NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Effects of iron and omega-3 fatty acid supplementation on physical activity of iron deficient primary school children residing in KwaZulu-Natal

J Greeff B.Sc. Hons. Nutrition

Dissertation in fulfilment of the requirements for the degree Magister Scientiae in Nutrition for the School of Physiology, Nutrition and consumer Sciences of the North-West University, Potchefstroom Campus

December 2011

Supervisor:	Prof CM Smuts
Co-supervisor:	Dr Jane Kvalsvig
Assistant supervisor:	Jeannine Baumgartner

Acknowledgement

I am sincerely and heartily grateful to my study leader, professor C.M. Smuts, for his wisdom, guidance and support.

This dissertation would not have been possible without the help and support of my co-supervisor and loyal friend, Jeannine Baumgartner. I would like to thank her for her continued support, guidance and patience, and for being the perfect example of a young, passionate researcher. Thank you so much Jeannine. I really enjoyed working with you.

I would also like to thank the following:

- Jane Kvalsvig for her input in the study and her contribution to interpretation of my results.
- Shumi Shelembe for coordinating the Conners' Teacher Rating Scale.
- All the principals, teachers and learners that participated in the study – these people are in essence what made the study possible. I owe them my sincere thankfulness.
- The North West University and the Centre of Excellence for Nutrition for providing me with opportunities and financial support.
- My parents (Ampie and Amanda), siblings (Nelia, Moller and Christof) for support in all areas.

I would also like to state very special thanks to my friend and soul mate, Bertus Schoeman, without whose support the writing of this dissertation would not have been possible.

I owe sincere and earnest thankfulness to you all.

Abstract

Background: Iron deficiency (ID) is the most prevalent nutritional deficiency in the world. In children, both inadequate iron and fatty acid (FA) status have been found to have an effect on cognitive and behavioural function, including physical activity behaviour and attention deficit hyperactivity disorder (ADHD)-related behaviour.

Aim: To investigate the effects of supplementation with iron and omega-3 fatty acids (n-3 FAs), alone and in combination, on spontaneous motor activity and ADHD-related behaviour in iron deficient primary school children in KwaZulu-Natal. An additional aim was to evaluate the use of the *Actical* accelerometer as a tool to assess physical activity behaviour.

Methods: The study design was a 2x2 factorial, randomized, double-blind and placebo-controlled trial. Iron deficient school children aged six to ten years with or without mild anaemia were included in the study (n = 321). Subjects were randomly assigned to receive one of the following supplement combinations: (1) 420mg docosahexaenoic acid (DHA)/80 mg eicosapentaenoic acid (EPA) + 50mg of iron as ferrous sulphate (Fe); (2) 420mg DHA/80mg EPA + placebo; (3) 50mg of Fe + placebo; (4) placebo + placebo. Supplements were provided four times a week for a duration of 8.5 months (excluding school holidays). Physical activity of a subgroup of subjects (n=98) was recorded on four random school days at baseline, midpoint and endpoint (12 days in total) during three different time periods namely *class time 1* (08h00–10h30)*, break time* (10h30-11h00) and *class time 2* (11h00-12h00). Classroom behaviour of study subjects was assessed by teachers at baseline and endpoint using the Conners' Teacher Rating Scale-Revised: Short Forms (CTRS). Iron status indicators and red blood cell (RBC) FA composition were measured at baseline and endpoint. Treatment effects were assessed for activity and CTRS scores. Furthermore, the relationship between activity, CTRS scores and iron/FA status indicators was determined using bivariate correlation and multivariate linear regression analysis.

Results: Overall activity of all subjects varied over time from baseline and midpoint to endpoint. A significant cycle x age interaction (P = 0.005) as well as a significant cycle x time period x gender interaction (P = 0.036) was observed on overall activity. There were no significant interactions of cycle or time period with treatment. However, there was a significant main effect of DHA/EPA supplementation for lower *class time 1* activity at endpoint (P = 0.014). Biological markers indicating better or poorer iron status were positively and negatively associated with activity at *break time,* respectively. Subjects in the group receiving both iron and DHA/EPA supplements showed a significant improvement from baseline to endpoint on the cognitive problems/inattention subscale (P = 0.005) of the CTRS. Hyperactivity scores increased

significantly from baseline to endpoint in all groups (*P* = 0.006). DHA (r = -.203; *P* = 0.040) and EPA (r = -.199; *P* = 0.044) content of RBC were negatively associated with activity at *class time 1*. No significant associations were observed between activity and CTRS scores at baseline. At endpoint, *class time 1* activity was positively associated with all CTRS subscale scores except for the cognitive problems subscale, which only bordered significance (correlation, *P* = 0.051; regression, *P* = 0.073).

Conclusions: These findings suggest that n-3 FA supplementation may have an influence on ADHD-related behaviour during class time. During school break time when subjects were allowed to move around freely, iron status was positively associated with spontaneous motor activity. Furthermore, the accelerometer might be a useful complimentary tool for assessing both classroom and break time activity behaviour in school children.

Key words: iron deficiency, iron supplementation, school children, motor activity, omega-3 fatty acid, attention deficit hyperactivity disorder, behaviour, accelerometer, Conners' Teacher Rating Scale

Opsomming

Agtergrond: Ystergebrek is die mees algemene nutriëntgebrek in die wêreld. In kinders is gevind dat beide yster- en vetsuur (FA)-status kognitiewe en gedragsfunksie, insluitende fisiese aktiwiteit en aandagtekort hiperaktiwiteitsversteuring (ADHD)-verwante gedrag, affekteer.

Doel: Om die effekte van supplementering met yster en omega-3 vetsure (n-3 FAs), alleen en in kombinasie, op spontane motoriese aktiwiteit en ADHD-verwante gedrag in yster-gebrekkige primêre skoolkinders in KwaZulu-Natal te ondersoek. 'n Bykomende doelwit was om die gebruik van die *Actical* versnellingsmeter as 'n instrument om fisiese aktiwiteit te meet, te evalueer.

Metodes: Die studie-ontwerp was 'n 2x2 faktoriale, gerandomiseerde dubbelblinde en plasebogekontroleerde ondersoek. Ystergebrekkige skoolkinders ses tot tien jaar oud met of sonder matige anemie is in die studie ingesluit (n = 321). Proefpersone is ewekansig toegewys om een van die volgende supplementkombinasies te ontvang: (1) 420 mg dokosaheksanoësuur (DHA)/80 mg eikosapentanoësuur (EPA) + 50 mg yster as ystersulfaat (Fe); (2) 420 mg DHA/80 mg EPA + plasebo; (3) 50 mg Fe + plasebo; (4) plasebo + plasebo. Supplemente is vier keer per week voorsien vir 8.5 maande lank (uitsluitend skool-vakansies). Fisiese aktiwiteit van 'n ondergroep proefpersone is gedurende vier ewekansige skooldae tydens basislyn, middel- en eindpunt van die studie (12 dae in total) genoteer gedurende drie verskillende tydsintervalle naamlik *klastyd 1* (08h00-10h30), *pouse* (10h30-11h00) en *klastyd 2* (11h00-12h00). Klaskamergedrag van proefpersone is deur onderwysers met basislyn en eindpunt geassesseer deur gebruik te maak van die hersiene Conners se onderwyser waardebepalingskaal: kort vorms (*Conners' Teacher Rating Scale-Revised: Short Forms*, CTRS). Ysterstatusindikatore en rooibloedsel (RBS)-FA-samestelling is tydens basislyn en eindpunt gemeet. Behandelingseffekte is gemeet vir aktiwiteit en CTRS-tellings. Verder is die verband tussen aktiwiteit, CTRS-tellings en yster/FA-statusindikatore gemeet deur gebruik te maak van bivariate korrelasie en multivariate regressieanalise.

Resultate: Algehele aktiwiteit van al die proefpersone het oor tyd van basislyn en middel- tot eindpunt van die studie gevarieer. 'n Betekenisvolle siklus x ouderdom interaksie (P = 0.005) asook 'n betekenisvolle siklus x tydperk x geslaginteraksie (P = 0.036) is op algehele aktiwiteit waargeneem. Daar was geen betekenisvolle interaksies van siklus of tydperiode met behandeling nie. Daar was egter 'n betekenisvolle hoofeffek van DHA/EPA-supplementasie vir laer *klastyd 1*-aktiwiteit aan die einde van die studie (P = 0.014). Biologiese merkers wat dui op beter of swakker ysterstatus was positief en negatief geassosieer met aktiwiteit tydens pouse, respektiewelik. Proefpersone in die groep wat beide Fe en DHA/EPA-supplemente ontvang het, het 'n betekenisvolle verbetering van basislyn na einde vertoon op die kognitiewe

probleem/aandaggebrek-subskaal (*P* = 0.005) van die CTRS. Hiperaktiwiteittellings het betekenisvol van basislyn na eindpunt in alle groepe vermeerder (*P* = 0.006). DHA (r = -.203; *P* = 0.040) en EPA (r = -.199; *P* = 0.044)-inhoud van RBS was negatief geassosieer met aktiwiteit met *klastyd 1*. Geen betekenisvolle assosiasies tussen aktiwiteit en CTRS-tellings met basislyn is gevind nie. Aan die einde van die studie was *klastyd 1*-aktiwiteit positief met alle CTRS-tellings geassosieer behalwe die kognitiewe probleem-subskaal, wat gegrens het aan betekenisvolle assosiasie (korrelasie, *P* = 0.051; regressie, *P* = 0.073).

Gevolgtrekkings: Hierdie bevindings suggereer dat n-3 FA-supplentering ‘n invloed mag hê op ADHD-verwante gedrag gedurende klastyd. Gedurende skoolpouse, wanneer die proefpersone toegelaat is om vryelik rond te beweeg, was ystertekort positief met spontane motoriese aktiwiteit geassosieer. Verder mag die versnellingsmeter ‘n bruikbare instrument wees om aktiwiteitsgedrag van kinders tydens beide klaskamertyd en pouse te meet.

Sleutelwoorde: ystergebrek, ystersupplementering, skoolkinders, motoriese aktiwiteit, omega-3-vetsuur, aandagtekort hiperaktiwiteitsversteuring (ADHD), gedrag, versnellings-meter, Conners se onderwyser waardebepalingskaal

Table of contents

List of tables

TABLES FOR CHAPTER I

TABLES FOR CHAPER II

TABLES FOR CHAPTER III

List of figures

FIGURES FOR CHAPER II

FIGURES FOR CHAPTER III

Addendum

Acronyms and abbreviations

5’ UTR	five prime untranslated region
AA	arachidonic acid
ADHD	attention deficit hyperactivity disorder
ALA	alpha-linolenic acid
ANCOVA	analysis of covariance
CO_2	carbon dioxide
CP	ceruloplasmin
CPRS	Conners’ Parent Rating Scale
CRS	Conners’ Rating Scale
CTRS	Conners’ Teacher Rating Scale
CTRS-R:L	Conners’ Teacher Rating Scale-Revised: long form
CTRS-R:S	Conners’ Teacher Rating Scale-Revised: short form
D5D	delta-5 desaturase
D6D	delta-6 desaturase
DCT1	divalent-citation transporter 1
DGLA	dihomogamma linolenic acid
DHA	docosahexaenoic acid
DMT1	divalent metal transporter 1DPA
DPA	docosapentaenoic acid
EID	early iron deficiency
EPA	eicosapentaenoic acid
ETA	eicosatetraenoic acid
FA	fatty acid
Fe	iron
FPN	ferroportin
Hb	haemoglobin
HCP1	heam carrier protein 1

HO-1	heam oxygenase
ID	iron deficiency/iron-deficient
IDA	iron deficiency anaemia
IRE-IRP	iron-responsive element-iron regulatory protein
LA	linoleic acid
LC	long chain
n-3	omega-3
n-6	omega-6
NEFA	non-esterified fatty acid
NFCS	National Food Consumption Survey
ns	not significant
PF	plasma ferritin
PND	postnatal day
PUFA	polyunsaturated fatty acid
RBC	red blood cell
RES	reticuloendothelial system
SF	serum ferritin
TBI	transferrin-bound iron
TfR	transferring receptor
TFR1	transferrin receptor 1
Trf	transferrin
TRF2	transferrin receptor 2
ZnPP	zinc protoporphyrin

Chapter I - Introduction

1.1 Problem statement and motivation

Dietary iron deficiency (ID) sufficient to cause anaemia (IDA) is commonly prevalent in underdeveloped countries (WHO, 2001; Beard, 2000), such as South Africa. ID is the most prevalent nutritional deficiency in the world (Nojilana *et al*., 2007). Young children are especially prone to ID with iron intake often being inadequate in combination with impaired absorption of iron and rapid growth rate (Labadarios & Louw, 2007). IDA occurs when ID is adequately severe to diminish erythropoiesis, leading to a decrease in the number of red blood cells (RBCs) in the blood, which results in anaemia (Nojilana *et al*., 2007; Stoltzfus, 2003). Even though nutritional deficiencies such as folate and vitamin B_{12} and conditions such as malaria, HIV and other chronic diseases may have a role in the causal path of anaemia, previous findings suggest that ID is responsible for about 25 - 50% of cases of anaemia in young children and pregnant women in developing countries (Nojilana *et al*., 2007; Stoltzfus *et al.*, 2004, Stoltzfus, 2003). The National Food Consumption Survey (NFCS) of South Africa in 2005 found that the prevalence of poor iron status has doubled at the national level since 1994 (compared to data from the South African Vitamin A Consultative Group; SAVACG) to one out of five children. Other data concluded that the prevalence of poor iron status was 13.3% in children and 18.2% in women (Labadarios & Louw, 2007). Studies in rural KwaZulu-Natal have found the prevalence of ID among preschoolers to be 19.8% (Oelofse *et al.,* 1999) and found 42-52% prevalence of anaemia among 6 – 74 year old individuals (Mayet *et al*., 1985).

ID and IDA may have several consequences including congestive cardiac failure, increased susceptibility to infections, poor physical growth, increased fatigue, reduced work and mental performance, retardation of psychomotor development, reduced learning ability and other attention deficit hyperactivity disorder (ADHD)-related behaviours (Schrimshaw, 1991 Labadarios & Louw, 2007, Konofal *et al*., 2005, 2008). Soloojee and Pettifor (2001) asserted that the most worrying associations are between ID and impaired development in behaviour, cognition and psychomotor skills.

With prevalence estimates ranging between 4-15%, ADHD is the most common developmental disorder of childhood (Costello *et al*., 2003; Wolraich *et al*., 1998; Richardson, 2006). It is characterized by a combination of inattention, impulsiveness and hyperactivity (Swanson *et al*., 1998). According to the Diagnostic and Statistical Manual of Mental Disorder- (DSM) IV, symptoms of ADHD may cause impairment in social and educational settings and family functioning; it could also have a profound impact on academic performance and quality of life (Loe & Feldmann, 2007; Klassen *et al*., 2004). Possible links between both Fe and omega-3 (n-3) FAs and ADHD have been studied (Konofal *et al.,* 2004, 2007; Gadoth, 2008; Burgess *et al*.,

2000). Richardson (2006) stated that the evidence for a causal contribution of n-3 FAs presently appears strongest in relation to disorders of mood and/or impulsivity, most of which show high co-morbidity with ADHD.

As indicated by a review of ID and IDA studies in humans and animals by McCann and Ames (2007), associations between IDA and deficits in cognitive or behavioural performance in children are consistently observed. As for ID without anaemia – several studies have showed significant effects on cognition and behaviour, even without anaemia. McCann and Ames (2007) have also indicated that a consistent association between decreased motor activity and IDA has been showed in animal studies. A considerable amount of research has been done to establish a possible causal relationship between ID/IDA and impaired child development, but a definitive link has been excluded due to the fact that anaemia is associated with many other disadvantages such as poverty, low birth weight, malnutrition, poor education among mothers and lack of stimulation in the home – all of which also affect child development (Stoltzfus, 2001; Soloojee & Pettifor, 2001).

As iron, n-3 FAs have also been linked with behaviour, cognitive function and motor activity in humans and animals, with a particular focus on ADHD-related behaviour and hyperactivity (Richardson, 2006). Evidence indicating the type of relationship between spontaneous activity (excluding hyperactivity) and n-3 FAs is scarce, and only a few animal studies (Levant *et al*., 2004, 2006, 2010), but no human studies have explored this. Several human studies have investigated the possible link between n-3 FA status and ADHD-related behaviour, but most of these studies have been observational, and only a few have studied the effects of n-3 FA supplementation on behaviour (Mitchell *et al*., 1987; Stevens *et al*., 1996; Antalis *et al*., 2006; Sinn and Bryan, 2007; Bélanger *et al*., 2009; Ka-Hung *et al*., 2009). Observational studies have shown that subjects with poorer n-3 FA status show more or greater severity of ADHD symptoms and related behaviour. Supplementation with n-3 FAs in children has been found to be successful in reducing these behaviours (Sinn and Bryan, 2007; Johnson *et al*., 2009).

Measurement of physical activity has made certain proficient advances in the past two decades with the development and use of activity monitors that provide real-time estimates of the frequency, intensity and duration of free-living physical activity, such as the accelerometer (Trost *et al.*, 2005; Freedson & Miller, 2000). However, very few studies have used these devices to measure spontaneous activity, and no studies could be found using accelerometers to measure activity in the context of ADHD-related behaviours. Since ADHD-related behaviours also include activity based behaviours such as squirminess and hyperactivity, a solid quantitative measurement as would be provided by and accelerometer could be a useful verification tool if used in combination with an ADHD behaviour-detecting scale, and vice versa.

1.2 Research and objectives

1.2.1 Aim

To investigate the effects of supplementation with iron and n-3 FAs, alone and in combination, on spontaneous motor activity and ADHD-related behaviour in iron deficient primary school children in KwaZulu-Natal. An additional aim was to evaluate the use of the *Actical* accelerometer as a tool to assess physical activity behaviour.

1.2.2 Objectives

- To investigate spontaneous physical activity of primary school children during class and break times using an accelerometer
- To assess ADHD-related behaviour of children using the CTRS.
- To assess children's physical activity behaviour over time at different seasons and different times of the school year.
- To determine the effects of the iron and n-3 FA supplements on spontaneous motor activity and CTRS scores of children during class and break times.
- To investigate potential relationships between CTRS scores and activity measurements and iron and FA-status indicators obtained through blood specimens.
- To assess the use of the *Actical* accelerometer as a tool for measuring behavioural outcomes in children.

1.3 Structure of dissertation

This dissertation is written in article format comprising of four chapters. Chapter one is the introductory chapter consisting of the problem statement, aims, objectives, an articulation of the structure of the dissertation and authors' contributions to the investigation. Chapter two is a literature review providing background information on iron, n-3 FAs, spontaneous motor activity and ADHD. This chapter provides general information on the topic and discusses prevalence statistics and biological mechanisms. A review of previous studies is included to construct a knowledge base of what research has found thus far. Chapter three is the article "Effects of iron and omega-3 fatty acid supplementation on spontaneous motor activity and ADHD-related behaviour in iron-deficient primary school children in KwaZulu-Natal". This article is written according to the guidelines of the journal *Physiology and Behaviour*. References for each chapter are provided at the end of each chapter. All references are written in Harvard Style according to the requirements of the North-West University except the references of chapter three, which are written according to the requirements of *Physiology and Behaviour.*

1.4 Contributions of the authors

This project was conducted by a team of researchers whose contributions to the work is detailed in Table 1.

Table 1. Qualifications and roles of the research team

Jani Greeff (M.Sc. student)	Responsible for all aspects concerning the planning and collection of spontaneous activity data as well as for the literature review, data analysis, interpretation and writing up of the results of all behaviour-focussed aspects of the study
Prof CM Smuts (Biochemist)	Project leader; provided supervision of the project and was the supervisor for Jani Greeff
Jeannine Baumgartner (PhD Nutrition student)	Primary investigator in the project; responsible for conducting the main study and for the analyses of iron and FA status indicators; assistant supervisor for Jani Greeff

I declare that I have approved the above-mentioned study that my role in the study as indicated above is representative of my actual contribution and I hereby give my consent that it be published as part of the M.Sc. dissertation of Jani Greeff.

1.5 References

ANTALIS, C.J., STEVENS, L.J., CAMPBELL, M., PAZDRO, R., ERICSON, K. & BURGESS, J.R. 2006. Omega-3 fatty acid status in attention-deficit/hyperactivity disorder. *Prostaglandins, leukotrienes and essential fatty acids,* 75(4-5):299-308.

BEARD, J.L. 2000. Iron requirements in adolescent females. *Journal of nutrition*, 130(suppl):440S-2S.

BÉLANGER, S.A., VANASSE, M., SPAHIS, S., SYLVESTRE, M., LIPPÉ, S., L'HEUREUX, F., GHADIRIAN, P., VANASSE, C. & LEVY, E. 2009. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. *Paediatrics & child health,* 14(2):89-98.

BURGESS, J.R., STEVENS, L., ZHANG, W. & PECK, L. 2000. Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. *The American journal of clinical nutrition,* 71(1):327S-330S.

COSTELLO, E. J., MUSTILLO, S., ERKANLI, A., KEELER, G., & ANGOLD, A. 2003. Prevalence and development of psychiatric disorders in childhood and adolescence*. Archives of general psychiatry,* 60:837–844.

FREEDSON, P.S. & MILLER, K. 2000. Objective monitoring of physical activity. *Research questions in exercise and sport,* 71:S21-S449.

GADOTH, N. 2008. On fish oil and omega-3 supplementation in children: The role of such supplementation on attention and cognitive dysfunction. *Brain & development,* 30(5):309-312.

JOHNSON, M., OSTLUND, S., FRANSSON, G., KADESJÖ, B. & GILLBERG, C. 2009. Omega-3/omega-6 fatty acids for attention deficit hyperactivity disorder: A randomized placebo-controlled trial in children and adolescents. *Journal of attention disorders,* 12(5):394-401.

KA-HUNG, N.G., BARBARA, J.M., LAUREN, R. & SINN, N. 2009. Dietary PUFA intakes in children with attention-deficit/hyperactivity disorder symptoms. *British journal of nutrition*, 102:1635-1641.

KLASSEN, A.F., MILLER, A. & FINE, S. 2004. Health-related quality of life in children and adolescents who have a diagnosis of attention-deficit/hyperactivity disorder. *Pediatrics*, 114:541–547.

