**Associations of maternal zinc and magnesium with offspring learning abilities and cognitive development at 4 years in GUSTO**

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**Associations of maternal zinc and magnesium with offspring learning abilities and cognitive development at 4 years in GUSTO**

Minerals deficiencies during pregnancy have been shown to be associated with poorer cognitive outcomes in offspring. This study aimed to investigate associations of maternal plasma zinc and magnesium concentrations with cognitive development in 4-year old children from the Growing Up in Singapore Towards healthy Outcome cohort. Maternal plasma zinc and magnesium concentrations were measured at 26-28 weeks’ gestation. The Lollipop test of school readiness, tests of working memory, number knowledge, receptive vocabulary, and phonological awareness were performed in children at 4 years. Associations were examined in 715 mother-offspring pairs using linear regressions adjusted for key confounders. Maternal plasma zinc and magnesium concentrations were 812±144µg/L and 19.9±1.8mg/L (mean±SD); 19% and 71% of mothers were zinc deficient and magnesium insufficient, respectively. After adjustment for multiple testing, higher maternal zinc concentrations (per SD increment) were associated with 0.35 higher scores in Lollipop subtest 2 of picture description and spatial identification (95% CI: 0.13, 0.58); higher maternal magnesium concentrations (per SD increment) were associated with 0.65 higher scores in Lollipop subtest 4 of letters and writing identification (95% CI: 0.23, 1.07). No significant associations were observed for other tests, suggesting little long term influences of maternal zinc and magnesium on child’s cognitive development.

Keywords: zinc, magnesium, pregnancy, cognition, preschool, child

# Introduction

Adequate maternal nutrition is important for normal brain growth and neurodevelopment of the offspring during the foetal period, as the foetal brain undergoes rapid structural and functional changes between 24 and 44 weeks’ after conception.1 In particular, brain regions (e.g. hippocampus, frontal lobe) that experienced most rapid growth and maturation during the intrauterine period were found to be associated with specific cognitive functions such as language, memory and emotional behaviour.1 Hence, the cognitive functions associated with these brain regions will likely be most sensitive to influences of maternal nutritional status.

Zinc is found in high concentrations in the hippocampus, amygdala and the cerebral cortex, which are brain regions involved in learning, memory and spatial ability.2 Animal studies have consistently found zinc-deficient rats and their offspring to have impaired spatial learning and working memory.3,4 The evidence in humans, however, is less clear. A few studies have found zinc supplementation in children to have positive effects on attention and learning (visual habituation task), working memory (digit span test), and non-verbal or performance intelligence, but these findings are not replicated in other studies.5 Studies examining effects of antenatal zinc supplementation or associations of maternal zinc concentrations/intakes with offspring cognitive outcomes have inconsistent findings.5 The inconsistencies in study findings may be due to the wide range of cognitive measurements used; whether or not the study finds significant associations may be dependent on whether the cognitive instruments used measures cognitive aspects most sensitive to nutritional influences.

Magnesium, on the other hand, contributes to the normal functioning of the N-methyl-D aspartate receptor which is associated with learning and memory functions.6 Animal studies have demonstrated the important role of magnesium in improving memory and spatial learning function.7,8 Specifically, offspring of pregnant rats supplemented with magnesium demonstrated improvements in learning ability and memory.9 No human studies to date have examined the influence of magnesium on cognitive development, or the relationship between maternal magnesium and offspring’s cognition.

Global estimates of micronutrients deficiencies during pregnancy is high including zinc and magnesium deficiencies (>15%).10 This underscores the importance of studies investigating the influence of maternal zinc and magnesium on cognitive development of their offspring, to help determine if interventions are needed to improve maternal zinc and magnesium status. In this study, we investigate the associations of maternal plasma zinc and magnesium concentrations and status during pregnancy, with their offspring general learning abilities and cognitive development at 4 years from a multi-ethnic Asian mother-offspring cohort.

# Materials and Methods

## Study population

Data from the Growing Up in Singapore Towards healthy Outcomes (GUSTO) study were used for the present analysis. GUSTO is a prospective mother-offspring cohort study in Singapore aimed at understanding influences of antenatal environmental factors on growth and development of the offspring.11 The methodology of the GUSTO study has been described in detail previously.11 Briefly, pregnant women (≥18 years) in their first trimester (<14 weeks) were recruited from the National University Hospital (NUH) and KK Women’s and Children’s Hospital (KKH) between June 2009 and September 2010. These women are Singapore citizens or permanent residents of Chinese, Malay or Indian ethnicity with homogenous parental ethnic background; intended to deliver in the two hospitals and to reside in Singapore for the next five years; and consented to donate birth tissues at delivery. Women receiving chemotherapy, psychotropic drugs or were diagnosed with type-1 diabetes were not eligible to participate. The GUSTO study has received ethical approval from the Institutional Review Board of NUH and KKH, and all procedures were conducted in accordance to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants at recruitment.

