregnancy was directly and repeatedly measured by hospital staff in both cohorts, with the exception of weight at delivery in Rhea, which was self-reported. Consequently, we were able to obtain precise estimates of GWG, comparable between cohorts, by considering the trends of weight gain during pregnancy.

A few limitations should also be considered. Pre-pregnancy BMI was based on first-trimester measured weight in MEFAB and on self-reported pre-pregnancy weight in Rhea. Although not ideal, these methods represent common practice in epidemiological studies and clinical settings, generally being considered reliable and comparable (19,47). In Rhea, delivery weight was self-reported 8-10 weeks postpartum. A systematic review has shown that the recall of delivery weight due to self-report is reproducible and valid (47), while underreporting of delivery weight, which tends to be more frequent than over-reporting, would most likely bias estimates toward the null (48). Given the linearity of GWG trajectories, we were not able to examine the three trimesters of pregnancy individually. The three trimesters are characterised by different metabolic processes – *i.e.*, a greater proportional accretion of fat mass characterises the first trimester, while the following two are

characterised by foetal growth and a greater increase in fat-free mass (49). Therefore, it could be hypothesised that trimester-specific GWG would be differentially associated with child development. Nonetheless, no evidence of significant differences in the association of trimester-specific GWG with childhood neurocognitive or behavioural traits is available (50,51). Furthermore, only a few participating women were classified as underweight or obese, precluding us from testing our hypotheses in these subgroups. Virtually all women included in this study were Caucasian, therefore these findings cannot be directly extended to other ethnic groups. Although the development of problem behaviours is influenced by several risk factors in the postnatal period, including parenting practices and infant's temperament (52), we did not assess any child- or family-specific factors exclusively related to infancy or childhood. These factors cannot be considered potential confounders of the association between GWG and problem behaviours, as they necessarily occur after the exposure (53), but might modify the association between GWG and childhood problem behaviours (*e.g.*, (54–56)). In addition, despite their possible association with weight status before pregnancy or GWG (57–60) and children's development and behaviour (61–63), we could not adequately control for maternal psychopathology, diet quality and physical activity before or during pregnancy. Finally, we were not able to adjust for common genetic influences between maternal pre-pregnancy BMI, GWG and childhood problem behaviours (64,65), which might have affected our results.

Conclusion

Increasing GWG, in combination with pre-pregnancy overweight/obesity, was associated with higher problem behaviours in school-age children. Less clear was the association between GWG and problem behaviours in children of women with a normal pre-pregnancy BMI. Future studies should examine further the relationship between GWG and childhood problem behaviours with a genetically informed study design, and adjusting for maternal psychopathology, diet quality and physical activity levels before or during pregnancy.

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Appendix to chapter 4

Text A4.1: Non-response analyses

Table A4.1A: Participants characteristics in MEFAB

		Included		Excluded	
Maternal characteristics	**n**	**n (%) or mean (SD) or median (IQR)**	**n**	**n (%) or mean (SD) or median (IQR)**	**p-value**
Age at delivery (years)	378	29.6 (4)	901	29.2 (4.4)	0.148
Ancestry (% Caucasian)	377	373 (98.9%)	884	860 (97.3%)	0.068
Pre-pregnancy BMI (kg/m^2)	378	22.9 (21.5, 25.2)	810	23.5 (21.2, 26.3)	0.030
Pre-pregnancy BMI (% Overweight/obese)	378	103 (27.3%)	810	277 (34.2%)	0.017
Smoking during pregnancy (% ever smokers)	376	94 (25%)	884	234 (26.5%)	0.586
Alcohol during pregnancy (% ever drinkers)	376	13 (3.5%)	884	22 (2.5%)	0.338
Parity	378		894		0.985
No children		281 (74.3%)		664 (74.3%)	
One child		78 (20.6%)		183 (20.5%)	
Two or more children		19 (5%)		47 (5.3%)	
Level of education	263		33		0.012
Low		67 (25.5%)		4 (12.1%)	
Middle		104 (39.5%)		22 (66.7%)	
High		92 (35%)		7 (21.2%)	
Weight in pregnancy (kg)					
First trimester	378	65.5 (10.9)	868	66.9 (12.6)	0.077
Second trimester	377	69.5 (10.8)	872	71 (12.6)	0.043
Third trimester	376	73.7 (10.8)	863	75.5 (12.8)	0.017
At delivery	375	76.9 (11.3)	873	78.6 (13.1)	0.033
Children's characteristics					
Gestational age (weeks)	378	39.9 (1.6)	828	39.8 (2.6)	0.807
Birth weight (g)	377	3304 (521)	892	3205 (583)	0.005
Sex (% male)	378	205 (54.2%)	896	492 (54.9%)	0.824
Breastfeeding (% ever breastfed)	268	125 (46.6%)	20	9 (45%)	0.887

Table A4.1B: Participants characteristics in Rhea

		Included		Excluded	
Maternal characteristics	**n**	**n (%) or mean (SD) or median (IQR)**	**n**	**n (%) or mean (SD) or median (IQR)**	**p-value**
Age at delivery (years)	413	30 (4.7)	1057	29.1 (5.2)	0.002
Ancestry (% Caucasian)	410	410 (100%)	1069	1060 (99.2%)	0.062
Pre-pregnancy BMI (kg/m^2)	414	23.6 (21.7, 26.7)	959	23.2 (20.8, 26.1)	0.006
Pre-pregnancy BMI (% Overweight/obese)	414	152 (36.7%)	959	312 (32.5%)	0.133
Smoking during pregnancy (% ever smokers)	371	65 (17.5%)	869	198 (22.8%)	0.038
Alcohol during pregnancy (% ever drinkers)	361	101 (28%)	759	216 (28.5%)	0.867
Parity	408		990		0.123
No children		185 (45.3%)		412 (41.6%)	
One child		157 (38.5%)		372 (37.6%)	
Two or more children		66 (16.2%)		206 (20.8%)	
Level of education	414		991		<0.001
Low		33 (8%)		181 (18.3%)	
Middle		213 (51.5%)		505 (51%)	
High		168 (40.6%)		305 (30.8%)	
Weight in pregnancy (kg)					
First trimester	342	67.5 (13.6)	793	67.1 (13.9)	0.672
Second trimester	-	-	-	-	-
Third trimester	362	77.6 (13.7)	817	75.8 (12.7)	0.031
At delivery	377	78.3 (13)	929	76.6 (12.8)	0.035
Children's characteristics					
Gestational age (weeks)	411	38.2 (1.6)	1063	38.12 (1.8)	0.672
Birth weight (g)	409	3213 (453)	1015	3121 (489)	0.001
Sex (% male)	414	232 (56%)	1108	535 (48.3%)	0.007
Breastfeeding (% ever breastfed)	401	344 (85.8%)	891	746 (83.7%)	0.346

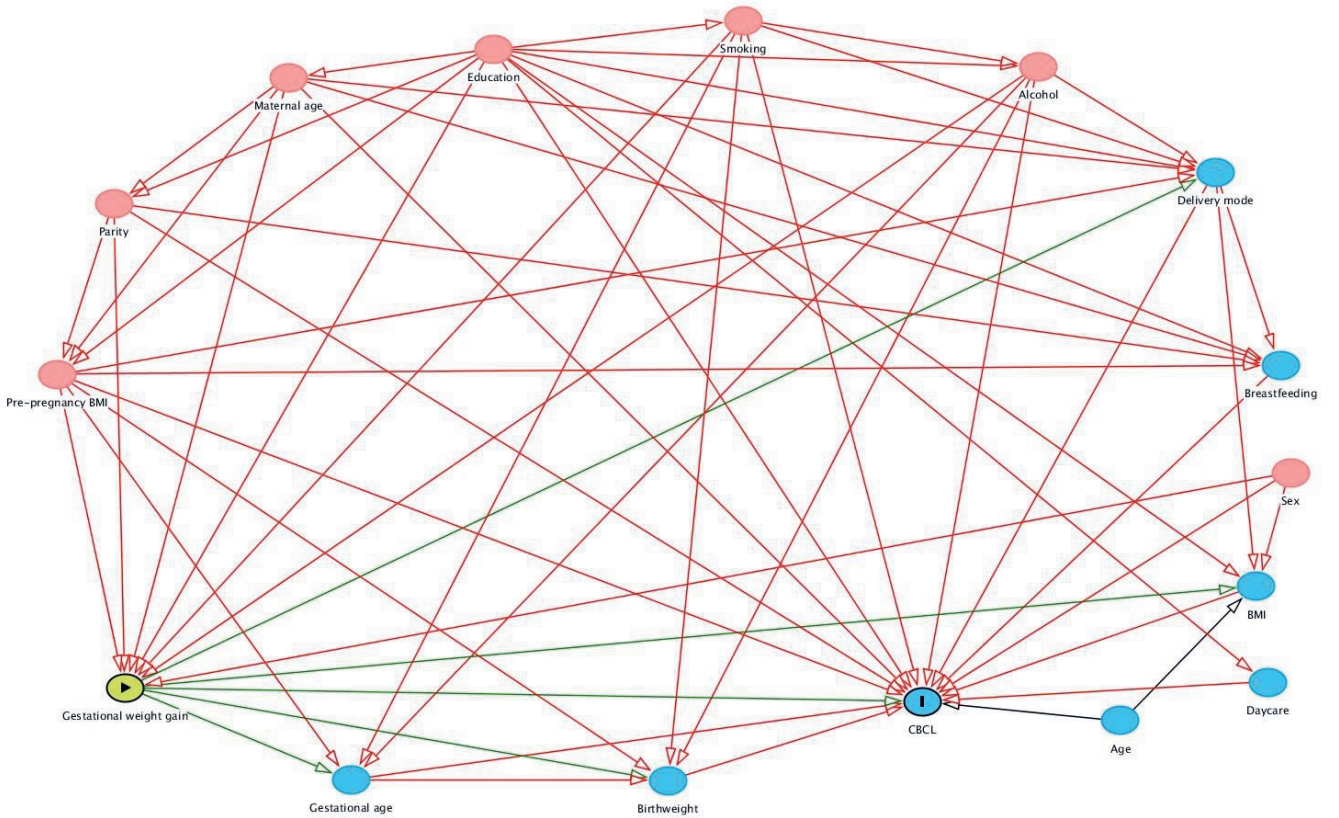

Figure A4.1: Directed acyclic graph (DAG)

Note: Green arrows represent causal paths from the exposure; red arrows represent biasing paths to the exposure and/or the outcome. Red ovals represent the minimal sufficient adjustment set for estimating the total effect of GWG on childhood problem behaviours. Maternal variables: Gestational weight gain (i.e., GWG), pre-pregnancy BMI, parity, maternal age, smoking, alcohol, education (maternal and paternal). Children's variables: CBCL (i.e., problem behaviours), sex, age, gestational age, birthweight, delivery mode, breastfeeding, BMI, day-care.

Text A4.2: Associations between gestational weight gain and problem behaviours in children of normal-weight women (pooled analysis)

The best-fitting fractional polynomial models for the association between gestational weight gain (GWG) and problem behaviours had the following form:

Male children:

Total problems = $\beta_0 + \beta_1 * GWG + \beta_2 * GWG^2 + \varepsilon$

Internalising = $\beta_0 + \beta_1 * \ln(GWG) + \beta_2 * \ln(GWG) * \ln(GWG) + \varepsilon$

Female children:

Total problems = $\beta_0 + \beta_1 * GWG^{-2} + \beta_2 * GWG^2 + \varepsilon$

Internalising = $\beta_0 + \beta_1 * GWG^3 + \beta_2 * GWG^3 * \ln(GWG) + \varepsilon$

Externalising = $\beta_0 + \beta_1 * GWG^2 + \beta_2 * GWG^2 * \ln(GWG) + \varepsilon$

were β_0 denotes the intercept, β_1 and β_2 denote the slopes and ε denotes the error term. Slope estimates are presented in table A4.2 below.

Table A4.2: Estimates for the associations between gestational weight gain and problem behaviour scales in children of normal weight women

	Total problems		Internalising behaviours		Externalising behaviours
	β (95% C.I.)		β (95% C.I.)		β (95% C.I.)
	Males	Females	Males	Females	
β_1	107.42 (2.74, 212.10)*	0.02 (-0.07, 0.10)	-13.33 (-42.89, 12.23)	27.07 (-41.56, 95.70)	-27.97 (-54.89, -1.04)*
β_2	-117.43 (-236.38, 1.51)§	-4.72 (-26.03, 16.59)	-11.48 (-25.42, 2.46)	99.15 (-61.43, 259.74)	-67.51 (-145.83, 10.80)

Note: n= 289 (males), 248 (females) or 537 (combined); all models were adjusted for maternal pre-pregnancy weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's age at assessment and cohort; for the externalising scale, the interaction of child's sex with GWG was not statistically significant, therefore estimates are presented for males and females combined and the model is additionally adjusted for child's sex; §: p≤ 0.06; *: p≤ 0.05.

Text A4.3: Associations between gestational weight gain and problem behaviour scales in children of overweight/obese women (pooled analysis)

The best-fitting fractional polynomial models for the association between gestational weight gain (GWG) and problem behaviours had the following form:

Total problems = $\beta_0 + \beta_1 * GWG^3 + \beta_2 * GWG^3 * \ln(GWG) + \varepsilon$

Internalising = $\beta_0 + \beta_1 * GWG^3 + \beta_2 * GWG^3 * \ln(GWG) + \varepsilon$

Externalising = $\beta_0 + \beta_1 * GWG^3 + \beta_2 * GWG^3 * \ln(GWG) + \varepsilon$

were β_0 denotes the intercept, β_1 and β_2 denote the slopes and ε denotes the error term. Slope estimates are presented in the table A4.3 below.

Table A4.3: Estimates for the associations between gestational weight gain and problem behaviour scales in children of overweight/obese women

	Total problems	**Internalising behaviours**	**Externalising behaviours**
	β (95% C.I.)	**β (95% C.I.)**	**β (95% C.I.)**
β1	90.60 (6.98, 174.21)*	95.18 (15.91, 174.45)*	60.15 (-21.80, 142.11)
β2	180.16 (14.08, 346.25)*	177.69 (20.09, 335.30)*	137.09 (-25.77, 299.95)

Note: n=255; all models were adjusted for maternal pre-pregnancy weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, and children's sex, age at assessment and cohort; *: p≤ 0.05.

Text A4.4: Problem behaviour score predictions by percentiles of weekly gestational weight gain

Table A4.4A: Total problem and internalising behaviour scores' predictions by percentiles of GWG and maternal pre-pregnancy BMI category

Weekly gestational weight gain			Total problems	Internalising behaviours
			Normal BMI	
Percentiles	**Kg/week**	**Children's sex**	**Prediction (95% C.I.)**	
5th	0.27	males	49.53 (46.50, 52.56)	63.90 (47.95, 79.84)
	0.24	females	49.83 (47.05, 52.60)	44.53 (35.39, 53.67)
25th	0.36	males	52.20 (50.81, 53.58)	56.45 (51.89, 61.01)
	0.36	females	49.50 (47.96, 51.05)	47.55 (43.93, 51.18)
50th	0.41	males	52.67 (51.29, 54.05)	53.79 (52.59, 55.00)
	0.41	females	49.35 (48.07, 50.63)	49.07 (47.75, 50.38)
75th	0.44	males	52.83 (51.37, 54.30)	50.89 (47.05, 54.73)
	0.45	females	49.23 (47.84, 50.63)	50.81 (47.83, 53.80)
95th	0.56	males	51.96 (49.45, 54.47)	40.83 (23.26, 58.41)
	0.52	females	49.00 (46.24, 51.76)	55.11 (44.16, 66.05)
			Overweight/Obese	
Percentiles	**Kg/week**		**Prediction (95% C.I.)**	
5th	0.22		40.95 (30.35, 51.55)	40.49 (30.43, 50.54)
25th	0.32		47.99 (43.71, 52.26)	47.57 (43.51, 51.63)
50th	0.38		51.80 (50.51, 53.10)	51.47 (50.24, 52.70)
75th	0.42		56.24 (52.67, 59.81)	55.94 (52.55, 59.33)
95th	0.52		66.13 (53.69, 78.57)	66.08 (54.28, 77.87)

Note: all models were adjusted for maternal first trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's age at assessment and cohort. Children's sex was additionally controlled for in non-stratified models.

Table A4.4B: Externalising behaviour scores' predictions by percentiles of GWG and maternal pre-pregnancy BMI category

Weekly gestational weight gain		**Externalising behaviours**
		Normal BMI
Percentiles	**Kg/week**	**Prediction (95% C.I.)**
5th	0.29	56.56 (51.66, 61.46)
25th	0.38	53.74 (51.58, 55.89)
50th	0.42	52.20 (51.25, 53.15)
75th	0.45	50.49 (48.91, 52.08)
95th	0.53	45.71 (39.38, 52.03)
		Overweight/Obese
Percentiles	**Kg/week**	**Prediction (95% C.I.)**
5th	0.22	45.73 (35.34, 56.12)
25th	0.32	50.89 (46.70, 55.08)
50th	0.38	53.58 (52.31, 54.85)
75th	0.42	56.82 (53.32, 60.32)
95th	0.52	63.77 (51.58, 75.97)

Note: all models were adjusted for maternal first trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's sex, children's age at assessment and cohort.

Text A4.5: Full-model estimates

Table A4.5.1: Full-model estimates for the association of GWG and total problems in the normal BMI group

	Males		Females	
Covariates	**β (95% C.I.)**		**β (95% C.I.)**	
GWG (β1)[a]	107.42	(2.74, 212.10)	0.02	(-0.07, 0.10)
GWG (β2)[a]	-117.43	(-236.38, 1.51)	-4.72	(-26.03, 16.59)
Pre-pregnancy weight	0.17	(-0.03, 0.37)	-0.17	(-0.37, 0.03)
Mother's age	-0.17	(-0.48, 0.14)	-0.32	(-0.71, 0.07)
Smoking (yes)	4.59	(1.25, 7.92)	1.18	(-2.16, 4.51)
Alcohol (yes)	0.98	(-2.94, 4.90)	-2.09	(-6.04, 1.87)
Education level (medium)	0.30	(-4.45, 5.05)	-2.06	(-7.24, 3.12)
Education level (high)	0.03	(-5.12, 5.18)	-4.17	(-9.50, 1.15)
Parity (one child)	-2.00	(-4.88, 0.88)	-0.41	(-3.60, 2.78)
Parity (two+ children)	-1.34	(-5.63, 2.95)	-1.49	(-6.88, 3.90)
Child's age	1.12	(-3.86, 6.10)	1.09	(-4.15, 6.32)
Cohort (Rhea)	3.31	(-1.30, 7.91)	0.88	(-4.19, 5.94)
Constant	13.69	(-31.82, 59.19)	64.68	(21.90, 107.47)

Note: a: Slopes of the power transformations of GWG. A detailed explanation of β1 and β2 is provided in the Appendix, Texts A4.2 and A4.3.

Table A4.5.2: Full-model estimates for the association of GWG and internalising behaviours in the normal BMI group

	Males		Females	
Covariates	**β (95% C.I.)**		**β (95% C.I.)**	
GWG (β1)[a]	-15.33	(-42.89, 12.23)	27.07	(-41.56, 95.70)
GWG (β2)[a]	-11.48	(-25.42, 2.46)	99.15	(-61.43, 259.74)
Pre-pregnancy weight	0.14	(-0.04, 0.33)	-0.05	(-0.25, 0.14)
Mother's age	-0.14	(-0.43, 0.15)	-0.05	(-0.42, 0.32)
Smoking (yes)	2.59	(-0.50, 5.67)	0.49	(-2.67, 3.66)
Alcohol (yes)	0.91	(-2.69, 4.51)	-1.86	(-5.60, 1.88)
Education level (medium)	0.00	(-4.26, 4.25)	-1.69	(-6.72, 3.34)
Education level (high)	0.08	(-4.48, 4.63)	-2.98	(-8.09, 2.13)
Parity (one child)	-3.40	(-6.08, -0.72)	-0.61	(-3.64, 2.41)
Parity (two+ children)	-3.16	(-7.16, 0.83)	-4.52	(-9.67, 0.63)
Child's age	1.93	(-2.74, 6.60)	0.29	(-4.72, 5.29)
Cohort (Rhea)	1.77	(-2.47, 6.01)	1.04	(-3.86, 5.95)
Constant	31.31	(-7.08, 69.70)	58.36	(16.89, 99.84)

Note: a: Slopes of the power transformations of GWG. A detailed explanation of β1 and β2 is provided in the Appendix, Texts A4.2 and A4.3.

Table A4.5.3: Full-model estimates for the association of GWG and externalising behaviours in the normal BMI group

Covariates	β (95% C.I.)
GWG (β1)[a]	-27.97	(-54.89, -1.04)
GWG (β2)[a]	-67.51	(-145.83, 10.80)
Pre-pregnancy weight	0.01	(-0.12, 0.15)
Mother's age	-0.30	(-0.53, -0.06)
Smoking (yes)	2.78	(0.50, 5.05)
Alcohol (yes)	0.01	(-2.63, 2.65)
Education level (medium)	0.81	(-2.56, 4.18)
Education level (high)	-0.60	(-4.24, 3.04)
Parity (one child)	-0.03	(-2.07, 2.01)
Parity (two+ children)	-0.02	(-3.24, 3.21)
Child's age	1.10	(-2.55, 4.74)
Cohort (Rhea)	-2.01	(-3.73, -0.28)
Constant	3.45	(0.06, 6.85)

Note: a: Slopes of the power transformations of GWG. A detailed explanation of β1 and β2 is provided in the Appendix, Texts A4.2 and A4.3.

Table A4.5.4: Full-model estimates for the associations of GWG and problem behaviours in the overweight/obesity group

	Total problems		Internalising		Externalising	
Covariates	**β (95% C.I.)**		**β (95% C.I.)**		**β (95% C.I.)**	
GWG (β1)[a]	90.60	(6.98, 174.21)	95.18	(15.91, 174.45)	60.15	(-21.80, 142.11)
GWG (β2)[a]	180.16	(14.08, 346.25)	177.69	(20.09, 335.30)	137.09	(-25.77, 299.95)
Pre-pregnancy weight	0.05	(-0.04, 0.14)	0.04	(-0.05, 0.13)	0.03	(-0.06, 0.12)
Mother's age	-0.32	(-0.58, -0.05)	-0.21	(-0.47, 0.04)	-0.35	(-0.61, -0.09)
Smoking (yes)	0.55	(-2.19, 3.30)	-0.74	(-3.39, 1.91)	0.60	(-2.12, 3.31)
Alcohol (yes)	2.54	(-0.62, 5.70)	2.68	(-0.32, 5.68)	1.72	(-1.39, 4.82)
Education level (medium)	-0.93	(-4.29, 2.43)	-0.89	(-4.04, 2.25)	-0.53	(-3.84, 2.78)
Education level (high)	-3.77	(-7.57, 0.04)	-2.87	(-6.43, 0.69)	-3.20	(-6.93, 0.54)
Parity (one child)	0.47	(-2.32, 3.25)	-0.99	(-3.63, 1.66)	2.03	(-0.71, 4.76)
Parity (two+ children)	-3.17	(-6.74, 0.40)	-4.50	(-7.89, -1.11)	-1.13	(-4.63, 2.38)
Child's age	-0.01	(-4.47, 4.44)	1.43	(-2.70, 5.56)	-0.47	(-4.91, 3.96)
Cohort (Rhea)	-2.20	(-4.50, 0.09)	-2.30	(-4.48, -0.12)	-1.38	(-3.63, 0.87)
Constant	1.46	(-2.87, 5.79)	2.65	(-1.40, 6.70)	2.03	(-2.22, 6.29)

Note: a: Slopes of the power transformations of GWG. A detailed explanation of β1 and β2 is provided in the Appendix, Texts A4.2 and A4.3.

Text A4.6: Sensitivity analyses

Table A4.6.1: Results of sensitivity analyses (outcome: total problems)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
1. Gestational age >36 complete weeks	Normal BMI, males	80.55 (-41.11, 202.21)	-94.45 (-230.04, 41.14)	51.15 (1.72)	51.09 (1.52)
	Normal BMI, females	0.02 (-0.07, 0.10)	-2.70 (-24.37, 18.96)	49.79 (1.44)	49.41 (1.44)
	Overweight	112.87 (25.00, 200.74)*	210.53 (35.43, 385.62)*	38.61 (5.66)	69.00 (6.66)
2. Complete gestational weight data	Normal BMI, males	102.26 (-3.54, 208.06)§	-108.00 (-228.25, 12.25)	49.01 (1.60)	51.91 (1.54)
	Normal BMI, females	0.01 (-0.07, 0.09)	-5.95 (-28.07, 16.16)	50.19 (1.50)	49.03 (1.48)
	Overweight	95.60 (9.01, 182.20)*	187.66 (13.77, 361.56)*	39.78 (5.89)	67.51 (6.89)
3. Analysis by cohort	MEFAB, Normal BMI, males	132.30 (19.27, 245.32)*	-141.05 (-269.23, -12.87)*	47.12 (1.87)	50.80 (1.94)
	MEFAB, Normal BMI, females	0.01 (-0.08, 0.11)	-5.27 (-31.68, 21.13)	50.42 (1.90)	49.26 (2.19)
	MEFAB, Overweight/Obese	98.07 (-9.20, 205.34)	196.18 (-24.83, 417.20)	36.06 (9.32)	74.04 (11.71)
	Rhea, Normal BMI, males	-86.64 (-525.09, 351.81)	68.84 (-428.11, 565.78)	55.47 (1.99)	51.60 (1.97)
	Rhea, Normal BMI, females	-0.84 (-7.47, 5.80)	-14.12 (-231.92, 203.68)	50.69 (10.17)	46.40 (12.30)
	Rhea, Overweight/Obese	39.51 (-375.21, 454.22)	114.07 (-523.72, 751.85)	48.64 (13.60)	56.49 (15.17)
4. Additional control for breastfeeding and day-care attendance	Normal BMI, males	103.62 (-0.14, 207.37)*	-114.86 (-232.76, 3.04)§	49.77 (1.53)	51.78 (1.27)
	Normal BMI, females	0.02 (-0.07, 0.10)	-6.51 (-28.15, 15.14)	50.02 (1.42)	48.82 (1.42)
	Overweight/obese	90.81 (7.00, 174.62)*	183.11 (16.50, 349.72)*	40.82 (5.39)	66.26 (6.33)

Table A4.6.1 (continued)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
5. Analysis excluding underweight and obese women	Normal BMI, males	99.96 (-9.87, 209.79)	-107.62 (-234.00, 18.75)	49.69 (1.50)	52.33 (1.26)
	Normal BMI, females	0.01 (-0.07, 0.10)	-3.77 (-25.57, 18.03)	49.68 (1.46)	49.02 (1.44)
	Overweight	112.30 (8.13, 216.48)*	280.10 (75.50, 484.70)**	35.93 (6.39)	70.24 (7.56)
6. Additional control for Mediterranean diet score (Rhea only)	Normal BMI, males	-138.83 (-584.74, 307.07)	126.83 (-378.27, 631.94)	55.80 (2.01)	51.63 (1.96)
	Normal BMI, females	-0.75 (-7.39, 5.90)	-11.55 (-229.73, 206.64)	50.43 (10.18)	46.71 (12.32)
	Overweight/obese	51.05 (-372.11, 474.21)	136.07 (-517.90, 790.04)	47.80 (13.91)	57.43 (15.52)
7. Complete-case analysis	Normal BMI, males	93.37 (-28.29, 215.03)	-93.75 (-233.63, 46.14)	49.14 (1.76)	53.03 (1.51)
	Normal BMI, females	0.19 (-0.16, 0.55)	14.23 (-14.38, 42.83)	46.49 (2.16)	51.75 (2.78)
	Overweight/obese	128.38 (8.44, 248.33)*	202.78 (-15.62, 421.19)	39.14 (6.74)	68.17 (7.93)

Note: a: values refer to predicted scores for the 5th and 95th percentiles of GWG; all models were adjusted for maternal first trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's age at assessment and cohort. Children's sex was additionally controlled for in non-stratified models; §: p<0.06; *: p<0.05; **: p<0.01.

Table A4.6.2: Results of sensitivity analyses (outcome: internalising behaviour)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
1. Gestational age >36 complete weeks	Normal BMI, males	-15.23 (-59.26, 28.79)	-10.67 (-34.95, 13.62)	63.94 (13.59)	41.44 (15.46)
	Normal BMI, females	26.84 (-42.97, 96.65)	95.81 (-67.81, 259.43)	44.73 (4.75)	55.07 (5.67)
	Overweight	117.27 (32.82, 201.72)**	219.76 (51.27, 388.25)*	37.75 (5.45)	69.41 (6.41)
2. Complete gestational weight data	Normal BMI, males	-8.54 (-36.84, 19.75)	-8.68 (-22.90, 5.55)	60.82 (8.72)	44.40 (9.74)
	Normal BMI, females	90.05 (9.66, 170.55)*	158.57 (-2.97, 320.11)§	40.85 (5.47)	65.54 (6.40)
	Overweight	90.05 (9.66, 170.44)*	158.57 (-2.97, 320.11)	40.85 (5.47)	65.54 (6.40)
3. Analysis by cohort	MEFAB, Normal BMI, males	-9.88 (-40.54, 20.77)	-9.84 (-25.03, 5.36)	62.71 (10.30)	42.03 (11.51)
	MEFAB, Normal BMI, females	25.44 (-55.11, 106.00)	87.16 (-108.14, 282.45)	44.62 (6.55)	55.63 (7.55)
	MEFAB, Overweight/Obese	89.84 (-0.47, 180.15)§	153.32 (-32.69, 339.33)	38.47 (7.85)	70.73 (9.86)
	Rhea, Normal BMI, males	-61.53 (-206.30, 83.24)	-28.33 (-112.14, 55.48)	71.12 (23.36)	34.62 (24.89)
	Rhea, Normal BMI, females	-243.00 (-837.75, 351.75)	-438.74 (-1616.58, 739.10)	63.44 (18.86)	32.85 (20.63)
	Rhea, Overweight/Obese	-126.88 (-565.07, 311.32)	-99.71 (-773.64, 574.22)	58.31 (14.37)	45.15 (16.03)
4. Additional control for breastfeeding and day-care attendance	Normal BMI, males	-14.97 (-42.35, 12.42)	-10.96 (-24.80, 2.88)	63.53 (8.04)	41.30 (8.86)
	Normal BMI, females	28.54 (-40.27, 97.35)	109.62 (-52.38, 271.62)	44.11 (4.66)	55.62 (5.59)
	Overweight/obese	95.77 (16.43, 175.10)*	182.70 (24.90, 340.50)*	40.27 (5.11)	66.31 (5.99)

Table A4.6.2 (continued)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
5. Analysis excluding underweight and obese women	Normal BMI, males	-10.34 (-39.63, 18.95)	-9.32 (-23.96, 5.30)	60.99 (8.22)	44.35 (8.97)
	Normal BMI, females	22.04 (-47.72, 91.80)	84.73 (-79.49, 248.94)	45.24 (4.75)	54.19 (5.71)
	Overweight	115.79 (14.56, 217.02)*	267.80 (68.66, 466.94)**	35.89 (6.21)	69.73 (7.35)
6. Additional control for Mediterranean diet score (Rhea only)	Normal BMI, males	-44.29 (-190.98, 102.41)	-17.74 (-102.78, 67.30)	65.44 (23.68)	40.67 (25.24)
	Normal BMI, females	-231.86 (-828.11, 364.38)	-415.82 (-1596.56, 764.93)	62.72 (18.90)	33.63 (20.68)
	Overweight/obese	-111.37 (-559.15, 336.42)	-71.02 (-763.20, 621.15)	57.20 (14.72)	46.39 (16.42)
7. Complete-case analysis	Normal BMI, males	-9.69 (-40.33, 20.95)	-9.34 (-24.45, 5.76)	61.19 (8.90)	44.21 (9.73)
	Normal BMI, females	42.53 (-30.90, 115.96)	108.12 (-67.49, 283.73)	42.81 (5.03)	56.10 (6.02)
	Overweight/obese	132.19 (18.13, 246.24)*	207.29 (-0.39, 414.98)*	38.13 (6.41)	67.92 (7.54)

Note: a: values refer to predicted scores for the 5th and 95th percentiles of GWG; all models were adjusted for maternal first trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's age at assessment and cohort. Children's sex was additionally controlled for in non-stratified models; §: p<0.06; *: p<0.05; **: p<0.01.