KONOFAL, E., CORTESE, S., LECENDREUX, M., ARNULF, I. & MOUREN, M.C. 2005. Effectiveness of iron supplementation in a young child with attention-deficit/hyperactivity disorder. *Pediatrics,* 116(5):e732-e734.

KONOFAL, E., LECENDREUX, M., DERON, J., MARCHAND, M., CORTESE, S., ZAÏM, M., MOUREN, M.C. & ARNULF, I. 2008. Effects of iron supplementation on attention deficit hyperactivity disorder in children. *Pediatric neurology,* 38(1):20-26.

LABADARIOS, D. & LOUW, R. 2007. Selected micronutrient status: Iron status. (In LABADARIOS, D., ed. National Food Consumption Survey-Fortification Baseline (NFCS-FB): South Africa, 2005. Tygerberg: University of Stellenbosh. 447-455.

LEVANT, B., OZIAS, M.K. & CARLSON, S.E. 2006. Sex-specific effects of brain LC-PUFA composition on locomotor activity in rats. *Physiology & behavior,* 89(2):196-204.

LEVANT, B., RADEL, J.D. & CARLSON, S.E. 2004. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behavioural brain research,* 152(1):49-57.

LEVANT, B., ZARCONE, T., & FOWLER, S. 2010. Developmental effects of dietary n-3 fatty acids on activity and response to novelty. *Physiology & behavior,* 101(1):176-183.

LOE, I.M. & FELDMAN, H.M. 2007. Academic and educational outcomes for children with ADHD. *Pediatrics,* 7:82–90.

MAYET, FG., SCHUTTE, CH. & REINACH, SG. 1985. Anaemia among the inhabitants of a rural area in northern Natal. *South African Medical Journal,* 67:458-462.

MCCANN, J.C. & AMES, B.N. 2007. An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. *American journal of clinical nutrition,* 85(4):931-945.

MITCHELL, E.A., AMAN, M.G., TURBOTT, S.H. & MANKU, M. 1987. Clinical characteristics and serum essential fatty acid levels in hyperactive children. *Clinical pediatrics,* 26(8):406-411.

NOJILANA, B., NORMAN, R., DHANSAY, M.A., LABADARIOS, D., VAN STUIJVENBERG, M.,E. & BRADSHAW, D. 2007. Estimating the burden of disease attributable to iron deficiency anaemia in south africa in 2000. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde,* 97(8):741-746.

OELOFSE, A., FABER, M., BENADE, JG. & KENOYER, DG. 1999. The nutritional status of a rural community in KwaZulu-Natal, South Africa: the Ndunakazi Project. *Central African journal of medicine,* 45(1):14-19.

RICHARDSON, A.J. 2006. Omega-3 fatty acids in ADHD and related neurodevelopmental disorders. *International review of psychiatry,* 18(2):155-172.

SALOOJEE, H. & PETTIFOR, J.M. 2001. Iron deficiency and impaired child development. 1377.

SCHRIMSHAW, N.S. 1991. Iron deficiency. *Scientific American,* October:24-30.

STEVENS, L.J., ZENTALL, S.S., ABATE, M.L., KUCZEK, T. & BURGESS, J.R. 1996. Omega-3 fatty acids in boys with behavior, learning, and health problems. *Physiology & behavior,* 59(4-5):915-920.

STOLTZFUS, R.J. 2003. Update on issues related to iron deficiency and anaemia control. International Nutritional Anaemia Consultative Group: Integrating to Move Iron Deficiency

Anaemia Control Forward Symposium. Marrakech, Morocco. International Life Sciences Institute.

STOLTZFUS, R.J., Mullany, L. & BLACK R.E. 2004. Iron Deficiency Anaemia. In: EZZI, M., LOPEZ, D., RODGERS, A., MURRAY, C.L.J. Comparitative quantification of health risks: global and regional burden of disease attributable to selected major risk factors. Vol 1. Geneva: World Health Organization. 163-208.

SWANSON, J.M., SERGEANT, J.A., TAYLOR, E., SONUGA-BARKE, E., JENSEN, P.S. & CANTWELL, D.P. 1998. Attention-deficit hyperactivity disorder and hyperkinetic disorder. *Lancet,* 351(9100):429.

TROST, S.G., MCIVER, K.L. & PATE, R.R. 2005. Conducting accelerometer-based activity assessments in field-based research. *Medicine & science in sports & exercise,* 37(11):S531-s543.

WHO. 2001. Iron deficiency anaemia: assessment, prevention, and control. A guide for programme managers. Geneva, Switzerland: World Health Organization. (WHO/NHD/01.3.)

WOLRAICH, M. L., LAMBERT, E. W., BICKMAN, L., SIMMONS, T., DOFFING, M. A., & WORLEY, K. A. 2004. Assessing the impact of parent and teacher agreement on diagnosing attention-deficit hyperactivity disorder. *Journal of developmental and behavioral pediatrics*, 25:41–47.

Chapter II – Literature Review

2.1 Introduction

This chapter will serve as a background on iron metabolism, ID, n-3 FAs, spontaneous activity and attention ADHD and related behaviours. Metabolic pathways of iron and n-3 FAs will be discussed as well as causes and consequences of ID. A review of the current literature on these topics will also be summarized, which will include explanations and possible biological mechanisms for the findings made.

2.2 Iron metabolism and homeostasis

2.2.1 Iron metabolism in humans: An overview

The body's iron content in adult humans is normally between 3-5 g, with generally higher values in men than in women. Most of this iron (about two thirds) is bound to the oxygen transport protein haemoglobin in circulating erythrocytes. Another 5-10% is found in muscle in the form of the oxygen storage protein myoglobin and an even smaller percentage in various tissues as other haemoproteins, iron-sulphur proteins and non-haem, non iron-sulphur proteins. Most of the remaining iron is stored as ferritin and haemosiderin (storage proteins) in the liver, spleen, bone marrow and muscle, while only a very small fraction of total body iron circulates in the plasma and other extracellular fluids bound to the iron transport protein transferrin (Bothwell *et al.,* 1979; Crichton, 2009; British Nutrition Foundation, 1998). Nevertheless, this transport compartment plays a central role in iron metabolism and is by far the most dynamic iron compartment in the body, as iron normally turns over at least ten times a day. It obtains dietary iron from the duodenum and recycled iron form the breakdown of effete RBCs. Storage reserves (mostly liver hepatocytes) can also provide iron to the circulation. This is the main source of iron for haemoglobin synthesis in erythroid cell precursors, and also provides iron to most other parts of the body (Crichton, 2009).

Iron absorption and excretion are mutually adjusted with unregulated iron loss due to sweat, dermal turnover, and incidental amounts excreted in urine and biliary secretion (Valerio, 2007; Andrews, 1999). This represents about 1 to 2 mg/day in each direction in the normal subject (Institute of Medicine and Food and Nutrition Board, 2001; Crichton, 2009). Transferrin is the glycoprotein in plasma and extracellular fluids which cycles iron to the bone marrow where it is incorporated into haem to supply the haemoglobin in newly formed red cell precursors (Crichton, 2009). The erythrocytes circulate in the peripheral blood stream until the end of its lifespan (~120 days) after which they are engulfed by the cells of the reticuloendothelial system (RES) located mainly in the liver, spleen and bone marrow. In this recycling process the iron is separated from haem and either stored in ferritin – the main storage protein in the body – or as haemosiderin (Crichton, 2009; MacPhail, 2007; Valerio, 2007). Ferrous iron is released back into circulation via transmembrane protein ferroportin (under the control of hepcidin) where it is

converted to ferric iron by caeruloplasmin (a ferroxidase enzyme) and again picked up by transferrin. It should be noted that most of the iron entering the circulation comes from recycled erythroid cells via the RES and not from iron absorption (ratio about 20:1) (MacPhail, 2007).

2.2.2 Dietary iron absorption

Absorption of dietary iron occurs in the intestinal duodenum (Andrews, 1999). There are two chemical forms of dietary iron – nonhaem and haem (Valerio, 2007). Nonhaem iron is the more prevalent (85% to 90%) in a regular diet than haem iron (10% to 15%), but with haem iron having a higher bioavailability than nonhaem iron, it may contribute to as much as one third to one half of dietary iron absorbed in iron-replete subjects (Anderson *et al.,* 2005; Carpenter and Mahoney, 1992; Beswoda *et al.*, 1983). Absorption of dietary iron is increased when body iron stores are insufficient and will decrease when the body is sufficient (Chua *et al.,* 2007). Absorption is also influenced by the chemical form of the iron present and potentially, some underlying factors of disease such inflammation, hypoxia, dysfunctional erythopoietic activity, alcoholic liver injury, hepatocellular carcinoma and other physiological factors, including predisposing genetic factors (British Nutrition Foundation, 1998; Valerio, 2007). Absorption of haem and nonhaem iron by the enterocyte is demonstrated by figure 1.

2.2.2.1 Absorption of non-haem iron

Uptake of dietary non-haem iron in the intestinal tract starts by reduction of Fe^{3+} (ferric oxidized form) to Fe^{2+} (ferrous form), which increases its solubility (Crichton, 2009; Valerio, 2007). However, more research needs to be done on the factors responsible for this conversion (Valerio, 2007). The first contender is the membrane-bound enzyme duodenal cytochromes b (Dcytb) and is described as the ferric reductase enzyme capable of biochemically reducing Fe^{3+} to Fe^{2+}. Dcytb is also appropriately localized and principally expressed at the brush border of duodenal enterocytes (McKie *et al*., 2002). Secondly, several studies have found evidence for nonenzymatic reduction of ferric to ferrous iron by means of circulating ascorbic acid, citrate and glutathione (Conrad, 1970; Dorey *et al.*, 1993; Han *et al.*, 1995; Thomas and Oates, 2004). Thirdly, an additional factor promoting the absorption of non-haem iron is the acidic environment in the gut lumen, although the exact effects of this environment are not clearly established (National Research Council, 1979; World Health Organization, 1983; Valerio, 2007). The next step of non-haem iron absorption is the transport of iron across the enterocyte apical plasma membrane. This process is facilitated by the ferrous specific divalent metal transporter 1 (DMT1, also known as divalent-citation transporter 1 [DCT1] and natural resistance-associated macrophage protein 2 [Nramp2]), which is a proton membrane protein that uses the acid microclima at the brush-border to provide the H^+ electrochemical gradient to drive transport of Fe^{2+} into the enterocyte (Gunshin *et al.,* 1997; Fleming, *et al*., 1997).

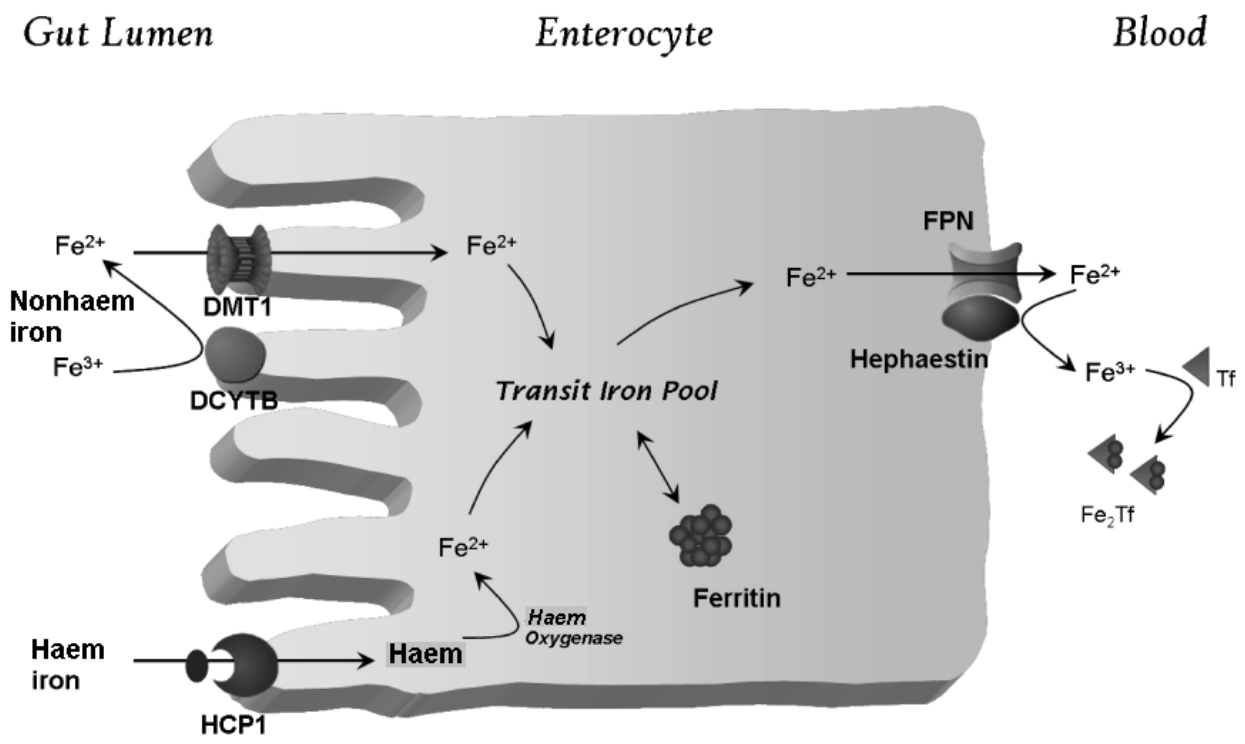

Figure 1. Iron absorption by the enterocyte. Non-haem iron in the intestinal lumen is reduced from Fe^{3+} to Fe^{2+} by the ferric reductase enzyme Dcytb and transported across the brush border via DMT1. Haem iron is taken up via haem carrier protein1 (HCP1), and the iron is released by haem oxygenase. In the enterocyte the iron from both sources enters a common transit pool and is either stored as ferritin or transferred across the basolateral membrane by ferroportin into the bloodstream, where it is oxidized by hephaestin and binds to circulating apotransferrin (adapted from Chuna *et al*., 2007).

2.2.2.2 Absorption of haem iron

Due to its crucial role in haemoglobin and as a prosthetic group of numerous enzymes, haem iron is vital for basic human physiological processes, especially metabolism. In the first step of absorption, haem iron is released from haemoglobin and myoglobin proteins in a process aided by proteolytic enzymes active in the lumen of the stomach and the small intestine (Valerio, 2007). Subsequently, haem is absorbed at the apical surface of enterocytes of the small intestine. Uncertainty exists around the specific transporters for haem on the apical surface of enterocytes (Crichton, 2009; Valerio, 2007), although a haem transport protein, termed haem carrier protein1 (HCP1) has been proposed to be involved in uptake of haem into the enterocytes due to its high expression in duodenal enterocytes (Beard and Han, 2009; Shayegi *et al*., 2005). It is also not clear whether the iron in haem is liberated prior to or following cellular uptake, but if the liberation from haem does occur intracellularly through enzymatic catalysis, the best candidate at this time is the inducible microsomal enzyme haem oxygenase (HO-1). HO-1 can catabolize the iron porphyrin ring to yield ferrous iron (Fe^{2+}), CO_2 and bilirubin (Raffin *et al*., 1974; Valerio, 2007). Once iron has been liberated from the protoporphyrin ring of haem via HO-1, it is proposed that “free-floating” haem-derived iron follows the same pathway of

intestinal iron absorption as non-haem dietary iron (Andrews, 2005). However, since "free-floating" haem is chemically unstable, some biochemical mechanism must exist for its stabilization so that cellular uptake can be readily achieved, but further research is needed in this area (Valerio, 2007). Furthermore, the observation that non-haem iron from the diet is able to suppress the absorption of dietary haem iron, and vice versa, adds support to the suggestion that the two forms of dietary iron may share common pathways for their absorption (Anderson *et al*., 2005; Hallberg and Sovell, 1967).

2.2.3 Cellular Iron Transport

2.2.3.1 Transferrin

After intestinal absorption of iron from haem or nonhaem sources has taken place it moves into the circulating bloodstream for cellular and tissue distribution for use and storage within the body (Chuna *et al*., 2007; Valerio, 2007). Within the circulatory system, the fate of nonhaem nonprotein-bound iron is controlled by its reversible binding to the plasma glycoprotein transferrin (Trf) – the major plasma protein that transports iron between sites of absorption, storage and utilization (Chuna *et al*., 2007; Valerio, 2007). The amount of iron bound to Trf adds up to about 0.1% of total body iron (Andrews, 2000). Due to the ample amounts of circulating Trf, most of the nonhaem iron circulating in the blood plasma is bound to Trf (Valerio, 2007). There is, nevertheless, low concentrations (~600 nM) of free-floating nontransferrin-bound iron available in the plasma of regular individuals (Valk *et al*., 2000). "Free-floating" iron is capable of stimulating the production of highly reactive oxygen species which are capable of inducing oxidative damage from the circulatory bloodstream (Darley-Usmar and Halliwell, 1996). A primary pathway for Trf in the circulatory system is to deliver iron to the developing erythroid precursors and the other major tissues of the body such as the liver, pancreas, heart and muscle. Trf thus serves a physiological role as an iron delivery transport protein for distributing iron throughout the body and in preventing the formation of toxic reactive oxygen species in the bloodstream (Valerio, 2007).

2.2.3.2 Transferrin Receptor 1

A fundamental aspect in the cellular transport of iron mediated by Trf is the existence of Trf receptors, transferrin receptor 1 (TFR1) and transferrin receptor 2 (TFR2), which are located at the cellular surface (Valerio, 2007). TRF1 is expressed in most cells, except for mature erythrocytes (Petrat *et al*., 2002; Rauen *et* al., 2007), with its expression being most prominent in developing erythrocytes, placental syncytiotrophoblasts, and rapidly proliferating cells (Epsztejn, 1999). It is regulated by the iron-responsive element-iron regulatory protein (IRE-IRP) system, and synthesis of TFR1 is promoted by low intracellular iron levels (Kaur *et al.,* 2003; Kaur and Anderson, 2009). TFR1 is involved in a process of receptor-mediated endocytosis, which occurs in almost all cell types (Chuna *et al*., 2007). First, TFR1 binds Trf

with high affinity at the cell surface (Wallander *et al*., 2006). This iron-TFR1 complex is taken into the cell contained within a vesicle where a fall in pH reduces the affinity of Trf and causes the iron to be released from Trf (MacPhail, 2007; Piccinelli and Samuelsson, 1998). This acidification step not only facilitates the release of iron from Trf for cellular storage or utilisation (Valerio, 2007), but also enables proton-coupled iron transport out of the endosomes to the cytoplasm through the action of DMT1 (Fleming *et al*., 1998). The net effect is that Trf without bound iron (apotransferrin) and the TFR1 recycle themselves by returning to the cell surface and separating to participate in another cycle of cellular iron uptake (Andrews, 2000b). This mechanism is known as the Trf cycle and occurs in most cell types including hepatocytes and erythroid cells (Valerio, 2007).

2.2.3.3 Transferrin Receptor 2

TRF2 is predominantly expressed in the liver and is localized mainly on the basolateral membrane domain of hepatocytes (Merle *et al.,* 2007). Low levels of the transcript have also been identified in the spleen, small intestine, heart, kidney and testes (Kawabata *et al.,* 1999; Fleming *et al.,* 2000). Similar to TFR1, the interaction between transferring receptor 2 (TFR2) and Trf is pH-dependent (Chuna *et al*., 2007). Both TFR1 and TFR2 bind diferric Trf but not apotransferrin at pH 7.4, suggesting that TFR2 may also take up Trf-bound iron (TBI) by a receptor-mediated endocytic pathway (Kawabata *et al.,* 2000). The deposition of TFR2-mediated transferrin uptake into multivesicular bodies suggests that TFR2 promotes the intracellular deposition of Trf (Robb, 2004). Kawabata *et al.* (2000) also showed that TFR2 promotes cell growth in iron-depleted conditions. As TFR2 is highly expressed in hepatocytes, it has been proposed that it may be involved in the TFR1-independent uptake of TBI (Kawabata *et al.,* 1999).

2.2.3.4 Divalent Metal Transporter 1

Divalent Metal Transporter 1 (DMT1) protein is expressed in most cells, with highest expression in the duodenum, brain, kidney and reticulocytes (Canonne-Hergaux *et al*., 1999), as well as in the liver (Trinder *et al*., 2000). DMT1 does not exclusively transport iron - it also transports several other divalent metals such as zinc, manganese, cobalt, cadmium, copper, nickel and lead (Gunshin *et al*., 1997). Highest activity of DMT1 occurs at a low pH (Gunshin *et al*., 1997), which is consistent with its role as an iron transporter in endosomes and in the intestine, where the pH is approximately 5 to 6 and DMT1 activity would be optimal (Chuna *et al*., 2007). At the subcellular level, DMT1 has been identified on the plasma membrane and also in recycling endosomes, co-localizing with transferrin (Su *et al.,* 1998), which proposes that it cycles between the endosomal membrane and the plasma membrane and may mediate iron uptake across both membranes.

2.2.3.5 Ferroportin

Ferroportin (FPN) has been identified and characterized as the transport protein that exports iron from the cells. The exact mechanism by which FPN mediates iron export is not clear (Chuna *et al*., 2007). It is confined to the basolateral membrane of the duodenal enterocytes in the villus and exports the iron taken up by the enterocytes from the intestinal lumen (McKie *et al.,* 2000; Abboud & Haile, 2000). FPN is highly expressed in macrophages in the liver, spleen, and bone marrow (Abboud & Haile, 2000), emphasizing the role of these cells in the recycling of iron after haemoglobin degradation from effete erythrocytes (Chuna *et al*., 2007). Over-expression of FPN results in greater iron release and depletion of cellular iron from both the cytosolic compartment and ferritin stores (Abboud & Haile, 2000; McKie *et al.,* 2000).

Translation of FPN is regulated by iron levels through the iron responsive element in the five prime untranslated region (5' UTR) (Liu, *et al.,* 2002; Lymboussaki *et al.*, 2003), but other regulatory mechanisms may also be involved, since both FPN, mRNA and protein expression are up-regulated in the duodenum but repressed in the liver in iron-deficient mice (Abboud & Haile, 2000).

2.2.3.6 Ceruloplasmin

Ninety-five percent of the copper present in the plasma is contained in the serum ferroxidase ceruloplasmin (CP; Hellman & Gitlin, 2002). CP is considered to have a role in iron homeostasis due to the observation that patients with aceruloplasminemia present with hepatic iron overload (Miyajima *et al.,* 1987). CP acts in combination with FPN to mediate iron release from hepatic cells. The released iron is oxidised to the ferric and picked up by circulating Trf (Chuna *et al*., 2007).