A total of 1247 pregnant women participated at baseline, of which 1152 conceived naturally and had singleton live births. The present analysis included mother-offspring pairs whereby the mothers provided sufficient blood for analysis of plasma zinc and magnesium concentrations at 26-28 week’s gestation, and the children had usable data for the relevant cognitive tests at 4 years (**Figure 1**). Children who did not participate in the cognitive tests were due to parents’ busy schedules, lack of interest, or drop out from the GUSTO study. As some children had unusable data owing to fatigue, poor cooperation or fussiness, the number of children with usable data for each cognitive test differed – 715 children had usable data for at least one cognitive test and were included in analysis.

[Figure 1 near here]

## Plasma minerals concentrations in mothers

Overnight fasting (8-10h) blood samples were obtained from pregnant women at the 26–28 weeks’ gestation follow-up visit using standard venepuncture technique. The samples were processed and stored at -80oC in EDTA tubes within 4h of collection and thawed just prior to analysis. Plasma zinc and magnesium concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS model NexIon 300D, Perkin Elmer; Rotkreuz, Switzerland). Plasma samples were defrosted and 50-100µl plasma was diluted to 5ml in a plastic tube using 0.14M HNO3 after addition of 100µl of 1 mg/l In as internal standard. The ICP-MS was calibrated using standards containing 25, 50, 75, 100µg/l for zinc, and 250, 500, 750 1000µg/l for magnesium, and NaNO3, Ca(NO3)2 and L-Cysteine hydrochloride monohydrate for matrix matching. Isotopes selected for analysis were 66Zn, 26Mg and 115ln. The ICP-Ms was equipped with a glass cyclonic spray chamber and Meinhard nebulizer (300µl/min sample uptake). Interferences were removed using the collision mode with a He cell gas flow of 3.5 ml/min. Lyophilized human control plasma (Seronorm Trace Element Plasma, Level 1 and 2, Sero AS, Billingstad, Norway, Ref. No: 201405 and 203105) was analysed for quality control together with the samples. Coefficient of variation of intermediate reproducibility CV (iR) of the method obtained with quality control samples Level 1 was 2.5% for zinc and 3.7% for magnesium. Coefficient of variation of intermediate reproducibility CV (iR) of the method obtained with quality control samples Level 2 was 2.0% for zinc and 3.4% for magnesium. Recovery for both elements at both concentration levels was in the range of 94-107%.

We also measured plasma ferritin, a reliable marker of iron status, as it has been shown to influence concentrations of other minerals especially zinc.12 Plasma ferritin concentrations were measured by using a commercial ELISA kit (AssayMax Human Ferritin ELISA, AssayPro). Briefly, 50 µl of sample or standard or control is added on a pre-coated micro plate and is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for ferritin, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The colour development is stopped and the intensity of the colour is measured immediately on a micro plate reader at a wavelength of 450 nm.

## Neurocognitive assessments in children at 4 years

Six cognitive tests were used to capture general learning abilities and to assess different dimensions of cognitive development including working memory, language and numeracy. They were administered to children at 4 years of age (48-50 months) at their homes. All the cognitive tests were administered in English and performed by research coordinators trained by GUSTO investigators with a background in psychiatry. Greater scores in these cognitive tests indicate better performance in the related cognitive domain.

We used the Lollipop test, a validated diagnostic screening test of school readiness in preschool children,13 to assess general learning abilities. This test assesses children’s ability in the following four areas: identification of colours and shapes (subtest 1); picture description and spatial identification (subtest 2); identification of numbers and calculations (subtest 3); identification of letters and writing (subtest 4).

The Random Object Span Test (ROST) and the Visually Cued Recall task (VCR) were commonly used to assess working memory in young children.14,15 In ROST, the children were presented with a set of pictures with the position of these pictures randomly changing from trial to trial, and the children were required to select a different picture each time.14 For VCR, children had to remember a gradually increasing number of items shown on a card.15 There were a total of 12 test sets, and the test is terminated if the child makes two errors on two subsequent test sets.

Two tests were used to assess language skills. The Peabody Picture Vocabulary Test, 4th edition (PPVT-4) was used to assess receptive vocabulary.16 The child was required to select one of four pictures that best illustrates the meaning of the given word. Children’s phonological awareness (a prerequisite to reading fluency) was assessed with the Comprehensive Test of Phonological Processing, 2nd edition (CTOPP-2).17 These tests were clinically used by Singaporean psychologists.

The Number Knowledge Test (NKT) measures basic knowledge and understanding of number concepts, which was administered in two levels: Level 0 assesses the child's ability to quantify objects that can be seen or touched, and is a prerequisite to the next level; Level 1 relies on the child’s ability to perform mental calculation with limited visual aids (e.g. how much is 2 plus 4?).18

## Covariates

Self-reported socio-demographic information of mothers was collected at the recruitment visit (<14 weeks’ gestation): age, ethnicity, highest education attained, monthly income and current occupation. As educational level was correlated to monthly income and occupational level, all statistical analysis used maternal education as indicator of socio-economic status. At the 26-28 weeks’ gestation follow-up visit, maternal mental wellbeing was assessed with the Edinburgh Postnatal Depression Scale (EPDS)19 and State-Trait Anxiety Inventory (STAI).20 Maternal pre-pregnancy BMI (kg/m2) were based on self-reported pre-pregnancy weights collected during recruitment, and height measured with a stadiometer (SECA model 213) at the 26-28 weeks’ gestation follow-up visit. Maternal diet including intake of vitamins and minerals supplement during pregnancy (at 26-28 weeks’ gestation) was assessed using a 24-hour recall obtained by trained clinical staff. Overall diet quality was determined with the Healthy Eating Index for pregnant women in Singapore (HEI-SGP).21