Table A4.6.3: Results of sensitivity analyses (outcome: externalising behaviour)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
1. Gestational age >36 complete weeks	Normal BMI	-27.22 (-54.44, -0.00)*	-56.46 (-137.11, 24.19)	56.32 (2.53)	46.57 (3.24)
	Overweight/obese	71.43 (-16.02, 158.89)	150.46 (-23.87, 324.79)	44.74 (5.64)	65.28 (6.63)
2. Complete gestational weight data	Normal BMI	-25.74 (-53.07, 1.58)	-65.79 (-145.08, 13.50)	56.28 (2.63)	45.56 (3.41)
	Overweight/obese	69.78 (-17.71, 157.27)	161.36 (-14.47, 337.18)	43.78 (5.95)	65.94 (6.97)
3. Analysis by cohort	MEFAB, Normal BMI	-27.71§ (-56.36, 0.93)	-74.24 (-157.33, 8.85)	56.22 (3.18)	41.96 (4.41)
	MEFAB, Overweight/obese	70.42 (-36.12, 176.95)	168.46 (-51.10, 388.01)	39.87 (9.26)	69.78 (11.63)
	Rhea, Normal BMI	-58.76 (-191.73, 74.21)	-116.06 (-514.56, 282.43)	58.48 (6.97)	47.69 (7.97)
	Rhea, Overweight/obese	52.74 (-350.92, 456.41)	92.82 (-528.02, 713.66)	50.93 (13.24)	58.72 (14.15)
4. Additional control for breastfeeding and day-care attendance	Normal BMI	-28.37 (-55.25, -1.50)*	-63.07 (-141.36, 15.22)	56.47 (2.49)	45.87 (3.21)
	Overweight/obese	60.59 (-21.55, 142.73)	140.66 (-22.71, 304.03)	45.57 (5.29)	63.94 (6.21)
	Overweight/obese	97.21 (-19.05, 213.47)	159.89 (-51.81, 371.58)	44.05 (6.53)	66.48 (7.68)

Table A4.6.3 (continued)

Sensitivity analyses	Stratification group	GWG estimates		Predicted scores (SE) range[a]	
5. Analysis excluding underweight and obese women	Normal BMI	-23.96 (-52.08, 4.35)	-60.65 (-141.26, 19.97)	55.93 (2.56)	46.67 (3.24)
	Overweight	64.09 (-37.92, 166.11)	186.91 (-13.60, 387.41)	43.42 (6.26)	64.97 (7.41)
6. Additional control for Mediterranean diet score (Rhea only)	Normal BMI	-51.58 (-185.43, 82.28)	-92.63 (-494.36, 309.10)	57.70 (7.02)	48.59 (8.03)
	Overweight/obese	87.77 (-322.20, 497.74)	154.79 (-479.06, 788.63)	48.48 (13.48)	61.46 (15.04)
7. Complete-case analysis	Normal BMI	-12.58 (-43.94, 18.77)	-41.05 (-132.14, 50.03)	53.95 (2.92)	48.25 (3.73)
	Overweight/obese	97.21 (-19.05, 213.47)	159.89 (-51.81, 371.58)	44.05 (6.53)	66.48 (7.68)

Note: a: values refer to predicted scores for the 5th and 95th percentiles of GWG; all models were adjusted for maternal first trimester (MEFAB) or pre-pregnancy (Rhea) weight, maternal age at delivery, smoking and alcohol consumption during pregnancy, parent's level of education, parity, children's sex, children's age at assessment and cohort; §: p<0.06; *: p<0.05; **: p<0.01

Chapter 5

The association of maternal polyunsaturated fatty acids during pregnancy with social competence and problem behaviours at 7 years of age: the MEFAB cohort

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Abstract

Background: The prenatal exposure to maternal n-6 and n-3 polyunsaturated fatty acids (PUFAs) might influence the development of childhood social competence and problem behaviours, because of the numerous functions PUFAs play within the nervous system.

Methods: To analyse the association of selected maternal PUFAs (*i.e.*, AA, EPA, DHA, total n-6, total n-3, and the n-6:n-3 ratio) measured during gestation with childhood social competence and problem behaviours, we examined 311 mother-child pairs from the Maastricht Essential Fatty Acid Birth cohort (MEFAB). For each woman, PUFA-specific changes in relative concentrations were calculated by identifying the best-fitting curve of PUFA concentration by linear splines of gestational age. The associations of changes in maternal PUFAs in early and late pregnancy with childhood social competence, total problems, internalising and externalising behaviours, measured with the Child Behaviour Checklist 4/18 at age 7, were investigated with linear regression analyses adjusted for maternal and children's socio-demographic characteristics.

Results: In late gestation (*i.e.*, from gestational week 30), an increase in arachidonic acid (AA) was associated with higher social competence, while a decrease in total n-6 was associated with lower externalising behaviours. However, important confounders such as maternal psychopathology and personality could not be adjusted for. No associations could be considered significant after controlling for multiple testing.

Discussion: Considering the modest clinical relevance of identified associations, the lack of statistical significance after controlling for multiple testing and the study limitations, this study provides limited evidence of an association of maternal gestational PUFAs and childhood social competence and problem behaviours.

Background

Low social competence and problem behaviours in childhood are important markers of psychological adjustment. Social competence is a multidimensional trait that includes the ability to interact with other people and manage different situations (1), and is considered a central component of an optimal development and healthy functioning (2). Internalising problem behaviours are characterised by extreme emotional reactions, with symptoms of anxiety, depression and social withdrawal, while externalising behaviours include problems with attention and self-regulation, as well as antisocial and aggressive behaviours (3–5). Both a lack of social competence and the presence of problem behaviours can have deleterious effects for the individual and the society, including a higher risk for psychiatric disorders and delinquency during adulthood (6–9). Different models have been proposed to describe the relationship between social competence and problem behaviours, which are often inversely related in children (6,10,11), but none was proven definitive (12). A study examining social competence and problem behaviours longitudinally during childhood found that social competence at 4 years was predictive of problem behaviours at 10 years (2). In addition, all three traits were largely stable over the 10-year follow-up period (2), suggesting that an early identification of at-risk children might have substantial public health benefits. Consequently, the perinatal environment is receiving increasing attention as it might play a central role in shaping future behaviour (13).

Inadequate amounts of polyunsaturated fatty acids (PUFAs) during brain development may be associated with suboptimal neurological outcomes. Specifically, three long-chain PUFAs – *i.e.*, the n-6 arachidonic acid (AA) and the n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) – play both structural and functional roles within the nervous system. DHA is the only n-3 PUFA present in significant amounts in the brain (14), representing, together with AA, 25%-30% of the brain's dry weight (14–17). DHA might affect processes such as synaptogenesis and synaptic activity (18), and might participate in the transduction of nervous signals (19). EPA has anti-inflammatory, neuroprotective and antiapoptotic properties (20), while AA mediates neuronal signalling, limits oxidative stress in the hippocampus and is likely involved in nerve growth and synaptogenesis (21–23). During gestation, long-chain PUFAs, as well as other fatty acids, are firstly released from maternal adipose tissue in a process denoted *fat mobilisation* (24,25), and subsequently transferred to the foetus through the placenta (26,27). Although all fatty acids are transferred, a preferential transport of DHA and AA has been suggested (26,27). Specifically, the transfer of AA exceeds that of DHA particularly in the first two trimesters, with the result that the brain of term infants contains

more AA than DHA (28). However, the rate accretion of DHA increases from week 30 of gestation, corresponding to the brain growth spurt (14,29) and the most prominent fat deposition in the foetus (26), and continues after birth (14). Sufficient PUFA concentrations in maternal plasma, coming from fat mobilisation and dietary intake, are therefore necessary to guarantee an adequate foetal brain development and have been hypothesised to be related with higher psychological traits in the offspring (30), possibly including problem behaviours and social competence.

Because of the brain growth spurt in late gestation (14,29), several studies have analysed PUFA concentrations in the umbilical cord blood in the attempt to examine their associations with child problem behaviours (31–33). Published results are, however, inconclusive, and the association of these fatty acids at birth with childhood behaviour is still unclear. No associations between cord DHA or AA and child problem behaviours at 9 years were found in 235 Dutch children (31). In contrast, higher cord DHA concentration was associated with lower internalising problems in 7-year-old Dutch children (n=393) who received artificial formula. However, no associations were found in children who were breastfed, or between DHA or AA status and childhood externalising behaviours (32). Besides, higher DHA levels in cord blood were related with lower total problems and hyperactivity/inattention, while higher AA was associated with lower emotional symptoms in 416 German children aged 10 years. By contrast, in the same population higher levels of α-linolenic acid (ALA, the essential n-3 fatty acid) were associated with greater peer-relation problems, while EPA was positively associated with conduct problems (33). However, even early-pregnancy PUFA availabilities might influence the foetus' developing brain (34,35), since all neuronal structures form during the embryonic stage (29). Nonetheless, early- and mid-pregnancy maternal long-chain PUFAs in relation to problem behaviours and emotional problems at 6 years of age have been analysed only in one cohort (36,37). Higher maternal plasma DHA concentrations and n-3:n-6 ratio in mid-pregnancy were associated with less emotional problems at age 6 years (n=2,828) (36); however, only a marginally significant association was observed when analysing DHA concentrations in early pregnancy (37). Moreover, AA concentrations in mid- but not early pregnancy were positively associated with problem behaviours in the same population of children (36,37).

Contrarily to problem behaviours, the development of social competence has been studied less extensively: to our knowledge, no studies have been published examining the potential association of prenatal maternal PUFA status with social competence development in humans. Nonetheless, an animal study reported that piglets whose mothers were fed a DHA-rich diet during pregnancy showed more social activities and played more, compared to piglets whose mothers were fed an

oleic acid-rich diet (38). Since pigs are considered good models for human nutrition and neurobiology (39), and given the relationships between social competence and problem behaviours (6,10,11), we hypothesised that higher maternal PUFAs during pregnancy might be associated with improved social competence in children as well.

The main objective of the present study was to analyse the association between the change relative to baseline levels in selected maternal PUFAs (*i.e.*, AA, EPA, DHA, total n-6, total n-3 and n-6:n-3 ratio) during pregnancy, which can be used as proxies for the child's prenatal fatty acids exposure (24), with childhood social competence and problem behaviours measured with the Child Behaviour Checklist (CBCL). Given the previously highlighted functions that these PUFAs have in children's neurological development, we hypothesised that each of them would be independently associated with social competence and problem behaviours. The possible role of the change in total n-6, total n-3 and the n-6:n-3 ratio on childhood outcomes was investigated due to the evidence of poorer health outcomes associated with an excessive intake of n-6 PUFAs compared to n-3 PUFAs (40). Given available evidences and the previously described physiological reduction of all PUFAs' relative concentrations during pregnancy (41), we hypothesised that a smaller reduction in AA, EPA, DHA and total n-3 concentrations during pregnancy would be associated with improved childhood social competence and problem behaviours. In contrast, we hypothesised that a greater reduction in total n-6 or n-6:n-3 ratio would be associated with better outcomes in children. Contrarily to the previously published literature, in which single measurements of maternal PUFAs during pregnancy were used to predict childhood outcomes, in the present study we calculated the subject- and PUFA-specific change in relative concentrations, by modelling subsequent measurements of maternal PUFA concentrations in plasma phospholipids. We therefore obtained subject- and PUFA-specific variations in concentration in two gestational periods (*i.e.*, early and late) with a novel approach that allows for a greater control of random variations in PUFA concentrations while providing a more accurate representation of foetal exposures to maternal fatty acids (42).

Materials and methods

Study population

The Maastricht Essential Fatty Acid Birth cohort (MEFAB) is a prospective birth cohort established in 1989 in the southern part of the Netherlands. The main aim was to analyse the variations in maternal PUFA concentrations during pregnancy and their correlations with infants' fatty-acid

concentrations and with various birth outcomes. Details of the study population and the data collection have been previously reported (43). Briefly, between 1989 and 1995, 1,203 pregnant women were recruited. In this period, maternal blood samples and socio-demographic data were collected as described below. A follow-up study was conducted when children were 7 years old (1997-2000). Seven hundred and fifty children were eligible, of whom 421 (56%) eventually participated. Children were further excluded from participation in this study if less than 2 maternal blood samples were collected over the course of pregnancy or at delivery, or if no information on child problem behaviours was obtained, resulting in 311 mother-child pairs included in the present analysis. The study was approved by the Medical Ethics Committee, University Hospital, Maastricht/ University of Maastricht, the Netherlands.

The non-response analysis revealed that participating women had a slightly lower body mass index (BMI) at study entry compared to non-participants (median: 22.9 kg/m2, interquartile range (IQR): 21.5; 25.1 in participants vs. 23.4 kg/m2, IQR: 21.3; 26.2 in non-participants; p=0.024). Furthermore, a higher birth weight of participating children compared to those non-participating (mean: 3347g, standard deviation (SD):515 vs. 3197g, SD:580; p<0.001). However, no other significant differences were found in other sociodemographic characteristics, including gestational weight gain, smoking status, birth weight by gestational age and parity. Furthermore, no differences were found in total n-6, total n-3 or the n-6:n-3 ratio between women included and excluded from the current study (Appendix, table A5.1). Hence, selection bias was not expected to be an issue.

Maternal PUFAs

Maternal blood samples were collected during clinic visits before week 16 of pregnancy (mean: 10.5; SD: 2.7 weeks), around week 22 (22 ± 1.3 weeks), around week 32 (32 ± 0.9 weeks), and at delivery (39.2 ± 1.8 weeks) to measure the phospholipid fatty acids profile as previously described (25,44). Briefly, maternal venous blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. Plasma was, then, separated from erythrocytes by centrifugation before being stored at -80° C under nitrogen. A modified Folch extraction (45,46) was used to prepare total lipid extracts from 100μl plasma samples after addition of L-α-Dinonadecanoyl lecithin as internal standard to calculate the absolute fatty acid amounts (mg/L). Aminopropyl-bonded phase columns were used to separate phospholipids from the total lipid extract. The fatty acid constituents of the phospholipids were transmethylated to the corresponding methylesters, which were separated and quantified on a nonpolar CP-Sil 5 CB column

(Chrompack, Middelburg, The Netherlands). For the purpose of the present analysis, plasma phospholipids were preferred over erythrocytes, because they are considered to be better indicators of recent PUFA intakes or mobilisation (25,47,48). Given the high correlation between fatty acids, we expressed all analysed PUFA concentrations as weight percentages (weight of fatty acids measured against total fatty acids present; wt%), which ensures an automatic control for all assayed fatty acids in maternal plasma.

All assayed n-6 PUFAs and n-3 PUFA concentrations were summed up to calculate the total n-6 and n-3 PUFAs, respectively. Furthermore, the total n-6 and the total n-3 were used to compute the n-6:n-3 ratio. More details regarding the specific PUFAs included can be found in the Appendix (table A5.2).

Child Behaviour Checklist

The Dutch version of the CBCL 4/18 was used to assess children's social competence and problem behaviours as perceived by their parents (5,49,50). The CBCL has demonstrated good psychometric properties, reliability and validity (51,52). The social competence scale of the CBCL 4/18 consists of 20 items grouped into three subscales: social skills, activity engagement and school functioning (which includes items related to academic performance and academic or other problems in school). As discussed in chapter 1 (pages 16, 17 and 21), social competence is of complex definition and the different assessment tools focus on specific aspects (53). The focus of the CBCL's social competence scale is on the child's ability to initiate and maintain successful social relationships and to handle academic requirements. Furthermore, three aspects of problem behaviours can be assessed through the CBCL: 1) internalising problems, which consists of the social withdrawal, somatic complaints and anxiety/depression scales; 2) externalising problems, which comprises the delinquent behaviour and aggressive behaviour scales; and 3) total problems, which is the sum of the internalising and externalising scales, together with additional syndrome scales focused on social, thought, attention and sex problems. Elements of the social competence scale can be scored 0-1, 0-2 or 0-3, with increasing scoring corresponding to higher competence, while each item of problem behaviours scales can be scored 0-2, with higher scores corresponding to greater problem behaviours. To allow comparability between studies, raw scores are usually converted into age-standardised T-scores (with a mean of 50 and a standard deviation of 10). This study assessed the T-scores for the social competence, total problem, internalising and externalising scales. High social competence T-scores (*i.e.*, >40) denote a normal development, while for total problems,

internalising and externalising behaviours higher T-scores (*i.e.*, ≥63) indicate clinical levels of symptomatology.

Covariates

Information about maternal age (years), BMI (kg/m^2), smoking during pregnancy (ever/never) and parity (primipara/multipara) was recorded by hospital staff at study entry. Maternal weight during pregnancy and at delivery (kg) was measured by hospital staff during follow-up visits. Data on child's sex (male/female) and birth weight (grams) were recorded by hospital staff at delivery. Gestational age at birth (weeks) was calculated from the date of the last menstrual period. Data regarding breastfeeding (exclusive breastfeeding/bottle feeding/combination) were collected by means of questionnaires at follow-up. Due to a low number of mothers who reported to have exclusively breastfed their children (n=21, 9.68%), this category was merged to the combination category (*i.e.*, breastfeeding and bottle-milk). Parents' level of education was categorised into low, middle or high; the highest category was chosen in case of discordance between parents (54). Parents' employment status was categorised as: both parents working full-time, one working full-time while the other part-time, and both working part-time. None of the parents reported being unemployed. Children's anthropometrics (in kg or cm, as appropriate) were measured by research staff when children were 7 years old. At follow-up, parents of 191 children (61.41%) gave informed consent for the assessment of their children's plasma PUFAs, which were measured following the same methodology used to assay maternal PUFAs. Finally, information regarding maternal postnatal depression (diagnosed/not diagnosed) was self-reported at follow-up.

Statistical analysis

Descriptive statistics

Maternal and children's characteristics are reported as mean and SD, median and IQR or number and percentage, as appropriate. Maternal PUFA concentrations at each measurement time-point are reported as mean (SD); to assess the differences between PUFA concentrations at different time-points, one-way analysis of variance (ANOVA) and Bonferroni's post hoc tests were used. PUFA-specific concentration changes in early and late pregnancy, calculated as explained below, are reported as mean (SD).

PUFA-specific concentration changes during pregnancy

Since maternal PUFA concentrations throughout pregnancy vary following non-linear patterns, we estimated the best-fitting curve of each fatty-acid concentration during gestation. To improve the prediction, all women with at least one PUFA measurement during pregnancy were included (n=1,285). First, we modelled PUFA concentrations throughout pregnancy as a function of gestational age using linear splines (42,55). For each PUFA, we aimed to identify a set of knots that allowed the construction of the best-fitting curve. We selected one knot per curve that was used to define two periods during pregnancy (*i.e.*, *early* and *late*). The identified splines were used in mixed-effect linear regression models with a random intercept for mother and a random slope, allowing for individual variations of maternal fatty acids. Subject- and PUFA-specific slopes, calculated considering both the fixed and the random portions of the regressions, were then extracted and used as main exposures in subsequent linear regression analyses. These slopes represent the change in PUFA concentration in specific periods of pregnancy, as defined by the previously identified knots.

Linear regression analyses

The associations between the change in maternal PUFA concentrations during pregnancy and social competence or problem behaviours were analysed by means of linear regression analyses. Two models were constructed, which always included both changes in each PUFA concentration in the two periods of pregnancy. First, we tested the crude, non-adjusted associations; then, we adjusted for the following *a priori* selected variables based on previous studies of perinatal PUFAs and child problem behaviours (32,33,36,37): maternal age, smoking during pregnancy, maternal BMI at study entry, parental educational level, parental employment status, parity, breastfeeding, child's sex and age at assessment. Furthermore, the measured PUFA concentration at the beginning of pregnancy was introduced into the model as a proxy for maternal habitual PUFA status. The interaction of maternal PUFA with child's sex was also examined. Since relative PUFA concentrations were used, no additional controlling for other fatty acids was applied.

We treated the changes in maternal PUFA concentrations as continuous variables in all analyses. However, to aid the interpretation of identified associations, we used the MIMRGNS (56) command in Stata to predict children's scores in social competence and problem behaviours at the 5^{th}, 25^{th}, 50^{th}, 75^{th} and 95^{th} percentiles of maternal PUFAs' change during late pregnancy, while keeping constant all other variables at their mean values.

To reduce bias due to missing values, multiple imputation of missing covariate data was performed. Fifty imputed datasets were generated using the multiple imputation of chained equation (MICE)

method (57). To increase the precision of the imputation, all outcomes, exposures, and confounders were included in the imputation model, together with additional auxiliary variables- *i.e.*, gestational weight gain, pregnancy outcomes, day-care attendance, delivery mode, gestational age, birth weight, postpartum depression, child's BMI and waist-to-hip ratio at follow-up, and child's PUFAs at follow-up. The appropriateness of the imputation model was confirmed by comparing the distribution of imputed values with that of observed values (58). Furthermore, complete-case analyses were performed by including only participants with no missing values on any confounding factor.

Sensitivity analyses were performed to assess the robustness of the results: 1) birth weight and gestational age, 2) maternal postpartum depression, or 3) child's BMI and PUFAs at follow-up were included in the adjusted the model separately to evaluate their effect on the coefficients for maternal PUFAs. Furthermore, in the analyses between maternal PUFAs and childhood social competence we additionally adjusted for day-care attendance, which has been shown to be an important factor for child's social competence development (59).

In secondary analyses we examined the associations between each PUFA, measured at 10, 22, 32 and 40 weeks of gestation, with social competence or problem behaviours to allow for an easier comparison with previously published studies. All linear regressions were controlled for the set of *a priori* confounding factors identified for the main analysis.

The Benjamini-Hochberg procedure (60) with a false discovery rate of 5% was used to correct for multiple testing. All statistical analyses were done in Stata v.14 (61).

Results

The study population's characteristics are reported in table 5.1. On average, mothers were 29.7 (3.9) years old and were predominantly non-smokers and primiparas. Children's mean T-scores were: 44.7 (8.6) for social competence, 50.7 (11.0) for total problems, 51.8 (10.3) for internalising problems and 50.9 (10.4) for externalising problems.

Mean (SD) for each PUFA concentration are presented in table 5.2. Mean PUFA concentrations varied over the course of pregnancy, with higher values found in the first or second periods except for the n-6:n-3 ratio. The mean, linear changes in PUFA concentrations in early and late pregnancy are shown in table 5.3. Higher absolute values indicate a greater change in concentration, compared

Table 5.1: Population's characteristics

Maternal characteristics	**n**	**Mean (SD), Median (IQR) or n (%)**
Age at delivery (years)	311	29.7 (3.9)
Smoking during pregnancy (% yes)	308	68 (22.08%)
Level of education	215	
High		78 (36.28%)
Middle		92 (42.79%)
Low		45 (20.93%)
Employment status	219	
Both full-time		12 (5.48%)
One full-time, one part-time		119 (54.34%)
Both part-time		88 (40.18%)
Parity (% primiparas)	311	221 (71.06%)
BMI at study entry	294	22.9 (21.5; 25.1)
Breastfeeding (% yes)	218	99 (45.41%)
Children's characteristics	**n**	**Mean (SD), Median (IQR) or n (%)**
Sex (% males)	311	163 (52.41%)
Gestational age (weeks)	311	39.2 (1.8)
Age at follow-up (years)	217	7.3 (0.3)
Birth weight (g)	310	3347 (515)
BMI (at 7 years)	216	15.2 (14.4; 16.4)

to no change. The definition of early and late depends on the specific PUFA's variation throughout pregnancy, and, therefore, on the selection of the best knots for the linear splines' construction. For AA and total n-6, late pregnancy would start at week 30, for EPA at week 22, for DHA at week 20, and for total n-3 and the n-6:n-3 ratio at week 10.

Tables 5.4-5.7 present the results of the linear regressions subdivided by CBCL scale; full-model estimates are shown in the Appendix (tables A5.3-A5.6). In the unadjusted model, we found a tendency for lower social competence with increasing DHA concentration in early pregnancy (β=-37.67; 95% C.I.: -75.53, 0.19). In the adjusted model, AA and total n-6 in late pregnancy were

associated with social competence (AA: β=25.13; 95% C.I.: 3.40, 46.85) and externalising behaviours (total n-6: β=83.05; 95% C.I.: 18.19, 147.90), respectively.

Table 5.2: Mean maternal gestational PUFA concentrations throughout pregnancy

PUFAs	Relative concentrations (wt%)*				
	Periods of pregnancy				
	First trimester	Second trimester	Third trimester	Partus	
	Mean (SD)				p-value
AA	9.59 (1.43)[a]	8.62 (1.31)[b]	8.15 (1.19)[c]	8.44 (1.39)[b,c]	<0.001
EPA	0.55 (0.40)[a]	0.41 (0.34)[b]	0.34 (0.18)[b,c]	0.34 (0.21)[c]	<0.001
DHA	4.09 (0.91)[a]	4.19 (0.85)[a,b]	3.99 (0.72)[a,b,c]	3.90 (0.76)[c]	<0.001
Total n-6	35.54 (2.11)[a]	35.22 (1.97)[a,b]	35.07 (1.78)[b]	34.29 (1.84)[c]	<0.001
Total n-3	5.79 (1.25)[a]	5.65 (1.18)[a]	5.32 (0.91)[b]	5.17 (0.99)[b]	<0.001
n-6:n-3	6.44 (1.49)[a]	6.48 (1.35)[a]	6.81 (1.32)[b]	6.90 (1.48)[b]	<0.001

Note: *: Except for the n-6:n-3 ratio. P-values refer to One-way Analysis of Variance (ANOVA)'s results; a-c: results of Bonferroni's post-hoc tests, with different letters representing a statistically significant difference (p<0.05) between PUFA concentrations in different periods of pregnancy.

Table 5.3: Change in concentrations in maternal PUFAs throughout pregnancy

PUFAs	Change in concentrations (wt%/week)	
	Periods of pregnancy	
	Early[a]	Late[a]
	Mean (SD)	
AA	-0.07 (0.03)	0.03 (0.06)
EPA	-0.01 (0.02)	-0.00 (0.00)
DHA	0.02 (0.03)	-0.02 (0.01)
Total n-6	-0.03 (0.04)	-0.07 (0.03)
Total n-3	0.06 (0.08)	-0.02 (0.01)
n-6:n-3	-0.07 (0.08)	0.02 (0.02)

Note: a: cut-off points for different PUFAs derived from the identified best-fitting linear splines. For AA and Total n-6, the "late" pregnancy starts at week 30, for EPA at week 22, for DHA at week 20, and for Total n-3 and the n-6:n-3 ratio at week 10.

An increase in maternal plasma AA in late pregnancy (0.15 wt%/week vs. -0.07 wt%/week, which correspond to the 95th and 5th percentiles, respectively) resulted in 5.50-point higher social competence T-score, after controlling for previously identified confounders and AA concentration change in early pregnancy (figure 5.1). Furthermore, a larger decrease in maternal plasma total n-6 in late pregnancy (-0.12 wt%/week vs. -0.03 wt%/week, corresponding to the 5th and 95th percentiles, respectively) resulted in lower externalising behaviours, with a reduction of 8.20 points

Table 5.4: Associations of maternal gestational PUFAs with social competence, presented by gestational period.

PUFAs	Early pregnancy[a]		Late pregnancy[a]	
	Beta (95% C.I.)		Beta (95% C.I.)	
	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]
AA	-27.95 (-57.37, 1.48)	-19.21 (-70.18, 31.76)	8.11 (-7.23, 23.44)	25.13 (3.40, 46.85)*
EPA	-14.60 (-64.22, 35.01)	67.98 (-315.03, 450.99)	-21.28 (-238.37, 195.81)	-125.91 (-929.76, 677.95)
DHA	-37.67 (-75.53, 0.19)§	-14.13 (-77.87, 49.61)	28.72 (-47.92, 105.37)	81.77 (-29.52, 193.06)
Tot n-6	20.89 (-11.50, 53.27)	23.65 (-17.54, 64.84)	-14.74 (-52.41, 22.94)	-15.63 (-68.97, 37.71)
Tot n-3	-2.50 (-15.44, 10.45)	-2.88 (-22.68, 16.92)	-28.96 (-99.42, 41.49)	108.36 (-48.59, 265.32)
n-6:n-3	4.99 (-7.82, 17.80)	10.63 (-7.70, 28.96)	7.26 (-48.09, 62.61)	-52.33 (-133.35, 28.69)

Note: Imputed dataset analysis, n=311; a: Cut-off points for different PUFAs derived from the identified best-fitting linear splines. For AA and Total n-6, the "late" pregnancy starts at week 30, for EPA at week 22, for DHA at week 20, and for Total n-3 and the n-6:n-3 ratio at week 10; b: All models included the following variables: change in the index PUFA in early and late pregnancy, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up; §: p≤0.06; *: p≤0.05.

Table 5.5: Associations of maternal gestational PUFAs with total problems, presented by gestational period

PUFAs	Early pregnancy[a]		Late pregnancy[a]	
	Beta (95% C.I.)		Beta (95% C.I.)	
	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]
AA	-3.11 (-40.68, 34.47)	-8.10 (-74.29, 58.09)	2.80 (-16.79, 22.39)	7.59 (-20.51, 35.69)
EPA	-5.84 (-68.64, 56.96)	322.59 (-168.09, 813.27)	30.96 (-243.83, 305.74)	561.34 (-470.54, 1593.21)
DHA	-12.35 (-60.49, 35.78)	-24.16 (-106.17, 57.86)	-24.81 (-122.25, 72.64)	10.76 (-130.84, 152.36)
Tot n-6	3.57 (-37.36, 44.50)	4.89 (-47.20, 56.97)	27.74 (-19.87, 75.36)	51.54 (-15.70, 118.77)
Tot n-3	-10.71 (-27.06, 5.64)	-4.53 (-29.81, 20.76)	-19.37 (-108.35, 69.61)	-41.18 (-241.59, 159.23)
n-6:n-3	11.13 (-5.03, 27.29)	7.64 (-15.58, 30.86)	13.90 (-55.93, 83.73)	12.67 (-90.52, 115.87)

Note: Imputed dataset analysis, n=311; a: Cut-off points for different PUFAs derived from the identified best-fitting linear splines. For AA and Total n-6, the "late" pregnancy starts at week 30, for EPA at week 22, for DHA at week 20, and for Total n-3 and the n-6:n-3 ratio at week 10; b: All models included the following variables: change in the index PUFA in early and late pregnancy, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up; §: p≤0.06; *: p≤0.05.

compared to a smaller decrease in maternal plasma total n-6 concentration (figure 5.2).

When analysing the association between maternal AA and social competence, there was some evidence of an interaction between AA in late pregnancy and child's sex (p=0.033). However, after stratification by child's sex, no clear associations were observed (Appendix, table A5.7). Sensitivity

Table 5.6: Associations of maternal gestational PUFAs with internalising problems, presented by gestational period

PUFAs	Early pregnancy[a]		Late pregnancy[a]	
	Beta (95% C.I.)		Beta (95% C.I.)	
	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]
AA	-3.90 (-38.90, 31.10)	-30.31 (-91.46, 30.84)	0.72 (-17.52, 18.96)	8.99 (-16.84, 34.82)
EPA	-27.90 (-86.28, 30.48)	129.43 (-328.52, 587.38)	20.87 (-234.58, 276.31)	147.55 (-812.19, 1107.29)
DHA	-11.21 (-56.08, 33.66)	-55.33 (-131.08, 20.43)	3.10 (-87.74, 93.94)	16.20 (-115.02, 147.42)
Tot n-6	-5.21 (-43.42, 33.00)	-2.93 (-51.34, 45.49)	19.14 (-25.31, 63.59)	35.71 (-26.69, 98.11)
Tot n-3	-9.65 (-24.88, 5.59)	-11.29 (-34.68, 12.09)	-6.68 (-89.61, 76.24)	-24.06 (-209.64, 161.53)
n-6:n-3	9.35 (-5.72, 24.43)	14.59 (-6.89, 36.07)	-0.87 (-66.02, 64.27)	7.78 (-87.76, 103.32)

Note: Imputed dataset analysis, n=311; a: Cut-off points for different PUFAs derived from the identified best-fitting linear splines. For AA and Total n-6, the "late" pregnancy starts at week 30, for EPA at week 22, for DHA at week 20, and for Total n-3 and the n-6:n-3 ratio at week 10; b: All models included the following variables: change in the index PUFA in early and late pregnancy, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up; §: p≤0.06; *: p≤0.05.

Table 5.7: Associations of maternal gestational PUFAs with externalising problems, presented by gestational period

PUFAs	Early pregnancy[a]		Late pregnancy[a]	
	Beta (95% C.I.)		Beta (95% C.I.)	
	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]
AA	3.18 (-32.83, 39.19)	-7.98 (-72.40, 56.44)	6.92 (-11.85, 25.68)	9.06 (-18.09, 36.21)
EPA	19.88 (-40.29, 80.05)	291.86 (-186.45, 770.17)	-13.85 (-277.12, 249.41)	348.91 (-655.19, 1353.00)
DHA	-6.56 (-52.59, 39.48)	-8.26 (-88.02, 71.51)	-59.42 (-152.62, 33.78)	-39.00 (-176.09, 98.08)
Tot n-6	-0.92 (-39.85, 38.01)	-11.05 (-60.98, 38.89)	51.47 (6.18, 96.76)	82.87 (18.21, 147.54)*
Tot n-3	-7.23 (-22.91, 8.45)	5.79 (-18.76, 30.34)	-35.99 (-121.34, 49.35)	-94.98 (-289.18, 99.22)
n-6:n-3	6.41 (-9.08, 21.90)	-3.97 (-26.45, 18.51)	38.38 (-28.54, 105.30)	34.80 (-64.92, 134.52)

Note: Imputed dataset analysis, n=311; a: Cut-off points for different PUFAs derived from the identified best-fitting linear splines. For AA and Total n-6, the "late" pregnancy starts at week 30, for EPA at week 22, for DHA at week 20, and for Total n-3 and the n-6:n-3 ratio at week 10; b: All models included the following variables: change in the index PUFA in early and late pregnancy, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up; §: p≤0.06; *: p≤0.05.