2.2.4 Regulation of systemic iron balance

Systemic iron balance involves several mechanisms including regulation at the level of iron absorption from the intestinal tract, regulation of iron recycling form macrophages and, finally, mobilisation of hepatic iron stores (Chuna *et al*., 2007; Crichton, 2009). The principal factors which are known to modulate the mechanism of systemic iron homeostasis are described below:

1) Iron requirements of the erythroid system
   This is described as the erythropoietic regulator and represents the iron requirements of the erythroid system for haemoglobin synthesis (Crichton, 2009). It is the regulatory pathway by which iron absorption is stimulated when a massive loss of iron occurs through haemorrhage or ineffective erythropoesis, where immature erythrocytes are destroyed in the

bone marrow (1958; Andrews, 2000b). An iron-deficient individual may absorb over 20mg of iron when there is a high demand for erythroid iron (Finch, 1994).

2) Total body iron store

   Individuals with a high iron status will absorb less iron consumed than an individual with poor iron status, and an individual with lower iron status will absorb more iron (Beard and Han, 2009).

3) Amount and form of iron compounds ingested (Crichton, 2009).

4) Inflammation

   Inflammatory processes such as microbial infections will induce a withdrawal of iron from the circulation in order to starve the invading microorganisms of the iron needed for their proliferation, and consequently decrease the risk of infection (Crichton, 2009).

Krause *et al.* (2000) and Park *et al.* (2001) established that the regulation of systemic iron balance is to a large extent controlled by hepcidin, an antimicrobial peptide hormone found in the circulation and produced essentially in the liver. Hepcidin expression is known to be influenced by each of the factors which affect systemic iron balance. When serum iron increases, hepcidin levels rise accordingly. Alternatively, levels are decreased in response to hypoxia and increased demand for erythropoiesis or ID. Hepcidin acts by blocking iron export from intestinal epithelial cells and from tissue macrophages, which suggests involvement with FPN, the only known iron export protein (Crichton *et al*., 2009). Nemeth *et al.* (2004) has demonstrated that hepcidin binds directly to ferroportin, provoking its internalization and degradation within the lysosomal compartment of the cell. Ferroportin can thus be seen as the membrane receptor for hepcidin (Crichton *et al*., 2009).

The regulation of hepatic hepcidin expression, and therefore of circulating hepcidin levels, must reflect iron requirements for erythropoiesis, which will be increased in conditions of anoxia, as well as by the level of iron stores. Furthermore, it is also recognized that hepcidin levels are regulated by inflammatory stimuli (Crichton *et al*., 2009).

2.3 Iron deficiency

Nutritional ID develops when physiological requirement cannot be met by iron absorption from the diet (Zimmermann & Hurrell, 2007). Smuts *et al*. (2005) demonstrates that onset of iron deficiency is characterized by sequential changes in the amount of storage iron in the various compartments of the body. First, iron becomes depleted, but there is still enough iron to meet the needs of red blood cell production. Next, circulating iron starts to drop, and red blood cell production becomes compromised (iron-deficient eythropoiesis). Finally, iron stores are exhausted and circulating iron is very low, red blood cell production drops drastically and anaemia develops.

2.3.1 Causes of ID

a) Iron intake/content of the diet

The risk for ID is highest when iron requirements are larger than energy needs (Zimmermann & Hurrell, 2007). Poor diet/inadequate intake of iron and/or the type of iron (haem or nonhaem) consumed in the diet can cause iron deficiency (Crichton, 2009). Nonhaem iron, contained in cereals, pulses, fruits and vegetables comprises the major and often exclusive source of iron in developing countries (WHO, 1988).

b) Low bioavailability

Adequate iron in the body not only depends on the iron content of the diet, but also, and to a much greater extent, on the bioavailability of the iron from the diet. Iron bioavailability can be described as the amount of ingested iron which is absorbed and utilised for metabolic functions (Hurrell, 1997a).

c) Parasitic infestations

In many developing countries ID often occurs in infants and young children and is affected by increased blood loss form gastrointestinal parasites aggravates dietary deficiencies (Zimmermann & Hurrell, 2007). Hookworms cause chronic intestinal blood loss by attaching themselves to the mucosa of the upper small intestine, ingesting blood tissue, and altering their feeding site every 4-6 hours. Blood loss occurs from both ingestion by the worm and through bleeding from the damaged mucosa (Staltzfus *et al*., 1997).

2.3.2 Consequences of ID

Iron deficiency adversely effects metabolic processes such as electron transport, catecholamine metabolism, DNA synthesis and several enzyme systems (Baynes and Bothwell, 1990). The final stage of iron deficiency is iron deficiency anaemia (IDA), a condition in which there is not enough red blood cells to transport oxygen to the body's tissues, and which is characterized by low haemoglobin concentrations. Severe IDA can cause increased risk of child and maternal mortality (Brabin *et al*., 2001). ID and IDA have also been reported by many studies to have an effect on mental and motor development and cognitive and behavioural function in children; these effects have been reviewed in Sachdev *et al*. (2005) and McCann & Ames (2007) and will be discussed in section 2.6.

2.4 Omega-3 fatty acid metabolism and regulation

2.4.1 An overview of omega-3 fatty acids in humans

Omega-3 fatty acids are part of the polyunsaturated fatty acid (PUFA) family, which has effects on many biological systems ranging from immune reactions, to blood platelets, endothelial cell function and growth regulation of several different cell types as well as being related to numerous health outcomes including cardiovascular disease morbidity and mortality, mental

health and psychiatric disorders (Glasier *et al.,* 2011). The n-3 FA with 18 carbon atoms (18:3n-3; alpha-linolenic acid [ALA]) is found primarily in vegetable foods, whereas very long-chain n-3 FAs (20 and 22 carbon atoms) are mainly found in marine products. In the human diet, the majority of very long-chain n-3 FAs are found in fatty fish such as herring, mackerel and salmon, or fatty fish products such as fish oil and cod liver oil (Drevon *et al*., 1995).

Fatty acids are categorized according to their length and degree of unsaturation of the hydrocarbon chain. Saturated fatty acids do not have any double bonds in the hydrocarbon chain, whereas unsaturated fatty acids contain at least one double bond. PUFA describes fatty acids with at least two double bonds, and PUFA with 20 or more carbon atoms are classified as long-chain polyunsaturated fatty acids (LC-PUFA). Three PUFA families may be distinguished: the omega-9, omega-6 and omega-3 series, which are so classified according to the location of the last double bond, most distant from the carboxyl group (the alpha carbon atom). The parent fatty acids of these three families are oleic acid (omega-9), linoleic acid (LA; omega-6) and ALA (omega-3). These parent unsaturated fatty acids can be converted into fatty acids with longer chain length and higher degree of unsaturation by a sequence of alternating desaturation and chain elongation steps. Existing knowledge is that the three metabolic pathways share the same enzymes for desaturation and elongation. Two of these parent PUFAs, LA and ALA, are essential nutrients that cannot be synthesized endogenously in humans and must therefore be provided by the diet (Glaser *et al*., 2011).

Many of the physiological effects of PUFA are considered to be principally mediated by tissue concentrations of LC-PUFAs, in particular arachidonic acid (AA; 20:4n-6), EPA (20:5n-3) and DHA (22:6n-3; Tang *et al.,* 1993). These LC-PUFAs can be directly supplied by the diet, but can also be synthesized in human metabolism starting from the precursor essential fatty acids, LA and ALA (which are also, as previously mentioned, supplied by the diet), via consecutive desaturation and chain elongation (Sprecher *et al*., 1995). Due to this, it is noted that the essentiality of ALA may lie primarily in it being a substrate for the synthesis of the long-chain, more unsaturated PUFAs EPA and DHA (Burge, 2006).

2.4.2 Absorption of ALA

Few information exists regarding the efficiency of absorption of ALA by the human gut, but findings have suggested that absorption of ALA across the gut is efficient in humans (>96%). Adipose tissue accounts for approximately 15% of body mass in men and 23% of body mass in women, and therefore incorporation of ALA into this storage pool represents a potentially important route of disposal of dietary ALA and a reserve pool which is available for mobilization during periods of increased demands (Burge, 2006). ALA altogether accounts for about 0.7% of total fatty acid in neutral lipids in adipose tissue in men and women (Tang *et al.,* 1993).

Exchange of ALA between the blood and adipose tissue compartments has not been characterized in detail in humans in vivo. Burge *et al.* (2002) found a rapid release of [U-^{13}C] ALA into the plasma non-esterified fatty acid (NEFA) pool (2h with a peak at 6h after ingestion) in male subjects, which would tend to facilitate supply of ALA to the liver (Burge, 2006). Also, McCoy *et al.* (2004) estimated that 15-81% of the administered dose of [^{13}C] ALA was present in adipose tissue at 6h following the ingestion of the tracer.

2.4.3 Omega-3 FA metabolism

The desaturation/elongation pathway supplies ALA metabolites to other tissues, which makes the activity of the pathway most important. All reactions occur in the endoplasmic reticulum, except for the final reaction, which results in the formation DHA (Burge, 2006). The pathway which n-3 FAs follow is described by Glasier *et al*. (2011) and Burge (2006) (figure 2) and starts with conversion of ALA (18:3n-3) is to 18:4n-3 by delta-6 desaturase (D6D). This is the rate-limiting reaction of the pathway after which 18:4n-3 can be further elongated to eicosatetraenoic acid (ETA, 20:4n-3) by delta-5 desaturase (D5D). Alternatively, ALA can be elongated to eicosatrienoic acid (ETE, 20:3n-3), which can be further desaturated to ETA by delta-8 desaturase (D8D) to ETA (Park *et al*. 2009). EPA (20:5n-3), which is the delta-5 desaturated product of ETA, is an important n-3 metabolite and serves as precursor of biologically potent eicosanoids. Finally, the major downstream product of the omega-3 family is DHA (22:6n-3). The conversion of EPA to DHA has been a matter of controversy. There is no evidence for the formerly assumed role of a delta-4 desaturation in conversion of EPA to DHA in mammals (Glasier *et al*., 2011). DHA synthesis by two chain elongations of EPA, followed by a delta-6 desaturation and a partial β-oxidation is a possible pathway (Sprencher, 1999). The low activity of D6D and the compartmental translocation to peroxisomes may explain the low conversion rate of omega-3 docosapentaenoic acid (DPA; 22:5n-3) to DHA in humans (Burge, 2004). There are also indicators of a higher conversion of

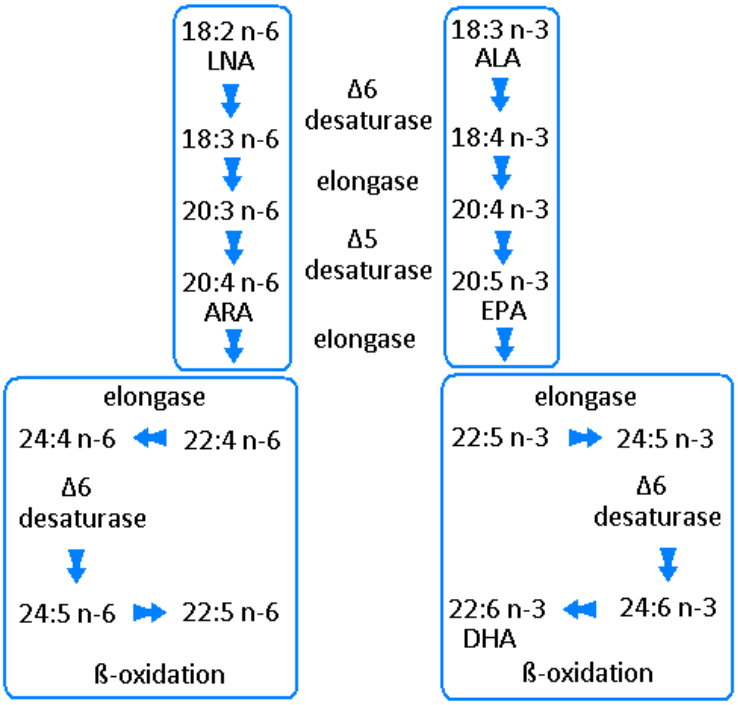

Figure 2. The general pathway for conversion of ALA to longer-chain PUFA. DPA, docosapentaenoic acid. Adapted from Glasier *et al*., 2011).

ALA to EPA and DHA in women than in men, which is presumed to be due to oestrogen effects (Burge, 2006).

2.4.4 Regulation of PUFA metabolism

Enzymes D5D and D6D are the key regulators of LC-PUFA synthesis (Nakamura & Nara, 2004). These are both membrane-bound desaturase enzymes produced in a majority of human tissues, with the highest activities found in liver. Significant activities of these enzymes are also found in adipose tissue, brain, heart and lung, whereas fewer amounts have been shown in placenta, skeletal muscle, kidney, pancreas and pregnant uterus. These two desaturates are proposed to be rate limiting in the metabolic pathways of all three PUFA series (Glasier *et al*., 2011).

2.5 The role of fatty acids in brain development

Lipids have essential structural and functional roles in the central nervous system. Phospholipids make up a large part of neuronal membranes, with each containing two fatty acids. The exact fatty acid composition of the membrane can affect the tertiary and quaternary structures of membrane-bound receptors and associated neurotransmitter functioning. Furthermore, most second-messenger systems depend on lipids such as free fatty acids, diacylglycerols, prostaglandins, leukotrienes, and hydroxyl-fatty acids. Hence, fatty acids can extensively influence key aspects of cell signalling (Richardson & Puri, 2000).

There are four particularly important fatty acids within the brain: AA and dihomogamma linolenic acid (DGLA) from the omega-6 (n-6) fatty acid series, and EPA and DHA from the n-3 series. AA and DHA have a structural role in neuronal membranes, making up 20% of the dry mass of the brain. EPA and DLGA have a smaller structural role, but are also vital for normal brain function. These compounds are of immense importance as they perform numerous regulatory functions in the brain and throughout the rest of the body (Richardson and Puri, 2000).

Essential fatty acids can have an impact on many aspects of brain development, including neural migration, axonal and dendritic growth, and the creation, remodelling and pruning of synaptic connections (Crawford, 1992). DHA appears to play a special role in highly active sites such as synapses and photoreceptors, and deficiencies have particularly been linked to visual and cognitive defects (Neuringer *et al*., 1994, 1986).

2.6 Iron, n-3 FA, spontaneous physical activity and ADHD-related behaviour: a review of the evidence

It is well-known that a healthy level of physical activity is beneficial to health and quality of life – producing benefits such as preventing and reducing overweight and obesity as well as other

short- and long-term health gains (Tucker *et al*., 2008). Conversely, biological factors also have an influence on daily physical activity – such as iron status, which has been shown to have an effect on fatigue, physical work capacity, physical performance and general overall activity (Table 1 and 2).

ADHD involves clinically diverse dysfunctions of sustained attention, with early onset overactivity and impulsiveness. It is prevalent, and is becoming more common, in children world-wide, possibly involving up to 10% of the school-age population (Vancassel *et al*., 2007). Children with ADHD have problems paying attention and completing tasks; they fidget and squirm, are impulsive, hyperactive and interrupt others, causing impaired function at home and school (Richardson & Puri, 2000; Swanson *et al*., 1998). The aetiology of ADHD is generally recognized to be multifactorial, involving both biological and environmental determinants, and with increasing attention being paid to the clinical heterogeneity of the disorder both iron and n-3 FAs have been studied in relation to ADHD (Konofal *et al.,* 2005, 2008; Richardson & Puri, 2000; Schuchardt *et al*., 2010).

Several studies concerning the cognitive and behavioural effects of ID (mostly IDA) and iron supplementation in humans have been conducted and reviewed (McCann & Ames, 2007; Sachdev *et al*., 2005; Grantham-McGregor & Ani, 2001). However, there are few existing studies that have assessed the effects of ID or IDA, as well as of iron supplementation on behaviour and activity and in humans. Most of these studies were conducted in animals (rats, mice and monkeys) and most are very old. Many studies also tend to be more focussed on endurance capacity, physical/work performance, physical work capacity and cognitive outcomes than on general physical activity and behavioural outcomes specifically. Also, not many studies have investigated the effects of ID without anaemia. Table 1 summarizes animal studies on the effects of ID/IDA on general physical activity, ADHD and other behavioural outcomes, whereas Table 2 reviews the human studies.

Animal studies summarized in Table 1 tested voluntary activity, daily activity, locomotion, and/or ADHD-related behaviours among other outcomes. All studies demonstrated association between ID and/or IDA and decreased activity, either by significant change/difference or trend. Earlier studies tend to have found the effects on activity to be related to Hb rather than iron status (Edgerton *et al*., 1972, 1977; Koziol *et al*., 1982). Edgerton *et al.* (1972; 1977) and other early studies have all shown positive effects, demonstrating that iron administration or correcting of Hb levels could increase activity and improve work performance (Glover & Jacobs, 1972; Finch *et al*., 1976). In contrast, latter studies have found irreversible changes in behaviours of iron deficient animals even after iron treatment (Felt & Lozoff, 1996; Kwik-Uribe *et al*., 1999, 2000; Piñero *et al*., 2001).

Table 2 shows the human studies on the effects of ID and IDA on activity and other behavioural outcomes such as ADHD. There have not been many human studies conducted measuring activity outcomes in relation to ID. In fact, only three studies were found: one measuring work capacity and level of physical activity in a subgroup of female tea plantation workers (Edgerton *et al*., 1979), one measuring spontaneous motor activity in infants with IDA using an actigraph (Angulo-Kinzler *et al*., 2002) and one measuring habitual activity of school children using a frequency questionnaire for physical activity (Wang *et al*., 2009). Edgerton *et al.* (1979) found the level of physical activity of anaemic female tea plantation workers in their everyday environment to be greater in iron-treated subjects. Angulo-Kinzler *et al*. (2002) found that infants with IDA generally spent less time in an alert-active state, and also demonstrated significantly less spontaneous activity when awake. The study also found that IDA was associated with reduced motor activity in infants even after iron treatment. Wang *et al*. (2009) determined that severe ID (IDA) impaired the habitual physical activity of school children. In the same study, aerobic activity and energy expenditure at leisure were significantly lower in the severe ID group than in the marginal ID and iron adequate groups.

The exact mechanisms through which iron affects activity has not been fully established. It has been proposed that IDA affects physical capacity by reducing the availability of oxygen from tissues, which, in turn, reduces maximal work capacity, endurance, productivity, energy expenditure and voluntary activity (Haas & Brownlie, 2001; Li *et al*., 1994; Gardner *et al*., 1977; Edgerton *et al*., 1979). In iron depletion without anaemia, the Hb value is greater than a specified cut-off point for anaemia and the oxygen carrying capacity of blood is not expected to be compromised (Wang *et al*., 2009). However, impairment of the ability to utilize oxygen may still exist as animal studies have shown that ID without anaemia can reduce work capacity and spontaneous physical activity (Beard *et al*., 2002; Koziol *et al*., 1982; Hunt *et al*., 1994; Finch *et al*., 1976).

Some behavioural effects have been demonstrated by animal and human studies involving ID. Behavioural changes in animals included poorer performance, attenuated startle responsiveness, decreased stereotypical behaviour, slower/lower rate of habituation to a novel environment, decreased exploratory behaviour, delays in gross and fine motor development and greater emotionality in association with ID (Felt & Lozoff, 1996; Kwik-Uribe *et al.*, 2000; Piñero *et al*., 2001; Beard *et al.*, 2002; Gulob *et al.*, 2005). It was also found that ID during early life resulted in persistent biochemical and behavioural differences in test animals even after iron treatment (Felt & Lozoff, 1996; Kwik-Uribe *et al.*, 2000; Piñero *et al*., 2001). Behavioural effects of ID have also been demonstrated in human studies. In two case-control studies (Konofal *et al.,* 2004; Juneja *et al.*, 2010) it was found that 84% and 92% of children with ADHD had low SF

(<30 ng/mL) compared to 18% and 0% of controls, respectively. Although SF was not correlated with all subscales of the scale used to measure ADHD during group comparisons, it was shown to be correlated with ADHD symptoms severity. In an intervention trial using the Conners' Rating Scales (CRS), ADHD Rating Scale and Clinical Global Impression Scales, Konofal *et al.* (2007) concluded that iron supplementation appeared to improve ADHD symptoms in children with low SF levels and suggested a need for future investigations with larger controlled trials.

A few observational studies have assessed the possible association between plasma and red blood cell concentration of fatty acids and symptoms of ADHD and other behavioural outcomes. Effects of supplementation with fatty acids (especially PUFAs) on these outcomes have also been assessed in several studies. Tables 3 and 4 below review these studies.

A number of studies have shown that subjects/test animals with ADHD or behavioural problems, including hyperactivity, had lower plasma/red blood cell levels of PUFAs, and especially n-3 FAs, than controls (Mitchell *et al*., 1987; Stevens *et al*., 1995; Antalis *et al*., 2006; Vancassel *et al*. 2007; Ku-Hang *et al*., 2009). Notably, the aetiologies of ADHD and related disorders are highly complex and multifactorial, involving biological, genetic and environmental factors (Richardson, 2004; Vancassel *et al*., 2007). In addition, LC-PUFAs have such profound and extensive influences on brain development and function that their potential roles in these conditions are countless (Richardson, 2004). There are currently, however, certain theories of possible mechanisms involved in the link between n-3 FAs and ADHD and behavioural problems.

Customary pharmacological treatment for ADHD involves stimulant medications that increase the availability of dopamine, as reflected in all current aetiological theories of this condition (Richardson, 2006). As shown by animal studies, chronic n-3 FA deficiencies can reduce dopamine and its binding to D2 receptors in the frontal cortex and other brain regions, which is associated with attentional and behavioural dysfunctions comparable to those involved in ADHD. Fatty acids and their metabolism can also influence the functioning of other major neurotransmitter systems implicated in ADHD and related psychiatric disorders. The effects of fatty acids on neural signalling can be mediated by means of a large variety of different mechanisms, direct and indirect, as LC-PUFAs not only affect membrane structure and function, but also help to regulate blood flow, endocrine and immune functions as well as modulate ion channels, neurotransmitter uptake, synaptic transmission, apoptosis and gene expression, among other biological processes (Richardson, 2006).