## Statistical analysis

Maternal plasma concentrations of zinc and magnesium were summarised according to characteristics of the 715 mother-offspring pairs with data for at least one cognitive test. Differences in concentrations between groups were compared using independent-sample t-test or one-way ANOVA. Bonferroni *post hoc* analysis was performed to identify groups which differed if the one-way ANOVA test was significant.

The values for maternal plasma zinc and magnesium were converted into SD scores to facilitate comparison across exposures. The effect sizes for child’s cognitive tests thus represent changes in scores per 1-SD increment in concentrations of maternal plasma zinc and magnesium. Associations of maternal plasma zinc and magnesium and cognitive outcomes in children were examined using linear regressions. We presented results for crude models without adjusting for potential confounders, and then with adjustment for maternal age, ethnicity, education, pre-pregnancy BMI, plasma ferritin concentrations, diet quality and multivitamins and minerals intake, and antenatal depression and anxiety levels (Model 1).

We repeated the analysis comparing child’s cognitive tests scores among mothers with different zinc and magnesium statuses adjusting for confounders as per Model 1 above. Maternal zinc status was defined as: zinc deficient (<700µg/L) based on cut-offs used in previous studies;22 and two groups of zinc sufficiency according to a median split of individuals with sufficient zinc concentrations (sufficient-low: 700 to <825 µg/L, sufficient-high: ≥825µg/L). Maternal magnesium status was defined as magnesium deficient (<18.25 mg/L), insufficient (18.25-20.68mg/L) and sufficient (>20.68mg/L) based on cut-offs commonly used in other studies.23 Mothers of sufficient-high zinc status or sufficient magnesium status were used as reference.

A large proportion of GUSTO children (90%) were attending child care at 4 years old, where the main educational language is English; thus should be able to follow the cognitive tests instructions as the tests mainly involved visual stimuli and required only basic understanding of English. However, the levels of English/non-English exposure may vary depending on the duration of child care attendance per week and what languages they were spoken to outside of child care. To minimise influences resulting from a poor understanding of tests instructions on tests performance, we conducted a sensitivity analysis excluding children with very low exposure to English at home. Parent-rated child’s exposure to English and other languages at home (expressed in percentages) were captured by a questionnaire, and a cut-off of ≤20% was treated as very low exposure. This cut-off was chosen to account for very poor understanding while maintaining sufficient statistical power. This sensitivity analysis was performed with continuous maternal plasma zinc and magnesium concentrations to maintain sufficient statistical power, and not with zinc and magnesium statuses.

Missing data were imputed using multiple imputation technique with chained equations (20 times) for the following confounding variables: *n*=6 education, *n*=18 EPDS, *n*=18 STAI. The results of the 20 imputations were pooled. All analyses were conducted using Stata version 14 (StataCorp LP, College Station, TX, USA). To account for multiple hypothesis testing, two-sided *P*<0.008 (6 cognitive tests) was considered statistical significant while *P*<0.05 was considered trending association.

# Results

Compared to the 715 mother-offspring pairs included in analysis, mothers of the 273 mother-offspring pairs who did not participate in the cognitive tests tended to have lower magnesium concentrations and to be younger (**Supplementary Table S1**), but other characteristics were comparable.

## Participant characteristics

**Table 1** summarises the concentrations of maternal plasma zinc and magnesium according to maternal characteristics for 715 mother-offspring pairs included in analysis. The mean ± SD concentrations of maternal plasma zinc and magnesium were 812 ± 144µg/L and 19.9 ± 1.8mg/L respectively. Mothers with lower zinc and magnesium concentrations tended to be Indian and be overweight or obese before pregnancy. Additionally, mothers with lower zinc concentrations were also more likely to have lower plasma ferritin concentrations, whilst mothers with lower magnesium concentrations also tended to have poorer diet quality and to not be taking multivitamins and minerals supplements. Approximately 19% of these 715 mothers were zinc deficient, 15.8% were magnesium deficient, and 55.5% were magnesium insufficient at 26-28 weeks’ gestation.

[Table 1 near here]

## Associations of maternal plasma zinc concentrations and status with child’s cognitive outcomes

The associations of maternal plasma zinc concentrations with cognitive outcomes in children at 4 years are presented in **Table 2**. We observed a statistically significant association between higher maternal plasma zinc concentrations (1-SD or 144 µg/L increment) and higher scores in picture description and spatial identification (Lollipop subtest 2) after adjusting for key confounders (Model 1: β 0.35; 95% CI: 0.13, 0.58). Likewise, we observed trending (*P*=0.037) association of maternal zinc deficiency and their children scoring lower in picture description and spatial identification (**Supplementary Table S2**). No significant associations were observed between maternal zinc and children’s abilities to identify colours and shapes, numbers and calculation, letters and writing (Lollipop subtests 1, 3-4).