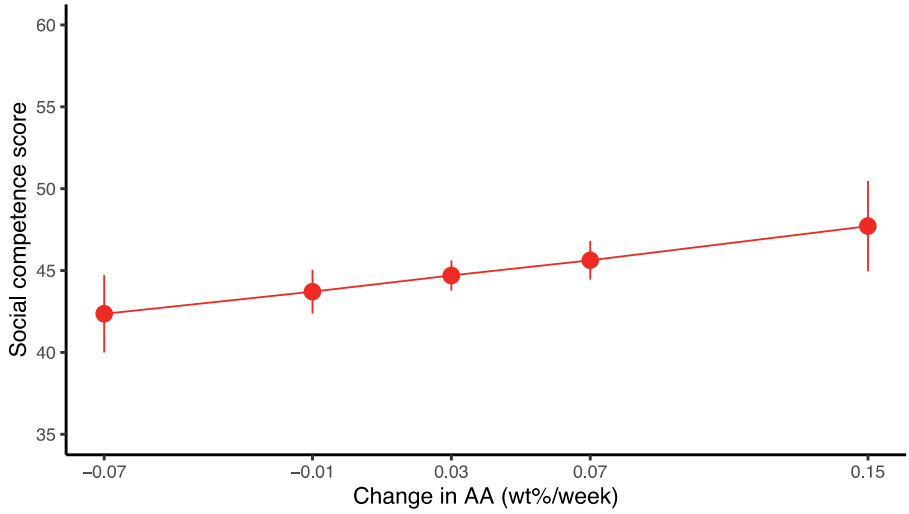

Figure 5.1: Predicted social competence score at the 5th, 25th, 50th, 75th and 95th percentiles of change in maternal AA concentration in late pregnancy.

Note: n= 311; the x- axis represents the change in AA in late gestation (*i.e.*, from week 30 to delivery) in the present population of pregnant women. Presented values correspond to the 5th (-0.07 wt%/week), 25th (-0.01 wt%/week), 50th (0.03 wt%/week), 75th (0.07 wt%/week) and 95th (0.15 wt%/week) percentiles. Adjusted for: change in AA in early pregnancy, AA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up.

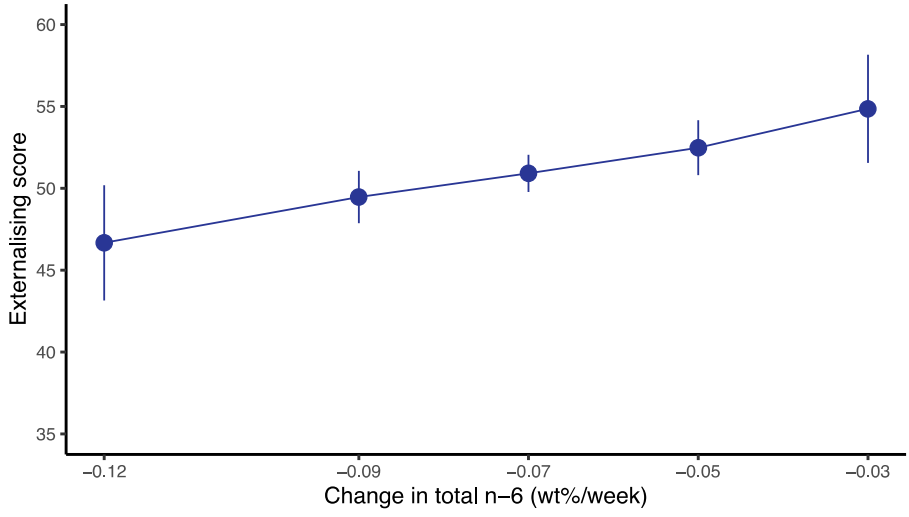

Figure 5.2: Predicted externalising behaviour score at the 5th, 25th, 50th, 75th and 95th percentiles of change in maternal total n-6 concentration in late pregnancy.

Note: n= 311; the x- axis represents the change in total n-6 in late gestation (i.e., from week 30 to delivery) in the present population of pregnant women. Presented values correspond to the 5th (-0.12 wt%/week), 25th (-0.09 wt%/week), 50th (-0.07 wt%/week), 75th (-0.05 wt%/week) and 95th (-0.03 wt%/week) percentiles. Adjusted for: change in total n-6 PUFAs in early pregnancy, total n-6 concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up.

analyses showed no significant changes in the coefficients after the inclusion of several possible mediators or postnatal confounders to the adjusted model (Appendix, tables A5.8-A5.11).

Tables A5.12-A5.15 (Appendix) show the results of the secondary analyses. We found positive associations between AA at 40 weeks of gestation and EPA at 32 weeks, respectively, and social competence (AA: β=1.63; 95% C.I.: 0.49, 2.77. EPA: β=8.04; 95% C.I.: 0.31, 15.77). Furthermore, AA at 10 and 40 weeks of gestation was associated with internalising behaviours (week 10: β=1.33; 95% C.I.: 0.01, 2.65. week 40: β=1.47; 95% C.I.: 0.15, 2.80), while total n-6 at 40 weeks was associated with externalising behaviours (β=1.13; 95% C.I.:0.05, 2.20). Finally, EPA in week 10 was negatively associated with externalising behaviours (β=-4.17; 95% C.I.: -8.05, -0.03).

However, none of the reported associations could be considered statistically significant after controlling for multiple testing.

Discussion

In this study, we investigated the association of maternal PUFAs during pregnancy with childhood social competence and problem behaviours using a novel approach to evaluate the change in PUFA concentrations throughout pregnancy. Present results showed that a larger increase in maternal AA during late pregnancy (*i.e.*, from week 30 of gestation) was associated with a modest increase in social competence at 7 years of age. Similarly, a larger reduction in total n-6 in the same period was associated with a small decrease in externalising behaviours at 7 years of age. In the sensitivity analyses, controlling for postnatal influences or possible mediators did not alter the results. In contrast, changes in other PUFAs during pregnancy were not associated with scores on the social competence or problem behaviour scales. Nonetheless, these findings should be interpreted with caution, particularly because none of the identified associations could be considered statistically significant after controlling for multiple testing. Furthermore, even regarding the identified associations as plausible, their clinical relevance could be deemed modest at most, since predicted scores of the social competence and externalising behaviour scales fall within the normal-development range.

Despite AA being part of the n-6 PUFA family, from the present results an increase in maternal AA concentration appears beneficial for an optimal social competence development, while a reduction in total n-6 seems more beneficial for externalising behaviours. In the present population, changes in late gestation of AA and total n-6 PUFAs were only modestly correlated (r=0.15, p=0.007), while

changes in LA, which constitutes the vast majority of total n-6 PUFAs (see table A5.2 in the Appendix for a summary of all PUFAs in this population), were strongly correlated with changes in total n-6 PUFAs (r=0.76, p>0.001). It could therefore be hypothesised that high LA concentrations might have a deleterious effect on foetal brain development. Although in post-hoc analyses we could not find an association between the change in LA concentration during pregnancy and childhood social competence or problem behaviours (Appendix, table A5.16), an effect of this n-6 PUFA on child psychological development should not be completely excluded. In fact, previous studies have reported that high LA concentrations in maternal plasma were associated with poorer psychomotor and mental development at 6 months (62), and more autistic traits at 6 years (63). In contrast, AA, the most abundant long-chain PUFA of the human brain with DHA (14,15), is considered a crucial enhancer of neurological development (22): it affects neuronal excitability and synaptic transmission (22), and is probably involved in nerve growth and synaptogenesis, processes in which it might play a crucial role by regulating the maturation of synaptic endings (23). Furthermore, it is particularly represented in brain regions responsible for sensorimotor integration and various language-implicated processes – *i.e.*, pre- and post-central cortices, basal ganglia, hippocampus and thalamus (15,64–66). Considering that social competence is related with sensory processing (67) and language development (68), a role of AA on the development of social competence might be plausible, despite the modest clinical significance of the reported association.

Although no studies analysing the association between maternal PUFAs during pregnancy and childhood social competence have been published to date, a few have explored the development of problem behaviours, reporting inconclusive results (31–33,36,37). Studies examining the association between PUFA concentrations in the umbilical-cord blood and childhood problem behaviours reported either no statistically significant association (31), a decrease in total problems and emotional problems for every unit increase in DHA and AA, respectively (33), or a reduction in internalising problems for every unit increase in DHA in children fed exclusively with artificial formula, but not in those breastfed (32). In the Generation R Study, maternal DHA and n-3:n-6 ratio in mid-pregnancy were negatively associated with child emotional problems, while a positive association between maternal AA in mid-pregnancy and total problems was observed (36). However, when considering maternal PUFAs in early-pregnancy in the same cohort, only a tendency for fewer emotional symptoms was observed with increasing DHA concentration (37). These results can be compared to those of the present study's secondary linear regression analyses, in which positive associations were observed between AA and internalising behaviours, and between total

n-6 PUFAs and externalising behaviours. Furthermore, EPA was negatively associated with externalising behaviours and positively associated with social competence. However, PUFA concentrations change over the course of pregnancy (41), and analysing single measurements at a time may not provide an appropriate representation of foetal exposure during gestation. With the analytical approach used in the present study we aimed to account for previously published studies' shortcomings. Instead of presenting a sequence of linear regressions using the four fatty-acid measurements as separate predictors, in the main analysis we constructed PUFA-specific concentration curves throughout gestation. In doing so, we assumed no significant deviations from the predicted values between measurements. However, since we noticed a general trend in PUFA concentrations among participants, we are confident this method provides an adequate representation of the change in maternal PUFA concentrations during pregnancy. Compared to any sequence of linear regression analyses, here the temporal variation of exposures is taken into account, while extreme values, which are likely due to temporary shifts in maternal diet, tend to be smoothed.

Other observational studies focused only on maternal fish and seafood intake, reporting small improvements in childhood hyperactivity and prosocial behaviour (69–71). Fish and seafood products are particularly rich in long-chain n-3 PUFAs compared to other food groups, so their intake is often used as proxy for EPA and DHA intake (47). In addition, considering that higher consumption of fish and seafood is often correlated with lower meat and higher fruit and vegetable intake, higher consumption of fish and seafood is also used as proxy for "healthy" dietary pattern (72). However, this approach could lead to biased results as it does not account for the correlations and interactions between food groups (73). In contrast, the use of biomarkers to assess gestational PUFA concentrations is a more reliable method to assess foetal exposure to PUFAs. In fact, relative PUFA concentrations automatically controls for all other assayed fatty acids in maternal blood. Furthermore, assessing PUFA concentrations in maternal plasma allows for the estimation of the whole foetal exposure, which results from PUFAs introduced with diet in the days before the assessment and those released from maternal adipose tissue (25,47,48). Conversely, questionnaires rely on self-report of maternal dietary intakes to estimate the amount of PUFAs consumed during pregnancy and are therefore less accurate than biomarkers (47).

All in all, the evidence of an association between prenatal PUFA exposure and childhood social competence or problem behaviours coming from observational studies is not strong nor consistent. A possible explanation might be that maternal PUFAs might not have a direct effect on child

psychological development. The few, small randomised controlled trials (RCTs) that investigated the role of prenatal PUFA exposure on infants' neurocognitive and behavioural outcomes support this hypothesis, as no effect of maternal supplementation was found (74). In this perspective, factors responsible for maternal dietary intake before conception and during pregnancy – including socioeconomic status, personality and propensity towards a healthy lifestyle (75,76) – might be responsible for the previously reported associations. In particular, maternal personality might affect dietary intake (76) and thus prenatal PUFA exposure, and might also affect child problem behaviours directly (77) and indirectly by modulating the risk of maternal psychopathology (78) and affecting the relationship with her children (79).

This study comes with a few limitations. Despite the large number of respondents at baseline, the final study population was affected by a high loss-to-follow-up rate, which might have introduced biases. Nonetheless, most sociodemographic characteristics were similar among participants and non-participants, as were maternal PUFA concentrations during pregnancy. Furthermore, although we controlled for numerous sociodemographic characteristics, residual confounding is still possible. Specifically, we were not able to control for important influences on the development of social competence and problem behaviours, such as parenting practices and parental psychopathology or personality. In fact, although we attempted to account for maternal psychopathology by controlling for maternal postpartum depression in one of the sensitivity analyses, we must note that postpartum depression represents only a limited facet of the complex pattern of parental psychopathology. In addition, although by using relative PUFA concentrations we automatically accounted for all assayed saturated and mono-unsaturated fatty acid concentrations in maternal blood during pregnancy, we were not able to control for other dietary macro- and micro-nutrients. Given the intercorrelation between food groups within a dietary pattern, it cannot be excluded that other nutrients have not affected the examined associations. Finally, no data were available regarding maternal physical activity during pregnancy, which are correlated with dietary patterns in pregnancy (75) and affect fatty-acid mobilisation from the adipose tissue (80). Physical activity during pregnancy might therefore have affected the relative change in PUFA levels over time, thus confounding the associations examined.

Conclusions

In conclusion, this study suggests that a rise in maternal AA and a reduction in total n-6 in late pregnancy might be associated with small improvements in childhood social competence and

externalising behaviours, respectively, at 7 years of age. In contrast, changes in other PUFAs during pregnancy were not associated with social competence or problem behaviour scale in school-age children. However, associations were not statistically significant after accounting for multiple testing and could therefore be spurious findings. Overall, there is limited evidence of an effect of prenatal PUFA exposure on psychological outcomes. Future studies investigating the possible influence of maternal nutrition on childhood social competence or problem behaviours should consider more comprehensive measures of prenatal exposure, and account for common determinants of maternal diet and child outcomes.

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Appendix to chapter 5

Non-response analysis

Table A5.1: Baseline maternal and children characteristics divided by participation status

	Participants	Non-participants	
Maternal characteristics	**Mean (SD), median (IQR) or n (%)**	**Mean (SD), median (IQR) or n (%)**	**p-value**
Age at delivery	29.7 (3.9)	29.2 (4.4)	0.052
Smoking during pregnancy	68 (22.1%)	258 (27.3%)	0.069
Level of education			0.084
High	78 (36.28%)	21 (25.93%)	
Middle	92 (42.79%)	34 (41.98%)	
Low	45 (20.93%)	26 (32.10%)	
Employment status			0.788
Both full-time	12 (5.48%)	3 (3.57%)	
One full-time, one part-time	119 (54.34%)	47 (55.95%)	
Both part-time	88 (40.18%)	34 (40.48%)	
Parity (% primiparas)	221 (71.06%)	724 (69.28%)	0.549
BMI at study entry	22.9 (21.5; 25.1)	23.4 (21.3; 26.2)	0.036
Weight gain	11.3 (3.8)	11.6 (4.5)	0.364
Pregnancy outcomes			0.142
Adequate for gestational age	243 (78.90%)	734 (79.61%)	
Small for gestational age	29 (9.42%)	110 (11.93%)	
Large for gestational age	36 (11.69%)	78 (8.46%)	
Breastfeeding (% ever)	99 (45.41%)	46 (53.49%)	0.204
Total n-6			
First trimester	35.54 (2.11)	35.57 (1.93)	0.823
Second trimester	35.22 (1.97)	34.98 (1.98)	0.079
Third trimester	35.07 (1.78)	34.88 (1.91)	0.135
Partus	34.29 (1.84)	34.25 (2.18)	0.816
Total n-3			
First trimester	5.79 (1.25)	5.64 (1.19)	0.072
Second trimester	5.65 (1.18)	5.53 (1.12)	0.123
Third trimester	5.32 (0.91)	5.28 (1.01)	0.565
Partus	5.17 (0.99)	5.14 (0.99)	0.657
n6:n3			
First trimester	6.44 (1.49)	6.61 (1.51)	0.102
Second trimester	6.48 (1.35)	6.60 (1.49)	0.225
Third trimester	6.81 (1.32)	6.87 (1.47)	0.532
Partus	6.90 (1.48)	6.95 (1.49)	0.660
Children's characteristics			
Sex (% males)	163 (52.41%)	534 (55.45%)	0.349
Gestational age (weeks)	40.01 (1.7)	39.75 (2.5)	0.101
Birth weight (g)	3347 (515)	3198 (579)	<0.001

Overview of all PUFAs assayed within MEFAB

Table A5.2: Assayed n-6 and n-3 PUFAs

PUFA (wt%)	First trimester	Second trimester	Third trimester	Partus
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
n-6				
18:2 n-6 (Linoleic acid)	21.44 (2.77)	21.64 (2.56)	22.02 (2.14)	20.81 (2.39)
18:3 n-6 (Gamma-linolenic acid)	0.03 (0.02)	0.03 (0.02)	0.03 (0.02)	0.03 (0.02)
20:2 n-6 (Eicosadienoic acid)	0.46 (0.09)	0.54 (0.08)	0.50 (0.08)	0.44 (0.08)
20:3 n-6 (Dihomo-gamma-linolenic acid)	3.09 (0.59)	3.34 (0.57)	3.33 (0.56)	3.45 (0.60)
20:4 n-6 (Arachidonic acid, AA)	9.59 (1.43)	8.62 (1.31)	8.15 (1.19)	8.44 (1.39)
22:2 n-6 (Docosadienoic acid)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
22:4 n-6 (Adrenic acid)	0.39 (0.08)	0.40 (0.09)	0.37 (0.08)	0.38 (0.08)
22:5 n-6 (Docosapentaenoic acid, Osbond acid)	0.35 (0.12)	0.46 (0.15)	0.48 (0.15)	0.53 (0.17)
24:2 n-6	0.17 (0.06)	0.17 (0.06)	0.17 (0.06)	0.18 (0.07)
n-3				
18:3 n-3 (Alpha-linolenic acid)	0.21 (0.14)	0.24 (0.12)	0.25 (0.11)	0.21 (0.10)
20:3 n-3 (Eicosatrienoic acid)	0.04 (0.03)	0.04 (0.02)	0.03 (0.02)	0.02 (0.02)
20:4 n-3 (Eicosatetraenoic acid)	0.14 (0.06)	0.15 (0.07)	0.14 (0.06)	0.13 (0.06)
20:5 n-3 (Eicosapentaenoic acid, EPA)	0.55 (0.40)	0.41 (0.34)	0.34 (0.18)	0.34 (0.21)
22:3 n-3	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
22:5 n-3 (Docosapentaenoic acid)	0.76 (0.17)	0.63 (0.13)	0.57 (0.11)	0.55 (0.12)
22:6 n-3 (Docosahexaenoic acid, DHA)	4.09 (0.91)	4.19 (0.85)	3.99 (0.72)	3.90 (0.76)

Full-model-estimate tables

Table A5.3A: Full-model estimates of the associations between maternal gestational AA and social competence, presented by gestational period

Variables	β	95% CI
AA "early"	-19.16	-70.16, 31.83
AA "late"	24.48	2.61, 46.36
Mother's age	-0.05	-0.34, 0.24
Smoking (yes)	0.79	-1.74, 3.32
Pre-pregnancy BMI	0.02	-0.26, 0.29
Child's age	-0.92	-5.12, 3.27
Child's sex (female)	2.09	-2.49, 6.67
Employment (full time/part time)	-1.39	-6.23, 3.45
Employment (both part time)	-3.13	-8.07, 1.80
Parent's education (medium)	4.62	1.68, 7.56
Parent's education (high)	5.77	2.38, 9.16
Parity (at least one child)	0.63	-1.66, 2.91
Breastfeeding (ever)	1.49	-0.80, 3.78
AA at baseline	-0.12	-1.20, 0.97
Constant	48.12	13.28, 82.95

Table A5.3B: Full-model estimates of the associations between maternal gestational EPA and social competence, presented by gestational period

Variables	β	95% CI
EPA "early"	48.11	-336.62, 432.84
EPA "late"	-167.72	-976.68, 641.24
Mother's age	-0.09	-0.38, 0.20
Smoking (yes)	0.64	-1.90, 3.18
Pre-pregnancy BMI	0.07	-0.21, 0.34
Child's age	-1.04	-5.26, 3.17
Child's sex (female)	3.48	-0.01, 6.97
Employment (full time/part time)	-1.36	-6.31, 3.60
Employment (both part time)	-2.83	-7.90, 2.25
Parent's education (medium)	4.73	1.76, 7.69
Parent's education (high)	5.93	2.51, 9.34
Parity (at least one child)	0.61	-1.66, 2.88
Breastfeeding (ever)	1.60	-0.71, 3.91
EPA at baseline	3.26	-15.82, 22.34
Constant	47.74	13.97, 81.51

Table A5.3C: Full-model estimates of the associations between maternal gestational DHA and social competence, presented by gestational period

Variables	β	95% CI
DHA "early"	-11.18	-75.08, 52.72
DHA "late"	79.92	-31.47, 191.31
Mother's age	-0.06	-0.35, 0.23
Smoking (yes)	0.82	-1.69, 3.32
Pre-pregnancy BMI	0.03	-0.25, 0.30
Child's age	-1.42	-5.55, 2.70
Child's sex (female)	0.33	-3.70, 4.36
Employment (full time/part time)	-1.69	-6.59, 3.20
Employment (both part time)	-3.44	-8.51, 1.64
Parent's education (medium)	4.55	1.63, 7.46
Parent's education (high)	5.75	2.39, 9.11
Parity (at least one child)	0.87	-1.40, 3.14
Breastfeeding (ever)	1.36	-0.93, 3.65
DHA at baseline	0.70	-0.76, 2.15
Constant	51.81	17.93, 85.68

Table A5.3D: Full-model estimates of the associations between maternal gestational total n-6 and social competence, presented by gestational period

Variables	β	95% CI
Total n-6 "early"	22.04	-19.11, 63.19
Total n-6 "late"	-16.45	-69.94, 37.05
Mother's age	-0.11	-0.41, 0.18
Smoking (yes)	0.61	-1.95, 3.16
Pre-pregnancy BMI	0.03	-0.26, 0.31
Child's age	-1.53	-5.82, 2.76
Child's sex (female)	1.23	-4.10, 6.57
Employment (full time/part time)	-1.60	-6.53, 3.33
Employment (both part time)	-3.19	-8.24, 1.87
Parent's education (medium)	4.74	1.79, 7.69
Parent's education (high)	5.98	2.63, 9.34
Parity (at least one child)	0.77	-1.53, 3.07
Breastfeeding (ever)	1.54	-0.76, 3.84
n-6 at baseline	-0.12	-0.71, 0.46
Constant	58.78	16.13, 101.43

Table A5.3E: Full-model estimates of the associations between maternal gestational total n-3 and social competence, presented by gestational period

Variables	β	95% CI
Total n-3 "early"	-2.09	-21.92, 17.73
Total n-3 "late"	105.71	-51.03, 262.46
Mother's age	-0.08	-0.37, 0.21
Smoking (yes)	0.46	-2.08, 3.00
Pre-pregnancy BMI	0.01	-0.26, 0.29
Child's age	-1.33	-5.50, 2.84
Child's sex (female)	0.65	-3.87, 5.16
Employment (full time/part time)	-1.69	-6.64, 3.26
Employment (both part time)	-3.30	-8.41, 1.82
Parent's education (medium)	4.45	1.49, 7.42
Parent's education (high)	5.60	2.22, 8.99
Parity (at least one child)	0.76	-1.53, 3.04
Breastfeeding (ever)	1.33	-0.98, 3.65
n-3 at baseline	1.40	-0.14, 2.94
Constant	47.72	14.47, 80.97

Table A5.3F: Full-model estimates of the associations between maternal gestational n-6:n-3 and social competence, presented by gestational period

Variables	β	95% CI
n-6:n-3 "early"	10.01	-8.36, 28.38
n-6:n-3 "late"	-55.01	-136.02, 26.00
Mother's age	-0.08	-0.37, 0.21
Smoking (yes)	0.50	-2.04, 3.03
Pre-pregnancy BMI	0.02	-0.26, 0.30
Child's age	-1.37	-5.53, 2.80
Child's sex (female)	-0.12	-3.82, 3.59
Employment (full time/part time)	-1.74	-6.71, 3.22
Employment (both part time)	-3.27	-8.38, 1.83
Parent's education (medium)	4.66	1.72, 7.60
Parent's education (high)	5.82	2.45, 9.18
Parity (at least one child)	0.86	-1.43, 3.15
Breastfeeding (ever)	1.31	-1.01, 3.62
n-6:n-3 at baseline	-0.68	-1.42, 0.06
Constant	59.35	24.68, 94.01

Table A5.4A: Full-model estimates of the associations between maternal gestational AA and total problems, presented by gestational period

Variables	β	95% CI
AA "early"	-8.09	-74.37, 58.19
AA "late"	7.10	-21.23, 35.42
Mother's age	-0.29	-0.67, 0.09
Smoking (yes)	4.25	0.97, 7.53
Pre-pregnancy BMI	0.00	-0.36, 0.37
Child's age	3.15	-2.94, 9.24
Child's sex (female)	-0.25	-6.14, 5.63
Employment (full time/part time)	-1.42	-8.18, 5.34
Employment (both part time)	-0.72	-7.53, 6.10
Parent's education (medium)	-1.54	-5.48, 2.40
Parent's education (high)	-1.98	-6.60, 2.63
Parity (at least one child)	-1.62	-4.56, 1.31
Breastfeeding (ever)	2.07	-1.08, 5.22
AA at baseline	0.38	-1.01, 1.78
Constant	33.63	-15.48, 82.74

Table A5.4B: Full-model estimates of the associations between maternal gestational EPA and total problems, presented by gestational period

Variables	β	95% CI
EPA "early"	336.14	-157.23, 829.51
EPA "late"	594.71	-442.98, 1632.41
Mother's age	-0.32	-0.69, 0.06
Smoking (yes)	4.10	0.82, 7.38
Pre-pregnancy BMI	0.05	-0.30, 0.40
Child's age	2.75	-3.19, 8.70
Child's sex (female)	-1.13	-5.59, 3.32
Employment (full time/part time)	-1.51	-8.17, 5.14
Employment (both part time)	-0.80	-7.50, 5.90
Parent's education (medium)	-1.53	-5.53, 2.48
Parent's education (high)	-1.66	-6.42, 3.10
Parity (at least one child)	-1.48	-4.38, 1.42
Breastfeeding (ever)	2.28	-0.86, 5.41
EPA at baseline	16.82	-7.61, 41.25
Constant	37.77	-8.58, 84.13

Table A5.4C: Full-model estimates of the associations between maternal gestational DHA and total problems, presented by gestational period

Variables	β	95% CI
DHA "early"	-24.93	-107.26, 57.40
DHA "late"	11.49	-130.42, 153.39
Mother's age	-0.28	-0.66, 0.09
Smoking (yes)	4.04	0.80, 7.28
Pre-pregnancy BMI	0.06	-0.30, 0.42
Child's age	2.67	-3.37, 8.70
Child's sex (female)	-3.83	-8.97, 1.30
Employment (full time/part time)	-1.53	-8.21, 5.15
Employment (both part time)	-0.81	-7.57, 5.95
Parent's education (medium)	-1.48	-5.41, 2.45
Parent's education (high)	-1.80	-6.40, 2.79
Parity (at least one child)	-1.73	-4.64, 1.18
Breastfeeding (ever)	2.24	-0.89, 5.37
DHA at baseline	-0.75	-2.63, 1.13
Constant	43.65	-4.68, 91.98

Table A5.4D: Full-model estimates of the associations between maternal gestational total n-6 and total problems, presented by gestational period

Variables	β	95% CI
Total n-6 "early"	5.68	-46.54, 57.91
Total n-6 "late"	51.03	-16.48, 118.54
Mother's age	-0.33	-0.71, 0.05
Smoking (yes)	4.16	0.87, 7.45
Pre-pregnancy BMI	0.06	-0.30, 0.43
Child's age	3.43	-2.59, 9.44
Child's sex (female)	-2.79	-9.53, 3.94
Employment (full time/part time)	-1.42	-8.20, 5.36
Employment (both part time)	-0.54	-7.33, 6.25
Parent's education (medium)	-1.51	-5.45, 2.43
Parent's education (high)	-1.79	-6.40, 2.81
Parity (at least one child)	-1.97	-4.88, 0.95
Breastfeeding (ever)	2.45	-0.66, 5.56
n-6 at baseline	0.71	-0.03, 1.46
Constant	14.17	-43.19, 71.53

Table A5.4E: Full-model estimates of the associations between maternal gestational total n-3 and total problems, presented by gestational period

Variables	β	95% CI
Total n-3 "early"	-4.90	-30.25, 20.45
Total n-3 "late"	-40.09	-240.76, 160.58
Mother's age	-0.32	-0.69, 0.06
Smoking (yes)	4.03	0.75, 7.32
Pre-pregnancy BMI	0.03	-0.33, 0.39
Child's age	2.71	-3.26, 8.69
Child's sex (female)	-3.45	-9.22, 2.31
Employment (full time/part time)	-1.65	-8.29, 5.00
Employment (both part time)	-0.91	-7.61, 5.79
Parent's education (medium)	-1.68	-5.67, 2.31
Parent's education (high)	-2.06	-6.72, 2.60
Parity (at least one child)	-1.75	-4.67, 1.16
Breastfeeding (ever)	2.34	-0.81, 5.49
n-3 at baseline	-0.68	-2.65, 1.30
Constant	44.98	-1.19, 91.14

Table A5.4F: Full-model estimates of the associations between maternal gestational n-6:n-3 and total problems, presented by gestational period

Variables	β	95% CI
n-6:n-3 "early"	8.17	-15.13, 31.47
n-6:n-3 "late"	13.02	-90.44, 116.48
Mother's age	-0.32	-0.70, 0.05
Smoking (yes)	3.99	0.72, 7.26
Pre-pregnancy BMI	0.05	-0.31, 0.42
Child's age	3.02	-2.96, 9.00
Child's sex (female)	-3.33	-8.03, 1.36
Employment (full time/part time)	-1.62	-8.29, 5.05
Employment (both part time)	-0.79	-7.48, 5.90
Parent's education (medium)	-1.64	-5.62, 2.33
Parent's education (high)	-1.98	-6.60, 2.64
Parity (at least one child)	-1.74	-4.65, 1.18
Breastfeeding (ever)	2.42	-0.70, 5.53
n-6:n-3 at baseline	0.54	-0.40, 1.49
Constant	35.72	-12.44, 83.88

Table A5.5A: Full-model estimates of the associations between maternal gestational AA and internalising behaviours, presented by gestational period

Variables	β	95% CI
AA "early"	-29.98	-91.27, 31.30
AA "late"	9.50	-16.59, 35.60
Mother's age	-0.17	-0.52, 0.18
Smoking (yes)	2.80	-0.23, 5.82
Pre-pregnancy BMI	-0.09	-0.43, 0.24
Child's age	1.90	-3.32, 7.12
Child's sex (female)	0.37	-5.06, 5.80
Employment (full time/part time)	-0.63	-6.64, 5.39
Employment (both part time)	-1.85	-7.89, 4.18
Parent's education (medium)	-0.86	-4.64, 2.92
Parent's education (high)	-0.77	-5.11, 3.58
Parity (at least one child)	-3.57	-6.28, -0.85
Breastfeeding (ever)	2.57	-0.30, 5.43
AA at baseline	0.30	-0.98, 1.58
Constant	43.17	0.62, 85.72

Table A5.5B: Full-model estimates of the associations between maternal gestational EPA and internalising behaviours, presented by gestational period

Variables	β	95% CI
EPA "early"	135.23	-325.90, 596.36
EPA "late"	159.23	-806.82, 1125.29
Mother's age	-0.19	-0.54, 0.16
Smoking (yes)	2.68	-0.36, 5.71
Pre-pregnancy BMI	-0.05	-0.37, 0.28
Child's age	1.55	-3.48, 6.58
Child's sex (female)	-3.04	-7.17, 1.09
Employment (full time/part time)	-0.53	-6.50, 5.44
Employment (both part time)	-1.72	-7.72, 4.27
Parent's education (medium)	-1.03	-4.87, 2.81
Parent's education (high)	-0.67	-5.15, 3.81
Parity (at least one child)	-3.38	-6.07, -0.69
Breastfeeding (ever)	2.71	-0.16, 5.58
EPA at baseline	7.74	-15.10, 30.59
Constant	48.26	8.50, 88.03

Table A5.5C: Full-model estimates of the associations between maternal gestational DHA and internalising behaviours, presented by gestational period