Clinical signs of essential fatty acid deficiency such as polydypsia, frequent urination, dry skin and hair and zinc deficiency are also often associated with ADHD (Vancassel *et al*., 2007). Due to the acceptance that both genetic and environmental factors play a role in ADHD, it should be considered that many other factors – both intrinsic and extrinsic – could influence an individual's fatty acid status. Burgess *et al*. (2000) applied this argument to a subpopulation of children with ADHD who exhibited frequent symptoms of essential fatty acid deficiency and low LC-PUFA status and considered the following as potential explanations for low levels of plasma LC-PUFAs found in the subjects:

1) Fatty acid intake

   Populations in developing countries, such as the population in this study, are at risk for poor quality of the total diet, which is probably more of a problem than are specific nutrient deficiencies. A diet low in energy, protein, or both would probably also be low in specific LC-PUFAs, such as DHA and EPA. N-6 FAs are much more plentiful than n-3 FAs in modern diets, and in the past century dramatic increases have been seen in the n-6 to n-3 FA ratio (Richardson, 2004).

2) Inefficient conversion of essential fatty acids to LC-PUFAs

   Another possible cause for the low LC-PUFA status of subjects may be impaired conversion of the fatty acid precursors LA and ALA to their longer and more highly unsaturated products such as DHA and EPA (Richardson and Puri, 2000; Burgess *et al*., 2000). *In vivo* studies have indicated that the conversion process is not very efficient in, but it can also be influenced by diet and lifestyle as well as constitutional factors (Richardson, 2004).

3) Enhanced cellular metabolism of LC-PUFAs

   Depletion of many LC-PUFAs in the rat liver has been demonstrated to be caused by enhanced nonenzymatic oxidation due to impaired cellular defence systems. Therefore, enhanced cellular metabolism of LC-PUFAs through nonenzymatic mechanisms is another possible explanation for lower blood LC-PUFA compositions (Brugess *et al.,* 2000).

Combinations of LC-PUFAs and n-3 FA treatment alone have been found to improve ADHD symptoms in children and adolescents with ADHD (Sinn and Bryan, 2007; Bélanger *et al*., 2009; Johnson *et al*., 2009). This finding deserves further investigation in larger trails. No studies could be found investigating the effects of n-3 FAs on spontaneous physical activity in humans.

Overall, there is evidence for both iron and n-3 FA status to have an impact on activity and behaviour in children. These findings have consistently been observed, but strong evidence is lacking for whether supplementation of these nutrients has considerable effects on the affected behavioural outcomes. It would therefore be of interest to assess effects of supplementation with iron and n-3 FAs in different populations and larger trials.

2.7 Accelerometers and Conners' Rating Scale as assessment tools

Accelerometers have become popular tools for physical activity research, being used to assess both exercise and free-living physical activity. Validity of multiple-axis accelerometers such as the Actical (omni-directional) accelerometer has been evaluated in several studies and reported to provide information comparable to each other as well as to observed physical activity and recorded heart rate (summarised in Trost *et al*., 2005). Accelerometers function by integrating a filtered digitalized acceleration signal over a user-specified time interval, generally referred to as and epoch (Trost *et al*., 2005). An epoch length of one minute is commonly used for free-living activity. Studies that have used accelerometers to assess activity and behaviour of children include Puyau *et al*., 2002, Janz *et al*., 1995 and Nilsson *et al*., 2002.

The CRS is a commonly used rating scale used for assessment of classroom behaviour problems related to ADHD (Epstein, 1998) and consists of both parent and teacher questionnaires of child behaviour (CTRS and CPRS). The scale is made up of subscales which each represent different areas of ADHD-related behaviours such as hyperactivity and cognitive problems. This is a commonly used research tool and has been used for identifying children at risk for ADHD in several studies including some involving iron and n-3 FAs (Sinn and Bryan, 2007; Juneja *et al.,* 2010; Konofal *et al*., 2007, 2004). Using the CRS and accelerometers in combination could prove to be useful in assessment of child behaviour involving activity-related assessments in a behavioural context.

Table 1. Animal studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes

Investigators	Study design	Test animals	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Edgerton *et al*., 1972	Controlled, parallel intervention involving four projects	Project 1) 60 male, 32-day-old rats Project 2) 60 male 18-day-old rats Project 3) 27 male 18-day-old rats Project 4) 24 male 18-day-old rats All rats were fed an iron-deficient diet and were anaemic by the beginning of intervention	18 weeks	Different regimes of iron administration with certain rats receiving iron repletion at different stages of the study.	Endurance capacity by a forced exhaustive run and voluntary activity on an activity wheel (weekly)	Motor-driven treadmill and activity wheel	- Anaemia caused a decrement in forced exercise performance while repletion of normal iron levels in anaemic rats improved their capacity to tolerate sustained exercise tests. - Anaemic rats maintained a lower level of voluntary activity than their controls; the difference dissipated upon repletion of normal Hb levels; difference in activity was more evident during the night - Anaemic groups showed an increase in voluntary activity whenever Hb levels were permitted to rise, and activity quickly decreased with corresponding reduction of Hb following phenylhydrazine injection - A close relationship was demonstrated between Hb level and performance by the quick recovery of performance upon repletion of normal Hb levels. - Conclusion: even moderate levels of anaemia induced by an iron-deficient diet, or hemolytically, produce decrements in voluntary and forced running which are alleviated upon iron repletion
Glover and Jacobs, 1972	Controlled pilot study	18 male Wistar rats: Group 1) normal iron status Group 2) IDA Group 3) Mild IDA	Diet treatment since weaning; 7 days of measurement	Group 1) normal diet since weaning; ferric ammonium sulphate in drinking water on days 3 and 4 (28 mg/100ml) Group 2) Iron-free diet since weaning; ferric ammonium sulphate in drinking water on days 4 and 5 (28 mg/100ml) Group 3) iron-deficient diet since weaning, normal diet 10 days before the beginning of the study; ferric ammonium sulphate in drinking water on days 3 to 6 (28 mg/100ml).	Total daily activity and the diurnal rhythm	Animex activity meter	- Considerable reduction in the total number of movements in both the mildly and severely iron-deficient rats - In severely iron-deficient rats there was a pronounced increase in activity after iron was given but a decline towards previous levels in the two days after iron was withheld - In mildly anaemic rats iron administration was continued and there was a smaller but sustained increase in activity - Conclusion: a pronounced change was present in behaviour in iron-deficient rats consisting partly in a reduction of total activity and partly in an alteration of diurnal rhythm; the cause is unknown but seems to be rapidly corrected by the administration of iron

Table 1. Animal studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes (continued...)

Investigators	Study design	Test animals	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Edgerton *et al*., 1976	Controlled, parallel study	20 anaemic and control adult rats	17 weeks of diet treatment and activity monitoring	Experimental rats were fed an iron-deficient diet and made anaemic by periodic bleeding and replacement of the volume of blood removed with serum plus 0.95 saline; control rats were fed the same diet but were supplemented with 1.68 Fe/kg of diet.	Weekly voluntary activity by revolutions on an activity wheel	Blood sampling; activity wheel	- Voluntary activity changed within a few days of when Hb was elevated or lowered (voluntary activity changed proportionally) beyond a critical point of approximately 11.0 g Hb/100 ml - Conclusion: Hb may be more critical than iron stores in causing the depressed voluntary activity
Finch *et al*., 1976	3 different studies containing 4-5 groups with 6 -15 animals per group	4 week old male rats	4 weeks (outcomes were measured 1 – 4 weeks after diet treatment)	Control animals were given a meal containing 382 mg of iron/kg; treatment groups received 8 mg of iron/kg diet, of which some of the animals received intraperitoneal injections of 5 mg of iron dextran.	Work performance on a treadmill	Treadmill	- At a haemoglobin compatible with normal work performance, iron-deficient animals showed a marked impairment of running ability compared to controls - Iron therapy corrected the disability within four days
Williamson & Ng, 1979	Observational study	Adult hooded rats	2, 8 and 12 weeks	Rats were placed on an iron-deficient diet for 2, 8 or 12 weeks	Memory and activity levels	A single taste-aversion task	- Memory was below normal for all three groups of rats and did not significantly differ from each other - Activity levels decreased with increasing ID
Koziol *et al*., 1982	Controlled, observational study consisting of severely and moderately anaemic and control rats	24 two month old rats	1 year	Anaemia was induced in experimental rats by blood removal, and they were fed a commercial diet containing 6 mg of iron/kg of diet; the anaemic group received the same diet plus 1.68g of ferrous sulphate.	Physical work capacity by exhaustive runs on a treadmill	Treadmill	- Physical work capacity was significantly lower in the moderate and severely iron deficient rats over the time coarse studied - Average mean run time to exhaustion was 64% and 18% of control values in the moderately and severely anaemic rats, respectively - Conclusion: the consistently decreased work tolerance with moderate IDA seems more closely related to the Hb concentration, while the greater decrease in work tolerance associated with the even lower Hb of severe IDA is pounded by the additive effect of the ID on skeletal muscle haemproteins

Table 1. Animal studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes (continued...)

Investigators	Study design	Test animals	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Hunt *et al*., 1994	Controlled, parallel intervention consisting of 4 groups	24 growing male rats, 1 week past weaning	60 days	Animals were placed on diets containing 4.6 (deficient), 19.9 (marginal) and 108.4 mg (adequate) Fe/kg of diet or a control diet.	Differentiation between the effects of iron deficiency and marginal iron nutriture; to determine whether specific aspects of behaviour were especially sensitive to iron status	Digiscan animal activity monitor; infrared beams	- ID resulted in less time and frequency of horizontal, vertical and stereotypic movements; less distance moved and less frequent rotations - Despite greater speed of movements in iron-deficient rats, they moved fewer times, for less total distance and spent less time in movement and more time at rest than iron-supplemented rats - Diurnal activity was not reversed by iron status
Felt and Lozoff, 1996	Controlled, parallel intervention consisting of 5 groups	6 – 10 rat litters per experimental group	16 days	Four periods of IDA were instituted during gestation and lactation in adolescent rat mothers by limiting iron available in the diet for brief periods to produce early and late ID and early and late lactation groups and controls.	Determination whether treatment of IDA with iron before weaning can normalize measures of brain iron concentration and behaviour in rats; exploration of the effects of the time of IDA and iron treatment during development	Activity wheel; swim test (Morris maze)	- The IDA of dams resulted in significantly lower pup brain iron concentrations at 3 months of age despite iron treatment of dams as early as mid-gestation - Dam IDA during lactation lowered pup brain iron concentration significantly more than IDA during gestation (21% lower) - All IDA groups had significantly poorer performance and lower activity compared with controls - Conclusion: results of the study raise the concern that iron sufficiency throughout the course of brain development is crucial to the achievement of normal brain iron concentration and behaviour in rats
Kwik-Uribe *et al*., 1999	Controlled, parallel intervention	20 mice	50 days	Dams were put on iron deficient diet for 8 weeks before mating; pups were fed the same diet as their dams: marginal diet – 12.5 µg Fe/g, control diet – 75 µg Fe/g.	Testing if the hypothesis that marginal iron diets fed during gestation and through a period of postnatal development would lead to persistent changes in behaviour later	Neurobehavioral test battery (grip strength); computer-controlled sound generation (startle testing); tissue sampling	- Marginal iron animals exhibited a 20-55% reduction in grip strength - Hematocrits were unaffected by dietary iron reductions - oxidative stress was indicated by markers in the cerebellum of marginal iron animals - Conclusions: chronic marginal ID during critical periods of growth can result in functional changes in motor development even in the absence of IDA; alterations in mineral status and oxidative stress may be mechanisms contributing to the observed changes

Table 1. Animal studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes (continued...)

Investigators	Study design	Test animals	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Kwik-Uribe *et al*., 2000	Controlled, parallel intervention	29 mice	50 days	Dams were fed either a control (75 µg Fe/g diet) or marginal (14 µg Fe/g diet) for 9 weeks before mating. Offspring of marginal ID rats were fed the same diet as their dams from postnatal day 21 throughout the study, whereas offspring of control dams received the control diet.	Investigation of the behavioural and cognitive outcomes associated with chronic marginal iron intakes during early development	Neurobehavioral test battery (grip strength); computer-controlled sound generation (startle testing); tissue sampling; Morris water maze (cognitive test)	- Marginal ID mice consistently demonstrated significantly lower grip strength, which was independent of differences in body weight - Marginal ID males demonstrated attenuated startle responsiveness, as well as altered performance in the Morris water maze; these differences in performance were found in association with lower brain iron concentrations - Postnatal iron supplementation did not reverse all of the disturbances because differences in brain iron concentrations and maze learning persisted - Conclusion: the study demonstrates that chronic marginal iron intakes during early development can result in persistent biochemical and behavioral changes in mice
Piñero *et al*., 2001	Controlled intervention with repletion period for some animals	8-15 rats per group	35 days	At approximately day 10 of pregnancy dams were placed either on an ID or iron supplemented diet. Pups were correspondingly made either made ID or supplemented with iron on postnatal day (PND) 10-21, PND 21-35 and PND 10-35. Some ID rats were iron depleted between PND 21-35.	Evaluation of the effects of dietary ID and excess iron on physical activity in rats	Digiscan Animal Activity Monitor	- Iron-deficient and iron-supplemented rats showed decreased activity and stereotypic behaviour - Recovery from ID did not normalize these functional variables, showing that deleterious effects of early ID persist despite subsequent adequate treatment - Conclusion: ID in early life leads to irreversible behavioural changes
Beard *et al*., 2002	Controlled observational study	20 iron-deficient and control male and female weanling rats	4 weeks of dietary treatment	Two previously prescribed diets: 35 mg Fe per kg diet (control); 3 mg Fe per kg diet (iron-deficient). Free access was allowed to food and water.	Association between brain iron measures of dopamine function, and behavioural measures of activity and reactivity	Digiscan Animal Activity Monitor; blood collection	- Iron-deficient rats evinced significantly decreased locomotion than controls - Rate of habituation in locomotor activity to a novel environment was significantly slowed in iron-deficient rats - ID resulted in a non-significant trend toward a decrease in exploratory behaviour - ID reduced repetitive movements in males and females

Table 1. Animal studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes (continued...)

Investigators	Study design	Test animals	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Golub *et al*., 2005	Observational study	38 pre- or postnatally iron deprived and control infant rhesus monkeys	Observation of the first 4 months of life	Infants were offspring of dams fed iron deprived (10 µg Fe/g) or iron adequate (100 µg Fe/g) diets. Postnatally deprived infants were fed 12 µg Fe/g while prenatally deprived and control infants were fed 93 µg Fe/g.	Differences in anthropometry, iron status, motor observations; cognition; emotionality; behaviour; activity	Blood drawing; anthropometry; Fagan Infant Intelligence Test (visual recognition memory); fine motor ability tests; other behavioural tests; acitimeters (activity/diurnal rhythms)	- Neither pre- or postnatal iron deprivation led to significant delays in growth, gross, or fine motor development -Prenatally deprived infants demonstrated a 20% reduced spontaneous activity level, lower inhibitory response to novel environments and more changes from one behaviour to another in weekly observation sessions - Postnatally deprived infants demonstrated poorer performance of an object concept task and greater emotionality relative to controls - no group differences were found in duration of wake and sleep periods - Data suggested prolonged periods of inactivity and very low average activity counts in several of the prenatally deprived monkeys in the home cage
Felt *et al*., 2006	Controlled intervention study	45 iron deficient and control rats	120 days	Pregnant dams were assigned to an EID or control group. Control dams and their pups were fed a 40 mg/kg Fe diet; EID group dams were fed a 4mg/kg Fe diet and pups were fed a 10 mg/kg diet until PND20 when all pups received the 40 mg/kg Fe diet	Haematology and behavioural assessments: sensorimotor function, general activity, response to novelty, spatial alteration, spatial water maize performance	Several behavioural tests; Morris water maze; brain tissue assessment	- Early ID rats had persisting sensorimotor deficits, were more hesitant in novel settings and had poorer water maize performance than controls - Early ID rats tended to rear less in a open field test (not significant) - General activity and spatial alteration were similar for the two groups Conclusion: Rats with chronic perinatal IDA showed behavioural impairments that suggest persistent brain dysfunctions despite normalization of haematology, growth and most brain measures
Beard *et al.,* 2006	Controlled observational study	20 iron deficient and control rats	25 days (birth to PND25)	Pregnant dams were assigned to an ID or control group. Control group dams and their pups were fed a 40 mg/kg Fe diet; ID group dams were fed a 4mg/kg Fe diet and after birth ID dams were fed 10 mg/kg until PND20 when all pups and dams received 40 mg/kg Fe	Determination of whether dietary induced gestational and lactational ID alters brain monoamine metabolism and behaviours dependent on that neurotransmitter system	Brain iron assessment; sensorimotor tests; number of sectors entered in an open field (general activity)	- The number of sectors entered in the open field did not differ by diet group at PND15, but was significantly less for ID group pups at PND25 - ID pups were delayed in the attainment of a number of sensorimotor milestones - Conclusion: similar developmental delays in ID human infants suggest that alterations in iron status during this developmental period likely affects developing brain monaminergic systems in these infants

Note: ID, iron-deficient/iron deficiency; EID, early iron deficiency; IDA, iron-deficiency anaemia/iron deficiency anaemic; Hb, haemoglobin; PND, post natal day

Table 2: Human studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes

Investigators	Study design	Subjects	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Edgerton *et al*., 1979	Controlled, single-crossover study	199 female tea plantation workers between 20 and 60 years of age (mean 35.3 years) with low Hb concentration	Two months, the placebo group was crossed over to treatment in the second month	200 mg ferrous sulphate or placebo (300 mg calcium lactate)	Work capacity by the quantity of tea picked per day	Scale (weight of tea picked); activity recording device strapped to the ankle	- After one month's treatment significantly more tea was picked when Hb concentration was increased by iron supplementation - The degree of improvement was greater in more-anaemic subjects (6-9 g Hb/dl) - After three weeks level of physical activity of anaemic subjects in their everyday environment was greater in iron-treated subjects - Conclusions: implications of increased work productivity with iron treatment is evident , particularly in developing countries; results provide strong evidence for clinical impression that people with IDA suffer from tiredness and weakness
Deinard *et al*., 1981	Observational study	212 nonamaemic infants: Group1) SF ≤9 ng/ml Group 2) SF 10-19 ng/mL Group 3) SF ≥20ng/mL	N/A	N/A	Effects of iron depletion on cognitive development and attending behaviour	Visual stimulus (habituation/attention); Bayley Scales of Infant Development; Uzgiris and Hunt Ordinal Scales of Physiological Development	- There were no significant differences between the results of any of the groups on any of the scales or the visual stimulus test - Group 1 subjects were noted to be less visually and auditorally attentive, to produce more vocal sounds and to mouth less toys, but differences were not statistically significant Conclusion: no relationship was found between iron depletion in the absence of anaemia and cognitive and behavioural outcomes in 1-year-old children
Angulo-Kinzler *et al*., 2002	Controlled intervention	17 six month old anaemic or control infants	1 year	15 mg of elemental iron as ferrous sulphate per day	Spontaneous motor activity	Finger stick haemoglobin monitor; piezoelectric sensor (actigraph) on right ankle	- IDA infants generally spent less time in an alert-active state - IDA infants demonstrated significantly less spontaneous activity when awake - The magnitude of the differences increased at 12 and 18 months of age Conclusion: IDA was associated with reduced motor activity in infants even after iron treatment
Konofal *et al*., 2004	Controlled group comparison study (case-control)	80 children 4-14 years: 53 DSM-IV diagnosed ADHD and 27 controls	N/A	N/A	Evaluation of ID in children with ADHD vs. ID in a control group	CPRS; blood drawing	- Mean SF levels were lower in ADHD children than controls - Serum iron, Hb and hematocrit levels were within normal ranges in both children with ADHD and controls - In the ADHD group 84% of children had abnormally low SF (<30 ng/mL) vs. 18% of controls - SF levels were correlated with ADHD symptoms severity in children with CPRS - SF was correlated with the cognitive subscore and tended toward correlation with the hyperactivity subscore, but did not correlate with the oppositional subscore - Control group SF levels did not correlate with CPRS scores - Conclusion: results suggest that low iron stores contribute to ADHD; ADHD children may benefit from iron supplementation

Table 2: Human studies on the effects of ID and IDA on activity, ADHD and other behavioural outcomes (continued...)