There was a trend towards higher maternal plasma zinc concentrations and higher scores in working memory task like ROST and VCR after adjusting for confounders (Model 1). A trend of higher maternal zinc concentrations and higher scores in receptive vocabulary (PPVT-4) was also observed but not with phonological awareness (CTOPP-2).

Maternal plasma zinc concentrations were not associated with children’s performance in number knowledge.

[Table 2 near here]

When analysed using maternal zinc statuses, no statistical significant associations with all cognitive tests except of Lollipop subtest 2 (as described above) were observed (Supplementary Table S2).

## Associations of maternal plasma magnesium concentrations and status with child’s cognitive outcomes

The associations of maternal plasma magnesium concentrations with cognitive outcomes in children at 4 years are presented in **Table 3**. Higher maternal plasma magnesium concentrations (1-SD or 1.8 mg/L increment) were observed to have statistical significant association with higher scores in identification of letters and writing (Lollipop subtest 4) after adjusting for key confounders (Model 1: β 0.65; 95% CI: 0.23, 1.07). Likewise, children of mothers with magnesium deficiency had lower scores in identification of letters and writing (trending in significance, *P*=0.037), compared to children of mothers with sufficient magnesium (**Supplementary Table S3**). Positive associations between maternal magnesium concentrations and children’s performance in identification of colours and shapes, picture description and spatial identification, and identification of numbers and calculations (Lollipop subtests 1-3), were attenuated after adjusting for confounders especially maternal education and pre-pregnancy BMI.

There were trending associations between higher maternal magnesium concentrations and higher scores in Level 1 number knowledge (Table 3) and between maternal magnesium deficiency and lower scores in Level 1 number knowledge (Supplementary Table S3).

[Table 3 near here]

Associations of maternal magnesium concentrations or statuses with other cognitive tests: working memory (ROST and VCR), receptive vocabulary (PPVT-4) and phonological awareness (CTOPP-2), were not statistically significant (Supplementary Table S3).

## Subgroup analyses

A total of 72 children were defined as having very low exposure to English, while 51 children did not have data for parent-rated English exposure and childcare attendance. These 123 children were excluded in the sensitivity analysis. Associations which were statistically significant or trending in significance in the main analyses remained in the subgroup analysis excluding children with very low exposure to English; but that of maternal magnesium concentrations and number knowledge were attenuated (**Supplementary Tables S4 and S5**).

# Discussion

In general, this study observed mostly null associations between maternal zinc or magnesium during pregnancy and tests of learning abilities and cognitive development in children at 4 years of age, aside from statistical significant associations with 2 subtests of the Lollipop (maternal zinc concentrations and children’s picture description and spatial identification, and maternal magnesium concentrations and children’s identification of letters and writing). Results across tests of same cognitive domains were largely inconsistent and lack robustness. This is contrary to the proposed biochemical mechanisms and findings from animal studies.

Higher maternal zinc concentrations showed statistically significant associations with subtest 2 of the Lollipop which contains a spatial ability component; although not with the other Lollipop subtests. This association is akin to findings from animal studies showing zinc supplementation during pregnancy to have an effect on spatial learning in the offspring,3,4 and another study showing zinc supplementation in children to improve their scores in a number of subtests of the Wechsler Intelligence Scale for Children – third edition (WISC-III) measuring spatial ability (picture completion, block design and object assembly).24 Zinc plays an important role in neurotransmission in neural pathways which relay spatial information to the hippocampus, and intracellular Zn2+ signalling in the dentate gyrus is required for object and space recognition.2 Keeping in mind, however, that the Lollipop subtest 2 is mainly a test of learning abilities and verbal skills thus may not be a sensitive test of spatial ability; hence this finding should be interpreted with caution.

There were suggestions that higher maternal zinc may be beneficial for offspring’s working memory, as indicated by trending associations with ROST and VCR. High concentrations of zinc were found in the hippocampus which plays a role in memory.2 Zinc predominantly exists in the form of free Zn2+, which serves as a signalling factor, involved in hippocampus-dependent memory.2 However, these associations were only trending in significance and there were no statistical significant/trending associations with both working memory tests for analyses based on maternal zinc statuses (deficient/sufficient). While a reduction in statistical power for analyses with maternal zinc statuses may explain the loss of statistical significance; the possibility that the significant associations with ROST and VCR are chance findings cannot be ruled out in view of the multiple hypotheses testing.

Although we observed a trending association between maternal zinc and receptive vocabulary, and a statistical significant association between maternal magnesium and identification of letters and writing; associations with other tests of language development (zinc with identification of letters and writing, magnesium with receptive vocabulary, and zinc and magnesium with phonological awareness) were not statistically significant. Thus, we cannot conclude at this point whether maternal zinc and magnesium have an influence on child’s early language development.