Variables	β	95% CI
DHA "early"	-56.52	-132.56, 19.52
DHA "late"	17.28	-114.25, 148.82
Mother's age	-0.16	-0.50, 0.19
Smoking (yes)	2.68	-0.32, 5.68
Pre-pregnancy BMI	-0.03	-0.36, 0.31
Child's age	1.15	-3.99, 6.29
Child's sex (female)	-5.96	-10.72, -1.20
Employment (full time/part time)	-0.60	-6.58, 5.39
Employment (both part time)	-1.87	-7.91, 4.16
Parent's education (medium)	-0.79	-4.57, 2.99
Parent's education (high)	-0.35	-4.70, 4.00
Parity (at least one child)	-3.68	-6.38, -0.98
Breastfeeding (ever)	2.63	-0.21, 5.48
DHA at baseline	-1.33	-3.07, 0.41
Constant	58.35	16.79, 99.92

Table A5.5D: Full-model estimates of the associations between maternal gestational total n-6 and internalising behaviours, presented by gestational period

Variables	β	95% CI
Total n-6 "early"	-2.41	-51.00, 46.17
Total n-6 "late"	36.46	-26.21, 99.13
Mother's age	-0.20	-0.55, 0.15
Smoking (yes)	2.93	-0.11, 5.98
Pre-pregnancy BMI	-0.01	-0.35, 0.32
Child's age	2.04	-3.08, 7.16
Child's sex (female)	-4.72	-10.98, 1.55
Employment (full time/part time)	-0.54	-6.58, 5.49
Employment (both part time)	-1.53	-7.54, 4.48
Parent's education (medium)	-0.76	-4.55, 3.03
Parent's education (high)	-0.40	-4.75, 3.96
Parity (at least one child)	-3.65	-6.36, -0.94
Breastfeeding (ever)	2.81	-0.02, 5.64
n-6 at baseline	0.51	-0.17, 1.20
Constant	30.06	-19.73, 79.86

Table A5.5E: Full-model estimates of the associations between maternal gestational total n-3 and internalising behaviours, presented by gestational period

Variables	β	95% CI
Total n-3 "early"	-11.59	-35.06, 11.88
Total n-3 "late"	-22.71	-208.70, 163.27
Mother's age	-0.20	-0.55, 0.15
Smoking (yes)	2.71	-0.33, 5.76
Pre-pregnancy BMI	-0.05	-0.38, 0.28
Child's age	1.35	-3.72, 6.42
Child's sex (female)	-5.40	-10.76, -0.04
Employment (full time/part time)	-0.68	-6.63, 5.27
Employment (both part time)	-1.90	-7.86, 4.06
Parent's education (medium)	-0.95	-4.79, 2.88
Parent's education (high)	-0.60	-4.98, 3.79
Parity (at least one child)	-3.60	-6.31, -0.88
Breastfeeding (ever)	2.72	-0.15, 5.58
n-3 at baseline	-0.41	-2.23, 1.41
Constant	54.57	14.81, 94.33

Table A5.5F: Full-model estimates of the associations between maternal gestational n-6:n-3 and internalising behaviours, presented by gestational period

Variables	β	95% CI
n-6:n-3 "early"	14.78	-6.79, 36.35
n-6:n-3 "late"	8.75	-87.13, 104.63
Mother's age	-0.20	-0.55, 0.14
Smoking (yes)	2.88	-0.13, 5.90
Pre-pregnancy BMI	-0.01	-0.34, 0.33
Child's age	1.75	-3.30, 6.80
Child's sex (female)	-6.45	-10.80, -2.09
Employment (full time/part time)	-0.79	-6.72, 5.14
Employment (both part time)	-1.74	-7.64, 4.16
Parent's education (medium)	-0.76	-4.56, 3.04
Parent's education (high)	-0.32	-4.65, 4.00
Parity (at least one child)	-3.46	-6.16, -0.76
Breastfeeding (ever)	2.76	-0.06, 5.58
n-6:n-3 at baseline	0.54	-0.33, 1.41
Constant	45.35	4.46, 86.24

Table A5.6A: Full-model estimates of the associations between maternal gestational AA and externalising behaviours, presented by gestational period

Variables	β	95% CI
AA "early"	-7.86	-72.44, 56.73
AA "late"	8.83	-18.60, 36.27
Mother's age	-0.27	-0.63, 0.10
Smoking (yes)	2.39	-0.78, 5.56
Pre-pregnancy BMI	0.19	-0.16, 0.54
Child's age	1.53	-4.52, 7.58
Child's sex (female)	1.20	-4.50, 6.90
Employment (full time/part time)	1.07	-5.32, 7.46
Employment (both part time)	2.69	-3.86, 9.24
Parent's education (medium)	-0.97	-4.79, 2.85
Parent's education (high)	-2.18	-6.74, 2.38
Parity (at least one child)	0.68	-2.15, 3.52
Breastfeeding (ever)	1.47	-1.68, 4.62
AA at baseline	-0.23	-1.60, 1.14
Constant	42.24	-6.53, 91.02

Table A5.6B: Full-model estimates of the associations between maternal gestational EPA and externalising behaviours, presented by gestational period

Variables	β	95% CI
EPA "early"	292.81	-188.98, 774.60
EPA "late"	352.52	-659.41, 1364.44
Mother's age	-0.27	-0.64, 0.09
Smoking (yes)	2.51	-0.65, 5.67
Pre-pregnancy BMI	0.22	-0.12, 0.55
Child's age	1.63	-4.32, 7.59
Child's sex (female)	0.75	-3.55, 5.05
Employment (full time/part time)	1.05	-5.30, 7.41
Employment (both part time)	2.92	-3.62, 9.45
Parent's education (medium)	-0.71	-4.56, 3.15
Parent's education (high)	-1.60	-6.28, 3.07
Parity (at least one child)	0.72	-2.07, 3.51
Breastfeeding (ever)	1.46	-1.69, 4.62
EPA at baseline	13.21	-10.65, 37.06
Constant	37.15	-9.27, 83.56

Table A5.6C: Full-model estimates of the associations between maternal gestational DHA and externalising behaviours, presented by gestational period

Variables	β	95% CI
DHA "early"	-7.96	-88.11, 72.19
DHA "late"	-38.86	-176.46, 98.74
Mother's age	-0.28	-0.64, 0.09
Smoking (yes)	2.24	-0.88, 5.37
Pre-pregnancy BMI	0.21	-0.13, 0.56
Child's age	1.48	-4.52, 7.48
Child's sex (female)	-0.80	-5.78, 4.17
Employment (full time/part time)	0.85	-5.48, 7.19
Employment (both part time)	2.65	-3.92, 9.21
Parent's education (medium)	-0.81	-4.58, 2.96
Parent's education (high)	-1.96	-6.47, 2.54
Parity (at least one child)	0.33	-2.47, 3.14
Breastfeeding (ever)	1.55	-1.57, 4.67
DHA at baseline	-0.50	-2.33, 1.32
Constant	42.67	-5.54, 90.88

Table A5.6D: Full-model estimates of the associations between maternal gestational total n-6 and externalising behaviours, presented by gestational period

Variables	β	95% CI
Total n-6 "early"	-11.01	-61.12, 39.11
Total n-6 "late"	82.65	17.71, 147.59
Mother's age	-0.30	-0.66, 0.07
Smoking (yes)	2.21	-0.93, 5.36
Pre-pregnancy BMI	0.20	-0.15, 0.55
Child's age	2.20	-3.83, 8.22
Child's sex (female)	-0.97	-7.43, 5.49
Employment (full time/part time)	0.97	-5.45, 7.38
Employment (both part time)	2.86	-3.73, 9.46
Parent's education (medium)	-0.95	-4.74, 2.83
Parent's education (high)	-2.10	-6.62, 2.41
Parity (at least one child)	0.08	-2.71, 2.87
Breastfeeding (ever)	1.76	-1.35, 4.87
n-6 at baseline	0.72	0.00, 1.44
Constant	17.00	-40.59, 74.60

Table A5.6E: Full-model estimates of the associations between maternal gestational total n-3 and externalising behaviours, presented by gestational period

Variables	β	95% CI
Total n-3 "early"	5.79	-18.84, 30.42
Total n-3 "late"	-94.92	-289.78, 99.93
Mother's age	-0.29	-0.66, 0.07
Smoking (yes)	2.30	-0.86, 5.47
Pre-pregnancy BMI	0.19	-0.16, 0.53
Child's age	1.65	-4.27, 7.58
Child's sex (female)	-0.40	-5.98, 5.18
Employment (full time/part time)	0.81	-5.51, 7.13
Employment (both part time)	2.62	-3.92, 9.17
Parent's education (medium)	-1.01	-4.84, 2.82
Parent's education (high)	-2.23	-6.81, 2.34
Parity (at least one child)	0.33	-2.48, 3.14
Breastfeeding (ever)	1.71	-1.45, 4.87
n-3 at baseline	-1.20	-3.12, 0.72
Constant	45.50	-0.34, 91.34

Table A5.6F: Full-model estimates of the associations between maternal gestational n-6:n-3 and externalising behaviours, presented by gestational period

Variables	β	95% CI
n-6:n-3 "early"	-3.85	-26.42, 18.72
n-6:n-3 "late"	34.49	-65.68, 134.67
Mother's age	-0.30	-0.67, 0.06
Smoking (yes)	2.10	-1.06, 5.25
Pre-pregnancy BMI	0.19	-0.16, 0.54
Child's age	1.73	-4.22, 7.69
Child's sex (female)	0.32	-4.22, 4.87
Employment (full time/part time)	0.91	-5.46, 7.27
Employment (both part time)	2.61	-3.94, 9.16
Parent's education (medium)	-1.14	-4.95, 2.68
Parent's education (high)	-2.35	-6.88, 2.19
Parity (at least one child)	0.25	-2.55, 3.06
Breastfeeding (ever)	1.77	-1.36, 4.89
n-6:n-3 at baseline	0.53	-0.40, 1.45
Constant	36.59	-11.64, 84.81

Associations between AA and social competence by children's sex

Table A5.7: Associations between AA and social competence by children's sex

Period of pregnancy	Males	Females
Early	1.77 (-60.63, 64.16)	-47.53 (-103.39, 8.33)
Late	21.60 (-2.65, 45.85)	-5.50 (-28.87, 17.87)

Note: n=163 (males) and 148 (females); estimates are presented as betas (95% C.I.); adjusted for: first trimester maternal AA concentration; mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, and child's age at follow-up.

Sensitivity analyses

Table A5.8: Results of sensitivity analyses (outcome: social competence)

Sensitivity analyses	Period of pregnancy	PUFAs		
		AA	**EPA**	**DHA**
Adjusted for birth weight and gestational age[a]	Early	-24.93 (-77.66, 27.79)	26.51 (-359.78, 412.80)	-12.03 (-75.80, 51.74)
	Late	25.06 (3.26, 46.86)*	-204.13 (-1012.65, 604.38)	75.94 (-36.71, 188.59)
Adjusted for maternal post-partum depression[a]	Early	-21.64 (-72.44, 29.17)	89.29 (-292.57, 471.15)	-12.53 (-76.29, 51.23)
	Late	26.81 (5.13, 48.49)*	-77.43 (-878.95, 724.10)	83.78 (-27.55, 195.10)
Adjusted for child PUFAs[a,b]	Early	-18.62 (-69.70, 32.43)	76.73 (-311.91, 465.38)	-12.18 (-76.38, 52.01)
	Late	25.16 (3.36, 46.96)*	-114.00 (-925.39, 697.39)	80.31 (31.49, 192.11)
Adjusted for day-care attendance[a]	Early	-14.23 (-65.70, 37.24)	27.64 (-359.20, 414.49)	-8.52 (-72.41, 55.37)
	Late	24.83 (2.96, 46.69)*	-209.72 (-1022.69, 603.24)	81.39 (-29.61, 192.39)
Complete-case analyses[c]	Early	-9.50 (-77.60, 58.59)	185.49 (-392.15, 763.13)	-15.60 (-99.95, 68.76)
	Late	31.54 (3.23, 59.85)*	-86.33 (-1250.89, 1078.22)	69.72 (-92.91, 232.36)

Table A5.8 (continued)

Sensitivity analyses	Period of pregnancy	PUFAs		
		Total n-6	**Total n-3**	**n-6:n-3**
Adjusted for birth weight and gestational age[a]	Early	26.54 (-14.70, 67.79)	-2.34 (-22.11, 17.43)	10.47 (-7.82, 28.76)
	Late	-15.61 (-68.79, 37.58)	102.47 (-54.42, 259.37)	-48.40 (-130.02, 33.22)
Adjusted for maternal post-partum depression[a]	Early	22.42 (-18.69, 63.54)	-2.56 (-22.31, 17.18)	10.52 (-7.77, 28.81)
	Late	-13.06 (-66.26, 40.14)	105.84 (-51.08, 262.75)	-52.61 (-133.58, 28.37)
Adjusted for child PUFAs[a,b]	Early	22.72 (-18.59, 64.03)	-2.05 (-22.06, 17.96)	10.25 (-8.28, 28.78)
	Late	-14.32 (-68.13, 39.50)	106.66 (-51.45, 264.77)	-52.91 (-135.33, 29.50)
Adjusted for day-care attendance[a]	Early	22.90 (-18.11, 63.91)	-0.56 (-20.44, 19.32)	9.29 (-9.02, 27.60)
	Late	-17.55 (-70.77, 35.68)	103.58 (-52.79, 259.95)	-57.42 (-138.38, 23.55)
Complete-case analyses[c]	Early	16.51 (-34.11, 67.13)	-5.24 (-30.51, 20.03)	18.10 (-5.61, 41.82)
	Late	-13.63 (-81.28, 54.03)	146.13 (-63.44, 355.71)	-74.02 (-182.77, 34.73)

Note: All models included first- and last-trimester index-PUFA change, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; a: imputed dataset (n=311); b: additionally adjusted for child PUFA; c: n=192; *: p<0.05; **: p<0.01

Table A5.9: Results of sensitivity analyses (outcome: total problems)

Sensitivity analyses	Period of pregnancy	PUFAs		
		AA	EPA	DHA
Adjusted for birth weight and gestational age[a]	Early	0.87 (-67.78, 69.51)	369.92 (-124.35, 864.19)	-26.85 (-109.08, 55.38)
	Late	8.73 (-19.58, 37.05)	645.96 (-391.46, 1683.39)	20.78 (-122.59, 164.15)
Adjusted for maternal post-partum depression[a]	Early	-6.75 (-73.14, 59.64)	309.36 (-183.39, 802.11)	-25.23 (-107.22, 56.76)
	Late	6.72 (-21.47, 34.91)	530.81 (-505.59, 1567.21)	9.78 (-132.02, 151.58)
Adjusted for child PUFAs[a,b]	Early	-8.13 (-74.36, 58.11)	345.12 (-148.42, 838.66)	-24.76 (-107.63, 58.11)
	Late	8.01 (-20.08, 36.09)	587.73 (-449.43, 1624.89)	12.63 (-130.36, 155.62)
Complete-case analyses[c]	Early	13.60 (-69.98, 97.18)	636.08 (-73.20, 1345.36)	-54.71 (-158.23, 48.81)
	Late	24.23 (-10.52, 58.99)	1407.43 (-22.51, 2837.37)	87.00 (-112.60, 286.59)

Table A5.9 (continued)

Sensitivity analyses	Period of pregnancy	PUFA		
		Total n-6	**Total n-3**	**n-6:n-3**
Adjusted for birth weight and gestational age[a]	Early	2.21 (-50.14, 54.57)	-4.86 (-30.21, 20.49)	7.74 (-15.48, 30.96)
	Late	51.79 (-15.52, 119.11)	-35.34 (-236.17, 165.49)	7.29 (-96.80, 111.39)
Adjusted for maternal post-partum depression[a]	Early	5.72 (-46.44, 57.88)	-4.78 (-30.07, 20.51)	7.78 (-15.47, 31.02)
	Late	49.80 (-17.58, 117.18)	-38.86 (-239.64, 161.93)	12.58 (-90.64, 115.79)
Adjusted for child PUFAs[a,b]	Early	4.00 (-48.27, 56.28)	-4.06 (-29.57, 21.45)	7.88 (-15.65, 31.41)
	Late	54.60 (-13.20, 122.39)	-34.30 (-237.73, 169.12)	7.95 (-97.50, 113.40)
Complete-case analyses[c]	Early	24.38 (-36.62, 85.37)	-20.44 (-51.32, 10.44)	22.56 (-6.46, 51.57)
	Late	63.78 (-17.74, 145.31)	64.78 (-191.34, 320.89)	-5.14 (-138.18, 127.91)

Note: All models included first- and last-trimester index-PUFA change, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; a: imputed dataset (n=311); b: additionally adjusted for child PUFA; c: n=192; *: p<0.05; **: p<0.01

Table A5.10: Results of sensitivity analyses (outcome: internalising behaviours)

Sensitivity analyses	Period of pregnancy	PUFAs		
		AA	EPA	DHA
Adjusted for birth weight and gestational age[a]	Early	-17.78 (-80.98, 45.42)	183.22 (-276.22, 642.66)	-59.17 (-134.88, 16.54)
	Late	11.00 (-15.00, 36.99)	240.49 (-721.10, 1202.09)	32.60 (-99.86, 165.05)
Adjusted for maternal post-partum depression[a]	Early	-30.83 (-92.09, 30.42)	133.48 (-326.16, 593.12)	-55.02 (-130.84, 20.79)
	Late	9.52 (-16.42, 35.46)	157.24 (-806.55, 1121.03)	17.04 (-114.44, 148.53)
Adjusted for child PUFAs[a,b]	Early	-30.69 (-91.93, 30.55)	141.57 (-317.84, 600.97)	-59.33 (-135.89, 17.22)
	Late	9.59 (-16.22, 35.40)	156.87 (-805.31, 1119.05)	22.21 (-110.09, 154.52)
Complete-case analyses[c]	Early	-14.17 (-91.80, 63.46)	375.31 (-293.53, 1044.14)	-54.94 (-152.44, 42.55)
	Late	27.99 (-4.29, 60.27)	742.96 (-605.44, 2091.36)	51.78 (-136.19, 239.75)

Table A5.10 (continued)

Sensitivity analyses	Period of pregnancy	PUFAs		
		Total n-6	**Total n-3**	**n-6:n-3**
Adjusted for birth weight and gestational age[a]	Early	-6.61 (-55.16, 41.93)	-11.11 (-34.51, 12.30)	14.60 (-6.84, 36.03)
	Late	36.31 (-26.03, 98.65)	-16.16 (-201.76, 169.45)	-1.56 (-97.72, 94.59)
Adjusted for maternal post-partum depression[a]	Early	-3.15 (-51.64, 45.34)	-11.25 (-34.67, 12.16)	14.60 (-6.93, 36.12)
	Late	36.18 (-26.47, 98.83)	-23.71 (-209.68, 162.25)	7.41 (-88.31, 103.14)
Adjusted for child PUFAs[a,b]	Early	-3.90 (-52.42, 44.63)	-11.77 (-35.31, 11.78)	15.52 (-6.20, 37.23)
	Late	39.13 (-23.56, 101.81)	-13.62 (-201.44, 174.20)	2.22 (-95.13, 99.56)
Complete-case analyses[c]	Early	7.69 (-50.21, 65.59)	-12.48 (-41.74, 16.78)	13.58 (-13.88, 41.04)
	Late	50.09 (-27.29, 127.47)	12.38 (-230.26, 255.02)	18.04 (-107.87, 143.95)

Note: All models included first- and last-trimester index-PUFA change, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; a: imputed dataset (n=311); b: additionally adjusted for child PUFA; c: n=192; *: p<0.05; **: p<0.01

Table A5.11: Results of sensitivity analyses (outcome: externalising behaviours)

Sensitivity analyses	Period of pregnancy	PUFAs		
		AA	**EPA**	**DHA**
Adjusted for birth weight and gestational age[a]	Early	-3.17 (-70.01, 63.66)	322.65 (-160.92, 806.22)	-9.51 (-89.73, 70.71)
	Late	9.73 (-17.70, 37.17)	403.16 (-609.94, 1416.26)	-34.18 (-173.46, 105.10)
Adjusted for maternal post-partum depression[a]	Early	-6.41 (-71.03, 58.21)	277.69 (-202.56, 757.94)	-9.42 (-89.09, 70.24)
	Late	8.04 (-19.22, 35.30)	315.33 (-692.73, 1323.40)	-40.11 (-177.51, 97.30)
Adjusted for child PUFAs[a,b]	Early	-7.42 (-71.97, 57.13)	311.71 (-170.81, 794.23)	-4.94 (-85.42, 75.54)
	Late	9.19 (-17.98, 36.36)	378.91 (-632.71, 1390.52)	-41.26 (-179.89, 97.37)
Complete-case analyses[c]	Early	26.65 (-51.50, 104.80)	607.88 (-43.49, 1259.26)	-19.87 (-116.21, 76.48)
	Late	20.45 (-12.05, 52.94)	1068.94 (-244.27, 2382.14)	54.14 (-131.62, 239.90)

Table A5.11 (continued)

Sensitivity analyses	Period of pregnancy	PUFAs		
		Total n-6	**Total n-3**	**n-6:n-3**
Adjusted for birth weight and gestational age[a]	Early	-12.43 (-62.70, 37.84)	5.59 (-19.07, 30.26)	-3.91 (-26.45, 18.63)
	Late	83.05 (18.19, 147.90)*	-91.56 (-286.62, 103.50)	31.84 (-69.02, 132.70)
Adjusted for maternal post-partum depression[a]	Early	-10.20 (-60.14, 39.73)	5.49 (-19.04, 30.02)	-3.81 (-26.30, 18.67)
	Late	81.21 (16.50, 145.91)*	-92.50 (-287.16, 102.17)	34.83 (-64.89, 134.55)
Adjusted for child PUFAs[a,b]	Early	-12.10 (-62.18, 37.98)	6.84 (-17.92, 31.60)	-4.35 (-27.16, 18.46)
	Late	85.42 (20.05, 150.78)*	-95.45 (-292.96, 102.06)	33.24 (-68.90, 135.38)
Complete-case analyses[c]	Early	-11.08 (-66.91, 44.75)	-5.99 (-34.63, 22.66)	9.87 (-17.12, 36.87)
	Late	104.67 (30.05, 179.29)**	55.95 (-181.57, 293.47)	-17.69 (-141.47, 106.08)

Note: All models included first- and last-trimester index-PUFA change, index-PUFA concentration in the first trimester, mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; a: imputed dataset (n=311); b: additionally adjusted for child PUFA; c: n=192; *: p<0.05; **: p<0.01

Secondary analyses

Table A5.12: Results of multiple linear regressions between PUFAs measured at 4 time-points and social competence

PUFAs	First trimester (n=193)	Second trimester (n=181)	Third trimester (n=187)	Partus (n=187)
AA	1.00 (-0.15, 2.15)	1.28 (-0.07, 2.65)	1.14 (-0.34, 2.62)	1.63 (0.49, 2.77)**
EPA	-0.10 (-3.54, 3.35)	1.63 (-9.05, 12.30)	8.04 (0.31, 15.77)*	7.15 (-1.57, 15.87)
DHA	0.49 (-1.30, 2.28)	1.09 (-1.18, 3.35)	0.32 (-2.04, 2.67)	1.74 (-0.66, 4.15)
Total n-6	-0.03 (-0.80, 0.75)	0.11 (-0.74, 0.96)	0.05 (-0.85, 0.94)	0.39 (-0.55, 1.34)
Total n-3	0.21 (-1.05, 1.47)	0.45 (-1.35, 2.25)	0.76 (-0.98, 2.50)	1.45 (-0.40, 3.31)
n-6:n-3	-0.17 (-1.27, 0.92)	-0.21 (-1.53, 1.11)	-0.15 (-1.39, 1.09)	-0.43 (-1.64, 0.77)

Note: Complete-case dataset. Adjusted for: mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; * p≤0.05; ** p≤0.01.

Table A5.13: Results of multiple linear regressions between PUFAs measured at 4 time-points and total problems

PUFAs	First trimester (n=193)	Second trimester (n=181)	Third trimester (n=187)	Partus (n=187)
AA	0.83 (-0.58, 2.24)	-0.04 (-1.75, 1.68)	0.78 (-1.02, 2.57)	1.22 (-0.22, 2.65)
EPA	-2.43 (-6.65, 1.79)	-0.94 (-14.33, 12.44)	-6.73 (-16.13, 2.67)	-2.47 (-13.39, 8.45)
DHA	-1.08 (-3.28, 1.12)	-1.93 (-4.76, 0.90)	-1.61 (-4.44, 1.22)	-0.83 (-3.84, 2.17)
Total n-6	0.39 (-0.55, 1.34)	-0.02 (-1.08, 1.04)	0.54 (-0.53, 1.61)	1.09 (-0.06, 2.25)
Total n-3	-1.05 (-2.59, 0.50)	-1.10 (-3.35, 1.15)	-1.56 (-3.64, 0.52)	-1.00 (-3.33, 1.32)
n-6:n-3	0.71 (-0.64, 2.05)	0.57 (-1.07, 2.22)	0.91 (-0.57, 2.38)	0.72 (-0.78, 2.21)

Note: Complete-case dataset. Adjusted for: mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up.

Table A5.14: Results of multiple linear regressions between PUFAs measured at 4 time-points and internalising behaviour

PUFAs	First trimester (n=193)	Second trimester (n=181)	Third trimester (n=187)	Partus (n=187)
AA	1.33 (0.01, 2.65)*	-0.01 (-1.57, 1.56)	0.95 (-0.73, 2.63)	1.47 (0.15, 2.80)*
EPA	0.38 (-3.58, 4.35)	2.62 (-9.59, 14.82)	-1.90 (-10.74, 6.93)	-0.48 (-10.59, 9.64)
DHA	-0.68 (-2.75, 1.39)	-1.62 (-4.21, 0.96)	-1.27 (-3.92, 1.39)	-0.76 (-3.55, 2.03)
Total n-6	0.17 (-0.72, 1.06)	-0.15 (-1.12, 0.82)	0.19 (-0.82, 1.19)	0.58 (-0.51, 1.66)
Total n-3	-0.40 (-1.86, 1.05)	-0.70 (-2.75, 1.36)	-0.90 (-2.86, 1.06)	-0.71 (-2.86, 1.45)
n-6:n-3	0.35 (-0.91, 1.61)	0.49 (-1.02, 1.99)	0.58 (-0.82, 1.97)	0.58 (-0.82, 1.97)

Note: Complete-case dataset. Adjusted for: mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; * p≤0.05.

Table A5.15: Results of multiple linear regressions between PUFAs measured at 4 time-points and externalising behaviour

PUFAs	First trimester (n=193)	Second trimester (n=181)	Third trimester (n=187)	Partus (n=187)
AA	0.13 (-1.19, 1.45)	0.05 (-1.57, 1.68)	0.24 (-1.48, 1.95)	0.76 (-0.59, 2.11)
EPA	-4.17 (-8.05, -0.30)*	1.94 (-10.69, 14.57)	-1.94 (-10.93, 7.04)	-0.06 (-10.24, 10.12)
DHA	-0.85 (-2.88, 1.19)	-1.08 (-3.77, 1.60)	-1.35 (-4.05, 1.34)	-0.24 (-3.04, 2.56)
Total n-6	0.46 (-0.42, 1.34)	-0.38 (-1.39, 0.62)	0.05 (-0.97, 1.07)	1.13 (0.05, 2.20)*
Total n-3	-1.11 (-2.53, 0.32)	-0.39 (-2.52, 1.74)	-0.98 (-2.97, 1.01)	-0.43 (-2.60, 1.74)
n-6:n-3	0.76 (-0.48, 2.01)	0.11 (-1.46, 1.67)	0.50 (-0.91, 1.91)	0.49 (-0.91, 1.88)

Note: Complete-case dataset. Adjusted for: mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and child's age at follow-up; * p≤0.05.

Post-hoc analyses

Table A5.16: Results of the associations between LA and childhood social competence and problem behaviours

	Early pregnancy	Late pregnancy
	Beta (95% C.I.)	Beta (95% C.I.)
Social competence	54.55 (-155.74, 264.83)	-2938.62 (-11996.80, 6119.57)
Total problems	33.45 (-224.79, 291.69)	98.86 (-11025.25, 11222.97)
Internalising behaviours	75.30 (-167.26, 317.87)	-3025.51 (-13474.30, 7423.29)
Externalising behaviours	-93.49 (-331.97, 144.99)	5128.94 (-5143.70, 15401.58)

Note: Complete-case analyses, n=192; Cut-off points for different PUFAs derived from the identified best-fitting linear splines. For LA, the "late" pregnancy starts at week 30; b: Adjusted for: mother's age, smoking during pregnancy, maternal BMI at study entry, parental education, parental employment status, breastfeeding, parity, child's sex and age at follow-up. All models included early- and late-pregnancy LA concentration changes.

Chapter 6

Does diet quality during pregnancy affect childhood behaviour? A pooled analysis of two South-European birth cohorts

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Abstract

Background: Evidence from human and animal studies suggests that maternal diet during pregnancy might be associated with childhood behaviour development. However, most epidemiological research focused on single food groups, while analysing the overall maternal diet quality might provide more insight into childhood development.

Aims: To examine the association between maternal diet quality during pregnancy, as assessed with the Mediterranean diet (MD) and the Dietary Approaches to Stop Hypertension (DASH) scores, and childhood problem behaviours in school years.

Methods: We harmonised and pooled individual data of 1,543 mother-child pairs from two South-European prospective birth cohorts – the Rhea Mother-Child birth cohort from Crete, Greece, and the INfancia y Medio Ambiente (INMA) birth cohort from Spain. Maternal food intake during pregnancy was assessed using validated food frequency questionnaires for pregnant women. We evaluated problem behaviours with the Child Behaviour Checklist (CBCL) at 6-10 years old. Age-standardised scores of the three CBCL broadband scales (*i.e.,* total problems, internalising and externalising behaviours) were categorised into normal *vs.* borderline/clinical development (cut-off score: ≥60). We examined associations between diet scores and childhood problem behaviour scales with multivariate generalised linear regressions.

Results: A one-point increase in maternal MD score (range: 0-8) was associated with lower odds of childhood externalising behaviours (OR: 0.88; 95% C.I.: 0.79, 0.98); a similar trend was observed when using continuous externalising scores. The estimated proportion of children with externalising behaviours decreased by 16.4% (*i.e.*, from 28.9% to 12.5%) in children whose mothers had the highest compared to lowest MD score. Results remained robust to several sensitivity analyses. No other associations were observed.

Conclusions: A higher adherence to the Mediterranean diet during pregnancy might be associated with a small reduction in childhood externalising behaviours in school years.

Background

Maternal diet during pregnancy is important for providing adequate sustainment for the expectant woman, as well as meeting the request of the developing foetus. Intake of specific nutrients, such as iron, zinc, calcium, folate and n-3 polyunsaturated fatty acids (PUFAs), has been associated with birth weight as well as maternal and foetal survival (1,2). In addition, the overall diet quality accounts for cumulative effects and interactions of all nutrients in the diet, hence providing a more comprehensive approach to study nutritional intakes and their health outcomes (2,3). Furthermore, it has been shown that improving the quality of overall dietary patterns would result in the adequate intake of all necessary nutrients (4,5).

Two main approaches for defining dietary patterns have been identified: *a posteriori* and *a priori* methods. Factor analysis, which includes the commonly used principal component analysis (PCA), and cluster analysis are considered *a posteriori* or data-driven methods because patterns are derived through statistical modelling (3,6). These methods are therefore largely dependent on the specific data and the analytic decisions applied to them (3,7). For this reason, data-driven dietary patterns might not be reproducible across studies (6–8), although patterns with similar characteristics have been identified in different studies (7). In contrast, *a priori* methods use dietary indices or scores, developed based on current nutritional knowledge, to assess the overall diet quality (3). If dietary indices measure the level of adherence to dietary recommendations, diet scores count the frequency or quantity of food items consumed that are considered beneficial or detrimental for health (6). The Mediterranean diet (MD) score (9) and Dietary Approaches to Stop Hypertension (DASH) score (10) are among the most commonly used diet scores. The two diet plans which they refer to are considered to be among the most healthy, practical and understandable diet plans and therefore are recommended for the general public (11–13).

Poor maternal diet during pregnancy has been associated with impaired development of the hippocampus, amygdala and pre-frontal cortex, via perturbations in the serotonergic and dopaminergic systems and reduced brain-derived neurotrophic factor (14). Consequently, it has been hypothesised that prenatal dietary exposure could program childhood psychopathology (15–17). Animal studies support this hypothesis, suggesting that high-fat or Western-style diets preconceptionally and during pregnancy might be associated with brain developmental abnormalities, epigenetic modifications and anxious or aggressive behaviours in the offspring (18–20).