Investigators	Study design	Subjects	Duration of the study	Intervention	Outcome(s) measured	Instruments for assessment	Outcomes and conclusions
Konofal *et al*., 2007	Double-blind, placebo-controlled, randomized pilot trial	23 nonanaemic children aged 5-8 years with serum ferritin levels <30 ng/mL with DSM-IV diagnosed ADHD	12 weeks	Oral iron (ferrous sulphate 80 mg/day) or placebo	Effects of iron supplementation on ADHD	CPRS, CTRS, ADHD Rating Scale (ADHD-RS), Clinical Global Impression-Severity (CGI-S), Clinical Global Impression-Improvement (CGI-I)	- CPRS and CTRS tended to improve in the iron group (non-significant) - ADHD RS severity significantly decreased after 12 weeks of treatment in the treatment group, particularly in the inattention subscore - The mean CGI-S significantly decreased at 12 weeks with iron treatment - Conclusion: iron supplementation appeared to improve ADHD symptoms in children with low serum ferritin levels suggesting a need for future investigations with larger controlled trials
Wang *et al*., 2009	Observational study	91 schoolchildren age 11-14 years with either severe or marginal iron deficiency or adequate iron status	N/A	N/A	Investigation of the relationship between ID of different degrees and physical performance and habitual activity of migrant schoolchildren	Electrically braked bicycle ergometer; portable cardiopulmonary breath-by-breath calorimetry system; food frequency questionnaire; frequency questionnaire for physical activity	- Severe ID (IDA) impaired the aerobic capacity and habitual physical activity - Aerobic activity and energy expenditure at leisure were significantly lower in the severe ID group than in the marginal ID and iron adequate groups - Conclusion: the functional effect of ID on physical performance and habitual physical activity rely on the degree of current ID; severe ID significantly impairs both aerobic capacity and habitual physical activity
Juneja *et al.,* 2010	Observational study	50 children 6-14 years: 25 DSM-IV diagnosed ADHD children with Hb >10g/dL and 25 controls without ADHD	N/A	N/A	Intelligence; ADHD symptoms	Wechsler Intelligence Scale for children; CPRS and CTRS; blood drawing	- SF was low in 92% cases with ADHD; none of the controls had low values - Mean SF levels were significantly lower in the ADHD group compared to controls - ADHD children with lower ferritin levels had more severe problems on the CPRS - CTRS oppositional scores were significantly correlated with SF level - No significant correlation was found between ferritin levels and CPRS inattentive scores or CTRS inattentive scores - No significant correlation of ferritin levels with CPRS or CTRS hyperactivity scores were found

Note: ID, iron-deficient/iron deficiency; IDA, iron-deficiency anaemia/iron deficiency anaemic; Hb, haemoglobin; ADHD, attention deficit hyperactivity disorder; CTRS, Conners' Teacher Rating Scale; CPRS, Conners' Parent Rating Scale

Table 3: Animal studies on the effects of omega-3 fatty acids on activity, ADHD and behavioural outcomes

Investigators	Study design	Animals	Duration of the study	Treatment	Outcome measures	Instruments used for assessment	Outcomes
Levant *et al.*, 2003	Parallel intervention study	20 rats: 7 controls, 7 deficient in ALA, 6 remediated	56 days	Dams of experimental animals were placed on an ALA deficient diet; pups were placed on the same diet accordingly; half of the deficient animals were treated after postnatal day 21 (weaning)	Locomotor activity in a novel environment	Observation by a trained observer	- Deficient animals exhibited 187% of the activity of control animals - Conclusions: rats exposed from conception to a diet that produces a relatively modest decrease in brain DHA content exhibit alterations in adult behaviour indicative of altered dopaminergic function; some of these behavioural alterations were reversed by dietary remediation at weaning
Levant *et al.*, 2006	Controlled intervention study	Four groups (control, high n-3, medium low n-3 and low n-3) containing 7-12 rats each	70 days	Dams were placed on either a control diet or a diet enriched with soybean oil and DHA (high n-3) or on a low n-3 diet with sunflower oil; pups were placed on the same diet for the duration of the study accordingly	Effects of variation in dietary availability of n-3 FA on brain FA composition and the consequent effects on locomotor activity	Coulbourn Tru-Scan activity monitors; brain composition testing	- In males, decreased brain DHA produced alterations in activity that were most pronounced post-adolescence and with the greatest decrease in DHA - The behavioral effects in males were not linearly related to brain DHA level - No significant effects of variation in brain fatty acid composition were observed in females Conclusion: This suggests that variation in brain DHA content produces sex-specific alterations in locomotor activity and that the neurochemical alterations underlying the observed behavioral changes vary depending on the degree of DHA depletion
Levant *et al.*, 2010	Controlled intervention study	31 male rats	70 days	Rats were raised from conception on diets containing ALA (control group) or being deficient in ALA (experimental group)	Assessment of activity, habituation and response to spatial change in a familiar environment	Modified force-plate actometers; brain composition testing	- Deficient pups exhibited a higher level of activity than controls during the first and second exposures to the test chamber, and less habituation during the first exposure, but were not more active after introduction of a novel spatial stimulus - Conclusions: the higher level of activity in a familiar environment, but not after a novel spatial stimulus is consistent with clinical observations of ADHD; observations also suggest that brain DHA content rather than dietary n-3 FA content likely underlies these effects

Note: ADHD, attention deficit hyperactivity disorder; DHA, docosahexaenoic acid; n-3 FA, omega-3 fatty acid

Table 4: Human studies on the effects of omega-3 fatty acids on activity, ADHD and behavioural outcomes

Investigators	Study design	Subjects	Duration of the study	Treatment	Outcome measures	Instruments used for assessment	Outcomes
Mitchell *et al*., 1986	Observational study	48 hyperactive children with 49 matched controls	N/A	N/A	Assessment of serum essential fatty acid levels	Blood drawing	- DHA significantly lower in hyperactive children - n-6 FA significantly lower in hyperactive children
Stevens *et al*., 1995	Observational study	Boys aged 6-12	N/A	N/A	Comparison of behaviour, learning and health problems of boys with lower plasma phospholipid total n-3 or total n-6 fatty acid levels of with boys with higher levels of these fatty acids	Parent and teacher behaviour questionnaires, Conners' Rating Scales (CRS), 3-day diet record	- Frequency of symptoms associated with essential fatty acid deficiency was higher in subjects with lower levels of plasma n-3 FAs - Subjects with lower total n-3 FA scored higher on many different behaviours and learning problems on the CRS and Health Questionnaire
Antalis *et al*., 2006	Observational study	12 ADHD-diagnosed students with 12 controls	N/A	N/A	Assessment of fatty acid composition of plasma and red blood cells	Blood drawing	- Higher saturated fatty acids in ADHD group - Higher monounsaturated fatty acids in ADHD group (only in plasma) - DHA 53% lower in plasma and 36% lower in red blood cells of ADHD group - Red blood cell ratio of AA to EPA 36% higher in ADHD group
Sinn and Bryan, 2007	Randomized, placebo-controlled, double blind parallel intervention	132 Australian children aged 7-12 y with scores ≥2 SD above the population average on the Conners' ADHD Index	15 weeks	1) PUFA 2) PUFA + micronutrients 3) Placebo	Testing for ADHD and related symptoms	CPRS and CTRS questionnaires	- Positive treatment effects for both PUFA and PUFA + micronutrient groups on parent ratings of core ADHD symptoms on CPRS - no additional effects with micronutrients

Table 4: Human studies on the effects of omega-3 fatty acids on activity, ADHD and behavioural outcomes (continued...)

Investigators	Study design	Subjects	Duration of the study	Treatment	Outcome measures	Instruments used for assessment	Outcomes
Bélanger *et al*., 2009	Randomized, placebo-controlled, double blind, one-way crossover trial	26 children aged 6 – 11 y with a DSM-IV diagnosis of ADHD based on results of the CPRS and CTRS and a clinical evaluation	16 weeks	20 – 25 mg/kg/day EPA and 8.5 – 10.5 mg/kg/day DHA + some phospholipids and tiny amounts of vitamin E	Determination the FA composition and the efficacy and safety of n-3 PUFA supplementation on ADHD clinical symptoms	Strength and Weakness in ADHD and Normal Behaviours (questionnaire); CPRS and CTRS questionnaires; dietary survey	- Supplementation with n-3 PUFA resulted in significant increases in EPA and DHA in the treatment group while the placebo group (supplemented with 500 ml sunflower oil) was enriched with alpha-linolenic, gamma linolenic and homo-gamma-linolenic acids - Statistically significant improvement in symptoms was noted based on the CPRS from baseline to end for impulsation and inattention in both groups, with greater improvement of symptoms in the n-3 PUFA group before crossover and greater improvement in the crossover group after crossover
Johnson *et al*., 2009	Randomized, placebo-controlled, double blind, one-way crossover trial	75 subjects aged 8 -18 y: 35 with ADHD combined subtype and 40 with ADHD mainly inattentive subtype	3 months + 3 month for all subjects	Omega-3/6 capsules; three capsules twice daily = daily dose of 558 mg EPA, 174 mg DHA, 60 mg gamma linoleic acid, 10.8 mg vitamin E	Assessment of ADHD core symptoms and differences between subgroups	Physical examination; neuromotor examination; Swanson, Nolan and Pelham Questionnaire; Five To Fifteen Parent/Teacher Scales; Brown's ADD Scale for Children and Adolescents, Brown's Self Report; Brown's Teacher Scale; other neurophysiological tests	- During Study Period 1 – a nonsignificant trend for reduction in ADHD Rating Scale scores in the active group versus placebo group for total ADHD scores and for inattentive and hyperactive/ impulsive subscores - Similar trend in Study Period 2 - Overall, results were not statistically significant
Ka-Hung *et al*., 2009	Observational study	86 children with ADHD symptoms above the 90th percentile on the 12-item ADHD Index from the Conners' Parent Rating Scales	N/A	N/A	Assessment of dietary PUFA intakes and food sources compared to previously published Australian National Nutrition Survey (NNS) data	Three-day-weighed food records	- Total n-3 PUFA intakes were significantly higher and n-6 PUFA were significantly lower in children with ADHD - Children with ADHD consumed half the amount of fish/seafood, meat and eggs when compared to the NNS - No significant correlations between FAs and ADHD symptoms - Children with ADHD met the adequate intake for LC n-3 PUFA but fell short of other recommendations

Note: ADHD, attention deficit hyperactivity disorder; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; n-3 FA, omega-3 fatty acid; PUFA, polyunsaturated fatty acid.

References

ABBOUD, S. & HAILE, D.J. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *Journal of biology and chemistry*, 275:19906–19912.

ANDERSON, G. J., FRAZER, D. M., MCKIE, A. T., VULPE, C. D. & SMITH, A. 2005. Mechanisms of haem and non-haem iron absorption: lessons from inherited disorders of iron metabolism. *BioMetals,* 18(4):339–348.

ANDREWS, N. C. 2000. Iron homeostasis: insights from genetics and animal models. *Nature reviews genetics,* 1(3):208–217.

ANDREWS, N. C. 2005. Understanding heme transport. *New England journal of medicine,* 353(23):2508–2509.

ANDREWS, N.C. 1999. Disorders of iron metabolism. *New England journal of medicine,* 341(26) 1986-1995.

ANTALIS, C.J., STEVENS, L.J., CAMPBELL, M., PAZDRO, R., ERICSON, K. & BURGESS, J.R. 2006. Omega-3 fatty acid status in attention-deficit/hyperactivity disorder. *Prostaglandins, leukotrienes and essential fatty acids,* 75(4-5):299-308.

BAYNES, R.D. & BOTHWELL, T.H. 1990. Iron deficiency. *Annual reviews in nutrition,* 10:133-148.

BEARD, J. & HAN, O. 2009. Systemic iron status. *Biochima et biophysica acta* 1790:584-588.

BEARD, J.L. 2000. Iron requirements in adolescent females. *Journal of Nutrition*, 130(suppl):440S-2S.

BÉLANGER, S.A., VANASSE, M., SPAHIS, S., SYLVESTRE, M., LIPPÉ, S., L'HEUREUX, F., GHADIRIAN, P., VANASSE, C. & LEVY, E. 2009. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. *Paediatrics & child health,* 14(2):89-98.

BEZWODA, W. R., BOTHWELL, T. H., CHARLTON, R. W., TORRANCE, J. D., MCPHAIL, A. P., DERMAN, D. P., AND MAYET, F. 1983. The relative dietary importance of haem and non-haem iron. *South African medical journal,* 64:552–556.

BOTHWELL, T.H., CHARLTON, R.W., COOK, J.D. & FINCH, C.A. 1979. Iron metabolism in man. Oxford:Blackwell Scientific Publications. 451-555p.

BRABIN, B.J., HAKIMI, M. & VERHOEF, F. 2001. Analysis of anaemia and child morbidity. *Journal of Nutrition,* 131:636S-648S.

British Nutrition Foundation. 1998. Iron: nutritional and physiological significance. London: Chapman & Hall. 1-2 p.

BRUGESS, J.R., STEVENS, L., ZHANG & W., PECK, L. 2000. Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. *American journal of clinical nutrition,* 71:327S-330S.

BURDGE, G.C. 2004. Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. *Current opinion in clinical nutrition and metabolic care* 7:137–144.

BURDGE, G.C. 2006. Metabolism of α-linolenic acid in humans. *Prostaglandins, leukotrienes and essential fatty acids,* 75(3):161-168.

BURGE, G.C., JONES, A.E. & WOOTTON, S.A. 2002. Eicosapentanoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men. *British journal of nutrition*, 88:335-363.

CANONNE-HERGAUX, F., GRUENHEID, S., PONKA, P. & GROS, P. 1999. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood,* 93:4406–4417.

CARPENTER, C. E., AND MAHONEY, A. W. 1992. Contributions of heme and nonheme iron to human nutrition. *Critical reviews in food science and nutrition,* 31(4):333–367.

CHUA, A.C.G., GRAHAM, R.M., TRINDER, D. & OLYNYK, J.K. 2007. The regulation of cellular iron metabolism. *Critical reviews in clinical laboratory sciences,* 44(5-6):413-459.

CONRAD, M. E. 1970. Factors affecting iron absorption. In: Hallberg, L., Harwerth, H. G., and Annotti, A. V. (Eds.), *Iron deficiency. Pathogenesis—clinical aspects—therapy.*, London and New York: Academic Press, pp. 87–114.

CRAWFORD, M. A. Essential fatty acids and neurodevelopmental disorder. In: Bazan N. G. ed. Neurobiology of Essential Fatty Acids. New York: Plenum Press, 1992; 307-314.

CRICHTON, R. 2009. Iron metabolism – from molecular mechanisms to clinical consequences. 3rd ed. Belgium: Wiley. 271-294 p.

DARLEY-USMAR, V. & HALLIWELL, B. 1996. Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharmaceutical research,* 13(5):649–662.

DOREY, C., COOPER, C., DICKSON, D. P. E., GIBSON, J. F., SIMPSON, R. J. & PETERS, T. J. 1993. Iron speciation at physiological pH in media containing ascorbate and oxygen. *British journal of nutrition,* 70(1):157–169.

DREVON, C.A., BAKSAAS, I. & KROKAN, H.E. 1995. Omega-3 fatty acids: metabolism and biological effects. Basel: Birkhäuser Verlag. 1-9 p.

DREVON, C.A., BAKSAAS, I. & KROKAN, H.E. 1995. Omega-3 fatty acids: metabolism and biological effects. Basel: Birkhäuser Verlag. 1-9 p.

EDGERTON, V.R., BRYANT, S.L., GILLESPIE, C.A. & GARDNER, G.W. 1972. Iron deficiency anemia and physical performance and activity of rats. *The journal of nutrition,* 102(3):381-399.

EDGERTON, V.R., DIAMOND, L.B. & OLSON, J. 1977. Voluntary activity, cardiovascular and muscular responses to anemia in rats. *The journal of nutrition,* 107(9):1595-1601.

EPSTEIN J.N., MARCH J.S., CONNERS C.K. & JACKSON D.L.. 1998. Racial differences on the Conners Teacher Rating Scale. *Journal of abnormal child psychology*, 26:109-118.

EPSZTEJN, S., GLICKSTEIN, H., PICARD, V., SLOTKI, I. N., BREUER, W., BEAUMONT, C. & CABANTCHIK, Z. I. 1999. H-Ferritin subunit overexpression in erythroid cells reduces the oxidative stress response and induces multidrug resistance properties. *Blood*, 94:3593–3603.

FELT, B.T. & LOZOFF, B. 1996. Brain iron and behavior of rats are not normalized by treatment of iron deficiency anemia during early development. *Journal of nutrition,* 126(3):693.

FINCH, C. 1994. Regulators of iron balance in humans. *Blood,* 84:1697–1702.

FINCH, C.A., MILLER, L.R., INAMDAR, A.R., PERSON, R., SEILER, K. & MACKLER, B. 1976. Iron deficiency in the rat. physiological and biochemical studies of muscle dysfunction. *The journal of clinical investigation,* 58(2):447-453.

FLEMING, M. D., ROMANO, M. A., SU, M. A., GARRICK, L. M., GARRICK, M. D. & ANDREWS, N. C. 1998. Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *PNAS.* 95(3):1148–1153.

FLEMING, M. D., TRENOR, C. C., III., SU, M. A., FOERNZLER, D., BEIER, D. R., DIETRICH, W. F. & ANDREWS, N. C. 1997. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature genetics,* 16(4):383–386.

FLEMING, R.E., MIGAS, M.C., HOLDEN, C.C., WAHEED. A., BRITTON, R.S., TOMATSU, S. & BACON, B.R., SLY, W.S. 2000. Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. *National academy of science US*; 97:2214–2219.

GARDNER, J.M.M. & GRANTHAM-MCGREGOR, S. 1995. Activity and behavioral development in stunted and nonstunted children and response to nutritional supplementation. *Child development,* 66(6):1785-1797.

GLASER, C., LATTKA, E., RZEHAK, P., STEER, C. & KOLETZKO, B. 2011. Genetic variation in polyunsaturated fatty acid metabolism and its potential relevance for human development and health. *Maternal & child nutrition,* 7 Suppl 227-40.

GLOVER, J. & JACOBS, A. 1972. Activity pattern of iron-deficient rats. *British medical journal,* 2(5814):627-628.

GOLUB, M.S., HOGREFE, C.E., GERMANN, S.L., CAPITANIO, J.P. & LOZOFF, B. 2006. Behavioral consequences of developmental iron deficiency in infant rhesus monkeys. *Neurotoxicology & teratology,* 28(1):3-17.

GRANTHAM-MCGREGOR, S. & ANI, C. 2001. A review of studies on the effect of iron deficiency on cognitive development in children. *Journal of nutrition,* 131(2):649S.

GRIFFINS, T.A.M., MAUK, A.G. & MACGILLIVRAY, R.T.A. 2005. Recombat expression and functional characterization of human hephaestin: a multicopper oxidase with ferroxidase activity. *Biochemistry,* 44(14):725-731.

GUNSHIN, H., MACKENZIE, B., BERGER, U. V., GUNSHIN, Y., ROMERO, M. F., BORON, W. F., NUSSBERGER, S., GOLLAN, J. L. & HEDLGER, M. A. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature,* 388(6641):482–488.

GUNSHIN, H., MACKENZIE, B., BERGER, U.V., GUNSHIN, Y., ROMERO, M.F., BORON, W.F., NUSSBERGER, S., GOLLAN, J.L. & HEDIGER, M.A. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature,* 388:482–488.

HAAS, J.D. & BROWNLIE IV, T. 2001. Iron deficiency and reduced work capacity: A critical review of the research to determine a causal relationship. *Journal of nutrition,* 131(2):676S.

HALLBERG, L. & SOLVELL, L. 1967. Absorption of hemoglobin iron in man. *Acta medica Scandinavica,* 181(3):335–354.

HAN, O., FAILLA, M. L., HILL, A. D., MORRIS, E. R., & SMITH, J. 1995. Reduction of Fe(III) is required for uptake of nonheme iron by Caco-2 cells. *Journal of nutrition,* 125(5):1291–1299.

HELLMAN, N.E. & GITLIN, J.D. 2002. Ceruloplasmin metabolism and function. *Annual reviews of nutrition,* 22:439–458.

HUNT, J., ZITO, C., ERJAVEC, J. & JOHNSON, L. 1994. Severe or marginal iron deficiency affects spontaneous physical activity in rats. *The American journal of clinical nutrition*, 59(2):413-418.

HURREL, R.F. 1997. Bioavailability of iron. *Europen journal of clinical nutrition,* 51:S4-S8.

Institute of Medicine and Food and Nutrition Board. 2001. Iron. Washington, D.C.: National Academy Press. 290-393p.

JANZ, K.F., WITT, J. & MAHONEY, L.T. 1995. The stability of children's physical activity as measured by accelerometry and self-report. *Medicine and science in sports and exercise,* 27:1326-1332.

JOHNSON, M., OSTLUND, S., FRANSSON, G., KADESJÖ, B. & GILLBERG, C. 2009. Omega-3/omega-6 fatty acids for attention deficit hyperactivity disorder: A randomized placebo-controlled trial in children and adolescents. *Journal of attention disorders,* 12(5):394-401.

KA-HUNG, N.G., BARBARA, J.M., LAUREN, R. & SINN, N. 2009. Dietary PUFA intakes in children with attention-deficit/hyperactivity disorder symptoms. *British journal of nutrition*, 102:1635-1641.

KAUR, D., RAJAGOPALAN, S. & ANDERSEN, J. K. 2009. Chronic expression of H-ferritin in dopaminergic midbrain neurons results in an age-related expansion of the labile iron pool and subsequent neurodegeneration: implications for Parkinson's disease*. Brain research*, 1297:17–22.

KAUR, D., YANTIRI, F., RAJAGOPALAN, S., KUMAR, J., MO, J. Q., BOONPLUEANG, R., VISWANATH, V., JACOBS, R., YANG, L. & BEAL, M. F. 2003. Genetic or pharmacological iron chelation prevents MPTP-induced neurotoxicity in vivo: a novel therapy for Parkinson's disease. *Neuron,* 37:899–909.

KAWABATA, H., GERMAIN, R.S., VUONG, P.T., NAKAMAKI, T., SAID, J.W. & KOEFFLER, H.P. 2000. Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and in vivo. *Journal of biology and chemistry,* 275:16618–16625.

KAWABATA, H., YANG, R., HIRAMA, T., VUONG, P.T., KAWANO, S., GOMBART, A.F. & KOEFFLER, H.P. 1999. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *Journal of biological chemistry,* 274:20826–20832.

KONOFAL, E., CORTESE, S., LECENDREUX, M., ARNULF, I. & MOUREN, M.C. 2005. Effectiveness of iron supplementation in a young child with attention-deficit/hyperactivity disorder. *Pediatrics,* 116(5):e732-e734

KONOFAL, E., LECENDREUX, M., DERON, J., MARCHAND, M., CORTESE, S., ZAÏM, M., MOUREN, M.C. & ARNULF, I. 2008. Effects of iron supplementation on attention deficit hyperactivity disorder in children. *Pediatric neurology,* 38(1):20-26.

KOZIOL, B.J., OHIRA, Y., EDGERTON, V.R. & SIMPSON, D.R. 1982. Changes in work tolerance associated with metabolic and physiological adjustment to moderate and severe iron deficiency anemia. *The american journal of clinical nutrition,* 36(5):830-839.

KRAUSE, A., NEITZ, S. & MAGERT, H.J. 2000. LEAP1, a novel highly disulfide-bondedhuman peptide, exhibits anti-microbial activity, *FEBS Letts*, 408:147-150.

KWIK-URIBE, C. & GOLUB, M.S. 2000. Chronic marginal iron intakes during early development in mice alter brain iron concentrations and behavior despite postnatal iron supplementation. *Journal of nutrition,* 130(8):2040.

KWIK-URIBE, C., GOLUBT, M.S. & KEEN, C.L. 1999. Behavioral consequences of marginal iron deficiency during development in a murine model. *Neurotoxicology and teratology,* 21(6):661-672.

LI, R., CHEN, X., YAN, H., DEURENBERG, P., GARBY, L. & HAUTVAST, J.G. 1994. Functional consequences of iron supplementation in iron-deficient female cotton mill workers in beijing, china. *The american journal of clinical nutrition,* 59(4):908-913.