Possible influence of maternal magnesium on child’s numeric skills was suggested by an observation of a trending association with number knowledge. Interestingly, a significant association was observed with the more difficult subtest (i.e. Level 1 which requires the child to rely on mental counting skills) but not with the easier subtest (i.e. Level 0 which assesses the child’s ability to quantify objects which can be seen and touched), implying that higher maternal magnesium may influence ability to perform more difficult numeric task. But there were no statistical significant/trending associations with number knowledge test for analyses based on maternal magnesium statuses. There were a number of positive associations between maternal magnesium and child’s Lollipop tests scores (general learning abilities) that were markedly attenuated after adjustment for potential confounders.

Having very low exposure to the English language appears to have minimal influence on the associations observed in the main analysis, but attenuated the trending association between maternal magnesium and number knowledge. Although we used an English exposure level of 20% as a cut off to minimise the loss in statistical power, the attenuation could still be a result of a smaller sample size. However, this may again imply that the latter study findings are not robust.

Our study is the first to report on the association of maternal plasma magnesium concentrations and cognitive outcomes in children; and the first to examine maternal plasma zinc and children’s cognitive outcomes in an affluent country. Most previous studies examining the role of maternal zinc on child’s cognition were conducted in populations who were malnourished with children growing up in poor living environment, which will likely compromise early cognitive development. The timing of assessment (4 years old) also allowed the use of more specific developmental measures than what is possible during infancy. The GUSTO study has collected a range of socio-demographic and environmental factors known to influence cognitive development, which allowed for adjustment of these factors in statistical analysis.

Several limitations should be considered. First, concentrations of zinc and magnesium in plasma were only measured once mid-to-late pregnancy, and may not have identified the most critical *in utero* period when maternal nutritional influences on brain and the related cognitive functions were strongest. Furthermore, zinc and magnesium concentrations were not measured in children; hence a better or poorer cognitive performance may be a reflection of nutritional status in children rather than of their mothers but studies have found dietary patterns of the offspring to be reflective of their mothers’.25 A number of important contributors to neurocognitive development in children such as maternal intelligence and home environment were not measured in the cohort, but we adjusted for maternal education which is often used as a proxy for maternal intelligence.26 We recognised that the cut-off used to define very low English exposure (≤20%) is arbitrary but the sensitivity analysis was done as a precaution to account for children who are not attending child care or attends for very few days/hours per week and have limited exposure to English at home. Lastly, the cognitive tests we used have been relatively unexplored in relation to dietary intake or concentrations of minerals, and we cannot be certain that these tests had the necessary specificity to truly elucidate the expected associations.

Overall, there were suggestions that maternal zinc and magnesium play a role in child’s cognitive outcomes, but the current results lack consistency and robustness. Future epidemiological and intervention studies should use a hypothesis driven approach in selecting cognitive outcomes sensitive to zinc and magnesium influences, to allow comparability of study findings. Although we found generally null associations, it was important to note that there was a high prevalence of zinc deficiency (19%) and magnesium deficiency and insufficiency (71%) in our cohort of pregnant women; hence there remains a need for dietary recommendations to increase intake of zinc- and magnesium-rich foods during pregnancy for prevention of other adverse health outcomes associated with these micronutrients deficiencies.

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# References

1. Seress L. Morphological changes of the human hippocampal formation from midgestation to early childhood. In: Nelson CA, Luciana M, editors. Handbook of developmental cognitive neuroscience. Cambridge, MA: MIT Press; 2001. p. 45-58.

2. Tamano H, Koike Y, Nakada H, Shakushi Y, Takeda A. Significance of synaptic Zn2+ signaling in zincergic and non-zincergic synapses in the hippocampus in cognition. J Trace Elem Med Biol. 2016;38:93-8. PubMed PMID: 26995290.

3. Tahmasebi Boroujeni S, Naghdi N, Shahbazi M, Farrokhi A, Bagherzadeh F, Kazemnejad A, et al. The effect of severe zinc deficiency and zinc supplement on spatial learning and memory. Biol Trace Elem Res. 2009;130:48-61.

4. Yu X, Chen W, Wei Z, Ren T, Yang X, Yu X. Effects of maternal mild zinc deficiency and different ways of zinc supplementation for offspring on learning and memory. Food Nutr Res. 2016;60:29467. PubMed PMID: 26829185; PubMed Central PMCID: PMC4734033.

5. Warthon-Medina M, Moran VH, Stammers AL, Dillon S, Qualter P, Nissensohn M, et al. Zinc intake, status and indices of cognitive function in adults and children: a systematic review and meta-analysis. Eur J Clin Nutr. 2015;69:649-61. PubMed PMID: 25920424.

6. Xiong W, Liang Y, Li X, Liu G, Wang Z. Erythrocyte intracellular Mg(2+) concentration as an index of recognition and memory. Sci Rep. 2016;6:26975. PubMed PMID: 27253451; PubMed Central PMCID: PMC4890594.

7. Slutsky I, Abumaria N, Wu LJ, Huang C, Zhang L, Li B, et al. Enhancement of learning and memory by elevating brain magnesium. Neuron. 2010;65:165-77. PubMed PMID: 20152124.