In humans, only a few studies have examined the association between maternal dietary patterns during pregnancy and childhood internalising and externalising problem behaviours (21–24), markers of mental disorders that affect an estimated 7% to 20% of children and adolescents worldwide (25–27). Internalising behaviours relate to anxious and depressive traits, while externalising behaviours are characterised by aggressivity and lack of attention (28). Two cohort studies in Norway and the Netherlands, respectively, showed that maternal adherence to a "Healthy" dietary pattern, characterised by intakes of fruit, vegetables, cereals and fish, was associated with a reduction in externalising behaviour problems across pre-school years (21,22). Similarly, low adherence to a "Healthy" dietary pattern during pregnancy was associated with symptoms of hyperactivity-inattention, a specific aspect of externalising behaviours, in French children aged 3 to 8 years (23). By contrast, adherence to an unhealthy, Western-style dietary pattern during pregnancy was associated with an increase in externalising behaviours (21–23). Only one recent study examined maternal diet quality with a dietary index, the prenatal diet quality index (characterised by high intakes of fruit, vegetables, fish and whole grains), and its results indicated that increasing diet quality might be associated with a small decrease in both internalising and externalising behaviours in pre-school Norwegian children (24).

Yet, little is known about the possible effects of prenatal exposure to the MD or the DASH score on childhood problem behaviours. Only one study was found that used the MD score (9) to assess the relationship between maternal diet quality and problem behaviours in Dutch preschool-age children, supporting the results obtained with PCA in main analyses (22). In addition, maternal adherence to the MD score at the time of conception was associated with fewer internalising and externalising behaviour symptoms in 2-year-old children from the USA (29). However, results might be specific to North-European pre-school children, since little evidence is available for other populations. Therefore, in the present study, we aimed to examine the association of maternal diet quality during pregnancy, assessed by the MD and DASH scores, with childhood problem behaviours. To this end, we pooled individual data from two South-European prospective birth cohorts – the Rhea Mother-Child birth cohort and the INfancia y Medio Ambiente (Childhood and Environment, INMA) cohort – that assessed problem behaviours in school-age children.

Methods

Study population

The Rhea Mother-Child cohort (30) recruited pregnant women during their first-trimester ultrasound examination during 2007–2008 in Crete, Greece. A total of 1,363 singleton pregnancies were followed up until delivery and were eligible for the six-year postnatal follow-up (mean age: 6.57 years, standard deviation (SD): 0.27). In total, 289 (21.2%) women provided complete data regarding maternal dietary intake in pregnancy and child behaviour at follow-up and were therefore included in this study.

The INMA cohort is a multicentre, prospective birth cohort that recruited pregnant women during their first-trimester prenatal visit between 2003 and 2008 (31,32). A total of 2,644 women were initially recruited. Only participants who provided dietary intake data at least once during pregnancy and child problem behaviour data at follow-up were retained, which resulted in 1,254 (47.4%) mother-child pairs included in the present study (n=396 from the region of Gipuzkoa, n=431 from Sabadell and n=427 from Valencia). Children's mean ages (SD) at follow-up were 7.8 years (0.1), 9 years (0.7) and 9.1 years (0.2), in Gipuzkoa, Sabadell and Valencia, respectively.

All participants provided written informed consent at recruitment and at follow-up. The Rhea study was approved by the Ethics Committee of the University Hospital in Heraklion. The INMA study was approved by relevant hospital and institutional ethics committees in each region.

Results of the non-response analyses showed that, in both cohorts, participating women were slightly older and highly educated compared to non-included women from the same cohort. Nevertheless, other important characteristics such as smoking during pregnancy and children's gestational age were similar between participants and non-participants (Appendix, tables A6.1 and A6.2).

Maternal diet quality during pregnancy

In Rhea, maternal dietary intakes were measured with a 250-item, semi-quantitative Food Frequency Questionnaire (FFQ) in the second trimester of pregnancy (weeks 14-18). Dietary intakes referred to the period from conception to the dietary assessment. In INMA, maternal diet was assessed twice over the course of pregnancy, in the first trimester (weeks 10-13) and in the third (weeks 28-32), using a 101-item, semi-quantitative FFQ. Dietary intakes referred to the period from conception to the first assessment, and from the first to the second assessment, respectively. In

case two dietary assessments were available for the same woman (n=1230, 98.09%), the mean of the MD and DASH scores for the two periods were used as a better proxy of maternal diet quality over pregnancy. Both versions of the FFQ have been validated for use in either the Greek or the Spanish population of pregnant women (33–35).

MD score

We evaluated the adherence to the Mediterranean diet using an adapted version of the MD score proposed by Trichopoulou and colleagues (9). Intakes of vegetables, legumes, fruits and nuts, cereals, fish and seafood, olive oil, dairy products and meat were assessed. For components considered to be beneficial (vegetables, legumes, fruits and nuts, dairy products, cereals, olive oil and fish), women who consumed less (in grams) than the cohort-specific median intake were assigned a value of 0, otherwise they obtained a value of 1. For components presumed to be detrimental (meat and meat products), consumptions below the median were given a value of 1, otherwise a value of 0 was assigned. Given the recommendation to omit alcoholic beverages during pregnancy and lactation (36), alcohol intake was excluded from the MD score; maternal alcohol use during pregnancy was added as a potential confounding factor in sensitivity analyses. The intake of dairy products was considered beneficial to account for the higher calcium requirement of pregnant women to support foetal growth (37). Summing up the scores for each food item, participants' individual score could range from 0 to 8, with 0 indicating lowest diet quality and 8 indicating highest diet quality. Cohort-specific median intakes are presented in the Appendix, table A6.3.

DASH score

Fung's score (10) was used to calculate maternal adherence to the DASH diet during pregnancy. For each food item, participants were ranked into cohort-specific quintiles of intake (calculated in number of servings per day, except for sodium that was calculated as mg/day) and assigned a score. Vegetables, nuts/legumes, fruits, low-fat dairy products and whole grains received increasing scores for increasing quintiles of intake (1 for the first quintile, up to 5 for the fifth quintile); sugar-sweetened beverages, red and processed meat, and sodium intakes were reverse scored (5 for the first quintile, up to 1 for the fifth quintile). Summing-up the scores for each food item, participants' individual score could range from 8 to 40. Cohort-specific intakes are presented in table A6.4 (Appendix).

Childhood problem behaviours

In both cohorts, we assessed child problem behaviours using the parent-completed Child Behaviour Checklist (CBCL) 6/18 (38,39). The CBCL has demonstrated good psychometric properties, reliability and validity (39).

We focused on the three broadband scales: total problems, internalising and externalising behaviours. To allow comparability between cohorts, age-standardized T-scores (with a mean of 50 and a standard deviation of 10 (38,39)) were used. All three outcomes were treated in a continuous and a categorical scale. The cut-off value taken for differentiating children with a normal development from those with a behaviour in the borderline or clinical range of symptomatology was T-score ≥60 (38,39).

Covariates

Information regarding parental socio-demographic and lifestyle characteristics were collected with questionnaires administered by trained interviewers. Maternal and child anthropometric data were collected from medical records.

Selection of suitable covariates for model adjustment was based on previous knowledge and a directed acyclic graph approach (DAG (40); Appendix, figure A6.1). The following covariates were included in the models: maternal pre-pregnancy BMI (Kg/m^2), parity before the index pregnancy (no children, one child, two or more children), maternal smoking during pregnancy (ever, never), maternal age at delivery (years) and parental level of education (primary school, secondary school, university degree, based on the highest level of education of either parent). Moreover, we included total maternal total energy intake during pregnancy (Kcal/day) (41), children's sex (male, female), children's age at assessment (years) and an indication of cohorts' geographic region (*i.e.*, Crete, Gipuzkoa, Sabadell and Valencia).

Statistical analysis

Multivariate linear models

The associations of MD and DASH scores during pregnancy with childhood total problems, internalising and externalising behaviours were assessed with multivariable generalised linear models and multivariable linear models for binary and continuous outcomes, respectively, using the pooled dataset (n=1543).

To estimate the strength of identified associations, we used the "margins" package in R (42) to calculate marginal effects – *i.e.*, the estimated probability of childhood problem behaviours if all women had the same diet-quality score, controlling for all other variables in the model (43).

Treatment of missing covariate data

We used the "howManyImputations" package in R (44,45) to determine the minimum number of imputations required in the two datasets with a coefficient of variation of 0.05 (*i.e.*, 45 in Rhea and 2 in INMA). We then raised this number to improve precision and generated 50 imputed datasets using the multiple imputation of chained equation (MICE) method (46,47). Cohort-specific imputation models were created that included exposure, outcomes, confounders and additional auxiliary variables (*i.e.*, maternal pre-pregnancy weight (kg), maternal height (m), maternal alcohol intake in pregnancy (ever, never), birthweight-per-gestational age (adequate for gestational age, small for gestational age, large for gestational age), birthweight (g), gestational age (weeks), delivery mode (vaginal, caesarean), breastfeeding duration (months), day-care attendance (ever, never) and child BMI at follow up (Kg/m^2)).

Sensitivity analyses

Several sensitivity analyses were performed to assess the robustness of our results. Firstly, we additionally controlled for maternal alcohol intake during pregnancy (ever, never) or breastfeeding duration, since they might affect child brain development (48,49). Secondly, we repeated the analyses in each cohort separately to evaluate potential heterogeneity in the effect estimates. Thirdly, complete-case analyses were performed to verify if result interpretation would change from those obtained with the imputed dataset. Fourthly, given the correlation between maternal diet quality and physical activity, we additionally controlled for maternal physical activity during pregnancy (metabolic equivalents/hour/day). These analyses were conducted using only data from the INMA cohort with complete-case data due to the small percentage of women who reported being physically active during pregnancy in Rhea (n=18, 6.23%). Lastly, possible effect modifications of maternal pre-pregnancy BMI, breastfeeding status and sex of the child on the associations between maternal MD or DASH score and child problem behaviours were examined by assessing the interaction between each potential effect modifier and each exposure. These interactions were tested based on previous evidence of differential effects of prenatal influences on child neurocognitive or behavioural outcomes in different subpopulations (*e.g.*, (50–52)).

All statistical analyses were done in R, version 3.5.3 (53).

Results

Characteristics of the study populations by quintile of adherence to the MD or the DASH scores are reported in table 6.1 and 6.2; population characteristics by MD or DASH adherence and by cohort are reported in the Appendix (tables A6.4-A6.7). In the pooled dataset, mean (standard deviation, SD) of maternal MD and DASH scores were 3.87 (1.44) and 23.17 (4.40), respectively. The correlation between MD and DASH scores was 0.39 (p< 0.001). Women who reported a higher adherence to the MD diet also reported greater total energy intake compared to women with lower MD adherence. No differences in parental level of education, parity, or children's characteristics such as gestational age and birthweight were observed between degrees of MD adherence (table 6.1). Conversely, women with a high DASH adherence were older, less likely to smoke during pregnancy and more likely to have a high level of education compared to women with a lower adherence to the DASH diet (table 6.2). In the pooled dataset, the prevalence of childhood total problems, internalising and externalising behaviours was 22.3%, 26.3% and 19.8%, respectively.

Table 6.1: Population characteristics by quintiles of maternal MD score adherence

	Q1 (n=348)	Q2 (n=376)	Q3 (n=225)	Q4 (n=326)	Q5 (n=268)	
			Mean (SD) or n (%)			p
MD score	1.96 (0.55)	3.25 (0.25)	4.00 (0.00)	4.79 (0.25)	6.03 (0.55)	<0.001
DASH score	20.87 (4.15)	22.29 (3.92)	23.43 (4.04)	24.43 (4.26)	25.65 (3.95)	<0.001
Age at delivery (years)	31.12 (4.10)	31.56 (4.12)	31.64 (3.93)	32.27 (3.92)	31.89 (4.37)	0.006
Total energy intake (Kcal/day)	1715 (404)	1932 (381)	2070 (455)	2170 (438)	2454 (552)	<0.001
Pre-pregnancy BMI (kg/m2)	23.14 (21.38, 26.37)	22.86 (20.89, 25.15)	22.21 (20.81, 24.55)	22.43 (20.92, 25.28)	22.66 (20.78, 25.20)	0.059
Parental education						0.485
Primary school	43 (12.39%)	40 (10.64%)	16 (7.14%)	42 (12.88%)	28 (10.53%)	
Secondary school	149 (42.94%)	169 (44.95%)	97 (43.30%)	135 (41.41%)	125 (46.99%)	
University degree	155 (44.67%)	167 (44.41%)	111 (49.55%)	149 (45.71%)	113 (42.48%)	
Smoking (% yes)	93 (27.51%)	113 (30.87%)	58 (27.10%)	74 (23.20%)	66 (25.98%)	0.259
Alcohol (% yes)	45 (13.16%)	40 (10.81%)	36 (16.22%)	39 (12.15%)	39 (14.72%)	0.347
Parity						0.331
No children	196 (56.32%)	201 (53.74%)	124 (55.11%)	178 (55.28%)	137 (51.31%)	
One child	117 (33.62%)	150 (40.11%)	87 (38.67%)	124 (38.51%)	106 (39.70%)	
Two + children	35 (10.06%)	23 (6.15%)	14 (6.22%)	20 (6.21%)	24 (8.99%)	

Table 6.1 (continued)

	Q1 (n=348)	Q2 (n=376)	Q3 (n=225)	Q4 (n=326)	Q5 (n=268)	
			Mean (SD) or n (%)			p
Sex (% female)	161 (46.3%)	192 (51.1%)	105 (46.7%)	150 (46%)	126 (47%)	0.644
Gestational age (weeks)	39.49 (1.56)	39.49 (1.58)	39.28 (1.73)	39.55 (1.47)	39.21 (1.84)	0.054
Birthweight (g)	3300 (457)	3241 (452)	3249 (438)	3297 (421)	3239 (483)	0.202
Breastfeeding duration (months)	3.0 (0.0, 12.5)	4.4 (0.0, 15.8)	5.0 (0.2, 14.3)	6.0 (0.0, 16.8)	3.9 (0.1, 13.7)	0.075
Children's age at follow-up	8.23 (1.05)	8.44 (0.96)	8.12 (1.13)	8.39 (1.06)	8.25 (1.19)	0.001
Total problems						
Continuous	52.63 (9.86)	51.27 (9.61)	50.8 (10.07)	51.53 (10.55)	52 (9.93)	0.212
Binary	85 (24.4%)	83 (22.1%)	41 (18.2%)	74 (22.7%)	61 (22.8%)	0.535
Internalising behaviours						
Continuous	53.47 (9.77)	52.49 (9.25)	52.11 (9.39)	52.87 (10.04)	53.25 (9.65)	0.444
Binary	104 (29.9%)	93 (24.7%)	46 (20.4%)	86 (26.4%)	77 (28.7%)	0.108
Externalising behaviours						
Continuous	52.09 (9.53)	51.03 (9.88)	50.93 (9.83)	51.21 (10.01)	51.59 (9.54)	0.551
Binary	78 (22.4%)	71 (18.9%)	39 (17.3%)	64 (19.6%)	54 (20.1%)	0.630

Note: Q1 (quintile 1): MD ≤2.5; Q2 (quintile 2): MD >2.5-3.5; Q3 (quintile 3): MD= 4; Q4 (quintile 4): MD= 4.5-5; Q5 (quintile 5): MD > 5.

Results of the pooled analyses on childhood problem behaviour scales are reported in table 6.3; full-model estimates are reported in the Appendix (tables A6.8-A6.11). A one-point increase in MD score resulted in reduced odds of externalising behaviours (odds ratio (OR): 0.88; 95% C.I.: 0.79, 0.98), but not of total problems or internalising behaviours (OR total problems: 0.93; 95% C.I.: 0.84, 1.03. OR internalising behaviours: 0.93; 95% C.I.: 0.84, 1.03). An inverse relationship was also observed between MD score and externalising behaviour scores (β: -0.48; 95% C.I.: -0.87, -0.09) or total problems (β: -0.48; 95% C.I.: -0.88, -0.08) treated continuously. Only a tendency for lower internalising scores was found with increasing maternal MD score (β: -0.33; 95% C.I.: -0.72, 0.05). As shown in figure 6.1, after controlling for important confounders, the estimated proportion of children with externalising behaviours decreased by approximately 2% for every point increase in maternal adherence to the MD during pregnancy, up to a total average reduction of 16.4% (*i.e.*, from 28.9% to 12.5%) in children whose mothers had the highest compared to lowest MD score.

No associations were observed between maternal DASH score and childhood problem behaviours in the binary scale (OR total problems: 1.00; 95% C.I.: 0.97, 1.03. OR internalising behaviours: 1.01;

95% C.I.: 0.97, 1.04. OR externalising behaviours: 1.00; 95% C.I.: 0.97, 1.04) or in the continuous scale (β total problems: -0.05; 95% C.I.: -0.17, 0.08. β internalising behaviours: -0.03; 95% C.I.: -0.15, 0.09. β externalising behaviours: -0.03; 95% C.I.: -0.15, 0.09; table 6.3).

No evidence of an effect modification by cohort, maternal pre-pregnancy BMI, children's sex or breastfeeding on the main associations was found (all p-values >0.05). Sensitivity analyses confirmed the main analyses' findings (tables A6.12-A6.13, Appendix). No significant heterogeneity was found between cohorts (figures A6.2-A6.5, Appendix).

Discussion

In this pooled analysis of two South-European cohorts, we examined the association between maternal diet quality during pregnancy, measured with the MD and the DASH scores, and problem

Table 6.2: Population characteristics by quintiles of maternal DASH score adherence

	Q1 (n= 333)	Q2 (n=338)	Q3 (n=275)	Q4 (n=316)	Q5 (n=281)	
			Mean (SD) or n (%)			p
MD score	3.11 (1.22)	3.64 (1.43)	3.90 (1.34)	4.16 (1.31)	4.72 (1.36)	<0.001
DASH score	17.29 (1.93)	21.07 (0.72)	23.25 (0.55)	25.74 (0.86)	29.71 (1.84)	<0.001
Age at delivery (years)	30.58 (4.16)	31.56 (4.13)	31.51 (3.75)	32.01 (3.96)	32.91 (4.14)	<0.001
Total energy intake (Kcal/day)	2185 (496)	2069 (503)	2023 (525)	1971 (503)	1953 (468)	<0.001
Pre-pregnancy BMI (kg/m2)	22.50 (20.43, 25.15)	22.95 (20.94, 25.33)	22.74 (21.06, 25.16)	22.62 (20.91, 25.68)	22.92 (21.30, 25.27)	0.487
Parental education						<0.001
Primary school						
Secondary school	59 (17.77%)	41 (12.17%)	28 (10.26%)	21 (6.65%)	20 (7.12%)	
University	171 (51.51%)	156 (46.29%)	116 (42.49%)	132 (41.77%)	100 (35.59%)	
degree	102 (30.72%)	140 (41.54%)	129 (47.25%)	163 (51.58%)	161 (57.30%)	
Smoking (% yes)	118 (36.09%)	104 (32.30%)	83 (31.20%)	62 (20.33%)	37 (13.65%)	<0.001
Alcohol (% yes)	40 (12.20%)	50 (15.20%)	32 (11.81%)	39 (12.46%)	38 (13.62%)	0.720
Parity						0.063
No children	186 (55.86%)	160 (47.76%)	155 (56.57%)	173 (55.27%)	162 (57.65%)	
One child	126 (37.84%)	147 (43.88%)	104 (37.96%)	117 (37.38%)	90 (32.03%)	
Two + children	21 (6.31%)	28 (8.36%)	15 (5.47%)	23 (7.35%)	29 (10.32%)	

Table 6.2 (continued)

	Q1 (n= 333)	Q2 (n=338)	Q3 (n=275)	Q4 (n=316)	Q5 (n=281)	
			Mean (SD) or n (%)			p
Sex (% female)	171 (51.4%)	161 (47.6%)	125 (45.5%)	143 (45.3%)	134 (47.7%)	0.544
Gestational age (weeks)	39.64 (1.49)	39.44 (1.68)	39.47 (1.64)	39.29 (1.65)	39.26 (1.66)	0.029
Birthweight (g)	3241 (450)	3285 (440)	3269 (461)	3247 (459)	3296 (445)	0.483
Breastfeeding duration (months)	4.4 (0.0, 14.1)	3.9 (0.0, 15.5)	4.0 (0.0, 14.6)	5.4 (0.8, 15.6)	4.0 (0.5, 13.8)	0.859
Children's age at follow-up	8.68 (0.87)	8.44 (1.05)	8.31 (1.06)	8.14 (1.13)	7.87 (1.07)	<0.001
Total problems						
Continuous	52.83 (9.85)	52.27 (9.20)	51.27 (10.14)	51.50 (10.07)	50.28 (9.92)	0.021
Binary	85 (25.5%)	81 (24%)	61 (22.2%)	65 (20.6%)	52 (18.5%)	0.246
Internalising behaviours						
Continuous	53.67 (9.51)	53.36 (9.50)	52.30 (9.86)	53.00 (9.63)	51.74 (9.59)	0.089
Binary	87 (26.1%)	98 (29%)	68 (24.7%)	86 (27.2%)	67 (23.8%)	0.621
Externalising behaviours						
Continuous	51.83 (10.06)	52.04 (9.28)	50.91 (9.95)	51.31 (9.80)	50.65 (9.72)	0.344
Binary	74 (22.2%)	67 (19.8%)	55 (20.0%)	62 (19.6%)	48 (17.1%)	0.636

Note: Q1 (quintile 1): DASH ≤ 19.5; Q2 (quintile 2): DASH >19.5-22; Q3 (quintile 3): DASH >22-24; Q4 (quintile 4): DASH >24-27; Q5 (quintile 5): DASH > 27.

behaviours in school-age children. After controlling for important maternal and childhood characteristics, we found that a unit increase in maternal MD scores was associated with lower odds for children's externalising behaviours (OR: 0.88; 95% C.I.: 0.79, 0.98). This resulted in an estimated 16.4% average reduction (*i.e.*, from 28.9% to 12.5%) in the probability of childhood externalising

Table 6.3: Associations between maternal diet quality during pregnancy and childhood problem behaviours

		Total problems		Internalising behaviours		Externalising behaviours	
		Estimates	95% C.I.	Estimates	95% C.I.	Estimates	95% C.I.
MD score	OR	0.93	0.84, 1.03	0.93	0.84, 1.03	0.88*	0.79, 0.98
	Beta	-0.48*	-0.88, -0.08	-0.33	-0.72, 0.05	-0.48*	-0.87, -0.09
DASH score	OR	1.00	0.97, 1.03	1.01	0.97, 1.04	1.00	0.97, 1.04
	Beta	-0.05	-0.17, 0.08	-0.03	-0.15, 0.09	-0.03	-0.15, 0.09

Note: n=1543. Odds ratios (ORs) and their 95% confidence intervals (95% C.I.) were computed using generalised linear models for binary outcomes; beta coefficients and their 95% C.I. were calculated using linear regression models. All models were adjusted for maternal age, pre-pregnancy BMI, parity, smoking during pregnancy, total energy intake during pregnancy, parental level of education, children's sex, children's age and geographic region. *: p<0.05.

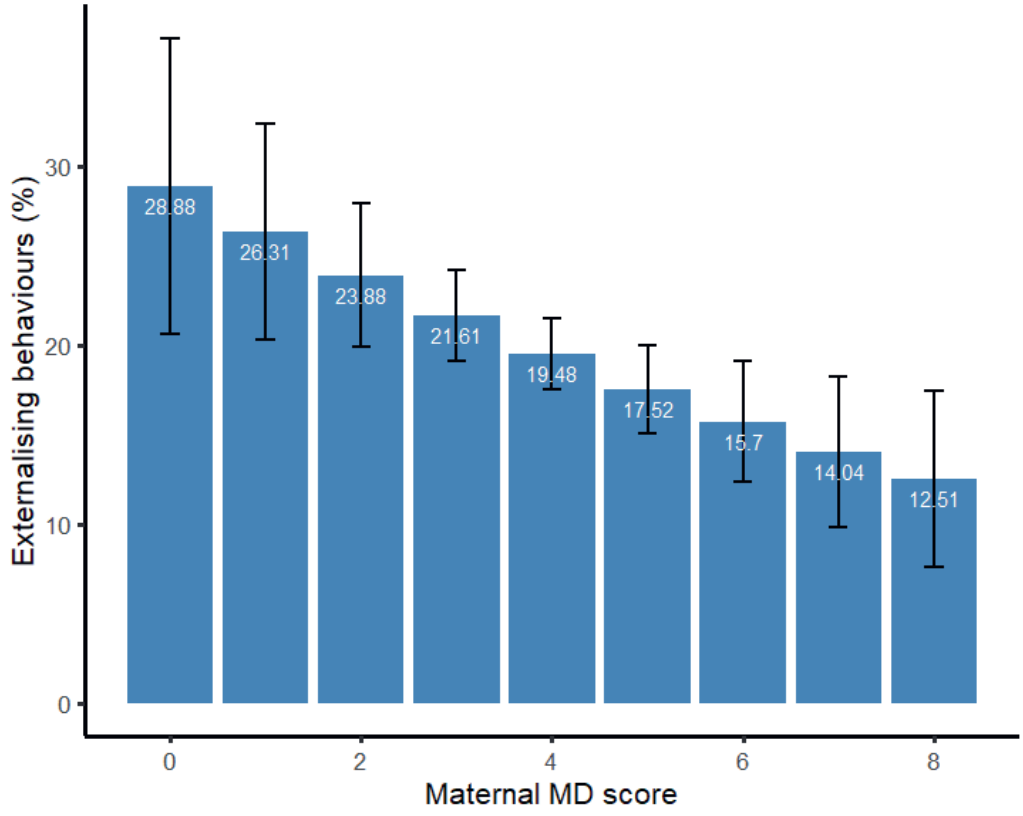

Figure 6.1: Estimated proportion of children with externalising behaviour problems by maternal MD score.

Note: n=1468. Adjusted for maternal age, pre-pregnancy BMI, parity, smoking during pregnancy, total energy intake during pregnancy, parental level of education, children's sex, children's age and geographic region.

behaviours in children whose mothers had the highest MD score compared to children exposed to the lowest-quality diet during gestation. An inverse association was also observed between maternal MD score during pregnancy and childhood total problem and externalising behaviour scores; however, no association was observed when examining total problems as a binary outcome. Conversely, no associations were observed between maternal MD score and childhood internalising behaviours, or between maternal adherence to the DASH diet and any problem behaviour scale.

Findings from previous studies from other European populations (*i.e.,* Norwegian, Dutch and French children) are generally in line with our results. Maternal adherence to a "healthy" dietary pattern – characterised by high intake of food groups commonly used to define adherence to the Mediterranean diet (*i.e.*, fruit, vegetables, cereals and fish) – was negatively associated with externalising behaviour scores in preschool-age children or hyperactivity-inattention symptoms up to the age of 8 years (21–23). A re-analysis in the Dutch Generation R cohort found a small reduction

in the odds of childhood externalising behaviours for increasing adherence to the MD (22). Furthermore, higher adherence to a diet quality index characterised by high intakes of fruit, vegetables, fish and whole grains, was associated with a small reduction in both internalising and externalising behaviours in preschool-age children from Norway (24). Similarly, we found a small decrease in externalising behaviours with increasing maternal MD adherence in pregnancy. Collectively, these results from various populations across Europe suggest that the adherence to a healthy dietary pattern during pregnancy that follows the Mediterranean-diet principles could have a small protective effect against externalising behaviours, with effects extending to late childhood.

Two reasons might be identified to explain why the effects of maternal diet quality during pregnancy might last up to late childhood and possibly beyond. Firstly, prenatal exposures to different dietary patterns might result in specific epigenetic modifications in the developing foetus, which would affect childhood behaviour. This hypothesis finds support in a study examining the methylation pattern and problem-behaviour symptoms in children in relation to their mothers' periconceptional diet quality (29). Maternal MD score was not only associated with symptoms of internalising and externalising behaviour in preschool years, but also with differences in the methylation patterns in the control regions of three imprinted genes, which were in turn associated with problem behaviours (29). Secondly, dietary intake or diet quality are markers of maternal lifestyle before, during and after pregnancy, and might therefore affect child mental health indirectly. Particularly important might be the child's diet, which is affected by maternal post-partum diet and correlated with maternal diet in pregnancy (58). The child's diet is therefore likely to be a mediator of the association between maternal diet in pregnancy and childhood psychopathology, sharing some influences with maternal diet. In this perspective, improving maternal diet quality in pregnancy would still have a beneficial effect on child's mental health. Moreover, since healthy lifestyle factors tend to cluster together (59), improvement in maternal diet quality might lead to a series of changes in other factors that have been associated with pregnancy outcomes and later child development (*e.g.*, gestational weight gain, and pregnancy and post-partum BMI and physical activity), with even greater benefits.

Maternal diet has received increasing attention as it can be considered a relatively easy-to-modify risk factor, especially in a highly receptive period for behaviour change such as pregnancy (54). To date, several nutrients, including vitamins (*e.g.,* folic acid), minerals (*e.g.,* iron and iodine) and polyunsaturated fatty acids, have been identified as potentially important for child neurodevelopment or behaviour (55,56). Nonetheless, the results from observational studies are

inconsistent and randomised controlled trials examining the effect of maternal supplementation with single nutrients have not showed consistent differences between supplementation and control (55,57). A possible reason might be found in the correlation between nutrients and food groups: the observed associations might be attributable to a nutrient correlated to the one examined (or the lack of it) or a combination of correlated nutrients (3). Furthermore, the few identified associations are of very small magnitude. Therefore, increasing the intake of a single factor while keeping the overall diet unchanged might not produce noticeable effects on the foetus. By contrast, measures of dietary patterns are considered useful to capture correlations between nutrients and food groups, and to better characterise the complexity of a person's diet (3).

Most previous studies examining maternal dietary pattern and childhood problem behaviours employed data-driven methods (*i.e.*, PCA) to assess overall diet quality (21–23). Such methods, although valuable for hypothesis generation, have been criticised for being excessively dependent on arbitrary decisions during data analysis and for limiting between-studies comparability (6,8). To account for these shortcomings and assess the generalisability of the evidence, we adopted two dietary indices, the MD and the DASH scores, that have shown to be associated with lower psychopathology in paediatric and adult populations (60–63). Moreover, indications included in these dietary indices are based on current recommendations, can be easily interpreted by the general population and, therefore, used for behaviour change (11–13). Together, results obtained with data-driven methods and diet quality indices can provide stronger evidence for an association.

Despite being recognised as a generally healthy diet (11–13), in the present study adherence to the DASH pattern during pregnancy was not associated with childhood problem behaviours. In contrast to the MD score, in the Fung's DASH score (10) there is a lack of attention towards fish and seafood intake, which has been positively associated with child neuropsychological and behavioural development (*e.g.*, (52,64–68)). In this study population, correlations of fish intake with DASH scores ranged between 0.08 ($p=0.108$) in Gipuzkoa and 0.17 ($p<0.001$) in Valencia, while correlations with a MD score calculated excluding fish intake were considerably higher (*i.e.*, from 0.15 in Gipuzkoa up to 0.29 in Sabadell, both $p<0.001$), suggesting that fish and seafood intake might be responsible, at least in part, of the observed association with childhood externalising behaviours. Fish and seafood consumption are among the largest sources of n-3 PUFAs, particularly of docosahexaenoic acid (DHA), which play central roles within the nervous systems and are considered crucial for brain development (69–74). To date, several studies have examined how prenatal exposure to PUFAs might affect childhood behaviour, suggesting a small beneficial effect of n-3 PUFAs, although with

some inconsistencies (57,75–79). Consequently, it needs to be clarified if fish and seafood provide specific nutrients that are beneficial for brain development, or if their intake is a good proxy for a diet (or an overall lifestyle) that promotes an optimal psychological development.

Major strengths of this study include the use of well-defined dietary scores previously associated with reduced risk of mental disorders in adult and adolescents (*e.g.,* (60–63)). Moreover, we assessed childhood problem behaviours with a validated, commonly used tool (39), thus allowing direct comparability of our results to other studies. Furthermore, we pooled individual data from two large, prospective, population-based birth cohorts to increase the statistical power. The absence of significant heterogeneity between estimates of individual cohorts additionally supports the robustness and generalisability of results.