LIU, X.B., HILL, P. & HAILE, D.J. 2002. Role of the ferroportin iron-responsive element in iron and nitric oxide dependent gene regulation. *Blood cells & molecules in disease,* 29:315–326.

LYMBOUSSAKI, A., PIGNATTI E., MONTOSI, G., GARUTI, C., HAILE, D.J. & PIETRANGELO, A. 2003. The role of the iron responsive element in the control of ferroportin1/IREG1/MTP1 gene expression. *Journal of hepatology,* 39:710–715.

MACPHAIL, P., MANN, J. & TRUSTWELL, A.S. 2007. Essentials of human nutrition. 3rd ed. New York: Oxford University Press. 125-137 p.

MCCANN, J.C. & AMES, B.N. 2007. An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. *American journal of clinical nutrition,* 85(4):931-945.

MCCLOY, U., RYAN, M.A., PENCHARZ, P.B., ROSS, R.J. & CUNNANE, S.C. 2004. Comarison of the metabolism of eighteen-carbon ^{13}C-unsaturated fatty acids in healthy women. *Journal of lipid research,* 45:474-485.

MCKIE, A. T., LATUNDE-DADA, G. O., MIRET, S., MCGREGOR, J. A., ANDERSON, G. J., VULPE, C. D., WRIGGLESWORTH, J. M. M. & SIMPSON, R. J. 2002. Molecular evidence for the role of a ferric reductase in iron transport. *Biochemical society transactions,* 30(4):722–724.

MCKIE, A.T., MARCIANI, P., ROLFS, A., BRENNAN, K., WEHR, K., BARROW, D., MIRET, S., BOMFORD, A., PETERS, T.J., FARZANEH, F., HEDIGER, M.A., HENTZE, M.W. & SIMPSON, R.J. 2000. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Molecules & cells,* 5:299–309.

MERLE, U., THEILIG, F., FEIN, E., GEHRKE, S., KALLINOWSKI, B., RIEDEL, HD., BACHMANN S., STREMMEL, W. & KULAKSIZ, H. 2007. Localization of the iron-regulatory proteins hemojuvelin and transferrin receptor 2 to the basolateral membrane domain of hepatocytes. *Histochemical cell biology,*127: 221–226.

MITCHELL, E.A., AMAN, M.G., TURBOTT, S.H. & MANKU, M. 1987. Clinical characteristics and serum essential fatty acid levels in hyperactive children. *Clinical pediatrics,* 26(8):406-411.

MIYAJIMA, H., NISHIMURA, Y., MIZOGUCHI, K., SAKAMOTO, M., SHIMIZU, T., HONDA N. 1987. Familial apoceruloplasmin eficiency associated with blepharospasm and retinal degeneration. *Neurology,* 37:761–767.

NAKAMURA, M.T. & NARA, T.Y. (2004) Structure, function, and dietary regulation of delta6, delta5, and delta9 esaturases. *Annual review of nutrition* 24:345–376.
National Research Council. 1979. Committee on medical and biological effects of environmental pollutants. Sub-committee on iron. *Iron.*, Baltimore, MD: University Park Publisher.

NEMETH, E., YUTTLE, M.S. & POWELSON, J. 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science,* 306:2090-2093.
Neuringer M., Reisbeck S., Janowsky J. The role of n-3 fatty acids in visual and cognitive development: Current evidence and methods of assessment. *J Pediatr* 1994; **125**: S39-47.

NEURINGER, M. D., CONNOR, W. E., LIN, D. S., BARSTAD L.& LUCK S. 1986. Biochemical and functional effects of prenatal and postnatal w-3 fatty acid deficiency on retina and brain in rhesus monkeys. *National academy of science USA,* 83: 4021-4025.

NILSSON, A., EKELUND, U., YNGVE, A. & SJOSTORM, M. 2002. Assessing physical activity among children with accelerometers using different time sampling intervals and placements. *Pediatric exercise science*, 14:87-96.

PARK, C.H., VALORE, E.V., WARING, A.J. & GANZ, T. 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Jouranl of biology and chemistry,* 267:7806-7810.

PARK, W.J., KOTHAPALLI, K.S., LAWRENCE, P., TYBURCZY, C. & BRENNA, J.T. 2009. An alternate pathway to long-chain polyunsaturates: the FADS2 gene product delta8- desaturates 20:2n-6 and 20:3n-3. *Journal of lipid research* 50:1195–1202.

PETRAT, F., DE GROOT, H., SUSTMANN, R. & RAUEN, U. 2002. The chelatable iron pool in living cells: a methodically defined quantity. *Biological chemistry*, 383: 89–502.

PICCINELLI, P. AND SAMUELSSON, T. 2007. Evolution of the iron-responsive element. *RNA,* 13:952–966.

PIÑERO, D., JONES, B. & BEARD, J. 2001. Variations in dietary iron alter behavior in developing rats. *The journal of nutrition,* 131(2):311-318.

PUYAU, MR, ALDOLPH, AL, VOHRA, FA, BUTTE, NF. 2002. Validation and calibration of physical activity monitors in children. *Obesity Research,* 10(3):150-157.

RAFFIN, S. B., WOO, C. H., ROOST, K. T., PRICE, D. C., SCHMID, R. 1974. Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. *Journal of clinical inverstigation,* 54(6):1344–1352.

RAUEN, U., SPRINGER, A., WEISHEIT, D., PETRAT, F., KORTH, H. G., DE GROOT, H. & SUSTMANN, R. 2007. Assessment of chelatable mitochondrial iron by using mitochondrion-selective fluorescent iron indicators with different iron-binding affinities. *ChemBioChem*, 8:341–352.

RICHARDSON, A.J. & PURI, B.K. 2000. The potential role of fatty acids in attention-deficit/hyperactivity disorder. *Prostaglandins, leukotrienes and essential fatty acids,* 63(1-2):79-87.

RICHARDSON, A.J. 2004. Long-chain polyunsaturated fatty acids in childhood developmental and psychiatric disorders. *Lipids,* 39(12):1215-1222.

RICHARDSON, A.J. 2006. Omega-3 fatty acids in ADHD and related neurodevelopmental disorders. *International review of psychiatry,* 18(2):155-172.

ROBB, A.D., ERICSSON, M. & WESSLING-RESNICK, M. 2004. Transferrin receptor 2 mediates a biphasic pattern of transferrin uptake associated with ligand delivery to multivesicular bodies. *American journal of physiology and cell physiology,* 287:C1769–C1775.

SACHDEV, H., GERA, T. & NESTEL, P. 2005. Effect of iron supplementation on mental and motor development in children: Systematic review of randomised controlled trials. *Public health nutrition,* 8(2):117-132.

SACHDEV, H., GERA, T. & NESTEL, P. 2005. Effect of iron supplementation on mental and motor development in children: Systematic review of randomised controlled trials. *Public health nutrition,* 8(2):117-132.

SCHUCHARDT, J.P., HUSS, M., STAUSS-GRABO, M. & HAHN, A. 2010. Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. *European journal of pediatrics,* 169(2):149-164.

SHAYEGHI, M., LATUNDE-DADA, G.O., OAKHILL, J.S., LAFTAH, A.H., TAKEUCHI, K., HALLIDAY, N., KHAN, Y., WARLEY, A., MCCANN, F.E., HIDER, R.C., FRAZER, D.M., ANDERSON, G.J., VULPE, C.D., SIMPSON, R.J. & MCKIE, A.T. 2005. Identification of an intestinal haem transporter. *Cell,* 122(5):789-801.

SIMOPOLOUS, A.P. & BAZAN, N.G. 2009. Omega-3 fatty acids, the brain and retina. Basil: Karger. p.99.

SINN, N. 2007. Physical fatty acid deficiency signs in children with ADHD symptoms. *Prostaglandins, leukotrienes and essential fatty acids,* 77(2):109-115.

SMUTS, C.M., LOMBARD, C.J., BENADÉ, A.J.S., DHANSAY, M.A., BERGER, J., LE, T.H., LÓPEZ, D.R., UNTORO, J., KARYADI, E., ERHARDT, J. & GROSS, R. 2005. Efficacy of a foodlet-based multiple micronutrient supplement for preventing growth faltering, anemia, and micronutrient deficiency of infants: The four country IRIS trial pooled data analysis. *Journal of nutrition,* 135(3):631S-638S.

SPRECHER, H. 1999. An update on the pathways of polyunsaturated fatty acid metabolism. *Current opinion in clinical nutrition and metabolic care* 2:135–138.

SPRECHER, H., LUTHRIA, D.L., MOHAMMED, B.S. & BAYKOUSHEVA, S.P. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of lipid research,* 36:2471-2477.

STALTZFUS, R.J., DREYFUSS, M.L., CHWAYA, H.M. & ALBONICO, M. 1997. Hookworm control as a strategy to prevent iron deficiency. *Nutrition Reviews,* 55:223-232.

STEVENS, J., QUITTNER, A.L., ZUCKERMAN, J.B. & MOORE, S. 2002. Behavioral inhibition, self-regulation of motivation, and working memory in children with attention deficit hyperactivity disorder. *Developmental neuropsychology,* 21(2):117-139.

SU, M.A., TRENOR, C.C., FLEMING, J.C., FLEMING, M.D., ANDREWS, N.C. 1998. The G185R mutation disrupts function of the iron transporter Nramp2. *Blood,* 92:2157–2163.

SWANSON, J.M., SERGEANT, J.A., TAYLOR, E., SONUGA-BARKE, E., JENSEN, P.S. & CANTWELL, D.P. 1998. Attention-deficit hyperactivity disorder and hyperkinetic disorder. *Lancet,* 351(9100):429.

TANG, A.B., NISHIMURA, K.Y. & PHINNEY, S.D. 1993. Preferential reduction in adipose tissue alpha-linolenic acid (18:3 omega-3) during low-calorie dieting despite supplementation woth 18:3 omega-3. *Lipids,* 28:987-993.

THOMAS, C. & OATS, P. S. 2004. Differences in the uptake of iron from Fe(II) ascorbate and Fe(III) citrate by IEC-6 cells and the involvement of ferroportin/IREG-1/MTP-1/SLC40A1. *Plfugers Archiv (European journal of physiology),* 448:431–437.

TRINDER, D., OATES, P.S., THOMAS, C., SADLEIR, J. & MORGAN, E.H. 2000. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. *Gut,* 46:270–276.

TUCKER, P. 2008. The physical activity levels of preschool-aged children: A systematic review. *Early childhood research quarterly,* 23(4):547-558.

UTHMAN, E. 1998. Understanding anemia. Mississippi: Jackson.

VALERIO, L.G. 2007. Mammalian iron metabolism. *Toxicology mechanisms and methods,* 17(9):497-517.

VALK, B., ADDICKS, M. A., GOSRIWATANA, I., LU, S., HIDER, R. C. & MARX, J. J. 2000. Non-transferrin-bound iron is present in serum of hereditary haemochromatosis heterozygotes. *European journal of clinical investigastion,.* 30(3):248–251.

VANCASSEL, S., BLONDEAU, C., LALLEMAND, S., CADOR, M., LINARD, A., LAVIALLE, M. & DELLU-HAGEDORN, F. 2007. Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. *Behavioural brain research,* 180(2):119-126.

WALLANDER, M. L., LEIBOLD, E. A. & EISENSTEIN, R. S. 2006. Molecular control of vertebrate iron homeostasis by iron regulatory proteins. *Biochimica et biophysica acta (BBA) - bioenergetics*,1763:668–689.

WANG, J., HUO, J., SUN, J. & NING, Z. 2009. Physical performance of migrant schoolchildren with marginal and severe iron deficiency in the suburbs of Beijing. *Biomedical and environmental sciences: BES,* 22(4):333-339.

WHO. 1998. Global database on anaemia. Geveva: World Health Organisation.
World Health Organization. 1983. Iron. In: Safety Evaluation of Certain Food Additives and Contaminants. WHO Food Additive Series 18. Geneva: Joint FAO/WHO Expert Group.

WOROBEY, J., TEPPER, B.J., KANAREK, R.B. & D'ANCI, K.E. 2005. Nutrition and behaviour: a multidisciplinary approach. Oxfordshire: CABI.

ZIMMERMANN, M.B. & HURRELL, R.F. 2007. Nutritional iron deficiency. *Lancet,* 370(9586):511-520.

Chapter III - Article

Effects of iron and omega-3 fatty acid supplementation on spontaneous motor activity and ADHD-related behaviour in iron-deficient primary school children residing in KwaZulu-Natal

Abstract

Objectives: To investigate the effects of iron and n-3 FA supplementation on spontaneous physical activity and attention deficit hyperactivity disorder ADHD-related behaviour in iron-deficient primary school children. Additionally, to evaluate the use of the *Actical* accelerometer as a behaviour assessment tool during school break and classroom time.

Design: A 2x2 factorial, randomized, double-blind, placebo-controlled trial.

Setting: Rural setting in KwaZulu-Natal Province, South Africa.

Subjects: Iron-deficient primary school children aged 6-10 years (n = 321).

Methods: Subjects were randomly assigned to receive one of the following supplement combinations: (1) 420mg DHA/80 mg EPA + 50mg of iron as ferrous sulphate; (2) 420mg DHA/80mg EPA + placebo; (3) 50mg of iron + placebo; (4) placebo + placebo. Supplements were provided four times a week for 8.5 months (excluding school holidays). Physical activity on four random school days was recorded at baseline, midpoint and endpoint (cycles 1, 2 and 3) during three different time periods namely *class time 1* (08h00–10h30)*, break time* (10h30-11h00) and *class time 2* (11h00-12h00). Classroom behaviour of study subjects was assessed by teachers' questionnaires at baseline and endpoint. Iron status indicators and RBC FA composition were measured at baseline and endpoint.

Results: Seasonal variation was observed on activity (significant cycle effect, P = 0.001). A significant cycle x age interaction (P = 0.005) as well as a significant cycle x time period x gender interaction (P = 0.036) was observed on overall activity. There were no significant interactions of cycle or time period with treatment. However, there was a significant main effect of DHA/EPA supplementation for lower *class time 1* activity at endpoint (P = 0.014). Biological markers indicating better or poorer iron status were positively and negatively associated with activity at *break time,* respectively. Subjects in the group receiving both Fe and DHA/EPA supplements showed a significant improvement from baseline to endpoint on the cognitive problems/inattention subscale (P = 0.005) of the CTRS. Hyperactivity scores increased significantly from baseline to endpoint in all groups (P = 0.006). DHA (r = -.203; P = 0.040) and EPA (r = -.199; P = 0.044) content of RBC were negatively associated with activity at *class time 1*. At endpoint, *class time 1* activity was positively associated with all CTRS subscale scores except for the cognitive problems subscale, which only bordered significance (correlation, P = 0.051; regression, P = 0.073).

Conclusion: These findings suggest that n-3 FA supplementation may have an influence on ADHD-related behaviour during class time. During school break time when subjects were allowed to move around freely, iron status was positively associated with spontaneous physical activity. Furthermore, the accelerometer might be useful tool for assessing both classroom and break time activity behaviour in school children.

Key words: iron deficiency, iron supplementation, school children, motor activity, omega-3 fatty acid, attention deficit hyperactivity disorder, behaviour, accelerometer, Conners' Teacher Rating Scale

Introduction

The National Food Consumption Survey (NFCS) of South Africa in 2005 found one out of five children in South Africa to have poor iron status [1]. Studies in rural KwaZulu-Natal have found the prevalence of ID among preschoolers to be 19.8% [2] as well as 42-52% prevalence of anaemia among 6 – 74 year old individuals [2,3]. Among other complications, studies have found important links between ID with or without anaemia and cognitive and behavioural function, including motor activity, with evidence for a possible causal relationship [4]. Similarly, associations have been found between n-3 FA status and spontaneous motor activity in animals [5,6,7]. Also, many characteristics associated with ADHD are consistent with deficiencies or imbalances in n-3/n-6 FA status [8,9].

It has been stated that physical activity plays an important role in learning from the environment and in cognitive development during infancy [10,11]. Black *et al*. [12] declared that a reduction in overall motor activity and engagement could produce changes in certain developmental processes, which in turn could affect the experimental processes of development. On the other hand, it is logical to presume that increased motor activity in a classroom setup could indicate presence of hyperactivity and other ADHD-related behaviours.

Although a number of observational studies have investigated the effect of ID on activity [13,14,15,16,17,18,19] and other behavioural outcomes [13,14,20], few previous studies have investigated the effects of Fe supplementation on spontaneous motor activity or ADHD-related behaviour [10,21]. A study by Angulo-Kinzler *et al.* [10] was the only study that could be found measuring the effects of Fe supplementation on spontaneous activity in humans (infants). Their study found that IDA was associated with reduced motor activity in infants even after iron treatment. Animal studies have shown mixed results for iron treatment, with some showing positive effects [22,23] and some showing no effects [24,25,26]. As for ADHD-related behaviour, some studies have observed significantly lower serum ferritin (SF) levels in ADHD diagnosed subjects compared to controls [14,20]. Konofal *et al.* [21] found that iron supplementation appeared to improve ADHD symptoms in children with low SF levels.

RBC and serum composition of DHA, EPA and other n-3 FAs have been found to be lower in ADHD diagnosed subjects [27,28,29]. Levant *et al*. [5,6,7] investigated the effects of n-3 FA

deficiency on activity-related behaviours in rats using activity monitors and found that changes in behaviour were consistent with clinical observations of ADHD. Treatment of ADHD-diagnosed subjects with n-3 FAs has shown positive results [30,31,32].

The aim of the present study was to assess the effects of iron and DHA/EPA treatment on spontaneous motor activity and ADHD-related behaviour in ID primary school children. The subjects were not diagnosed with ADHD. Activity behaviour of subjects was measured using an accelerometer and an ADHD-rating scale (the CTRS). The ability of the accelerometer as a behavioural assessment tool was also evaluated.

Methods

Data that were used in this study were part of a larger randomised, double blind and placebo-controlled intervention investigating effects of Fe and DHA/EPA supplementation on cognition and behaviour. This part of the study dealt specifically with the behavioural effects, using an accelerometer to measure spontaneous motor activity and the CTRS to assess behaviour related to ADHD. The study protocol was reviewed and approved by the ethics committee of ETH Zurich and the North-West University Potchefstroom, and informed consent was obtained from parents/guardians of study subjects.

Study site and selection of subjects

The study sample consisted of iron-deficient children from four primary schools in KwaZulu-Natal, South Africa, located in the Valley of a Thousand Hills – a rural area situated approximately 40 km north-west of the coastal city of Durban. This community is described as having low socio-economic status and a high prevalence of nutrient deficiencies.

A total of 926 subjects were registered into the study and took part in the baseline screening at the beginning of November 2009. For enrolment into the study, subjects had to be 6-10 years of age, have no chronic medical illnesses, and make no use of iron or n-3 fatty acid-containing supplements (excluding enriched or fortified food, like iron-enriched flour). ID as an inclusion criteria in this study was defined as either serum ferritin (SF) <20 µg/l or zinc protoporphyrin (ZnPP) >60 µmol/mol haem in washed erythrocytes or serum transferrin receptor >8.3 mg/L in combination with haemoglobin (Hb) ≥8.0 g/dL. Severe anaemia (Hb <8.0 g/dL) was an exclusion criteria. After the baseline screening, 321 iron-deficient children with or without mild anaemia were included in the study.

Intervention

Subjects enrolled in the study were randomly assigned to receive one of the following supplement combinations: (1) 420 mg DHA + 80 mg EPA and 50 mg of iron as ferrous sulphate (Fe); (2) 420 mg DHA + 80 mg EPA and placebo; (3) 50 mg of Fe and placebo; (4) placebo and placebo. Supplements were administered four times per week by trained field workers. The Fe or placebo tablets were administered together with a vitamin C-enriched drink in the morning when the children arrived at school. Administration of the fish oil and placebo capsules took place at the mid-morning meal. To avoid the possible influence of parasite infections, subjects were de-wormed at baseline and midpoint. They were given a chewable de-worming tablet containing 400 mg mebendazole under supervision of the fieldworkers. Supplementation was continued for the duration of the school year (nine months), except during school holidays.

Data collection

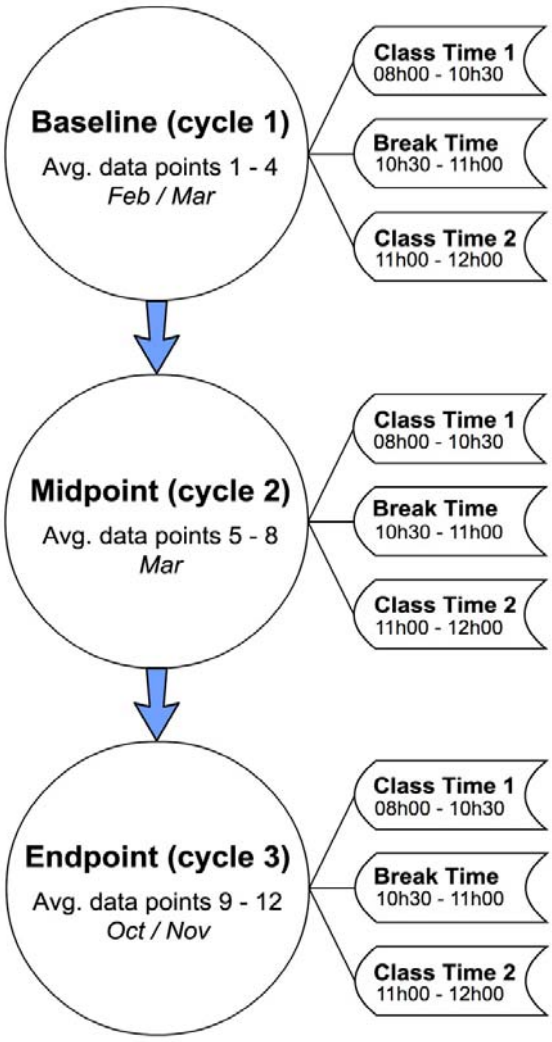

Figure 1: Activity data collection

Blood drawing

Baseline and end blood drawing took place at the schools and blood samples were processed at a small field laboratory close to the school. The experimental analysis was performed at the research laboratory of the Centre of Excellence for Nutrition, North-West University (Potchefstroom Campus) and ETH Zurich, Switzerland.