8. Boanca M, Popa EG, Lupusoru RV, Poroch V, Mititelu-Tartau L, Lupusoru CE. The effects of magnesium nanovesicle formulations on spatial memory performance in mice. Rev Med Chir Soc Med Nat Iasi. 2014;118:847-53. PubMed PMID: 25341311.

9. Lamhot VB, Khatib N, Ginsberg Y, Anunu R, Richter-Levin G, Weiner Z, et al. Magnesium sulfate prevents maternal inflammation-induced impairment of learning ability and memory in rat offspring. Am J Obstet Gynecol. 2015;213:851.e1-8. PubMed PMID: 26232507.

10. Gernand AD, Schulze KJ, Stewart CP, West KP, Jr., Christian P. Micronutrient deficiencies in pregnancy worldwide: health effects and prevention. Nat Rev Endocrinol. 2016;12:274-89. PubMed PMID: 27032981.

11. Soh S-E, Tint MT, Gluckman PD, Godfrey KM, Rifkin-Graboi A, Chan YH, et al. Cohort Profile: Growing Up in Singapore Towards healthy Outcomes (GUSTO) birth cohort study. Int J Epidemiol. 2014;43:1401-9.

12. Lynch S, Pfeiffer CM, Georgieff MK, Brittenham G, Fairweather-Tait S, Hurrell RF, et al. Biomarkers of Nutrition for Development (BOND)—Iron Review. J Nutr. 2018;148:1001S-67S.

13. Chew AL, Morris JD. Validation of the Lollipop Test: A Diagnostic Screening Test of School Readiness. Educ Psychol Meas. 1984;44:987-91.

14. Hongwanishkul D, Happaney KR, Lee WS, Zelazo PD. Assessment of hot and cool executive function in young children: age-related changes and individual differences. Dev Neuropsychol. 2005;28:617-44. PubMed PMID: 16144430.

15. Zelazo PD, Jacques S, Burack JA, Frye D. The relation between theory of mind and rule use: evidence from persons with autism‐spectrum disorders. Infant Child Dev. 2002;11:171-95.

16. Dunn LM, Dunn DM. Peabody Picture Vocabulary Test, fourth edition. Circle Pines, MN: American Guidance Service; 2007.

17. Richard KW. CTOPP-2 : comprehensive test of phonological processing. 2nd edition. Austin, Tex: Pro-Ed; 2013.

18. Okamoto Y, Case R. Exploring the microstructure of children's central conceptual structures in the domain of number. Monogr Soc Res Child Dev. 1996;61:27-58. PubMed PMID: 8657168.

19. Cox JL, Holden JM, Sagovsky R. Detection of postnatal depression. Development of the 10-item Edinburgh Postnatal Depression Scale. Br J Psychiatry. 1987;150:782-6. PubMed PMID: 3651732.

20. Spielberger CD. State-trait Anxiety Inventory: A Comprehensive Bibliography. Consulting Psychologists Press; 1984.

21. Han CY, Colega M, Quah EPL, Chan YH, Godfrey KM, Kwek K, et al. A healthy eating index to measure diet quality in pregnant women in Singapore: a cross-sectional study. BMC Nutrition. 2015;1:39.

22. World Health Organisation. Trace elements in human nutrition and health. Geneva, Switzerland: WHO, 1998.

23. Elin RJ. Assessment of magnesium status for diagnosis and therapy. Magnes Res. 2010;23:S194-8. PubMed PMID: 20736141.

24. de Moura JE, de Moura EN, Alves CX, Vale SH, Dantas MM, Silva Ade A, et al. Oral zinc supplementation may improve cognitive function in schoolchildren. Biol Trace Elem Res. 2013;155:23-8. PubMed PMID: 23892699.

25. Davison B, Saeedi P, Black K, Harrex H, Haszard J, Meredith-Jones K, et al. The association between parent diet quality and child dietary patterns in nine- to eleven-year-old children from Dunedin, New Zealand. Nutrients. 2017;9:483. PubMed PMID: PMC5452213.

26. Ritchie SJ, Tucker-Drob EM. How much does education improve intelligence? A meta-analysis. Psychol Sci. 2018;29:1358-69. PubMed PMID: 29911926.