Despite these strengths, results should be interpreted after considering some limitations. Dietary information was self-reported and based on food frequency questionnaires, which are prone to measurement errors, although the prospective design and the use of validated tools might have reduced the likelihood of measurement bias and exposure misclassification. Both the MD and the DASH scores were calculated using cut-off points based on each population's food intakes, which might not reflect recommended dietary intakes. However, this approach guarantees enough discriminating power between subjects and equal contribution of each item in the score, which would not be guaranteed if the intake of a certain food group was too low for the majority of the study population (80). The use of population-based cut-off values might also limit the comparability between populations. However, the MD and the DASH scores have been validated and used in several populations, and comparable diet-score ranges and estimates of disease risks have been reported (*e.g.*, (60,81,82)). Child problem behaviours were reported by children's parents, which might have resulted in biased assessments (83). However, while no gold standard for assessing child problem behaviours exists, we used a well-established, validated instrument, which has shown good psychometric properties (39). Moreover, although we controlled for a large set of important covariates, including total energy intake during pregnancy, parental level of education, maternal smoking during pregnancy and pre-pregnancy BMI, residual confounding is still possible. Specifically, we could not control for maternal mental health status before or during pregnancy. Since maternal mental illness is associated with lower quality of maternal diet (84) and higher childhood problem behaviours (85), a lack of adjustment is likely to result in the overestimation of the effect estimates. Furthermore, although no studies to date have examined the association between maternal physical activity during pregnancy and childhood psychopathology, physical

activity is correlated with diet quality in pregnancy (59) and might therefore be a confounding factor in the associations examined in this study. Although we attempted at controlling for physical activity in the INMA cohort, these data were not available for the vast majority of women in the Rhea cohort and residual confounding cannot be completely ruled out. Finally, although the development of problem behaviours is influenced by several risk factors in the postnatal period, including parenting practices and infant's temperament (86), we did not assess any postnatal influences. These factors cannot be considered potential confounders of the association between maternal diet quality in pregnancy and problem behaviours, as they necessarily occur after the exposure (87), but might modify the association between diet quality and childhood problem behaviours (*e.g.*, (88–90)).

Conclusions

To conclude, in this pooled analysis of individual data from two Southern European birth cohorts (*i.e.,* Rhea and INMA), higher maternal adherence to the Mediterranean diet during pregnancy was associated with a small reduction in externalising behaviours in their school-age children. Future studies are warranted to confirm this association in different populations in which maternal mental health status before or during pregnancy and physical activity in pregnancy are known. In addition, mediation analyses investigating the role of child's diet should be performed in order to clarify the potential benefits of a high-quality maternal diet during pregnancy.

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Appendix to chapter 6

Non-response analyses

Table A6.1: Non-response analysis, Rhea cohort

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	n	**Mean (SD), median (IQR) or n (%)**	**n**	**Mean (SD), median (IQR) or n (%)**	**p-value**
Maternal characteristics					
Age at delivery (years)	288	30.16 (4.74)	1182	29.16 (5.16)	0.002
MD score	289	4.12 (1.79)	615	3.94 (1.90)	0.224
DASH score	289	25.29 (4.20)	592	24.06 (4.34)	<0.001
Gestational age at diet assessment (weeks)	277	19.46 (5.15)	817	22 (17, 24)	<0.001
Total energy intake (Kcal/day)	289	1909.20 (1561.80, 2388.60)	615	1995.26 (1554.86, 2520.80)	0.069
Pre-pregnancy BMI	286	23.72 (21.76, 27.12)	1087	23.19 (20.83, 26.17)	0.002
Gestational weight gain	245	13.76 (5.99)	970	12.68 (8.21)	0. 773
Gestational diabetes (% yes)	288	34 (11.81%)	1083	111 (10.25%)	0.512
Parental education	288		1117		<0.001
Primary school		24 (8.33%)		190 (17.01%)	
Secondary school		145 (50.35%)		573 (51.30%)	
University degree		119 (41.32%)		354 (31.69%)	
Smoking (% yes)	252	46 (18.25%)	988	217 (21.96%)	0.230
Parity	284		1114		0.118
No children		119 (41.90%)		478 (42.91%)	
One child		120 (42.25%)		409 (36.71%)	
Two or more children		45 (15.85%)		227 (20.38%)	
Caesarean section (% yes)	287	146 (50.87%)	1202	620 (51.58%)	0.881
Children's characteristics					
Sex (% male)	289	172 (59.52%)	1233	595 (48.26%)	<0.001
Gestational age (weeks)	287	38.19 (1.59)	1187	38.11 (1.76)	0.481
Birth weight (g)	284	3256.00 (446.50)	1143	3120.46 (485.10)	<0.001
Pregnancy outcomes	277		1125		0.002
AGA		219 (79.06%)		856 (76.09%)	
LGA		53 (19.13%)		185 (16.44%)	
SGA		5 (1.81%)		84 (7.47%)	
Breastfeeding					
Status (% yes)	276	238 (86.23%)	1016	852 (83.84%)	0.385
Duration (months)	276	2.00 (0.75, 6.00)	981	2.00 (0.75, 6.00)	0.332
Day-care attendance (% yes)	289	172 (59.52%)	1049	147 (14.01%)	0.002

Note: AGA: adequate for gestational age; LGA: large for gestational age; SGA: small for gestational age.

Table A6.2: Non-response analysis, INMA cohorts (Subcohort: Gipuzkoa)

Gipuzkoa	Included participants		Excluded participants		
	n	Mean (SD), median (IQR) or n (%)	n	Mean (SD), median (IQR) or n (%)	p-value
Maternal characteristics					
Age at delivery (years)	396	32.57 (3.23)	217	32.62 (4.23)	0.871
MD score	396	4.18 (1.33)	242	4.07 (1.31)	0.317
DASH score	396	23.86 (3.97)	242	22.93 (3.83)	0.004
Gestational age at diet	395	14.05 (1.46)	242	14.18 (2.04)	0.345
assessment (weeks)	384	32.91 (2.44)	215	33.02 (2.82)	0.611
Total energy intake (Kcal/day)[a]	380	1882.00 (1662.00, 2104.00)	205	1883.13 (1657.83, 2134.94)	0.205
Pre-pregnancy BMI	396	22.21 (20.70, 24.30)	242	22.27 (20.66, 24.44))	0.497
Gestational weight gain	368	12.59 (4.29)	194	12.02 (4.25)	0.138
Gestational diabetes (% yes)	377	17 (4.51%)	203	5 (2.46%)	0.219
Parental education					
Primary school	395		242		<0.001
Secondary school		12 (3.04%)		22 (9.09%)	
University degree		133 (33.67%)		101 (41.74%)	
		250 (63.29%)		119 (49.17%)	
Smoking (% yes)	385	84 (21.82%)	214	59 (27.57%)	0.138
Parity	396		242		0.081
No children		225 (56.82%)		120 (49.59%)	
One child		151 (38.13%)		101 (41.74%)	
Two + children		20 (5.05%)		21 (8.68%)	
Caesarean section (% yes)	377	47 (12.47%)	204	27 (13.24%)	0.893
Children's characteristics					
Sex (% male)	396	196 (49.49%)	215	111 (51.63%)	0.675
Gestational age (weeks)	396	39.75 (1.45)	213	39.74 (1.53)	0.947
Birth weight (g)	392	3298.00 (440.38)	209	3299.11 (487.88)	0.984
Pregnancy outcomes					
AGA	388	322 (82.99%)	208	165 (79.33%)	0.278
LGA		38 (9.79%)		20 (9.62%)	
SGA		28 (7.22%)		23 (11.06%)	
Breastfeeding status (% yes)	372	327 (82.58%)	168	136 (80.95%)	0.032
Breastfeeding duration (months)	372	4.13 (1.11, 5.44)	168	3.80 (0.43, 5.39)	0.327
Day-care attendance (% yes)	341	229 (67.16%)	126	84 (66.67%)	1.000

Table A6.2 (continued. Subcohort: Sabadell)

Sabadell	Included participants		Excluded participants		
	n	Mean (SD), median (IQR) or n (%)	n	Mean (SD), median (IQR) or n (%)	p-value
Maternal characteristics					
Age at delivery (years)	430	31.81 (4.09)	304	30.65 (4.92)	0.001
MD score	431	4.26 (1.23)	222	4.43 (1.35)	0.121
DASH score	431	23.37 (3.85)	225	23.21 (4.19)	0.641
Gestational age at diet assessment (weeks)	429 425	13.65 (1.83) 34.43 (1.93)	222 185	13.44 (1.70) 34.55 (2.24)	0.135 0.512
Total energy intake (Kcal/day)[a]	431	2031.00 (1787.00, 2283.00)	187	2216.28 (1944.35, 2522.24)	<0.001
Pre-pregnancy BMI	431	22.83 (20.96, 25.40)	319	22.59 (20.96, 25.59)	0.928
Gestational weight gain	418	14.11 (4.99)	181	14.83 (5.25)	0.122
Gestational diabetes (% yes)	420	14 (3.33%)	216	9 (4.17%)	0.757
Parental education					
Primary school	429	64 (14.92%)	338	91 (26.92%)	<0.001
Secondary school		186 (43.36%)		156 (46.15)	
University degree		179 (41.72%)		91 (26.92%)	
Smoking (% yes)	427	118 (27.63%)	298	96 (32.21%)	0.183
Parity					
No children	429	250 (58.28%)	224	115 (51.34%)	0.095
One child		155 (36.13%)		88 (39.29%)	
Two + children		24 (5.59%)		21 (9.38%)	
Caesarean section (% yes)	421	66 (15.68%)	301	49 (16.28%)	0.909
Children's characteristics					
Sex (% male)	431	224 (51.97%)	307	151 (49.19%)	0.502
Gestational age (weeks)	431	39.76 (1.36)	305	39.68 (1.74)	0.540
Birth weight (g)	431	3272.00 (411.61)	302	3234.60 (513.73)	0.288
Pregnancy outcomes	420		183		0.092
AGA		340 (80.95%)		145 (79.23%)	
LGA		46 (10.95%)		14 (7.65%)	
SGA		34 (8.10%)		24 (13.11%)	
Breastfeeding status (% yes)	431	343 (79.58%)	290	215 (74.14%)	0.108
Breastfeeding duration (months)	430	3.98 (0.50, 4.89)	290 278	3.29 (0.00, 4.39)	0.018
Day-care attendance (% yes)	397	229 (57.68%)	189	95 (50.26%)	0.110

Table A6.2 (continued. Subcohort: Valencia)

Valencia	Included participants		Excluded participants		
	n	Mean (SD), median (IQR) or n (%)	n	Mean (SD), median (IQR) or n (%)	p-value
Maternal characteristics					
Age at delivery (years)	425	31.75 (4.11)	275	30.62 (4.78)	0.001
MD score	427	4.25 (1.35)	393	4.32 (1.28)	0.448
DASH score	427	23.39 (3.99)	400	23.03 (4.13)	0.211
Gestational age at diet assessment (weeks)	427	12.67 (1.36)	400	13.02 (1.86)	0.003
Total energy intake (Kcal/day)[a]	427	32.47 (1.72)	360	32.85 (2.43)	0.013
Pre-pregnancy BMI	427	2095.00 (1834.00, 2439.00)	358	2269.94 (1895.85, 2634.84)	<0.001
Gestational weight gain	427	22.58 (20.79, 25.40)	398	22.88 (20.53, 25.71)	0.328
Gestational diabetes (% yes)	424	13.95 (5.37)	355	14.57 (5.70)	0.120
Parental education	427	28 (6.56%)	374	19 (5.08%)	0.461
Primary school			400		<0.001
Secondary school		69 (16.16%)		125 (31.25%)	
University degree		211 (49.41%)		189 (47.25%)	
Smoking (% yes)	427	147 (34.43%)	360	86 (21.50%)	0.006
Parity		156 (36.53%)		166 (46.11%)	
No children	427		400		0.056
One child		242 (56.67%)		216 (54.00%)	
Two or more children		158 (37.00%)		140 (35.00%)	
Caesarean section (% yes)	419	27 (6.32%)	359	44 (11.00%)	0.727
		96 (22.91%)		87 (24.23%)	
Children's characteristics					
Sex (% male)	427	217 (50.82%)	360	200 (55.56%)	0.210
Gestational age (weeks)	427	39.62 (1.67)	360	39.37 (2.22)	0.080
Birth weight (g)	427	3240.00 (497.52)	360	3211.33 (560.30)	0.459
Pregnancy outcomes	426		360		0.662
AGA		341 (80.05%)		282 (78.33%)	
LGA		39 (9.15%)		40 (11.11%)	
SGA		46 (10.80%)		38 (10.56%)	
Breastfeeding status (% yes)	427	329 (77.05%)	278	213 (76.62%)	0.967
Breastfeeding duration (months)	427	3.71 (0.50, 5.39)	278	2.71 (0.26, 4.39)	0.107
Day-care attendance (% yes)	423	304 (71.87%)	262	185 (70.61%)	0.790

Cut-off values used to construct the MD and the DASH scores

Table A6.3: Cut-off levels used to compute the MD score

Food item	Cohort						
	Rhea	INMA-Gipuzkoa		INMA-Sabadell		INMA-Valencia	
		First assessment	Second assessment	First assessment	Second assessment	First assessment	Second assessment
	Median (g/day)	Median (g/day)	Median (g/day)	Median (g/day)	Median (g/day)	Median (g/day)	Median (g/day)
Vegetables	211.33	217.10	214.11	228.79	215.67	213.60	209.61
Legumes	7.75	60.23	60.23	20.08	20.08	20.08	20.08
Fruit and nuts	401.65	304.25	281.07	273.69	305.90	240.04	267.13
Dairy products	332.10	353.60	471.45	333.73	450.00	396.45	528.60
Cereals	141.45	156.16	145.49	135.33	133.88	181.19	168.73
Fish	19.31	66.59	68.52	65.29	62.96	57.29	54.51
Olive oil	40.63	8.65	8.65	27.50	27.50	27.50	11.00
Meat[a]	84.17	93.60	93.36	123.88	123.31	129.73	118.20

Note: A score of 1 was assigned for intakes equal to or higher than the reported cut-offs; a score of 0 was given otherwise; a: reversed score.

Table A6.4: Cut-offs levels used to compute the DASH score

Food item		Cohort						
		Rhea	INMA-Gipuzkoa		INMA-Sabadell		INMA-Valencia	
			First assessment	Second assessment	First assessment	Second assessment	First assessment	Second assessment
	Q	Portion/d	Portion/d	Portion/d	Portion/d	Portion/d	Portion/d	Portion/d
Vegetables	1st	<2.43	<1.39	<1.42	<1.47	<1.47	<1.30	<1.21
	5th	>5.42	>3.02	>2.81	>3.31	>3.15	>3.26	>3.26
Nuts and legumes	1st	<0.15	<0.16	<0.19	<0.14	<0.14	<0.13	<0.11
	5th	>0.52	>0.56	>0.47	>0.45	>0.45	>0.45	>0.44
Fruit	1st	<0.72	<1.43	<1.50	<1.20	<1.24	<1.03	<1.14
	5th	>3.43	>3.79	>3.56	>3.79	>3.93	>3.43	>3.43
Dairy products	1st	0.00	<1.60	<1.95	<1.43	<1.82	<1.71	<1.85
	5th	>1.00	>3.85	>4.10	>3.79	>4.04	>4.00	>4.29
Whole grains	1st	<0.03	0.00	0.00	0.00	0.00	0.00	0.00
	5th	>1.71	>0.79	>1.00	>0.79	>0.79	>1.00	>1.00
Red meat[a]	1st	<0.30	<0.14	<0.14	<0.29	<0.29	<0.28	<0.21
	5th	>1.13	>0.50	>0.50	>0.57	>0.64	>0.64	>0.57
Sugary drinks[a]	1st	<0.03	0.00	0.00	<0.07	<0.07	<0.07	<0.07
	5th	>0.92	>0.57	>0.50	>1.14	>1.07	>1.43	>1.14
Sodium[a,b]	1st	<1344.76	<2353.27	<2357.06	<2390.25	<2351.98	<2469.85	<2305.39
	5th	>2845.27	>3528.61	>3442.27	>3612.96	>3588.63	>4053.89	>3784.16

Note: Q: quintiles; Increasing scores were assigned for increasing quintiles of intake (1: first quintile up to 5: fifth quintile); a: reversed score; b: sodium is reported as mg/day.

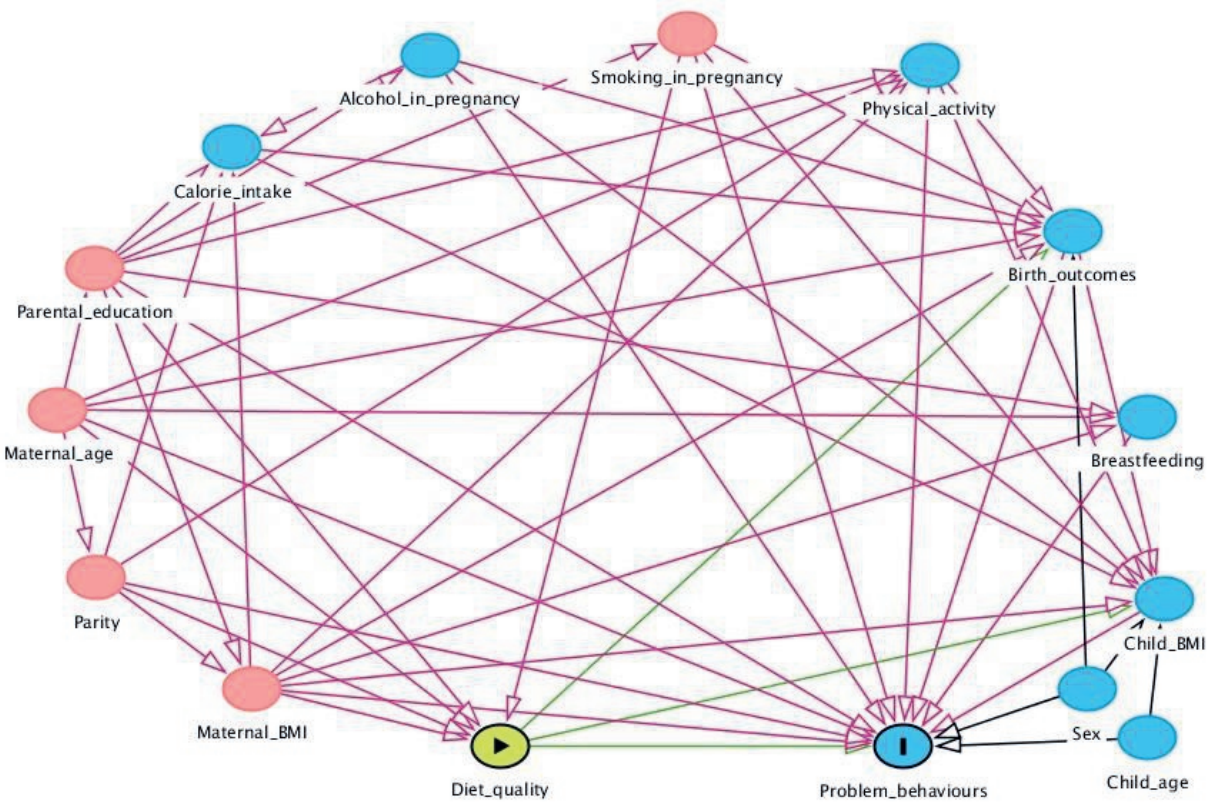

Figure A6.1: Directed Acyclic Graph (DAG)

Note: Green arrows represent causal paths from the exposure; pink arrows represent biasing paths to the exposure and/or the outcome. Red ovals represent the minimal sufficient adjustment set for estimating the total effect of maternal diet quality on childhood problem behaviours. Maternal variables: Diet quality, maternal (pre-pregnancy) BMI, parity, maternal age, smoking and alcohol during pregnancy, total calorie intake, education (maternal and paternal), physical activity during pregnancy. Children's variables: problem behaviours, sex, age at follow-up, birth outcomes (*i.e.*, gestational age and birth weight), breastfeeding, child BMI.

Study population characteristics

Table A6.5: Population characteristics by quintiles of adherence to the MD diet in the Rhea cohort

	Q1 (n=63)		Q2 (n=43)		Q3 (n=59)		Q4 (n=53)		Q5 (n=71)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
MD score	1.62	0.58	3	0	4	0	5	0	6.45	0.58	<0.001
DASH score	23.21	4.12	24.44	3.87	24.54	4.06	26.85	3.69	27.13	3.85	<0.001
Age at delivery (years)	29.32	4.3	29.6	5.34	31.21	4.67	30.96	4.42	29.8	4.89	0.118
Total energy intake (Kcal/day)	1,470	473	1,687	500	2,144	597	2,179	598	2,531	763	<0.001
Pre-pregnancy BMI (kg/m2)	24.84	22.21, 29.10	23.51	22.48, 28.27	23.63	21.93, 25.34	22.34	21.54, 26.18	24.34	21.51, 27.06	0.292
Parental education											0.121
Primary school	9	14.3%	6	14%	2	3.4%	4	7.5%	3	4.3%	
Secondary school	35	5.6%	23	53.5%	29	49.2%	23	43.4%	35	50%	
University degree	19	30.2%	14	32.6%	28	47.5%	26	49.1%	32	45.7%	
Smoking (% ever)	11	19%	6	17.1%	11	21.6%	6	12.5%	12	20%	0.807
Alcohol (% ever)	20	31.7%	16	37.2%	21	35.6%	12	22.6%	20	28.2%	0.497
Parity											0.074
No children	22	34.9%	15	36.6%	25	42.4%	28	54.9%	29	41.4%	
One child	23	36.5%	19	46.3%	28	47.5%	19	37.3%	31	44.3%	
Two + children	18	28.6%	7	17.1%	6	10.2%	4	7.8%	10	14.3%	

Table A6.5 (continued)

	Q1 (n=63)		Q2 (n=43)		Q3 (n=59)		Q4 (n=53)		Q5 (n=71)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
Children's sex (% female)	23	36.5%	14	32.6%	24	40.7%	26	49.1%	30	42.3%	0.518
Gestational age (weeks)	38.27	1.72	38.19	1.52	38.05	1.78	38.48	1.23	38.01	1.58	0.524
Birthweight (g)	3,341	385	3,333	506	3,207	452	3,252	437	3,177	457	0.173
Breastfeeding duration (months)	3.44	3.53	3.52	3.92	3.84	4.44	4.73	4.87	3.98	4.29	0.55
Children's age (years)	6.55	0.28	6.62	0.33	6.61	0.25	6.6	0.27	6.59	0.3	0.753
Total problems											
Continuous	52.4	8.02	52.16	9.86	50.73	10.05	51	9.58	51.34	8.5	0.838
Binary	10	15.9%	13	30.2%	8	13.6%	8	15.1%	11	15.5%	0.197
Internalising behaviours											
Continuous	52.52	8.88	51.98	9.15	51.31	8.8	51.32	9.64	50.76	8.66	0.833
Binary	19.00	30.2%	10.00	23.3%	9.00	15.3%	11.00	20.8%	14.00	19.7%	0.363
Externalising behaviours											
Continuous	54.97	7.36	54.28	10.27	53.15	9.9	52.87	9.26	53.27	8.44	0.686
Binary	13.00	20.6%	12.00	27.9%	14.00	23.7%	9.00	17%	17.00	23.9%	0.753

Note: Q1 (quintile 1): MD ≤2.5; Q2 (quintile 2): MD >2.5-3.5; Q3 (quintile 3): MD= 4; Q4 (quintile 4): MD= 4.5-5; Q5 (quintile 5): MD > 5.

Table A6.6: Population characteristics by quintiles of adherence to the MD diet in the INMA cohort

	Q1 (n=162)		Q2 (n=269)		Q3 (n=187)		Q4 (n=305)		Q5 (n=331)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
MD score	1.68	0.42	2.77	0.25	3.5	0	4.23	0.25	5.52	0.56	<0.001
DASH score	19.69	3.92	21.62	3.82	22.07	3.95	23.41	4.01	24.69	4.12	<0.001
Age at delivery (years)	30.96	3.98	32.1	3.94	31.68	3.71	32.2	3.69	32.53	3.87	<0.001
Total energy intake (Kcal/day)	1,777	394	1,855	343	1,983	360	2,080	391	2,342	446	<0.001
Pre-pregnancy BMI (kg/m2)	23.04	21.32, 25.75	22.94	20.87, 24.88	22.58	20.73, 25.16	22.06	20.57, 24.91	22.35	20.77, 24.86	0.035
Parental education											0.824
Primary school	21	13%	26	9.7%	21	11.2%	35	11.5%	42	12.7%	
Secondary school	68	42.2%	108	40.1%	84	44.9%	125	41.1%	145	43.9%	
University degree	72	44.7%	135	50.2%	82	43.9%	144	47.4%	143	43.3%	
Smoking (% ever)	48	30.6%	85	31.8%	56	29.9%	78	25.9%	91	27.8%	0.571
Alcohol (% ever)	12	7.7%	25	9.5%	12	6.4%	30	9.9%	31	9.6%	0.662
Parity											0.768
No children	96	59.3%	162	60.2%	102	54.5%	181	59.5%	176	53.3%	
One child	57	35.2%	93	34.6%	75	40.1%	105	34.5%	134	40.6%	
Two + children	9	5.6%	14	5.2%	10	5.3%	18	5.9%	20	6.1%	

Table A6.6 (continued)

	Q1 (n=162)		Q2 (n=269)		Q3 (n=187)		Q4 (n=305)		Q5 (n=331)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
Children's sex (% female)	71	43.8%	147	54.6%	98	52.4%	142	46.6%	159	48%	0.143
Gestational age (weeks)	39.81	1.38	39.75	1.52	39.55	1.42	39.74	1.41	39.68	1.66	0.511
Birthweight (g)	3,334	441	3,229	484	3,233	428	3,293	421	3,269	467	0.115
Breastfeeding duration (months)	7.4	8.69	8.31	8.68	8.67	8.65	9.68	8.92	9.44	9.05	0.149
Children's age (years)	8.62	0.75	8.62	0.75	8.67	0.73	8.69	0.77	8.81	0.75	0.017
Total problems											
Continuous	53.24	10.14	51.63	9.98	51	9.53	51.1	10.45	52.08	10.49	0.192
Binary	48	29.6%	58	21.6%	39	20.9%	67	22%	82	24.8%	0.251
Internalising behaviours											
Continuous	53.85	10.29	53.14	9.61	52.3	8.92	52.58	9.96	53.9	9.82	0.265
Binary	50	30.9%	71	26.4%	47	25.1%	75	24.6%	100	30.2%	0.389
Externalising behaviours											
Continuous	52.13	9.45	50.52	9.98	50.72	9.83	50.44	9.85	50.98	10	0.455
Binary	37.00	22.8%	55.00	20.4%	32.00	17.1%	50.00	16.4%	67.00	20.2%	0.412

Note: Q1 (quintile 1): MD ≤2.5; Q2 (quintile 2): MD >2.5-3.5; Q3 (quintile 3): MD= 4; Q4 (quintile 4): MD= 4.5-5; Q5 (quintile 5): MD > 5.

Table A6.7: Population characteristics by quintiles of adherence to the DASH diet in the Rhea cohort

	Q1 (n=58)		Q2 (n=69)		Q3 (n=74)		Q4 (n=44)		Q5 (n=44)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
MD score	3.14	1.75	3.94	1.76	4.15	1.75	4.82	1.40	4.93	1.66	<0.001
DASH score	19.43	1.5	23.09	0.8	26.14	0.83	28.52	0.51	31.84	1.72	<0.001
Age at delivery (years)	27.93	4.2	29.2	4.07	30.95	4.57	31.36	4.36	32.05	5.65	<0.001
Total energy intake (Kcal/day)	2,227	790	2,059	798	1,927.10	690	2,109	702	1,823	456	0.037
Pre-pregnancy BMI (kg/m2)	23.52	21.76, 26.90	23.63	21.66, 26.57	23.34	21.58, 27.12	23.71	22.10, 26.22	24.92	22.85, 28.48	0.440
Parental education											0.027
Primary school	6	10.3%	6	8.8%	4	5.4%	5	11.4%	3	6.8%	
Secondary school	40	69%	35	51.5%	35	47.3%	15	34.1%	20	45.5%	
University degree	12	20.7%	27	39.7%	35	47.3%	24	54.5%	21	47.7%	
Smoking (% ever)	13	26.5%	14	23.7%	9	14.1%	6	15.4%	4	9.8%	0.172
Alcohol (% ever)	15	25.9%	26	37.7%	24	32.4%	15	34.1%	9	20.5%	0.317
Parity											0.37
No children	25	44.6%	23	33.8%	29	40.3%	20	45.5%	22	50%	
One child	19	33.9%	37	54.4%	32	44.4%	18	40.9%	14	31.8%	
Two + children	12	21.4%	8	11.8%	11	15.3%	6	13.6%	8	18.2%	

Table A6.7 (continued)

	Q1 (n=58)		Q2 (n=69)		Q3 (n=74)		Q4 (n=44)		Q5 (n=44)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
Children's sex (% female)	24	41.4%	30	43.5%	28	37.8%	14	31.8%	21	47.7%	0.588
Gestational age (weeks)	38.19	1.39	38.12	1.96	38.27	1.52	38	1.67	38.34	1.22	0.851
Birthweight (g)	3,247	401	3,248	487	3,276	453	3,198	473	3,306	410	0.834
Breastfeeding duration (months)	3.64	4.44	3.23	3.59	4.38	4.43	4.04	4.22	4.35	4.55	0.504
Children's age (years)	6.57	0.28	6.58	0.28	6.55	0.22	6.68	0.34	6.64	0.31	0.102
Total problems											
Continuous	52.16	10.4	51.54	8.71	52.34	8.83	49.8	9.48	50.91	8.01	0.62
Binary	13	22.4%	9	13%	14	18.9%	7	15.9%	7	15.9%	0.701
Internalising behaviours											
Continuous	52.31	9.9	51.41	8.37	51.99	9.05	51.32	8.47	50.2	9.07	0.805
Binary	18	31.0%	10	14.5%	18	24.3%	9	20.5%	8	18.2%	0.221
Externalising behaviours											
Continuous	54.81	9.5	53.67	9.3	54.58	8.77	51.27	9.21	53.18	7.43	0.289
Binary	17	29.3%	15	21.7%	19	25.7%	7	15.9%	7	15.9%	0.381

Note: Q1 (quintile 1): DASH ≤ 19.5; Q2 (quintile 2): DASH >19.5-22; Q3 (quintile 3): DASH >22-24; Q4 (quintile 4): DASH >24-27; Q5 (quintile 5): DASH > 27.

Table A6.8: Population characteristics by quintiles of adherence to the DASH diet in the INMA cohort

	Q1 (n=357)		Q2 (n=298)		Q3 (n=211)		Q4 (n=195)		Q5 (n=193)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
MD score	3.21	1.25	3.72	1.3	3.9	1.22	4.18	1.14	4.65	1.28	<0.001
DASH score	17.6	2.06	21.55	0.68	23.69	0.56	25.88	0.69	29.5	1.68	<0.001
Age at delivery (years)	30.86	4.06	32.22	3.63	32.25	3.87	32.23	3.52	33.46	3.55	<0.001
Total energy intake (Kcal/day)	2,176	459	2,041	447	1,986	416	1,991	423	1,947	390	<0.001
Pre-pregnancy BMI (kg/m2)	22.35	20.43, 25.04	22.86	20.97, 25.14	22.66	20.85, 24.95	22.41	20.82, 25.48	22.39	21.02, 24.34	0.452
Parental education											<0.001
Primary school	63	17.7	36	12.1	25	11.9	9	4.6	12	6.2	
Secondary school	172	48.3	127	42.8	86	41	80	41	65	33.7	
University degree	121	34	134	45.1	99	47.1	106	54.4	116	60.1	
Smoking (% ever)	131	37.4	93	31.5	62	29.7	45	23.2	27	14.1	<0.001
Alcohol (% ever)	42	12	30	10.3	11	5.3	13	6.8	14	7.3	0.044
Parity											0.335
No children	198	55.6	159	53.4	125	59.2	115	59.3	120	62.2	
One child	136	38.2	124	41.6	76	36	70	36.1	58	30.1	
Two + children	22	6.2	15	5	10	4.7	9	4.6	15	7.8	

Table A6.8 (continued)

	Q1 (n=357)		Q2 (n=298)		Q3 (n=211)		Q4 (n=195)		Q5 (n=193)		
	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	Mean, median or n	SD, IQR or %	p
Children's sex (% female)	179	50.1	147	49.3	102	48.3	90	46.2	99	51.3	0.869
Gestational age (weeks)	39.73	1.45	39.70	1.59	39.70	1.41	39.64	1.55	39.76	1.51	0.949
Birthweight (g)	3,250	454	3,281	455	3,254	446	3,257	456	3,317	445	0.497
Breastfeeding duration (months)	7.75	8.51	9.28	9.16	8.58	8.63	9.70	8.57	9.68	9.38	0.141
Children's age (years)	8.83	0.66	8.79	0.73	8.69	0.77	8.58	0.8	8.42	0.81	<0.001
Total problems											
Continuous	52.84	9.96	52.41	10.18	50.57	9.94	51.41	10.47	50.25	10.44	0.014
Binary	95	26.6%	78	26.2%	43	20.4%	40	20.5%	38	19.7%	0.148
Internalising behaviours											
Continuous	53.74	9.59	53.74	9.82	52.22	9.63	53.28	9.81	52.18	9.94	0.181
Binary	97	27.2%	90	30.2%	51	24.2%	55	28.2%	50	25.9%	0.632
Externalising behaviours											
Continuous	51.62	10.10	51.52	9.46	49.82	9.59	50.52	9.89	49.93	10.21	0.103
Binary	80	22.4%	57	19.1%	35	16.6%	35	17.9%	34	17.6%	0.433

Note: Q1 (quintile 1): DASH ≤ 19.5; Q2 (quintile 2): DASH >19.5-22; Q3 (quintile 3): DASH >22-24; Q4 (quintile 4): DASH >24-27; Q5 (quintile 5): DASH > 27.