Biochemical indicators

Hemoglobin (Hb) concentration was measured on site on an aliquot of whole blood by direct cyanmethemoglobin method (Ames Mini-Pak Hb test pack and AmesTM Minilab), using Drabkins solution and a standard mini-photometer. The remaining blood samples were centrifuged at 500 g for 15 min at room temperature and plasma and serum were aliquoted and stored at -20°C for the duration of the fieldwork (4 days). RBCs were washed twice with 0.15 mol/L NaCl, and centrifuged at 500 g for 10 min to remove the buffy coat. Zinc protoporphyrin (ZnPP) was measured on washed RBC using a hematofluorometer (Aviv Biomedical, Lakewood, NJ, USA) and 3-level control material provided by the manufacturer on

the same day of blood sampling. Serum ferritin (SF) and C-reactive protein (CRP) were measured using an automated chemiluminescent immunoassay system (IMMULITE, DPC Bühlmann GmbH, Aschwil, Switzerland). Soluble transferrin receptor (TfR) was measured using an enzyme immunoassay (Ramco Laboratories, Inc, Stafford, TX). Body iron was calculated from the ratio of TfR:SF according to the equation of Cook et al. (26). Inflammation was defined as CRP > 5 mg/L. SF values of subjects with CRP > 5 mg/L were excluded from the analysis, due to the confounding effects of inflammation on SF.

Phospholipids were extracted from RBC membranes with chloroform:methanol (2:1 v/v; containing 0.01% BHT) by a modification of the method of Folch et al. (30). Total phospholipid FA fractions were isolated by thin layer chromatography, trans-methylated to yield FAME and analyzed by quadrupole GC-EI-MS on an Agilent Technologies 7890 A GC system equipped with an Agilent Technologies 5975C VL mas selective detector.

Spontaneous motor activity measurement

We selected all subjects from the largest of the four schools to participate in the activity sub-study. This school was the only school in the study with a large playing area (approximately 30 x 50 meters). The other three schools did not contain any playing space, and were therefore not used for activity measurements.

Daily school time physical activity was collected using 48 *Actical* devices. This omni-directional accelerometer is a small square object, about the size of a wrist watch, which could be securely strapped around the waist of a subject with an elastic waist band. The device was set to collect activity data at an epoch length of 60 seconds, which produced an output of activity counts per minute.

Subjects were randomized to wear the accelerometer on different days of the week and accelerometers were randomized between subjects. One day of activity data collection represented one data point for each individual. Each day of data collection was further divided into three time periods. Time periods 1, 2 and 3 described respectively *class time 1*, *break time* and *class time 2* (Figure 1). Data were collected at baseline, midpoint and endpoint. These three phases of data collection are also referred to as cycles. Each cycle comprised four data points per individual and, therefore, a total of 12 data points per individual were obtained after completion of the study. Completion of one cycle required approximately 8-12 school days, depending on whether subjects were absent from school or if the normal school day was interrupted. Four data points, respectively four replicates were chosen per time period in order to obtain a more representative sample, considering potential day-to-day variation in activity. Ultimately, the four data points collected during each cycle were combined to form an average for each time period of the school day.

ADHD-related behaviour assessment

Since we hypothesized that higher spontaneous motor activity during class time could be an indication of presence of behavioural problems in the classroom, activity measurements during class time were considered a measure of subject behaviour related to ADHD. The Conners' Teacher Rating Scale–Revised: Short Forms (CTRS-R:S) was the second and main assessment tool used for collection of ADHD-related behavioural data. The CTRS is a commonly used rating scale employed for assessment of classroom behaviour problems related to ADHD [33]. The general purpose of the scale is to provide information at a screening level to assist clinicians and researchers in understanding several important domains of child behaviour [34]. The CTRS-R:S were developed from the most clinically useful subscales (oppositional, cognitive problems/inattention, hyperactivity) of the CTRS-R:L (long form) for use when several administrations over time were desired. A fourth subscale, the ADHD index was also included for assessment of ADHD symptoms based on the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV) [35]. Ultimately, the CTRS-R:S is a teacher form containing 28 items concerning the behaviour of a child which are scored using a Likert-type scale scored from 0-4 (0 being "not true at all" and 4 being "very much true"). Higher scores indicate greater severity of symptoms. In this study, the CTRS-R:S subscales were not used to determine ADHD status of subjects, but to compare symptoms related to ADHD between different supplementation groups. For the purpose of this paper the term CTRS refers to the CTRS-R:S.

The CTRS was administered to teachers at the beginning of intervention and after they had been given a briefing on how to fill in the sheets. At endpoint a trained fieldworker was assigned to interview the teacher, asking him/her the questions appearing on the CTRS for each child included in the study. The administration of a fieldworker at endpoint was done in an effort to improve the quality of the data collected, since there was some difficulty in collection of data from the teachers at baseline.

Data analysis

Activity data were recorded and processed using the accelerometer device software (*Actical* version 2.1.2) to obtain activity counts per minute. All data were analysed and expressed by using PASW (formerly SPSS) software (version 18.0), and Microsoft Excel 2010. Data were checked for normal distribution and for the presence of outliers (± 3 SD from the mean, boxplots) prior to data analysis. No suitable transformation could be found for behavioural data from the CTRS, and therefore non-parametric tests were applied for these variables. One-way (supplementation group as between-subject variable) and two-way (iron and DHA/EPA verum vs. placebo) repeated-measures analysis of covariance (ANCOVA) was conducted on activity data. Cycles and time periods were used as repeated-measures variables, and age and gender as individual level covariates. Treatment effects on endpoint activity (cycle 3) and endpoint

CTRS behavioural data were investigated using one-way and two-way ANCOVA including age, gender and respective baseline activity or behavioural scores as covariates. Treatment effects on endpoint CTRS data was tested using Kruskal-Wallis ANOVA. Within-group differences between time points were assessed using paired t-tests or Wilcoxon matched-pairs test. Bivariate Pearson's or Spearman's correlations (for Conners' data) and multivariate regression analysis were used to study associations between continuous variables. *P* values < 0.05 were considered significant.

Results

Table 1: Subject characteristics at baseline

Variable	All subjects	Activity study subjects
N	321	104
Age (y)	8.9 ± 1.3	8.4 ± 1.0*
Ratio male:female (%)	51:49	50:50
Height (m)	1.28 ± 0.09	1.26 ± 0.8
Weight (Kg)	27.8 (17.9 - 48.1)	27.5 (18.1 - 43.2)
Height-for-age Z-scores	-0.62 ± 1.02	-0.50 ± 1.06
Weight-for-age Z-scores	-0.002 (-2.43 - 23.13)	0.050 (-2.15 - 2.70)
BMI-for age Z-scores	0.46 ± 0.94	0.54 ± .092
Anthropomentric indices [*n*(%)]		
Stunting (HAZ < -2SD)	18 (5.9)	6 (6.1)
Mildly stunted (HAZ < -1SD ≥ -2SD)	100 (32.9)	29 (29.6)
Underweight (WAZ < -2SD > -1SD)	7 (3.0)	1 (1.1)
Overweight (BAZ > 1SD < 2SD)	63 (21.1)	23 (24.0)
Obese (BAZ ≥ 2SD)	20 (6.7)	6 (6.3)
Blood hemoglobin (g/dL)	12.1 ± 0.8	12.1 ± 0.8
Plasma ferritin (µg/L)	19.1 (3.1-73.1)	19 (3.8 - 63.5)
Plasma transferrin receptor (mg/L)	5.7 (2.38-11.75)	5.5 (2.38 - 11.75)
Body iron stores (mg/kg)	3.0 ± 2.4	3.4 ± 2.3
Zinc protoporphyrin (µmol/mol haem)	75.0 (33.0-215.0)	76.0 (39.0 - 215.0)
C-reactive protein (mg/L)	0.35 (0.00-17.80)	0.49 (0.00 - 17.6)
Deficiencies [*n*(%)]		
Anaemia (Hb < 11.5 g/dL)	65 (20.6)	19 (18.6)
Iron deficiency based on PF (< 15.0 µg/L)[4]	78 (28.1)	21 (23.3)
Iron deficiency based on TfR (< 8.3 mg/L)	36 (11.3)	12 (11.5)
Iron deficiency based on ZnPP (>70 µmol/mol haem)	227 (72.3)	75 (75.8)
Shortage of body iron stores (negative values)	30 (10.7)	7 (7.7)
Iron deficiency anaemia (Hb <11.5 g/dL and SF <15 µg/L)	25 (9.2)	7 (8)
Acute-phase protein [*n*(%)]		
C-reactive protein (>5 mg/L)	22 (7.2)	9 (9)

Note: BMI, body mass index; Hb, haemoglobin; PF, plasma ferritin; TfR, transferrin receptor; ZnPP, zinc protoporphyrin; SF, serum ferritin.
*P < 0.001; independent-samples t-test

Table 1 shows the subject characteristics at baseline. Values are shown for all subjects as well as for subjects from the activity study only, which included children from only one of the four schools. Except for age, characteristics of the subjects used in the activity study are similar to those of the entire study population, which indicates that this sample is a good representation of the entire population. Mean age of the activity study population was slightly younger than that of the entire population ($P < 0.001$); this is because the activity study school did not include students from senior primary as the other schools did. No significant differences were found between supplementation groups at baseline. The mean adherence to treatment (observed capsules and tablets swallowed during the 105 d trial) was 95.4% and did not differ between treatment groups.

Spontaneous motor activity

Figure 2 shows mean activity of all subjects (n = 98) at baseline, midpoint and endpoint (cycles 1-3). A significant cycle effect was observed on activity ($P = 0.001$). Also, a significant cycle x age interaction ($P = 0.005$) and a significant cycle x time period x gender interactions ($P = 0.036$) were observed on overall activity (repeated measures). *Class time 1* activity did not change significantly over time. *Break time* activity was significantly lower at endpoint when compared with both baseline and midpoint ($P < 0.0001$). Also, class time 2 activity was significantly higher at endpoint than at baseline and midpoint ($P < 0.0001$, t-tests).

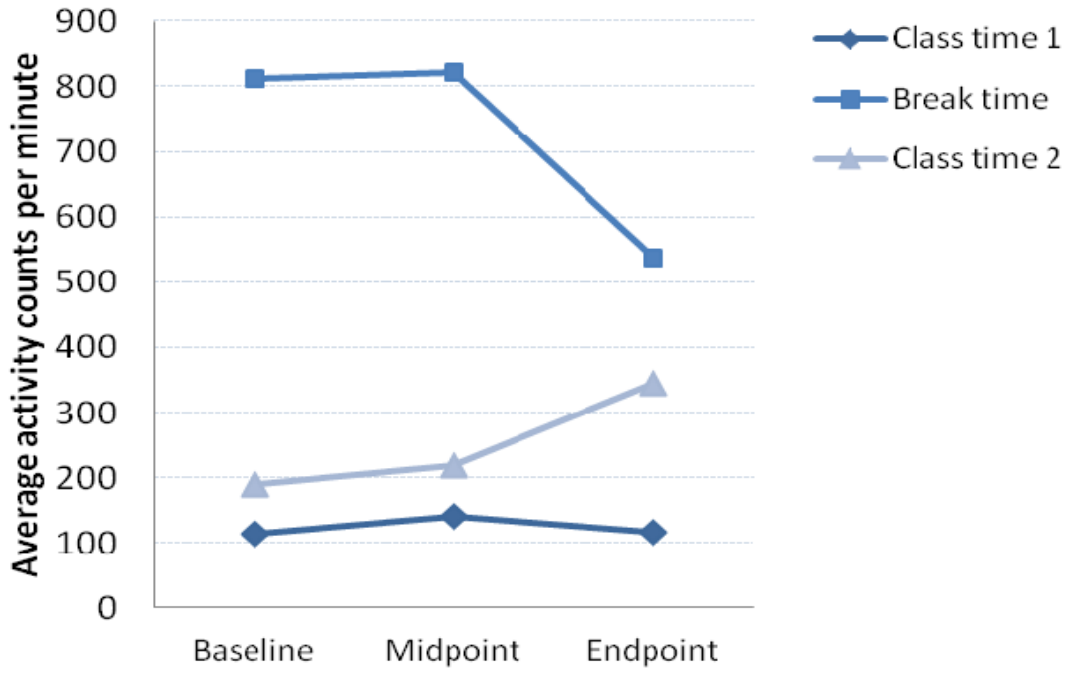

Figure 2: Overall mean activity at baseline, midpoint and endpoint for all groups combined. Cycles and time periods were used as repeated measures variables, and age and gender as individual level covariates.

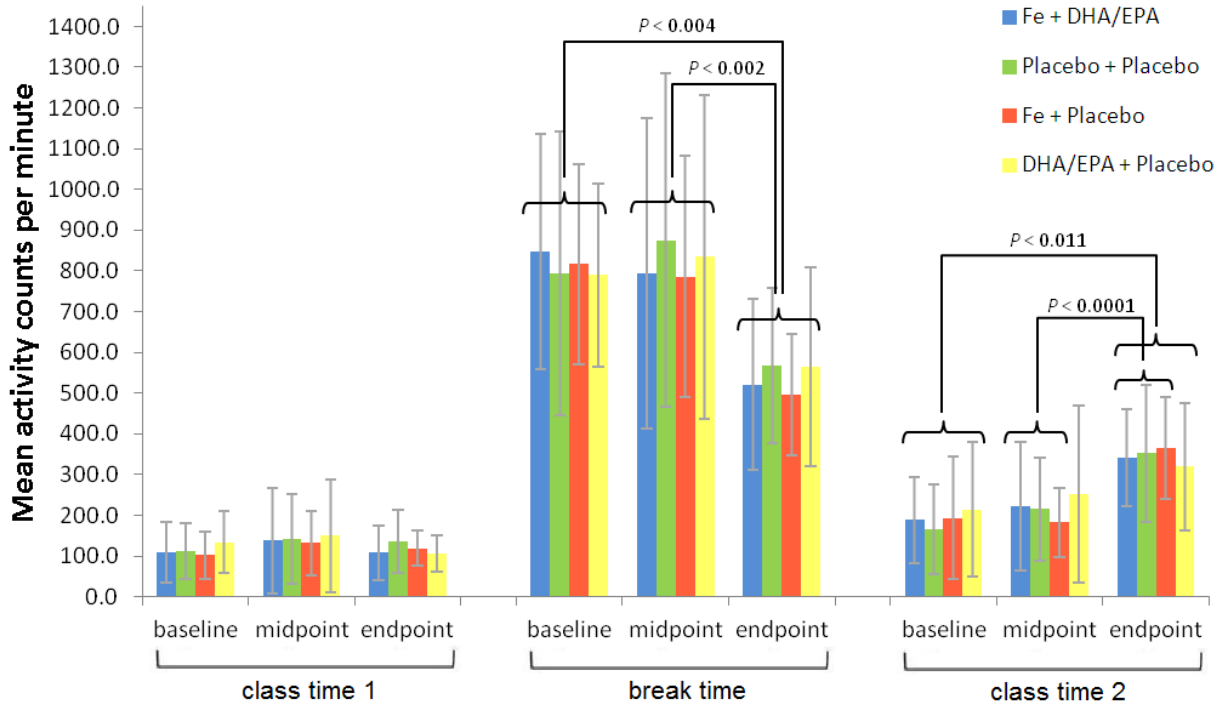

Figure 3: Mean activity for time periods across the study. Cycles and time periods were used as repeated-measures variables, and age and gender as individual level covariates. Dependent samples t-tests were used to observe within-group change between specific cycles (baseline, midpoint and endpoint).

Table 2: Mean activity counts per minute for time periods at all cycles (mean ± SD)

		Group				Significance		
	Cycle	Fe + DHA/EPA (n = 23-24)	Placebo + Placebo (n = 24-25)	Fe + Placebo (n = 21-24)	Placebo + DHA/EPA (n = 21-25)	Fe	EPA/ DHA	Fe x EPA/ DHA
Class time 1	*baseline*	110.6 ± 74.7	112.9 ± 68.7	102.2 ± 59.0	133.5 ± 75.8			
	midpoint	138.6 ± 129.8	142.9 ± 110.7	131.9 ± 79.0	149.6 ± 138.8			
	endpoint	108.1 ± 67.2	135.3 ± 77.2	119.7 ± 44.4	105.0 ± 44.7	*ns*	0.014	*ns*
	Time effect (*P*)	*ns*	*ns*	*ns*	*ns*			
Break time	*baseline*	847.2 ± 287.5	794.0 ± 346.9	817.5 ± 245.3	790.6 ± 224.6			
	midpoint	792.8 ± 381.0	875.7 ± 409.5	786.5 ± 294.8	834.6 ± 397.4			
	endpoint	520.6 ± 209.8	568.7 ± 190.0	496.9 ± 148.9	564.7 ± 244.4	*ns*	*ns*	*ns*
	Time effect (*P*)	<0.0001	<0.0001	<0.0001	0.001			
Class time 2	*baseline*	189.4 ± 105.7	166.4 ± 109.7	193.2 ± 150.2	214.1 ± 164.6			
	midpoint	221.5 ± 158.0	215.9 ± 126.2	183.5 ± 85.0	251.7 ± 218.1			
	endpoint	341.4 ± 118.5	352.9 ± 168.2	365.4 ± 124.8	319.6 ± 155.8	*ns*	*ns*	*ns*
	Time effect (*P*)	<0.0001	<0.001	<0.0001	0.025			

Note: Time effects (cycle effects) are indicated for each time period (repeated measures) and main effects are indicated in the last three columns (two-way ANCOVA). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ns, not significant. Treatment effects on endpoint activity were investigated using one-way and two-way ANCOVA including age, gender and respective baseline activity or behavioural scores as covariates.

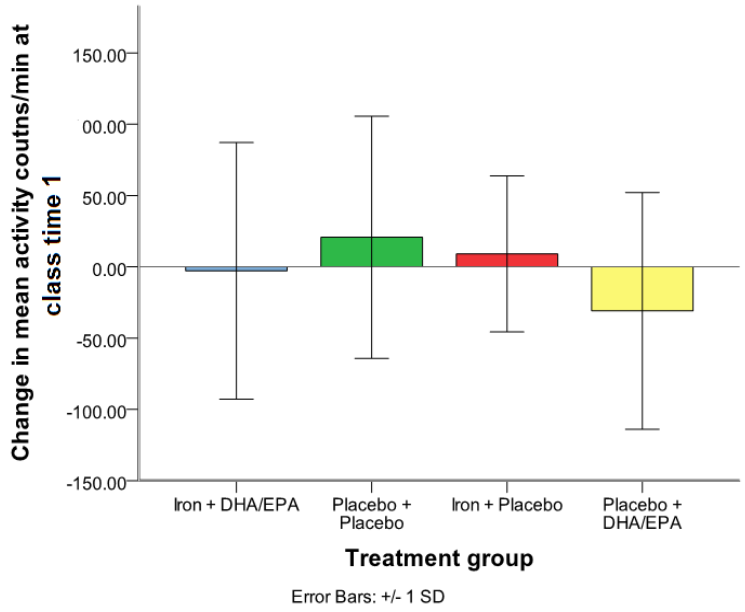

Figure 4: Change in mean activity from baseline to endpoint at class time 1. In a two-way ANCOVA including age, gender and respective baseline activity as covariates, there was a significant main effect of DHA/EPA supplementation for lower class time 1 activity at endpoint (*P* = 0.014).

Mean activity for all time periods at baseline, midpoint and endpoint is shown by supplementation group in *Figure 3* and *Table 2*. *Break time* and *class time 2* both showed cycle effects (repeated measures), and thus differences were investigated using t-tests. Activity of all groups increased significantly from baseline to endpoint for *class time 2.* Interestingly, all groups except the group receiving DHA/EPA + placebo showed a significant increase in activity from midpoint to endpoint at *class time 2* (figure 3). No between group differences or single group intervention effects were found. A two-way repeated measures ANCOVA on endpoint activity controlling for age, respective baseline activity and compliance indicated a trend towards a cycle x DHA/EPA interaction (p = 0.054). A two-way ANCOVA controlling for the same variables showed a significant main effect of DHA/EPA supplementation for lower *class time 1* activity at endpoint (*P* = 0.014; *figure 4*).

Associations between activity and biological iron/FA status indicators were assessed using Pearson's correlations and linear regression analysis. Correlations were only done at baseline and endpoint, as no blood was drawn and no behavioural tests done at midpoint. Log transformed activity count values were used for analysis and transformed iron/FA-status indicators were used when values were not normally distributed. All regression analyses included age, gender and respective baseline activity (only at endpoint) in the model as covariates. All FA status indicators were expressed as percentage of total FAs.

Table 3: Associations with break time activity at baseline

Fe/FA marker	Bivariate correlations		Multivariate linear regressions*		
	r	*P*-value	R^2 (model)	ß	*P*-value
Hb	.217	.034	.147	.231	.020
TfR	-.245	.015	.161	-.258	.008
ZnPP	-.271	.009	.159	-.254	.011
ARA	-.232	.024	.135	-.206	.042

Note: Hb, haemoglobin; TfR, transferrin receptor; ZnPP, zinc protoporphyrin; ARA, arachidonic acid. All multivariate linear regressions have been controlled for age and gender.
*gender was a significant predictor in all models, p < 0.012

Table 4: Associations with class time 2 activity at baseline

Fe/FA marker	Univariate correlations		Multivariate linear regressions		
	r	*P*-value	R^2 (model)	ß	*P*-value
n-6:n-3 FA	.286	.006	.091	-.292	.006

Note: omega-6 fatty acid; n-3 FA; omega-3 fatty acid. All multivariate linear regressions have been controlled for age and gender.