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| **Table 1**: Concentrations of maternal plasma zinc and magnesium at 26-28 weeks gestation according to maternal characteristics of the Growing Up in Singapore Towards healthy Outcomes cohort |
|   | **n (%)1** | **Zinc, µgL** | **P** | **Magnesium, mgL** | **P** |
| All participant | 715 (100) | 812 ± 144 |  | 19.9 ± 1.8 |  |
| **Maternal characteristics** |  |  |  |  |  |
| Age |  |  |  |  |  |
| <35 years | 538 (75.2) | 807 ± 130 | 0.112 | 19.8 ± 1.6 | 0.050 |
| ≥35 years | 177 (24.8) | 827 ± 180 |  | 20.1 ± 2.2 |  |
| Ethnicity |  |  |  |  |  |
| Chinese | 399 (55.8) | 835 ± 155 | <0.001\* | 20.1 ± 1.7 | <0.001\* |
| Malay | 184 (25.7) | 794 ± 130a |  | 19.7 ± 1.8a |  |
| Indian | 132 (18.5) | 771 ± 110a |  | 19.4 ± 1.9a |  |
| Highest education |  |  |  |  |  |
| ≤Secondary | 208 (29.1) | 807 ± 124 | 0.794 | 19.8 ± 1.8 | 0.052 |
| Post-secondary | 248 (34.7) | 815 ± 140 |  | 19.7 ± 1.6 |  |
| University  | 253 (35.4) | 816 ± 163 |  | 20.1 ± 1.9 |  |
| Pre-pregnancy BMI, kg/m2 |  |  |  |  |  |
| Underweight (<18.5) | 69 (9.7) | 813 ± 130a | 0.022\* | 20.2 ± 1.6a,b | <0.001\* |
| Normal weight (18.5-22.9) | 331 (46.3) | 829 ± 140a,b |  | 20.1 ± 1.6a,c |  |
| Overweight (23-27.4) | 158 (22.1) | 790 ± 168a,c |  | 19.7 ± 1.8b,c,d |  |
| Obese (≥27.5) | 157 (21.9) | 799 ± 128a,b,c |  | 19.3 ± 1.9d |  |
| Plasma ferritin concentrations |  |  |  |  |  |
| <75th percentile (<30.6ng/ml) | 536 (75.0) | 802 ± 147 | 0.001\* | 19.9 ± 1.78 | 0.723 |
| ≥ 75th percentile (≥30.6ng/ml) | 179 (25.0) | 844 ± 130 |  | 19.8 ± 1.68 |  |
| Diet quality (HEI-SGP) |  |  |  |  |  |
| <75th percentile | 530 (75.1) | 810 ± 148 | 0.494 | 19.8 ± 1.69 | 0.034\* |
| ≥ 75th percentile | 176 (24.9) | 819 ± 131 |  | 20.1 ± 1.94 |  |
| Multivitamins and minerals supplement intake |  |  |  |  |  |
| Yes | 110 (17.1) | 817 ± 126 | 0.983 | 20.5 ± 1.92 | <0.001\* |
| No | 534 (82.9) | 816 ± 150 |  | 19.7 ± 1.68 |  |
| EPDS score |  |  |  |  |  |
| Normal (<15) | 648 (90.6) | 814 ± 142 | 0.461 | 19.9 ± 1.8 | 0.929 |
| Depression (≥15) | 49 (6.9) | 799 ± 167 |  | 19.8 ± 1.5 |  |
| STAI state-score |  |  |  |  |  |
| Normal (<41) | 521 (72.9) | 813 ± 144 | 0.976 | 19.9 ± 1.8 | 0.718 |
| Anxiety (≥41) | 176 (24.6) | 814 ± 146 |  | 19.8 ± 1.7 |  |
| EPDS, Edinburgh Postnatal Depression Scale; HEI-SGP, Healthy Eating Index for Singapore Pregnant women; STAI, State-Trait Anxiety Inventory. |
| Values are means ± SDs or *n* (%). *P*-values (\**P*<0.05) are for independent-sample t-test or one-way ANOVA test with Bonferroni *post hoc* analysis (a, b, c, d groups without a common superscript letter differ, *P*<0.05).  |
| 1 Number of missing values: n=6 education, n=9 diet quality, n=71 multivitamins and minerals supplement intake, n=18 EPDS, n=18 STAI-state. |