Full-model-estimate tables

Table A6.9: Full-model estimates of the association between maternal MD score and childhood problem behaviours (binary outcomes)

	Total problems		Internalising problems		Externalising problems	
	OR	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
MD score	0.93	0.84, 1.03	0.93	0.84, 1.03	0.88	0.79, 0.98
Pre-pregnancy BMI	1.04	1.01, 1.06	1.03	1.01, 1.06	1.05	1.02, 1.08
Parity (one child)	0.66	0.50, 0.88	0.52	0.40, 0.69	0.79	0.59, 1.07
Parity (two or more children)	0.72	0.43, 1.23	0.55	0.33, 0.92	0.66	0.37, 1.15
Smoking (ever)	1.50	1.14, 1.98	1.32	1.01, 1.72	1.62	1.21, 2.16
Age at delivery	0.98	0.95, 1.02	1.01	0.97, 1.04	0.99	0.95, 1.02
Education (secondary school)	0.68	0.47, 1.00	0.71	0.49, 1.03	0.81	0.55, 1.21
Education (university degree)	0.44	0.29, 0.67	0.44	0.30, 0.67	0.55	0.36, 0.85
Children's sex (female)	0.63	0.49, 0.81	0.43	0.34, 0.55	0.56	0.43, 0.73
Children's age	1.10	0.81, 1.50	1.30	0.96, 1.76	0.89	0.64, 1.25
Region (Gipuzkoa)	0.97	0.54, 1.73	0.94	0.55, 1.61	0.85	0.47, 1.54
Region (Sabadell)	1.43	0.59, 3.45	0.73	0.31, 1.74	1.23	0.49, 3.09
Region (Valencia)	1.26	0.52, 3.05	0.68	0.29, 1.61	1.09	0.43, 2.77
Total energy intake	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00
Constant	0.15	0.01, 1.76	0.04	0.00, 0.44	0.24	0.02, 3.37

Table A6.10: Full-model estimates of the association between maternal DASH score and childhood problem behaviours (binary outcomes)

	Total problems		Internalising problems		Externalising problems	
	OR	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
MD score	1.00	0.97, 1.03	1.01	0.97, 1.04	1.00	0.97, 1.04
Pre-pregnancy BMI	1.04	1.01, 1.06	1.03	1.01, 1.06	1.05	1.02, 1.08
Parity (one child)	0.67	0.50, 0.89	0.53	0.40, 0.70	0.81	0.60, 1.09
Parity (two or more children)	0.74	0.44, 1.26	0.57	0.35, 0.95	0.69	0.40, 1.21
Smoking (ever)	1.52	1.15, 2.01	1.35	1.03, 1.76	1.66	1.24, 2.22
Age at delivery	0.98	0.95, 1.01	1.00	0.97, 1.03	0.98	0.95, 1.02
Education (secondary school)	0.68	0.47, 0.99	0.70	0.48, 1.02	0.80	0.54, 1.19
Education (university degree)	0.44	0.29, 0.66	0.44	0.29, 0.66	0.54	0.35, 0.83
Children's sex (female)	0.63	0.49, 0.81	0.43	0.34, 0.55	0.56	0.43, 0.73
Children's age	1.08	0.80, 1.48	1.27	0.94, 1.72	0.87	0.62, 1.21
Region (Gipuzkoa)	1.01	0.56, 1.82	1.00	0.58, 1.74	0.94	0.52, 1.71
Region (Sabadell)	1.52	0.61, 3.77	0.81	0.34, 1.98	1.42	0.55, 3.67
Region (Valencia)	1.35	0.55, 3.34	0.76	0.32, 1.83	1.27	0.49, 3.30
Total energy intake	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00
Constant	0.17	0.01, 2.12	0.04	0.00, 0.50	0.31	0.02, 4.38

Table A6.11: Full-model estimates of the association between maternal MD score and childhood problem behaviours (continuous outcomes)

	Total problems		Internalising problems		Externalising problems	
	Beta	**95% CI**	**Beta**	**95% CI**	**Beta**	**95% CI**
MD score	-0.48	-0.88, -0.08	-0.33	-0.72, 0.05	-0.48	-0.87, -0.09
Pre-pregnancy BMI	0.26	0.14, 0.37	0.22	0.11, 0.33	0.25	0.14, 0.36
Parity (one child)	-2.03	-3.11, -0.94	-2.23	-3.28, -1.18	-1.13	-2.20, -0.07
Parity (two or more children)	-3.46	-5.43, -1.49	-2.82	-4.73, -0.91	-2.78	-4.71, -0.84
Smoking (ever)	1.24	0.11, 2.37	0.94	-0.16, 2.03	1.39	0.28, 2.50
Age at delivery	-0.07	-0.20, 0.06	-0.02	-0.15, 0.10	-0.08	-0.21, 0.05
Education (secondary school)	-1.15	-2.80, 0.51	-1.04	-2.64, 0.57	-0.42	-2.04, 1.21
Education (university degree)	-3.59	-5.31, -1.86	-2.89	-4.56, -1.22	-2.61	-4.30, -0.91
Children's sex (female)	-1.85	-2.81, -0.89	-2.51	-3.44, -1.58	-1.77	-2.71, -0.82
Children's age	-0.11	-1.35, 1.12	0.22	-0.98, 1.42	-0.26	-1.47, 0.96
Region (Gipuzkoa)	-1.08	-3.24, 1.08	0.23	-1.86, 2.32	-2.94	-5.06, -0.81
Region (Sabadell)	1.04	-2.45, 4.53	1.88	-1.50, 5.26	-2.37	-5.79, 1.06
Region (Valencia)	1.54	-1.97, 5.05	1.54	-1.87, 4.94	-1.05	-4.50, 2.40
Total energy intake	0.00	0.00, 0.00	0.00	0.00, 0.00	0.00	0.00, 0.00
Constant	49.99	40.01, 59.97	47.22	37.55, 56.89	52.74	42.95, 62.54

Table A6.12: Full-model estimates of the association between maternal DASH score and childhood problem behaviours (continuous outcomes)

	Total problems		Internalising problems		Externalising problems	
	Beta	**95% CI**	**Beta**	**95% CI**	**Beta**	**95% CI**
MD score	-0.05	-0.17, 0.08	-0.03	-0.15, 0.09	-0.03	-0.15, 0.09
Pre-pregnancy BMI	0.26	0.14, 0.37	0.22	0.11, 0.33	0.25	0.14, 0.36
Parity (one child)	-2.00	-3.10, -0.91	-2.21	-3.27, -1.15	-1.09	-2.17, -0.02
Parity (two or more children)	-3.35	-5.32, -1.37	-2.74	-4.65, -0.83	-2.65	-4.58, -0.71
Smoking (ever)	1.30	0.16, 2.43	0.98	-0.12, 2.08	1.47	0.35, 2.59
Age at delivery	-0.08	-0.22, 0.05	-0.03	-0.17, 0.10	-0.10	-0.24, 0.03
Education (secondary school)	-1.20	-2.86, 0.46	-1.07	-2.68, 0.53	-0.47	-2.10, 1.15
Education (university degree)	-3.64	-5.37, -1.91	-2.93	-4.61, -1.26	-2.68	-4.38, -0.98
Children's sex (female)	-1.88	-2.84, -0.92	-2.53	-3.46, -1.60	-1.79	-2.74, -0.85
Children's age	-0.17	-1.41, 1.08	0.18	-1.03, 1.39	-0.33	-1.56, 0.89
Region (Gipuzkoa)	-0.89	-3.08, 1.30	0.38	-1.74, 2.49	-2.69	-4.83, -0.54
Region (Sabadell)	1.18	-2.41, 4.77	2.00	-1.48, 5.47	-2.11	-5.63, 1.42
Region (Valencia)	1.75	-1.85, 5.35	1.71	-1.77, 5.19	-0.72	-4.26, 2.81
Total energy intake	0.00	0.00, 0.00	0.00	0.00, 0.00	0.00	0.00, 0.00
Constant	51.49	41.44, 61.54	48.22	38.50, 57.95	54.05	44.19, 63.92

Sensitivity analyses

Table A6.13: Sensitivity analyses (binary outcomes)

Sensitivity analyses		Total problems		Internalising behaviours		Externalising behaviours	
		OR	95% C.I.	OR	95% C.I.	OR	95% C.I.
Additional control for alcohol intake[a]	MD	0.93	0.84, 1.03	0.93	0.85, 1.03	0.88	0.80, 0.98
	DASH	1.00	0.97, 1.03	1.01	0.98, 1.04	1.01	0.97, 1.04
Additional control for breastfeeding duration[a]	MD	0.94	0.85, 1.04	0.94	0.85, 1.04	0.89	0.80, 0.99
	DASH	1.00	0.97, 1.03	1.00	0.97, 1.04	1.00	0.97, 1.04
Complete-case analyses[b]	MD	0.91	0.82, 1.02	0.91	0.82, 1.01	0.87	0.78, 0.97
	DASH	1.00	0.97, 1.03	1.01	0.98, 1.04	1.01	0.97, 1.04
Additional control for physical activity in pregnancy[c]	MD	0.92	0.82, 1.04	0.98	0.87, 1.10	0.87	0.77, 1.00
	DASH	1.01	0.97, 1.05	1.02	0.99, 1.06	1.03	0.99, 1.07

Note: a: n= 1543; b: n=1468; c: n= 1205 (complete-case analyses with INMA data only).

Table A6.14: Sensitivity analyses (continuous outcomes)

Sensitivity analyses		Total problems		Internalising behaviours		Externalising behaviours	
		Beta	95% C.I.	Beta	95% C.I.	Beta	95% C.I.
Additional control for alcohol intake[a]	MD	-0.47	-0.87, -0.08	-0.33	-0.71, 0.06	-0.47	-0.86, -0.08
	DASH	-0.04	-0.17, 0.08	-0.03	-0.15, 0.09	-0.02	-0.15, 0.10
Additional control for breastfeeding duration[a]	MD	-0.43	-0.82, -0.03	-0.29	-0.67, 0.10	-0.44	-0.82, -0.05
	DASH	-0.05	-0.17, 0.08	-0.03	-0.15, 0.09	-0.03	-0.15, 0.09
Complete-case analyses[b]	MD	-0.57	-0.98, -0.16	-0.43	-0.82, -0.03	-0.56	-0.96, -0.16
	DASH	-0.05	-0.17, 0.08	-0.01	-0.14, 0.11	-0.03	-0.16, 0.09
Additional control for physical activity in pregnancy[c]	MD	-0.50	-0.98, -0.01	-0.17	-0.63, 0.29	-0.50	-0.98, -0.03
	DASH	-0.03	-0.17, 0.12	0.01	-0.12, 0.15	0.00	-0.14, 0.14

Note: a: n= 1543; b: n=1468; c: n= 1205 (complete-case analyses with INMA data only).

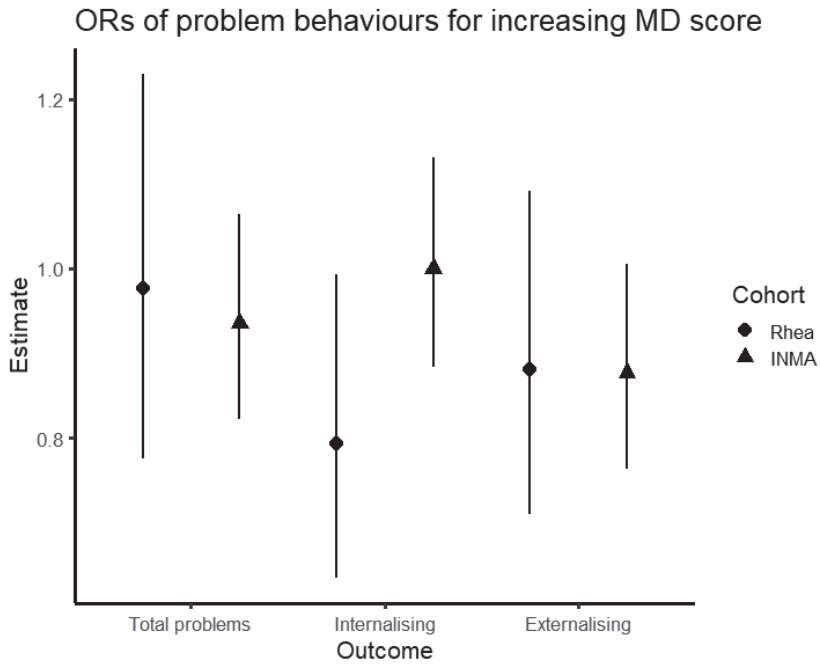

Figure A6.2: Estimate plots by cohort and outcome (MD score, binary outcomes)

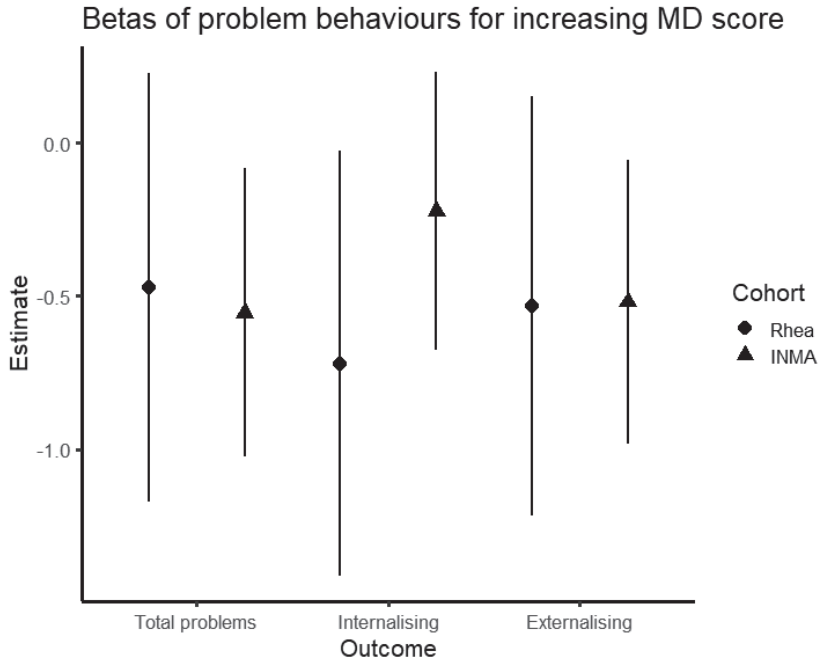

Figure A6.3: Estimate plots by cohort and outcome (MD score, continuous outcomes)

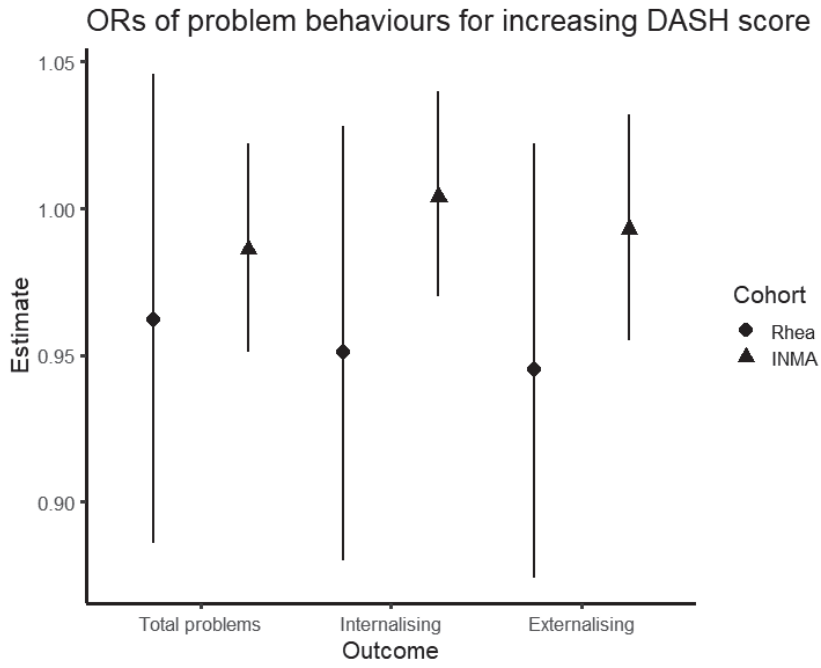

Figure A6.4: Estimate plots by cohort and outcome (DASH score, binary outcomes)

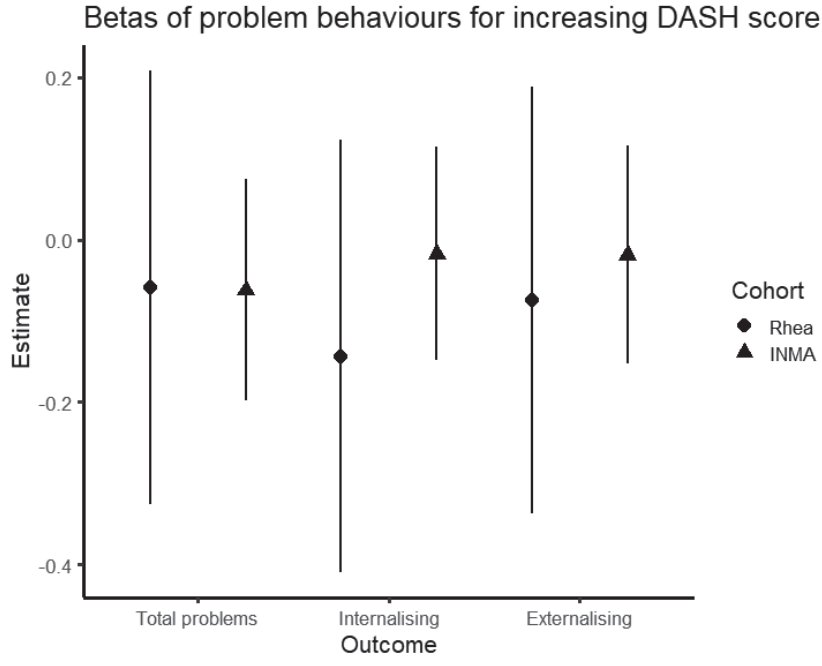

Figure A6.5: Estimate plots by cohort and outcome (DASH score, continuous outcomes)

Chapter 7

General discussion

Affecting 10%-20% of children and adolescents worldwide, mental disorders are the leading cause of disability in this population (1–5). Considering that treatment of young psychiatric patients would result in considerable economic and personal burden (6,7), primary prevention through the promotion of an optimal psychological development appears the best strategy towards a healthy life. Mounting evidence that followed the publication of the Developmental Origin of Health and Disease (DOHaD) hypothesis (8–10) suggests that several intrauterine factors might influence the psychological development of the child.

This thesis aimed to assess the influence of prenatal, environmental factors on childhood psychological development. In **chapter 2** we examined the association between intrapair birth-weight difference and the difference in problem behaviours of preschool-age twins from the UK-based Twins and Multiple Birth Association Heritability Study (TAMBAHS). From **chapter 3** to **chapter 6** we explored the possible influence of maternal lifestyle factors, associated with children's birth weight, on psychological development: in **chapter 3** we evaluated the relationship between maternal pre-pregnancy body mass index (BMI) and infants' temperament using data from TAMBAHS, while in **chapter 4** we described the association between gestational weight gain (GWG) and childhood internalising and externalising problem behaviours. To do so, we pooled together individual data from the Maastricht Essential Fatty Acids Birth cohort (MEFAB) from Limburg, the Netherlands, and the Rhea Mother-Child birth cohort from Crete, Greece. We, then, examined the associations between maternal dietary factors and child psychological development (**chapters 5 and 6**). Firstly, we assessed the relationship between changes in maternal polyunsaturated fatty acid (PUFA) levels during pregnancy and childhood social competence or problem behaviours in the MEFAB cohort (**chapter 5**). Secondly, in **chapter 6** we explored the association between maternal diet quality, measured with the Mediterranean Diet (MD) and the Dietary Approaches to Stop Hypertension (DASH) scores, and problem behaviours in school-age children from two Southern European birth cohorts: Rhea and INMA (INfancia y Medio Ambiente, from Spain).

This last chapter provides a general overview and interpretation of research findings, placing them into a wider context. Moreover, it provides general methodological considerations, future research directions and a discussion of the implications of research findings.

Summary of main findings

Birth weight and childhood psychological development

In **chapter 2**, we used multivariate linear regression analyses adjusted for maternal and childhood sociodemographic characteristics to investigate the association between the intrapair birth-weight difference and the difference in each problem behaviour scale in preschool-age monozygotic (MZ) and dizygotic (DZ) twins. The discordant-twin study design automatically controls for common genetic and environmental factors between co-twins, allowing the investigation of the effects of non-shared influences (which are exclusively environmental in nature in MZ twins, but genetic and environmental in DZ twins) responsible for the difference in birth weight (11,12). In the subgroup of MZ twins, we found that increasing birth-weight difference was associated with increasing differences in total problems (β= -5.95; 95% confidence interval, CI: -11.08, -0.82), driven by internalising behaviours (β= -4.17; 95% CI: -7.65, -0.69) and, specifically, emotional reactiveness (β= -2.70; 95% CI: -5.23, -0.17). Furthermore, increasing birth-weight difference was associated with larger predicted-score differences (*i.e.*, more negative predicted-score differences) in the three previously identified scales in MZ twins. This suggests that infants with a low birth weight might be more at risk of developing internalising problem (note that differences in problem-behaviour scores were calculated by subtracting the score of the smaller-at-birth twin from the score of the larger-at-birth twin; see **chapter 2** for details). Conversely, no associations were observed when examining DZ twins or MZ and DZ twins combined. Although none of the identified associations remained statistically significant after controlling for multiple testing, these results are in line with other studies done both in older twins (13–15) and singletons (*e.g.*, (16–19)), which reported an inverse relationship between birth weight and internalising behaviours. A similar trend is sometimes reported for externalising behaviours, although with smaller effect sizes (15,20–22). By contrast, a few other studies did not report any association between birth weight and childhood (23) or adolescence (24) problem behaviours in singletons.

Maternal weight before and during pregnancy and childhood psychological development

Maternal pre-pregnancy body mass index

The study described in **chapter 3** aimed to examine the association between maternal pre-pregnancy BMI and infant twins' temperament. The twin study design accounted for genetic and

environmental factors shared between co-twins (25), while allowing the assessment of the overall effect of maternal pre-pregnancy BMI on three temperamental scales (*i.e.,* activity level, distress to limitation and duration of orienting). We found a negative association with DZ twins' distress to limitation (β= -0.04; 95% CI: -0.065, -0.013), indicating that children born to women with a higher pre-pregnancy BMI would display fewer distress when confined in a place or position. However, the analyses in DZ twins are susceptible to residual confounding, as genetic influences cannot be completely controlled for. Furthermore, the estimate was very close to the null value and not significant after controlling for multiple testing. By contrast, no associations were found when examining the other temperamental scales or in MZ twins.

Previous studies in singletons reported poorer regulatory behaviours in infants born to women with pre-pregnancy overweight or obesity (26) or pre-pregnancy obesity in combination with excessive GWG (27). Maternal pre-pregnancy BMI was also associated with infants' negative affectivity (28) and negative behaviours (29), although in the last study the effect was reduced by high total n-3 PUFA concentrations in the last trimester of pregnancy. By contrast, maternal pre-pregnancy BMI was not found to be related with offspring temperament in children aged 1 year (30). However, while in two cases results should be considered preliminary due to the small sample size (*i.e.*, n=16 and n=68, (28,29)), overall no attempt to control for genetic or common environmental factors was made, which might have affected previous studies' results.

Gestational weight gain

The purpose of the study presented in **chapter 4** was to investigate the association between GWG and problem behaviours in school-age children. In addition, we assessed the possible effect modification of maternal pre-pregnancy BMI status, since previous studies reported a higher likelihood of excessive GWG in women with pre-pregnancy overweight or obesity (31). With this pooled analysis of individual data from the MEFAB and Rhea cohorts, we found evidence for higher problem behaviours associated with excessive GWG in pre-pregnancy overweight or obese women, while no evidence of an association was found in the pre-pregnancy normal-weight group. On average, children born to overweight/obese women who gained 0.5 kg/week scored 25 points higher (on a 0-100 scale) in both total problems and internalising behaviours than children whose mothers gained only 0.2 kg/week. Similarly, children born to overweight or obese women who gained 0.5 kg/week tended to have higher externalising problems, although the difference with children of women who gained less weight was smaller (*i.e.*, 18 points). Notably, being overweight

or obese and gaining 0.5 kg/week was predictive of problem behaviour scores in the clinical range of symptomatology as defined by the Child Behaviour Checklist (CBCL, *i.e.*, over 63/100).

These results are supported by previous findings showing higher infants' neurobehaviour and childhood attention deficit/hyperactivity disorder (ADHD) risk in children of pre-pregnancy overweight or obese women who gained excessive weight during pregnancy (27,32,33). Higher hyperactivity/impulsivity symptoms were also observed in children of women with insufficient compared to adequate GWG (33). By contrast, the only study that assessed specifically childhood problem behaviours, as well as ADHD symptoms, did not find any evidence of an association with GWG (34). However, the latter study examined data from a high-risk population, comprising a large proportion of young, never-married women with a low income who made use of illicit drugs, alcohol and marijuana during pregnancy. As we will discuss below (pages 239-240), the peculiarity of this study population precludes direct comparisons with our study, whose population can be considered at low risk, being largely composed of highly educated, double-parent families.

Maternal diet during pregnancy and childhood psychological development

Maternal PUFA concentrations

In the study presented in **chapter 5**, we focused on maternal PUFAs during pregnancy in relation to social competence and problem behaviours in school-age children from the MEFAB cohort. Since maternal PUFA concentrations change physiologically during pregnancy (35,36), we calculated the trend of change of maternal PUFAs during pregnancy. The results indicated that increasing maternal arachidonic acid (AA, a n-6 PUFA) concentration in late pregnancy (*i.e.,* defined as the period after the 30th week of gestation) might be associated with a small improvement in social competence. Furthermore, a larger decrease in total n-6 PUFAs in late gestation was associated with a small decrease in externalising behaviours. The clinical relevance of the two associations was modest, with predicted scores falling within the normal-development range as defined by the CBCL (*i.e.,* over 40/100 and less than 63/100 for social competence and externalising behaviours, respectively). In addition, associations fell short of statistical significance after controlling for multiple testing.

Previous observational studies analysed PUFA concentrations in the umbilical cord or in maternal blood at specific time-points, and reported largely inconsistent results (37–41). The only attempt to examine the whole prenatal exposure longitudinally was done by estimating maternal n-3 PUFA levels by assessing fish and seafood intake during pregnancy. Small improvements in hyperactivity and prosocial behaviour were reported in children of women with the highest fish or seafood intake

(42–44). However, assessing maternal fish and seafood intake alone limits the investigation to maternal n-3 PUFAs, while fatty acids of the n-6 family might also have a role. Furthermore, examining maternal PUFA concentration at a specific time-point or estimating n-3 PUFA intake cannot provide an adequate representation of prenatal PUFA exposure, which changes over time based on both maternal dietary intakes and the release of fatty acids from maternal adipose tissue (35,36,45). Finally, the role of prenatal PUFA exposure on neurocognitive and behavioural outcomes has been investigated by only a few, small randomised controlled trials (RCTs), which reported no effect of maternal supplementation on infants' outcomes (46).

Maternal diet quality

In **chapter 6**, we examined the association between maternal diet quality during pregnancy, measured with the MD or the DASH scores, and problem behaviours in 1,543 school-age children from the Rhea and the INMA cohorts. These two dietary scores were selected because they are based on healthy, practicable and understandable diet plans (47–49) that have been associated with reduced risk of psychiatric disorders in children and adults (*e.g.,* (50–52)). The results showed that a one-point increase in Mediterranean diet (MD) score, which could range from 0 to 8, was associated with lower odds of externalising behaviours (OR: 0.88; 95% C.I.: 0.79, 0.98). Overall, we estimated that the prevalence of childhood externalising behaviours in this population would be 16.4% lower (*i.e.*, from 28.9% to 12.5%) in children whose mothers had the highest compared to lowest MD score. Conversely, maternal diet quality assessed by adherence to the DASH diet was not associated with childhood problem behaviours. Compared to the MD, in the DASH score used for these analyses, intakes of fish and seafood are not assessed, while it is an integral part of the MD score and the MD dietary pattern in general. Furthermore, intake of fish and seafood was more modestly correlated with DASH score in this study population than with MD score, suggesting that prenatal exposure to fish and seafood might be beneficial to prevent later externalising behaviours.

Previous studies that examined the association between maternal dietary patters during pregnancy and childhood problem behaviours in other European populations found reduced odds of externalising behaviours in children of women following a "healthy" dietary pattern that resembled the MD, reporting overall similar effect estimates compared to those observed in our study (53–55). In addition, a re-analysis of the data to support findings obtained with principal component analysis found a small reduction in the odds of childhood externalising behaviours associated with higher adherence to the MD score (54). Moreover, higher adherence to a diet quality index characterised by high intakes of fruit, vegetables, fish and whole grains, was associated with a small reduction in

both internalising and externalising behaviours in preschool-age Norwegian children (56). Finally, maternal adherence to the MD at the time of conception was associated with fewer internalising and externalising behaviour symptoms in 2-year-old children from the USA. These associations corresponded to changes in the methylation patterns in the control regions of several imprinted genes, suggesting a possible mechanism underlying the association between maternal diet quality prenatally and childhood problem behaviours (57).

Methodological considerations

In this thesis we examined the relationships between a few prenatal factors and childhood psychological development. Because of ethical and practicability reasons, most studies assessing prenatal exposure, including all studies presented in this thesis, make use of an observational study design. Consequently, the interpretation of research findings as evidence of causality cannot prescind from a discussion of confounding as well as the risk of misclassification of exposures and outcomes that derives from the use of self-reported data.

Confounding

A confounder is generally defined as a factor associated with the exposure that also influences the outcome, generating a spurious association; adequately controlling for confounding variables is crucial to ensure the validity of study results. Several methods have been proposed for an efficient selection of potential confounding variables to control for, all with strengths and pitfalls (58). In **chapters 2, 3 and 5** of this thesis, potential confounders were selected based on previous evidence of their associations with the exposures or outcomes of interest. Although often used, this method of confounder selection may lead to unnecessary adjustment or, even worse, to over-adjustment, which would increase bias or affect precision (59). Furthermore, it could lead to adjustment for mediators (*i.e.,* variables that derive from the exposure and that lie in the causal path between the exposure and the outcome), potentially generating biased or paradoxical results (60). To avoid these drawbacks, postnatal factors were generally excluded from the main analyses, although additional control for important factors was done in sensitivity analyses in **chapter 5** to test the strength of the estimates. By contrast, in **chapters 4 and 6** we used literature-supported directed acyclic graphs (DAGs) to model the relationship between variables and identify the minimally sufficient set of variables to adjust for in the models (61,62). Additional variables considered to have an independent

effect on the outcome (*e.g.,* child's sex and age at follow-up), were controlled for in the main models or in sensitivity analyses. Despite the effort put in identifying the correct set of confounders, residual confounding is still possible. Specifically, maternal psychopathology, genetic influences, maternal physical activity during pregnancy and family environment are potential confounders that deserve further discussion.

Maternal psychopathology

Maternal psychopathology before and during pregnancy has been long considered an important foetal programming factor that is also related to maternal weight and diet during pregnancy (63–67). Numerous studies examined the association between maternal psychopathology and pregnancy outcomes (*i.e.,* birth weight, preterm birth and intrauterine growth restriction) or childhood emotional and behavioural development, and a fairly strong evidence of the importance of maternal prenatal psychopathology exists (68,69). Nonetheless, recent studies have suggested that the association between maternal psychopathology or pre-pregnancy BMI and childhood problem behaviours might be explained in large part by genetic factors, hence questioning the foetal programming mechanisms that have been postulated to date (70–77).

Genetic influences

Genetics is the single most important factor influencing several psychological traits, including those discussed in this thesis. In fact, heritability estimates based on twin studies have shown that about 50% of the variance in problem behaviours, temperament and social competence between co-twins is explained by genetic factors (*e.g.,* (77–82)). In addition, traits such as BMI, GWG and dietary patterns are genetically influenced. According to a systematic review and meta-regression of twin and family studies (total participants: n= 183,493), an average of 75% of the variability in BMI is genetically influenced, although higher heritability can be observed in children and adolescents compared to adults (83). Moderate heritability (*i.e.*, explaining 30%-40% of the variability) has been reported for GWG (84) as well as various dietary patterns (85,86). Consequently, it could be hypothesised that, due to the pleiotropic effect of genes, maternal genetic susceptibility to dietary intake or weight would affect child's genetic susceptibility to psychopathology, confounding previously reported associations. To this end, recent evidence suggests that the correlation observed between BMI and psychopathology might be in part due to shared genetic susceptibilities (*e.g.*, (87,88)). While no studies were found examining the genetic correlation between GWG or dietary patterns and psychopathology, such relationships are possible, given the phenotypic correlation between traits.