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| **Table 2:** Associations of maternal plasma zinc concentrations at 26-28 weeks gestation with neurocognitive outcomes in 4-year old children in GUSTO |
|  | Crude Model | Model 1 |
| Cognitive tests | β (95% CI) | P | β (95% CI) | P |
| Lollipop Test – general learning abilities (n=690) | 1.48 (0.41, 2.54) | 0.006+ | 1.09 (0.02, 2.16) | 0.046\* |
| Subtest 1 – identification of colours & shapes | 0.23 (-0.01, 0.47) | 0.058 | 0.20 (-0.03, 0.44) | 0.092 |
| Subtest 2 – picture description and spatial identification | 0.46 (0.23, 0.70) | <0.001+ | 0.35 (0.13, 0.58) | 0.002+ |
| Subtest 3 – identification of numbers and calculations | 0.32 (0.06, 0.57) | 0.016\* | 0.24 (-0.03, 0.51) | 0.079 |
| Subtest 4 – identification of letters and writing | 0.46 (-0.09, 1.02) | 0.103 | 0.29 (-0.29, 0.87) | 0.326 |
| Working memory – ROST (n=680) | 0.24 (-0.006, 0.49) | 0.056 | 0.28 (0.05, 0.51) | 0.017\* |
| Working memory – VCR (n=686) | 0.10 (0.01, 0.19) | 0.032\* | 0.10 (0.01, 0.18) | 0.034\* |
| Receptive vocabulary – PPVT-4 (n=674) | 2.11 (0.50, 3.72) | 0.010\* | 1.90 (0.53, 3.07) | 0.009\* |
| Phonological awareness – CTOPP-2 (n=627) | 0.44 (-0.25, 1.12) | 0.214 | 0.29 (-0.45, 1.03) | 0.438 |
| Number Knowledge (n=673) | 0.34 (0.02, 0.65) | 0.039\* | 0.25 (-0.07, 0.57) | 0.125 |
| Level 0 | 0.08 (-0.02, 0.18) | 0.102 | 0.06 (-0.04, 0.16) | 0.208 |
| Level 1 | 0.25 (-0.01, 0.51) | 0.058 | 0.19 (-0.08, 0.45) | 0.166 |
| \**P*<0.05, +*P*<0.008CTOPP-2, Comprehensive Test of Phonological Processing – 2nd edition; GUSTO, Growing Up in Singapore Towards healthy Outcomes; PPVT-4, Peabody Picture of Receptive Vocabulary – 4th edition; ROST, Random Object Span Test; VCR, Visually Cued Recall. Model 1 adjusted for: maternal age, ethnicity, education, pre-pregnancy BMI, diet quality, multivitamins and minerals supplement intake, plasma ferritin concentrations, antenatal depression and anxiety levels |
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| **Table 3:** Associations of maternal plasma magnesium concentrations at 26-28 weeks gestation with neurocognitive outcomes in 4-year old children in GUSTO |
|  | Crude Model | Model 1 |
| Cognitive tests | β (95% CI) | P | β (95% CI) | P |
| Lollipop Test – general learning abilities (n=690) | 2.10 (1.10, 3.09) | <0.001+ | 0.99 (0.05, 1.92) | 0.038\* |
| Subtest 1 – identification of colours & shapes | 0.35 (0.10, 0.59) | 0.006+ | 0.14 (-0.10, 0.39) | 0.246 |
| Subtest 2 – picture description and spatial identification | 0.33 (0.10, 0.57) | 0.006+ | 0.10 (-0.13, 0.34) | 0.388 |
| Subtest 3 – identification of numbers and calculations | 0.35 (0.07, 0.62) | 0.009\* | 0.10 (-0.15, 0.35) | 0.450 |
| Subtest 4 – identification of letters and writing | 1.06 (0.63, 1.50) | <0.001+ | 0.65 (0.23, 1.07) | 0.003+ |
| Working memory – ROST (n=680) | 0.05 (-0.16, 0.25) | 0.663 | 0.04 (-0.17, 0.25) | 0.720 |
| Working memory – VCR (n=686) | 0.06 (-0.05, 0.16) | 0.284 | 0.02 (-0.09, 0.13) | 0.770 |
| Receptive vocabulary – PPVT-4 (n=674) | 2.54 (0.97, 4.11) | 0.002+ | 1.32 (-0.11, 2.75) | 0.070 |
| Phonological awareness – CTOPP-2 (n=627) | 0.96 (0.12, 1.80) | 0.025\* | 0.63 (-0.25, 1.50) | 0.159 |
| Number Knowledge Test (n=673) | 0.55 (0.26, 0.83) | <0.001+ | 0.27 (-0.02, 0.55) | 0.064 |
| Level 0 | 0.06 (-0.04, 0.16) | 0.264 | -0.02 (-0.12, 0.08) | 0.709 |
| Level 1 | 0.49 (0.26, 0.72) | <0.001+ | 0.29 (0.05, 0.53) | 0.018\* |
| \**P*<0.05, +*P*<0.008CTOPP-2, Comprehensive Test of Phonological Processing – 2nd edition; GUSTO, Growing Up in Singapore Towards healthy Outcomes; PPVT-4, Peabody Picture of Receptive Vocabulary – 4th edition; ROST, Random Object Span Test; VCR, Visually Cued Recall. Model 1 adjusted for: maternal age, ethnicity, education, pre-pregnancy BMI, diet quality, multivitamins and minerals supplement intake, plasma ferritin concentrations, antenatal depression and anxiety levels |

Dropout due to stress, inconvenience, or other personal reasons

(n=88)

Mothers without sufficient blood for plasma zinc and magnesium assays

(n=65)

Non-participation due to busy schedules, lack of interest, dropout from GUSTO study; or had non-usable data for cognitive tests

(n=273)

Mothers at recruitment

(n=1247)

Mothers excluded if multiple births or received IVF treatment

(n=95)

Mothers who conceived naturally and had singleton live births

(n=1152)

Mothers with plasma zinc and magnesium concentrations

(n=988)

Children who completed cognitive tests at 4 years

(n=715 had usable data for at least 1 test)

ROST n=680

VCR n=689

NKT n=673

Lollipop n=690

PPVT n=678

CTOPP-2 n=627

# Figure caption

Figure 1: Participant flow diagram for the analysis of associations of maternal plasma zinc and magnesium with neurocognitive outcomes in children in the Growing Up in Singapore Towards healthy Outcomes (GUSTO) cohort study. CTOPP-2, Comprehensive Test of Phonological Processing – 2nd edition; IVF, In-vitro Fertilisation; NKT, Number Knowledge Test; PPVT-4, Peabody Picture of Receptive Vocabulary – 4th edition; ROST, Random Object Span Test; VCR, Visually Cued Recall.