To control for genetic factors, in **chapters 2 and 3** we examined data from young twins and their mothers. However, although valuable to clarify the relative importance of genetics and the environment, results from twin studies are poorly generalisable to singletons, because of the differences in the early-life environment experienced by twins and singletons (89). Another important limitation of twin studies stems from the fact that they are often population-based cohorts with volunteer participation, which tends to favour the inclusion of middle-class individuals. Apart from reducing the generalisability of findings to individuals belonging to low or high socioeconomic status (SES), this recruitment approach might also affect heritability estimates. In fact, the environment in which children develop might modify gene expression – phenomenon known as gene-environment interaction (GxE). As a consequence, a higher heritability for a given trait could be found in children from a disadvantaged *vs*. a favourable background, or *vice versa* (90). For instance, it has been shown that the heritability of ADHD is significantly higher in children developed in risky backgrounds compared to more favourable ones (90). The GxE observed in ADHD is highly relevant to the studies presented in this thesis, and might explain why an association between GWG and child problem behaviours was found when examining low-risk but not high-risk populations (see results from (27,32–34) and the study reported in **chapter 4**), in which environmental influences are less important compared to genetic influences.

Overall, controlling for genetic influences would produce more precise results, and the lack of such a control in the studies presented in **chapters 4 to 6** of this thesis should be considered an important limitation. Nonetheless, it is important to remember that genetic susceptibility does not imply genetic determination: the environment still plays a crucial role on children development and should be carefully examined. Yet, as we will discuss in the next section, in some cases the environment is itself genetically influenced – phenomenon known as gene-environment correlation (rGE) – and contributes to the effects of genetics on the development of psychopathology. Family studies of psychological development in singleton children certainly represent a step forward in order to improve the characterisation of the exposure-outcome relationship and identify groups of at-risk children with an increased precision. In addition, detailed knowledge about the specific genetic susceptibility would allow personalised prevention strategies to be developed. Although previous direct examinations of genes considered implicated in several mental health outcomes (*i.e.,* candidate-gene studies) yielded inconclusive results (*e.g.,* (91)), in the future, the combination of large genome-wide association studies (GWASs) and polygenic risk score (PRS) analyses is likely to provide valuable insights.

Maternal physical activity during pregnancy

Maternal physical activity during pregnancy is known to be correlated with GWG, PUFA concentrations and diet quality in pregnancy (67,92,93), and, according to animal studies and a few human studies on childhood cognition, might have beneficial effects on offspring development, possibly by reducing chronic inflammation, increasing the blood flow through the placenta and promoting neuroplasticity – effects observed in adult populations but yet to be investigated in foetus and children (*e.g.* (94)). Specifically, data from a study analysing rats suggested that physical activity during pregnancy might have a beneficial effect in the offspring, reducing anxiety and depression (95), while the little data available in human populations focused on childhood cognition and suggested a small beneficial effect on language development (*e.g.,* (96)). Nonetheless, no studies have been published that specifically investigated its association with childhood psychological development. In the cohorts analysed in this thesis, maternal physical activity during pregnancy was not adequately assessed, with the exception of the INMA cohort. Consequently, we were able to control for physical activity during pregnancy only in a sensitivity analysis of the study presented in **chapter 6**, excluding data from the Rhea cohort. Although the estimates of the associations between maternal MD or DASH score and childhood problem behaviours were not substantially affected by adding physical activity in the model, residual confounding due to the lack of proper adjustment for maternal physical activity cannot be ruled out.

Family environment and postnatal influences

The relationship between the (postnatal) family environment and childhood psychological development can be examined under numerous viewpoints, including the socioeconomic status, parenting behaviours and the relationship between parents and children. In addition, we will discuss an indirect measure of the postnatal family environment that is related to maternal lifestyle factors examined within this thesis (*i.e.,* child's diet).

The socioeconomic status

The family's socioeconomic status (SES) is a comprehensive measure that includes education level, income and employment status (97). SES is considered an important influence on both lifestyle and health outcomes. Previous studies reported that children who grow up in socially disadvantaged families or communities are more at risk of maladaptive psychological development (98), while women from a low SES are more likely to be overweight or obese and to eat less healthily (99,100). In addition, as we discussed in the previous paragraph, and in line with the diathesis-stress model of psychological development (90), the family's SES might interact with pre-existing susceptibilities

such that biologically vulnerable children exposed to a disadvantaged family environment might be more at risk of developing psychopathologies. Despite these well-recognised associations, in the studies presented in this thesis we were not able to completely account for SES, since data on family income and employment status were not available in most cohorts. This resulted in the use of parental education level as proxy of SES. Furthermore, other factors, such as ethnicity or marital status, are often related and contribute to SES (*e.g.,* (101–103)). Nonetheless, in this thesis we were not able to investigate the effects of marital status or ethnicity, since the four cohorts examined in this thesis were characterised by elevated homogeneity regarding both ethnicity and marital status, comprising mostly double-parent households and individuals of the same ethnic background (*i.e.,* White European). This allowed an easier comparison between cohorts and study results, but also precluded the possibility of exploring the associations in different populations, hence limiting study generalisability.

The postnatal environment: child's diet

SES and family structure might affect child psychological development indirectly by influencing parents' choices regarding the way children should be reared, thus shaping the postnatal environment. For example, children who grow up in socially disadvantaged families are less likely to be breastfed (104) and more likely to eat a low-quality diet (105), while a systematic review reported that children who were breastfed for at least three months were less likely to have symptoms of total problem behaviours and conduct disorders (106). Similarly, adherence to a healthier dietary pattern have been associated with fewer symptoms of internalising and externalising behaviours in preschool years, independently of maternal dietary pattern in pregnancy (53).

Despite their potential role in child psychological development, these factors cannot be considered confounders in the associations presented in this thesis, as they necessarily occur after the exposures (58). Consequently, their inclusion in the models would depend on the hypothesised causal path. In the case of child's diet, it could be assumed that it mediates the associations between maternal gestational weight or diet and child psychopathology. In fact, although still limited to some specific flavours, growing evidence is available suggesting a role for maternal prenatal diet in shaping child's food preferences and therefore their dietary intakes (107), hence supporting the postulated mediatory role of child's diet. In this perspective, not including a measure of child's diet in the model would provide an estimate of the *total effect* of maternal prenatal factors on child development (60). By contrast, adjusting for child's diet in presence of an unmeasured common cause between the intermediate itself and the outcome would result in the so-called collider

stratification bias with consequent paradoxical results (60). Estimates of the total effect of prenatal weight or diet on childhood psychological development might be particularly important for the development of prevention strategies with multiple effects on the well-being of the mother and the future child. In fact, assuming that the diet quality and the overall lifestyle of the expecting mother result in better child's diet and fewer problem behaviours, an improvement of maternal diet and lifestyle during pregnancy could be promoted to reduce the likelihood of childhood psychopathology, while reducing the risk for the mother and the child to develop other chronic conditions (50,52,108,109).

On the other hand, it can be hypothesised that maternal genetic influence, possibly affecting her personality and her propensity for healthy behaviours, would, at least to some extent, affect child breastfeeding, maternal and child dietary patterns and childhood psychopathology. Adjusting for maternal personality would therefore be required to obtain an unbiased estimate of either the *total* or the *direct effect* of maternal prenatal diet or weight on child psychopathology. Consequently, the lack of data regarding maternal personality should be considered a limitation of the studies included in this thesis, as well as the vast majority of available literature on the topic, as it precluded the possibility to test this hypothesised causal path. Although previous studies have sometimes controlled for maternal psychopathology, which is related to maternal personality (110), the latter is a relatively stable trait and is more likely to affect maternal lifestyle choices during pregnancy and the post-partum period (111), as well as the relationship with her children (112,113). In contrast, maternal psychopathology might manifest in a specific period of life but be absent in less stressful times, hence affecting lifestyle choices and relationships to a lesser extent. The role of maternal personality on the associations between prenatal maternal lifestyle and child psychopathology should, therefore, be investigated before any conclusion can be made.

The postnatal environment: parenting behaviours

Maternal personality is also related to another important measure of family environment in childhood: the relationship between parents and children. The parents-child relationship, and in particular parenting behaviours (also denoted parenting practices), has been extensively investigated as a possible risk factor for poor child psychological development (114,115). While not representing a source of potential confounding for the associations presented in this thesis (58), parenting practices and the whole family environment could be viewed as resulting from parental genetic susceptibility to negative personality or psychopathology. The family environment in which the child develops would therefore represent an example of rGE, which denotes genetic differences

in the *exposure* to a particular environment (in contrast to GxE, which refers to genetic differences in the *susceptibility* to a given environment (116)). Previous studies summarised in a systematic review revealed modest heritability estimates for various measures of parenting behaviours and family environment (*i.e.*, ranging from 12% to 37%), with more frequent incidents likely to be more heritable than occasional events (117). Different types of rGE have been described. *Passive* rGE refers to the situation in which the child inherits both the genetic vulnerability and the detrimental environment from the parents. *Evocative* or *reactive* rGE refers to the situation in which the child's genetic susceptibility to a difficult behaviour influences parental reactions to the child's behaviour, leading to harsh parenting practices and an overall detrimental family environment. Finally, *active* rGE refers to the situation in which the child actively selects the environmental niche that relates to their genetically influenced behaviours. While active rGE is considered less relevant for the family environment and especially in childhood, since younger children have fewer opportunities to actively select their environments (116), passive and evocative rGEs might be relevant. Specifically, passive rGE might be in play and confound the associations described in this thesis if maternal genetic susceptibility to negative personality affected both her weight or diet during pregnancy and her relationship with her children. In this perspective, maternal personality would be a marker of genetic risk (116) that should be controlled for in order to adequately investigate the association between maternal weight or diet during pregnancy and child psychopathology in the absence of genetic data. Failing to include maternal personality in these models likely resulted in an overestimation of the estimates.

Relationships between family characteristics

All in all, various characteristics of the family environment could explain at least in part the associations between prenatal factors and child psychological development. Nonetheless, their inclusion in statistical models should be carefully evaluated in order to avoid over-adjustment due to their interconnection – for example, women of low SES often come from ethnic minorities, are more likely to be never-married, be mentally ill or face stressful situations that might affect their relationships with their children and their rearing choices (118). In addition, because of this high intercorrelation, it could be found that the effect of one factor could be explained at least to some extent by other family characteristics. For example, a prospective study examining the possible effects of economic deprivation, maternal depression, and various mother-child relationship markers on child problem behaviours showed that a substantial part of the effect of economic deprivation of child behaviour was mediated by maternal depression (119). Furthermore, maternal

depression was found to negatively affect parenting practices and mother-child relationship, which were in turn associated with child problem behaviours (119). Similarly, a cross-sectional study investigating the roles of maternal depression and family environment in high-risk, low-income families found no independent effect of family environment on child problem behaviours (120). Yet, none of these studies controlled for genetic influences on psychopathology, which, as we previously discussed, might explain the associations between maternal depression, family environment and child problem behaviours. In addition, specific aspects of the family environment might also interact with child's genetic susceptibility to psychopathology even in presence of rGE, such that genetically predisposed children would be more likely to develop the outcome if raised in a risky environment, which would also be genetically favoured, resulting in a significantly higher risk for susceptible children (121,122). A higher-order model that accounts for genetic susceptibilities and time-dependent relationships between family characteristics might therefore help clarifying the role of the family environment on child psychopathology.

Risk of misclassification

Except for maternal weight during pregnancy that was directly measured by hospital staff, exposures and outcomes of this thesis are self-reported. Whenever possible, validated questionnaires were used to minimise drawbacks, but misclassification is still possible.

Exposures

TAMBAHS is a retrospective study, thus children's birth weight, zygosity and all other characteristics related to their birth were reported by their mothers at assessment, which might introduce recall bias. However, maternal reports of birth weight and perinatal outcomes are considered reliable and sufficiently accurate for using in epidemiological research (123). Furthermore, to ascertain zygosity the Goldsmith's zygosity questionnaire (124) was used, which accurately identifies zygosity in 95% of cases. Consequently, although collecting data from medical reports should be preferred, maternal recall within a few years after delivery can be considered sufficiently accurate and reliable.

The assessment of maternal pre-pregnancy weight often relies on self-reported data in both clinical and epidemiological settings, given the many economic and practical difficulties associated to alternative data collection processes (125). Yet, social desirability and recall bias have been proven to affect self-reported weight in some populations, with greater BMI underestimation in obese or female individuals (126). Nonetheless, a high level of accuracy has been observed comparing self-reported pre-pregnancy weight *vs.* pre-pregnancy or first-trimester measured weight. A systematic

review investigating accuracy and misclassification of self-reported pregnancy weight reported high correlations between self-reported and measured weights (r=0.90 to r=0.99), with 86.7% to 91% of women falling in the correct BMI category using self-reported data (125). However, a higher prevalence of misclassification was reported in women classified as underweight (23.5%), overweight (16.5% to 27%) or class-I obese (24.3%), although reporting error did not seriously bias estimates of several short-term pregnancy outcomes (125). Thus, overall self-reported pre-pregnancy weight data can be considered accurate, although whenever possible it should be compared to first-trimester measured weight to identify possible inaccuracies.

The food frequency questionnaire (FFQ) is a commonly used tool to assess dietary intakes in large epidemiological studies, thanks to a good compromise between costs and reliability. Nonetheless, the FFQ has been largely criticised over the years due to the well-recognised issues related to reporting portion size and recalling past food intake (127). To avoid these biases, in both Rhea and INMA cohorts, validated, semi-quantitative FFQs with indications of portion sizes were used (128,129). Furthermore, data collection was scheduled during pregnancy to avoid the recalling of remote food intake. In addition, in INMA dietary intake was measured twice (*i.e.,* during the first and the third trimesters of pregnancy) to assess dietary changes and misreports (130–132).

Outcomes

The Infant Behaviour Questionnaire- Revised (IBQ-R) and the CBCL were chosen for the assessment of infants' temperament and children's problem behaviours, respectively, by virtue of their validity and reliability (133–140). However, some previous studies suggested that reports done by women affected with psychopathology might be biased, since these women might be more prone to consider as problematic normal behaviours (*e.g.,* (141,142)). By contrast, other studies have suggested that children of women with psychopathology might indeed be more likely to manifest internalising or externalising problems and, therefore, that maternal psychopathology would not be a cause of bias (*e.g.,* (143)). If anything, depressed mothers might be more accurate in assessing their children's behaviour (144). A key difference between these studies concerns the standards against which maternal assessments were compared. These were either care-workers' and teachers' reports (141), children's self-report (142), trained interviewers' reports (143), or a clinical diagnosis and children's self-report (144). Although no gold standard for the assessment of childhood psychopathology exists, it could be argued that a clinical diagnosis is more reliable than teachers' and care-workers' reports, and certainly more objective than the child's self-report, especially if very young such as the children included in this thesis. Considering true this argument

would mean accepting maternal reports of childhood temperament or behaviour as reliable. Nonetheless, more research is necessary to clarify to what extent, if any, maternal psychopathology might influence the ability to judge their children's behaviour and, therefore, which assessment tool is preferable in epidemiological research.

Causal inference

Establishing cause-effect relationships in perinatal epidemiology is complicated by the ethical and practical limitations to the use of interventions. As previously mentioned, observational studies are subject to several types of bias, including residual confounding, misclassification and inaccuracy of variable assessment, report and recall bias. Nonetheless, well-conducted observational studies can still provide valuable insight on disease aetiology and can be useful for causal inference (145). A critical evaluation, which can be based on Bradford Hill's criteria for causal inference (146), should thus be carried out before accepting an association as causal. We will now apply Bradford Hill's criteria on the associations presented in this thesis to critically evaluate the available evidence.

The first criterion for causality is the *strength of associations*. The larger an association between exposure and outcome, the more likely it is to be causal. Weaker associations are more likely due to residual confounding or biases, although in some cases even weak associations might denote causal relationships (146). This might be especially true in case of multifactorial disorders, determined by several risk factors that might have small individual effects. The associations discussed in this thesis had generally a moderate-to-weak magnitude, but investigated outcomes are known to be multifactorial. However, studies were limited by residual confounding and strengths of identified associations are likely lower.

The second criterion identified by Bradford Hill is *consistency*: triangulation of evidence derived from different study designs, methods and populations is necessary to exclude that an identified association is due to some error, bias or residual confounding. The association between birth weight and problem behaviours has been supported by several studies examining singletons as well as twins, in which common genetic and environmental factors between co-twins were controlled for, reducing the likelihood of residual confounding (13,15,18,20–22,147,148). We found associations between intrapair birth-weight difference and total problems, internalising problems and emotional reactiveness, respectively, which however fell short of statistical significance after controlling for multiple testing. Yet, the association is supported by the vast majority of published studies (which

however often did not control for multiple testing), and the consistency criterion might be considered fulfilled. Similarly, consistency can be found regarding the association between maternal diet quality and problem behaviours, with available studies (including our own) suggesting that consuming a healthy diet rich in fish and seafood during pregnancy might reduce the risk of externalising problems in children (53–57,149). Moreover, available evidence largely supports the association between GWG and problem behaviours (*i.e.*, (27,32,33) and our study), while only one publication does not (34). However, it should be considered that all above-mentioned studies, with the exception of those analysing twin data, did not control for genetic influences. Consistency between study findings might therefore be due to this important limitation. By contrast, little-to-no consistency can be found between studies investigating maternal pre-pregnancy BMI in relation to infants' temperament, or prenatal PUFA exposure in relation to child psychopathology.

The criterion of *specificity* states that an exposure could cause only one disease, although diseases could have multiple causes. This criterion is now largely considered weak or irrelevant, especially in epidemiological or psychiatric research (150,151). Specifically, in developmental psychology it is generally accepted that not only different risk factors might contribute to the development of a given trait (*i.e.*, equifinality), but also that a single risk factor might lead to multiple outcomes (*i.e.*, multifinality) (152). The final outcome will depend on the characteristics of the child and the environment in which risk factors operate.

The *temporality* criterion indicates that the exposure should always precede the outcome. Consequently, it can be considered a necessary condition for causality to be established. The designs and the hypothesis underlying our studies guarantee the fulfilment of this criterion, as exposures refer to the prenatal period. Nonetheless, with a retrospective study design (such as the studies described in **chapters 2 and 3**), where exposures are measured after the outcome has developed, it might sometimes be difficult to ascertain temporality because recall and attribution biases might affect the exposure measurements. However, as we previously discussed, while self-report of maternal pre-pregnancy BMI might be more prone to bias (126), maternal report of children's birth weight is considered reliable even after long time (123). By contrast, the prospective design of the other four studies ensures that the temporality criterion is fulfilled.

The fifth criterion states that if the association could have a *biological gradient* or a dose-response curve, then this should be identified for causality to be inferred. We found dose-response

associations between birth-weight difference, GWG, PUFA concentration trends and maternal MD score, respectively, and child outcomes, generally fulfilling this criterion.

The reported association should have *plausible* underlying mechanisms of action and should be *coherent* with known biological mechanisms and disease aetiology. Nonetheless, plausibility and coherence are considered helpful, but not necessary, since they are largely dependent on the current biological knowledge (146). Yet, plausible hypothetical mechanisms of action have been identified for all investigated associations, which are coherent with biological knowledge and based on existing animal or human studies.

Although *experiments* would help strengthen the evidence of a cause-effect relationships, they are not always applicable. For example, it would not be ethically possible to test the effects of pre-pregnancy obesity, while to randomly assign a specific diet to expectant mothers would be rather impracticable. Yet, the association between maternal diet quality in pregnancy and child problem behaviours could be tested by comparing the effects of the MD with those of general indications to improve diet quality. Differences in children's behaviour would be then related to their mothers' diet, if all maternal and child characteristics were equally distributed between groups. Nonetheless, such an experiment would require several years before any outcome could be investigated, and even then, the exact cause-effect relationship might remain unclear. As previously mentioned, to date only the association between maternal n-3 PUFA concentrations and neurocognitive and behavioural development has been tested with RCTs, reporting no difference between intervention and control groups (46).

The last criterion is the *analogy* of the identified association with previously reported relationships. For instance, the effects of maternal alcohol or smoking use or of maternal psychopathology during pregnancy on childhood outcomes have been often cited as analogues of the hypothesised effect of maternal gestational weight or diet. However, in developmental psychology and perinatal epidemiology hypothetical aetiological factors are largely correlated and their effects might be difficult to be disentangled. Furthermore, a causal relationship with child problem behaviours has not been demonstrated yet even for likely plausible risk factors such as smoking, alcohol and psychopathology (*e.g.*, (72,111–114)). Besides, the criterion of analogy has been widely criticised, and could be considered more important for proposing and testing hypotheses, rather than for establishing causal relationships (151).

All in all, to date causality has not been proven for any of the associations discussed in this thesis, as the (partial) fulfilment of the criteria of strength and consistency is often based on studies with critical limitations. The association between birth weight and problem behaviours fulfils the largest number of criteria (at least 6 out 9), despite no statistically significant associations were observed in our study after controlling for multiple testing. By contrast, little support was found for the association between maternal pre-pregnancy BMI in relation to infants' temperament, or the association between maternal PUFA status and child psychopathology. Finally, the associations between maternal GWG or diet quality during pregnancy and child problem behaviours are promising, but they should be investigated more thoroughly, accounting for genetic influences, before any assumption of causality could be made.

Implications

The first 1,000 days of children's lives, from conception to the second birthday, are considered crucial to shape their future health (155). Over the last decades, researchers have investigated a whole range of factors that might have an impact on this extremely sensitive period, including maternal low SES, malnutrition, and obesity (156–158). The results reported in this thesis suggest that exposure to excessive GWG and poor diet quality during gestation might also increase the risk of internalising and externalising problem behaviours in childhood.

Even more, suffering from mental disorders increases the likelihood of eating poorly, being more sedentary and having an excessive body weight (159). Consequently, mental disorders exacerbate the risk of several metabolic diseases, including cardiovascular diseases, diabetes and cancer (160). Female children with internalising or externalising problem behaviours are, therefore, more likely to reach adulthood and enter pregnancy in poor health, both physical and mental, thus passing their susceptibilities on to the next generation.

This intergenerational transmission can and must be interrupted. As we noted earlier, environmental factors are important even in presence of high genetic influence on disease risk. Studies employing genetically informed designs, such as adoption or children-of-twin studies, have shown that this intergenerational transmission is in large part due to transmission of the risk environment (*e.g.*, (161–164)). Consequently, improving the rearing environment might help interrupting this vicious cycle of disease. Women of childbearing age should therefore be informed of the real risks for them and their future children that results from poor lifestyle choices. Pregnancy

is a highly receptive period, during which women tend to be more prone to change their behaviour in order to reduce negative consequences for their children's health (165). Yet, a large proportion of them, often corresponding to the least educated, more at-risk women, might not be completely aware of the risks. Clinicians and the scientific community have the responsibility to mediate between research and population, informing about the benefits of a healthy lifestyle at all life stages. Moreover, monitoring women's pregnancies could help them switch to a healthier dietary pattern, such as the Mediterranean one that has been proven beneficial to prevent several mental and physical conditions (*e.g.,* (50,52,108)), and limit GWG as recommended (165–167).

An alternative approach towards the interruption of the multigenerational transmission of disease risk concerns the early identification of at-risk groups. Children born preterm and with a low birth weight undeniably represent the most vulnerable population, but offspring of women with overweight or obesity who gained excessive weight or had a low-quality diet during gestation may also necessitate professional care to develop to their full potential. These children and their families might benefit from prevention programmes such as the World Health Organisation (WHO) Caregiver Skills Training programme, which aims to teach families of children with psychological disorders how to create an emotionally supportive and protective environment to help children develop optimally (168). In addition, a focus on better lifestyle choices might reduce the risk of psychopathology in both children and their parents (50,52,108,109).

To conclude, the high plasticity and vulnerability that characterise the first 1,000 days of life can be turned into our favour and exploited for health promotion. In line with current recommendations for adult populations, evidence is now growing of the importance of a healthy maternal lifestyle for promoting both physical and mental health in the offspring.

Future perspectives

The study of prenatal influences on childhood psychological development is a relatively new field of research that will benefit from the further elaboration of a few research topics.

Genetics

As discussed above, genetics is likely to have a profound influence on childhood development. Several family-based study designs have been used to explore possible genetic confounding in associations between prenatal factors and childhood psychological development, often reporting

some genetic influence (70–77). In the two twin studies published to date investigating the association between maternal pre-pregnancy BMI and infants' temperament (**chapter 3**) or childhood problem behaviours (77), maternal BMI was considered as part of the twins' common environment. However, BMI and psychopathology might share part of their genetic influence (*e.g.*, (87,88)) due to the pleiotropic effect of genes. Future studies should, therefore, examine further the mechanisms underneath the relationship between maternal weight before and during pregnancy and childhood psychological development.

Apart from family-based studies, GWASs will continue to provide more insights on the specific genetic variations and especially single-nucleotide polymorphisms (SNPs) associated with physical and psychological traits. It can be foreseen that SNPs and their aggregations, PRSs (169), will increasingly be used to calculate the genetic confounding in the association between prenatal factors and childhood outcomes when novel techniques and algorithms, as well as the genetic material itself, will become more accessible to the whole scientific community. To this regard, extremely promising techniques have been recently implemented to estimate the portion of phenotypic covariance between exposures and outcomes that is explained by genetic factors. Child's genetic confounding has been found in the association between maternal pre-pregnancy BMI and child weight (*i.e.,* birth weight and BMI in childhood and adolescence) (170); similar applications could be useful to investigate the relationship between maternal weight or diet and childhood psychopathology. Finally, genetic data (*i.e.,* either single SNPs or PRSs) are increasingly been used in Mendelian randomisation (MR) analyses to test the causality of associations between modifiable risk factors and health outcomes, by using genetic variants as instrumental variables (171). To date, MR analyses have identified causal relationships between maternal BMI and offspring birth weight (172) and adiposity (173), respectively, but no studies have assessed childhood psychopathological traits as outcomes.

Maternal physical activity and overall lifestyle during pregnancy

Maternal physical activity during pregnancy has received insufficient attention, but the few available studies suggest that offspring of women who are more active during pregnancy might have better language skills (*e.g.,* (96)) and be less at risk of developing symptoms of anxiety and depression (95). Future studies should therefore examine more deeply the association between maternal physical (in)activity and child psychopathology to strengthen the evidence and clarify the aetiological pathway.

Similarly to what happens to different food items and nutrients that interact to produce a unique diet, different lifestyle factors such as weight, diet, physical activity, smoking and alcohol consumption are likely to interact to create a person-specific, overall lifestyle. The end-result is likely to be a more complex factor, with potentiated effects compared to the sum of its combining parts. Future studies applying this holistic approach to the investigation of the relationship between maternal lifestyle and childhood psychopathology could provide novel insights onto childhood development.

Paternal influences

To date, the research on the effects of prenatal exposures on childhood psychological development focused on maternal factors, leaving research on possible paternal influences to the post-partum period (174). However, recent animal studies suggest that preconceptional stress or hazardous behaviours (both maternal and paternal) might have an impact on the offspring's psychological development (*e.g.,* (175–177)). Little epidemiological research has been done using paternal variables as additional exposures or negative controls, reporting no association of paternal BMI before pregnancy (178,179) or paternal antidepressant treatment before conception (180) on childhood psychological outcomes. However, higher risk of intellectual disability was observed in children whose fathers started antidepressant medication during the gestational period, suggesting that a depressive outbreak (or a paternal predisposition to antidepressant use) during conception might have negative effects on the child (180). Furthermore, paternal periconceptional BMI and diet have been associated with altered methylation patterns at imprinted genes in the sperm's and in the offspring's DNA (181,182). Similar effects might be produced by folate deficiency or over-supplementation, although current evidence is based solely on animal studies (181,183). Larger paternal research involvement and careful study design are, therefore, warranted to clarify the role of the paternal influence on childhood development.

Conclusion

In this thesis we examined the association between prenatal exposures and childhood psychological development. The results indicate that childhood problem behaviours might be more likely in children with a low birth weight, as well as in children of women with pre-pregnancy overweight/obesity who gained excessive weight during pregnancy. A beneficial effect on the risk of externalising behaviour might derive from high maternal adherence to the Mediterranean diet

during pregnancy. Little-to-no evidence of an association was found between maternal pre-pregnancy BMI and infants' temperament, and between maternal PUFA concentrations during pregnancy and childhood social competence or problem behaviours. The public health implications of these findings may in some cases be substantial, although the evidence should be confirmed as it is based on observational studies for which residual confounding, especially related to uncontrolled genetic or family-based (postnatal) influences, cannot be excluded. To provide further insight on the associations between prenatal exposures and childhood psychological development, future studies assessing additional environmental factors such as maternal physical activity and paternal influences, with a genetically informed design are warranted.

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Valorisation addendum

Mental health problems affect 10%-20% of children and adolescents worldwide, remaining unrecognised or untreated in the majority of cases (1,2). The consequences of (untreated) childhood mental disorders are widespread, including substantial economic burden to the society, lifelong psychiatric conditions and overall poor quality of life. Identifying risk factors associated with the development of mental conditions in childhood that could represent targets for novel and efficacious prevention strategies is, therefore, of extreme importance.

The work presented in this thesis, which has been published in internationally acknowledged journals and presented in well-known scientific conferences, suggests that excessive gestational weight gain and poor maternal diet quality during pregnancy might be two risk factors of internalising and externalising problems in childhood. Even though findings should be confirmed in larger observational or intervention studies, the research presented in this thesis can be used in clinical practice to inform women of childbearing age of the effects of their diets and weight gain on their future offspring's mental health. In fact, since during pregnancy women tend to be more prone to change their behaviours in order to promote their children's health, with adequate support and accessible information, they would likely adopt a healthier lifestyle.

The results of this thesis can also be used to identify at-risk children before any behavioural symptom appear. We showed that apart from children born preterm and/or with a low birth weight, who undeniably represent the most vulnerable population, offspring of women with overweight or obesity who gained excessive weight or children prenatally exposed to a low-quality diet may necessitate professional care to develop to their full potential. Based on all the available evidence, multidisciplinary prevention programmes, with a focus on the creation of an emotionally supportive and protective family environment and on healthier lifestyle choices, could promote the well-being of the whole family.

In addition, this thesis discusses the role of genetic influences on childhood psychological development, identifying in the scarcity of studies with genetic information a limitation of the literature regarding prenatal influences on childhood mental health. The academic community can, therefore, use the work and considerations presented in this thesis as a starting point of future investigations, which should take advantage of the increasing availability and accessibility of genetic material. Nonetheless, however large the influence of genetics, environmental factors, including parental lifestyle, are still relevant. In fact, for complex characteristics such as psychological

disorders, a genetic influence does not imply genetic determination, and any given condition can be prevented or treated by changing the environment in which children develop.

To conclude, the prevention of childhood psychopathologies represents a unique opportunity to promote lifelong health, which should be considered as equally important as the prevention of other non-communicable diseases. Indeed, if health is more than the absence of disease but rather a "state of complete physical, mental and social well-being" (3), the pursuit of public health must include the full promotion of psychological well-being. The results of this thesis suggest that by tackling lifestyle choices we could promote not only physical but also mental health.

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About the author

Elena Tore was born in Oristano, Italy, on 24 January 1991. In 2009, after graduating from secondary school with a curriculum on social sciences at the Liceo socio-psico-pedagogico, she went on to study Biological Sciences and received her BSc from Università degli Studi di Torino (University of Turin, Italy) in 2013. Subsequently, Elena continued her studies with a Master in Nutrition and Food Sciences at the University of Reading (United Kingdom), which she completed with Distinction in 2015.

During her Master studies, Elena developed a passion for epidemiology, and especially the relationships between genetic influences, lifestyle and diseases. After graduating, she worked under the supervision of Prof Zeegers and Dr Antoniou on the development of a consortium of twin studies with the aim of investigating the genetic and environmental influences on dietary intake and physical activity. This experience gave her the opportunity to work first-hand on the development of a scientific project and strengthened her will to pursue a career in academia.

In 2016, Elena started a joint PhD project between the University of Birmingham and Maastricht University to study prenatal influences on childhood psychological development. During her time at Maastricht University, Elena also worked part-time as lead scientist for the EIT-Health funded "Personalised Prevention Counsellor" (PEPRECO) project.

In 2021, Elena started working as research assistant on the "Sulphur amino acids, energy metabolism and obesity" (STAY) project at the Department of Internal Medicine, Maastricht University, where she investigates the associations between sulphur amino acids, obesity and related metabolic diseases.