Table 5: Associations with class time 1 activity at endpoint

Fe/FA marker	Univariate correlations		Multivariate linear regressions*		
	r	*P*-value	R^2 (model)	ß	*P*-value
ZnPP	.233	.032	.250	.229	.022
DHA	-.203	.060	.239	-.203	.040
EPA	-.173	.112	.238	-.199	.044
n-6:n-3 FA	.203	.061	.248	.222	.024

Note: ZnPP, zinc protoporphyrin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, n-6 FA; omega-6 fatty acid; n-3 FA; omega-3 fatty acid. All multivariate linear regressions have been controlled for age, gender and respective baseline activity.
*baseline activity was a significant predictor in all models, $p < 0.0001$

At baseline (Table 3), Hb concentration was positively associated with *break time* activity, and iron status indicators transferrin receptor (TfR) and zinc protoporphyrin (ZnPP) were negatively associated with *break time* activity. The n-6 FA indicator arachidonic acid (ARA) was negatively associated with activity at break time. For all these associations, gender was a significant predictor of activity. The only class time association at baseline was observed between the omega-6 (n-6) to n-3 FA ratio value and *class time 2* activity (Table 4). At endpoint, positive associations were found between ZnPP and n-6 to n-3 FA ratio values and activity at *class time 1*; EPA and DHA status values were both negatively associated with activity at *class time 1* (Table 5). For all significant endpoint regressions, respective baseline activity was a significant predictor of endpoint activity (*P* < 0.0001).

ADHD-related behaviour

Table 6 displays the scores of each of the subscales of the CTRS at baseline and endpoint. Subjects in the group receiving both Fe and DHA/EPA supplements showed a significant decreased from baseline to endpoint on the cognitive problems/inattention subscale (*P* = 0.005). Furthermore, hyperactivity scores increased significantly from baseline to endpoint in all groups (*P* = 0.006). No between group differences were observed at endpoint, but a two-way ANCOVA controlling for respective baseline score, age and gender showed a trend towards a significant main effect of DHA/EPA supplementation for reduced scores on the cognitive problems/inattention subscale at endpoint (*P* = 0.062).

For all regressions, age gender and respective baseline (only at endpoint) were entered as covariates in the model. No significant associations between activity and CTRS scores were found a baseline. At endpoint, however, significant associations were observed (refer to Table

7) between *class time 1* activity and all CTRS subscale scores except for the cognitive problems subscale, which bordered significance (correlation, *P* = 0.051; regression, *P* = 0.073). For all significant associations respective baseline activity was a significant predictor of endpoint activity (*P* > 0.015). *Figure 5* shows correlation plots for oppositional, hyperactivity and ADHD index subscales of the CTRS with mean activity counts per minute for *class time 1*.

Table 6: CTRS subscale scores (mean ± SD)

CTRS subscale	Cycle	Group: Fe + DHA/EPA (n = 36-72)	Placebo + Placebo (n =35-71)	Fe + Placebo (n = 33-68)	Placebo + DHA/EPA (n = 31-69)
Opposition					
	baseline	0.83 ± 2.69	0.54 ± 1.63	1.25 ± 2.03	1.00 ± 2.13
	endpoint	1.19 ± 1.87	1.11 ± 2.09	1.91 ± 2.82	1.19 ± 1.99
Cognitive Problems/ Inattention					
	baseline	3.70 ± 3.51	3.11 ± 3.45	4.37 ± 4.67	4.33 ± 4.97
	endpoint	3.08 ± 3.63[1]	3.31 ± 4.36	4.04 ± 4.43	3.27 ± 4.15
Hyperactivity					
	baseline	2.73 ± 4.45	1.97 ± 3.39	2.06 ± 2.94	2.45 ± 3.21
	endpoint	4.86 ± 3.82[2]	4.41 ± 3.15[3]	5.11 ± 3.36[4]	4.33 ± 2.96[5]
ADHD Index					
	baseline	5.83 ± 7.92	3.03 ± 4.96	6.40 ± 6.53	6.03 ± 6.98
	endpoint	5.15 ± 7.29	5.49 ± 7.26	6.62 ± 7.63	5.55 ± 6.79

Note: Significant differences from baseline to endpoint are indicated with superscripts (Wilcoxon matched pairs). SD, standard deviation; CTRS, Conners' Teacher Rating Scale–Revised: Short Forms; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid.
1 p = 0.005; 2 p = 0.006; 3 p = 0.001; 4 p < 0.0001; 5 p = 0.004

Table 7: Associations between class time 1 activity and CTRS subscale scores at endpoint

CTRS subscale	Univariate correlations		Multivariate linear regressions*		
	r_S	*p*-value	R^2 (model)	ß	*p*-value
Opposition	.280	.009	.253	.375	<.0001
Hyperactivity	.419	<.0001	.336	.476	<.0001
Cognitive problems/inattention	.211	.051	.148	.189	.073
ADHD Index	.313	.003	.288	.430	<.0001

CTRS, Conners' Teacher Rating Scale-Revised: Short Forms. ADHD, attention deficit hyperactivity disorder. All multivariate linear regressions have been controlled for age, gender and respective baseline activity.
*baseline activity was a significant predictor, p < 0.015

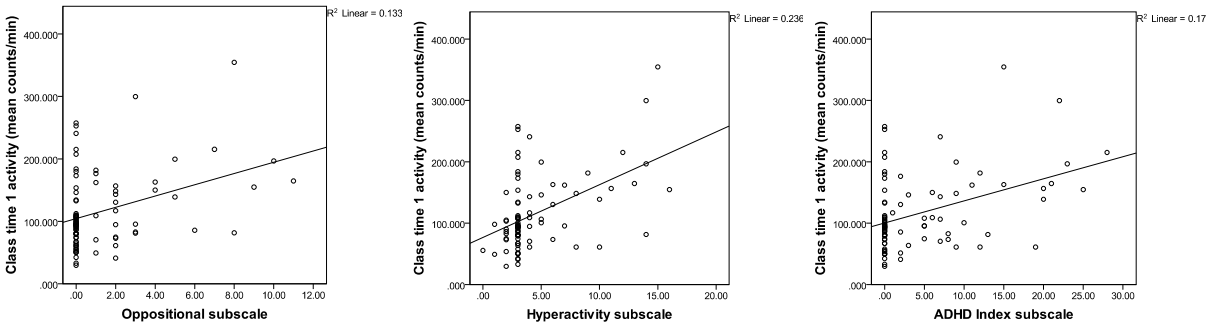

Figure 5: Significant correlations between class time 1 activity and CTRS subscales (Spearman's correlation)

Discussion

To our knowledge, the present study is the first randomized, double blind, placebo-controlled trial to investigate the effects of either Fe or EPA/DHA supplementation on spontaneous activity and/or ADHD-related symptoms in non-ADHD diagnosed ID, school-aged children. The lack of research in this area is quite surprising, since ID/IDA has constantly been associated with reduced motor activity in humans and animals [24,16,26,18,10,19]. Likewise, low n-3 FA status has been associated with behavioural alterations and specifically ADHD-related behaviours in humans and animals [5,6,7,27,28,29,30,31,32].

Even though effects of iron status on spontaneous motor activity have been investigated in observational studies, Angulo-Kinzler *et al*. [10] conducted the only intervention study evaluating the effects of Fe supplementation on spontaneous activity in IDA infants. They found that IDA was associated with reduced motor activity even after 18 months of Fe treatment. In the present study, no group effects were found with iron treatment. However, it should be noted that Angulo-Kinzler *et al*. [10] used non-IDA controls in their study. In this study, all subjects were ID at baseline, and consequently the results are not entirely comparable. Notably, indicators such as higher Hb and lower TfR and ZnPP values, which all indicate better Fe status, were associated with higher spontaneous activity during school break time at baseline – supporting evidence about the relationship between ID/IDA and motor activity. These associations were no longer observable at endpoint, which proposes that the variance in iron status at baseline which made the associations evident was wiped out by the intervention.

Although the relationship between Fe and ADHD-related behaviour has been pointed out by observational studies [20,14], studies on the effects of Fe supplementation on these behaviours are scarce, and the effects have not yet been clearly established. Konofal *et al*. [21] claimed to be the first double-blind, randomized, placebo-controlled trial evaluating the effects of oral administration of iron on ADHD symptoms in ID non-anaemic children with ADHD. Their study found that Fe supplementation decreased ADHD symptoms, but with some of the tests failing to reach significance. In this study, the effects of Fe supplementation alone failed to improve ADHD-related behaviour. Although Fe and DHA/EPA supplementation in combination did show improvement on the cognitive problems subscale, it could not be determined whether this improvement was due to iron supplementation. Moreover, it is important to note that subjects in this study were not ADHD-diagnosed and simply a regular sample of school children.

Levant *et al*. [5,6,7] have demonstrated some activity-behavioural effects related to PUFA and particularly n-3 FAs. These studies have indicated that increased and decreased motor activity could be observed in ALA/n-3 FA deficient rats, depending on the type of environment. Levant

et al. [7] concluded that behavioural observations were consistent with clinical observations of ADHD. There appears to be no existing human studies examining an association between n-3 FA status and/or n-3 FA supplementation and motor activity behaviours. However, several human studies have demonstrated that n-3 FAs are lower in subjects with ADHD [27,28,29], and a few studies have found positive effects of n-3 supplementation on ADHD behaviour [30,31,32]. Subjects supplemented with DHA/EPA in this study demonstrated less motor activity during the *class time 1* period between 08h00 and 10h30, which may reflect on less hyperactivity during this period. It is, however, necessary to consider whether crude measurements of motor activity, as when using an accelerometer, can be compared to an observational measurement such as 'hyperactivity', which is used in the context of ADHD-related behaviour – a matter which will be discussed later.

Class time 1 activity tended to be associated with several FA status indicators at endpoint. Since we would ideally expect children to be less physically active during class times, it was appealing to find that higher DHA/EPA concentrations were significantly associated with calmer behaviour during this period. On the other hand, a higher n-6 to n-3 FA ratio was significantly associated with higher motor activity during this class time period, which may suggest presence of restlessness, squirminess and/or disturbing of classmates. What these associations have suggested corresponds with previous findings from studies assessing FA composition of plasma and red blood cells of ADHD and control subjects [28,29] Even though the associations cannot be used to draw conclusions regarding the effects of the supplementation, the fact that these associations only became evident at endpoint suggests that the intervention may have changed the blood FA concentrations in such a way that associations became evident.

In this study, the hyperactivity subscale on the CTRS significantly increased from baseline to endpoint in all the groups (indicates more hyperactivity). From the activity data it is evident that overall activity (all groups combined) of subjects did not change significantly over time for the *class time 1* period, but that a significant increase of *class time 2* activity was observed at endpoint. It is possible that the increase observed in the hyperactivity subscale could thus be confounded by the overall increase in activity observed at endpoint. It is also likely that a change in behaviour of subjects was present during the *class time 2* period at endpoint, since teachers themselves have plenty of administrative work to take care of at the end of the school year, and they may let the children play or keep themselves busy in the class time at the end of the school day (information from teacher interview).

The CTRS was an important tool used to measure ADHD-related behaviour in this study. Although the purpose of this study was not to diagnose or evaluate ADHD, it has been reported that parent and teacher rating scales are the most efficient method for assessing ADHD

compared with other diagnostic methods. Notably however, it is recommended that both be used in combination [36]. Due to certain logistic and other limitations, it was not possible for our study to include parent questionnaires, and only the teacher rating scales were used. This is a considerable limitation of the study, since at least two intervention studies which have examined effects of n-3 FA supplementation on ADHD behaviour using both the parent and teacher rating scales have found significant results only on the parent rating scales [30,31,32]. It should also be taken into account that baseline CTRS scores were recorded by teachers at the beginning of the school year – when they may not have had enough time to get to know the child well enough to be able to give accurate scores. For this reason it would be safe to say that the endpoint CTRS scores were more reliable than baseline scores.

One of the objectives of this study was to evaluate the use of the accelerometer as a tool for collecting activity data in a behaviour-focussed study, such as the present one. In choosing a device that will be suitable for the kind of data required, it is important to consider whether the device is both valid and reliable [37]. Validity for the use of accelerometers in measurement of motor activity, in a behavioural context such as a classroom setup, has not yet been established. Although the CTRS has been found to have certain limitations, it is a general tool which has been used to measure ADHD behaviour in children for many years [38,39]. Hence, it was of interest in the present study to compare observations of accelerometer data with those of the CTRS. Increased motor activity during class time may reflect on behaviours such as restlessness, squirminess, inability to remain still and distracting others – all of which are ADHD-related behaviours. Accordingly, we expected that children with higher activity scores during class times would also have higher CTRS scores. At endpoint the oppositional, hyperactivity and ADHD index scores were all significantly associated with higher *class time 1* activity, while the cognitive problems subscale showed a trend towards significant association. This proposes that the use of an accelerometer could be helpful in assessing ADHD-related behaviour of school children – even during class time.

Trost *et al*. [37] describe that using accelerometers in field-based research is “not a plug and play proposition”. Indeed, using the accelerometers in this study did require careful, comprehensive planning and raised a certain number of important questions about a) the type of device used; b) how long the device should be worn for; c) how and where the device should be positioned on the body; d) how the devices needed to be distributed and collected from subjects; e) the epoch length, unit of measurement used, how the measurements needed to be gathered from the devices and stored; and f) the amount of days/weeks needed to collect comprehensive measurements. Unlike the CTRS, which is based on observation, the accelerometer is able to provide a tangible and unbiased measurement of motor activity. This means that, if used appropriately, the accelerometer can provide accurate and reliable data.

Though the accelerometer is able to provide an unbiased measurement, it is obvious that the measurements may certainly be subject to influence by confounders such as human intervention, seasonal variability and physical environment. Ridgers *et al.* [40] investigated day-to-day and seasonal variability of physical activity during school recess in 19 school children aged 6-11 years. They observed a trend for physical activity levels to be higher in winter than summer, although this trend was not significant. Similarly, our study found break time activity to be higher during autumn (baseline) and winter (midpoint) than during spring (endpoint). The physical environment of subjects was either the classroom or the school grounds, which included large open spaces in which they could move around. All subjects in the activity study were in the same school and therefore the impact of physical environment was the same for all subjects.

In the present study it was also observed that activity overall showed large variation. Since it was not possible for us to observe all the subjects during the school day, we were not able to know what activity-impacting tasks may have been expected of them. However, being around the school area on a daily basis during data collection periods provided the opportunity for us to make certain observations which may have impacted the data. Firstly, it was noted that unconventional or out-of-class activities were sometimes performed during the *class time 2* period, and that activities varied from classroom to classroom during this time. The *class time 1* period seemed to be a more academically-focussed period. In addition to the fact that recording time of the *class time 1* period was more than twice as long as the *class time 2* period, this observation may clarify or explain why more consistent observations were made during *class time 1*. On the other hand, it should be taken into consideration that our study may have lacked adequate power to show stronger or more results.

Considering the explorative nature of the study as well as its limitations and possible influences, the present study may serve as groundwork for further exploration in larger studies. Findings of this study suggest that there appears to be definite link between iron status and spontaneous motor activity which may or may not be altered by iron supplementation. Activity measurements obtained from use of the accelerometer was able to demonstrate intervention effects of DHA/EPA supplementation on activity. It has thus been shown that accelerometer-based data is sensitive enough to pick up behaviours in a classroom setup that corresponded with the CTRS. In addition to showing good consistency with a generally used behavioural assessment tool, this suggests that the accelerometer can be used to measure activity in a behavioural context. The accelerometer was also able to pick up seasonal variability in activity.

In conclusion, the present study has found some evidence supporting the proposed link between n-3 FAs and behaviour associated with ADHD. Iron status was found to have an impact on spontaneous motor activity during school break time when subjects could move around freely. Overall, the small sample size of the study was limiting, and thus no definite conclusions could be drawn regarding the effects of iron and n-3 FA supplementations *per se*. Observations made regarding the use of the accelerometer in the behavioural context of the study can be viewed as useful information for future trials.

References

[1] Labadarios D, Louw R. Selected micronutrient status: Iron status. In: Labadarios D, National Food Consumption Survey-Fortification Baseline (NFCS-FB): South Africa, 2005. Tygerberg: University of Stellenbosh; 2007. p. 447-55.

[2] Oelofse A, Faber M, Benade JG, Kenoyer DG. The nutritional status of a rural community in KwaZulu-Natal, South Africa: the Ndunakazi Project. Cent Afr J Med 1999;45:14-9.

[3] Mayet FG, Schutte CH, Reinach SG. Anaemia among the inhabitants of a rural area in northern Natal. S Afr Med J 1985;67:458-62.

[4] McCann JC, Ames BN. An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. Am J Clin Nutr 2007;85:931-45.

[5] Levant B, Ozias MK, Carlson SE. Sex-specific effects of brain LC-PUFA composition on locomotor activity in rats. Physiol Behav 2006;89:196-204.

[6] Levant B, Radel JD, Carlson SE. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. Behav Brain Res 2004;152:49-57.

[7] Levant B, Zarcone T, Fowler S. Developmental effects of dietary n-3 fatty acids on activity and response to novelty. Physiol Behav 2010;101:176-83.

[8] Richardson AJ. Long-chain polyunsaturated fatty acids in childhood developmental and psychiatric disorders. Lipids 2004;39:1215-22.

[9] Richardson AJ, Puri BK. The potential role of fatty acids in attention-deficit/hyperactivity disorder. Prostaglandins, Leukotrienes Essent Fat Acid 2000;63:79-87.

[10] Angulo-Kinzler R, Peirano P, Lin E, Garrido M, Lozoff B. Spontaneous motor activity in human infants with iron-deficiency anemia. Early Hum Dev 2002;66:67-70.

[11] Bertenthal B, Campos J. A systems approach to the organizing effects of self-produced locomotion during infancy. Advance Infancy Res 1990;6:1-60.

[12] Black JE, Jones TA, Nelson CA, Greenough WT. Neural plasticity and developing brain. In: The Handbook of Child and Adolescent Psychiatry. NY: Wiley; 1998. p. 31–53.

[13] Deinard A, Gilbert A, Dodds M, Egeland B. Iron deficiency and behavioral deficits. Pediatrics 1981;68:828-33.

[14] Konofal E, Cortese S, Lecendreux M, Arnulf I, Mouren MC. Effectiveness of iron supplementation in a young child with attention-deficit/hyperactivity disorder. Pediatrics 2005;116:e732-4.

[15] Golub MS, Hogrefe CE, Germann SL, Capitanio JP, Lozoff B. Behavioral consequences of developmental iron deficiency in infant rhesus monkeys. Neurotox Teratol 2006;28:3-17.

[16] Felt BT, Lozoff B. Brain iron and behavior of rats are not normalized by treatment of iron deficiency anemia during early development. J Nutr 1996;126:693.

[17] Beard JL, Erikson KM, Jones BC. Neurobehavioral analysis of developmental iron deficiency in rats. Behav Brain Res 2002;134:517.

[18] Beard JL, Felt B, Schallert T, Burhans M, Connor JR, Georgieff, MK. Moderate iron deficiency in infancy: Biology and behavior in young rats. Behav Brain Res 2006;170:224-32.

[19] Wang J, Huo J, Sun J, Ning Z. Physical performance of migrant schoolchildren with marginal and severe iron deficiency in the suburbs of Beijing. BES 2009;22:333-9.

[20] Juneja M, Jain R, Singh V, Mallika V. Iron deficiency in Indian children with attention deficit hyperactivity disorder. Indian pediatrics 2010;47:955-8.

[21] Konofal E, Lecendreux M, Deron J, Marchand M, Cortese S, Zaïm M, Mouren MC, Arnulf I. Effects of iron supplementation on attention deficit hyperactivity disorder in children. Pediatric Neurol 2008;38:20-6.

[22] Edgerton VR, Bryant SL, Gillespie CA, Gardner GW. Iron deficiency anemia and physical performance and activity of rats. J Nutr 1972;102:381-99.

[23] Glover J, Jacobs A. Activity pattern of iron-deficient rats. Br Med J 1972;2:627-68.

[24] Hunt J, Zito C, Erjavec J, Johnson L. Severe or marginal iron deficiency affects spontaneous physical activity in rats. Am J Clin Nutr 1994;59:413-18.

[25] Felt B, Beard J, Schallert T, Shao J, Aldridge J, Connor J, Georgieff M, Lozoff B. Persistent neurochemical and behavioral abnormalities in adulthood despite early iron supplementation for perinatal iron deficiency anemia in rats. Behav Brain Res 2006;171:261-70.

[26] Piñero D, Jones B, Beard J. Variations in dietary iron alter behavior in developing rats. J Nutr 2001;131:311-18.

[27] Mitchell EA., Aman MG, Turbott, SH, Manku M. Clinical characteristics and serum essential fatty acid levels in hyperactive children. Clin Pediatrics 1987;26:406-11.

[28] Stevens LJ, Zentall SS, Abate ML, Kuczek T, Burgess JR. Omega-3 fatty acids in boys with behavior, learning, and health problems. Physiol behav 1996;59:915-20.

[29] Antalis CJ, Stevens LJ, Campbell M, Pazdro R, Ericson K, Burgess JR. Omega-3 fatty acid status in attention-deficit/hyperactivity disorder. Prostaglandins, Leukotrienes Essential Fat Acid 2006;75:299-308.

[30] Sinn N, Bryan J. Effect of supplementation with polyunsaturated fatty acids and micronutrients on learning and behavior problems associated with child ADHD. J Develop Behav Pediatrics 2007;28:82-91.

[31] Bélanger SA, Vanasse M, Spahis S, Sylvestre M, Lippé S, L'Heureux F et al. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. Paediatrics Child Health 2009;14:89-98.

[32] Johnson M, Ostlund S, Fransson G, Kadesjö B, Gillberg C. Omega-3/omega-6 fatty acids for attention deficit hyperactivity disorder: A randomized placebo-controlled trial in children and adolescents. J Atten Disord 2009;12:394-401.

[33] Epstein JN, March JS, Conners CK, Jackson DL. Racial differences on the Conners Teacher Rating Scale. J Abnorm Child Psychol 1998;26:109-118.

[34] Conners CK, Sitarenios G, Parker JDA, Epstein JN. Revision and restandardization of the Conners Teacher Rating Scale (CTRS-R): Factor structure. J Abnorm Child Psychol 1998;26:279.

[35] American Psychiatric Association. Diagnostic and statistical manual of mental disorders (DSM-IV). 4th ed. Washington, DC: Author; 1994.

[36] Pelham WEJ, Fabiano GA, Massetti GM. Evidence-based assessment of attention deficit hyperactivity disorder in children and adolescents. J Clin Child Adol Psychol 2005;34:449–76.

[37] Trost SG, Mciver KL, Pate RR. Conducting accelerometer-based activity assessments in field-based research. Med Sci Sport Exercise 2005;37:S531-43.

[38] Charach A, Chen S, Hogg-Johnson S, Schachar RJ. Using the Conners' Teacher Rating Scale–Revised in school children referred for assessment. Can J Psychiatry 2009;54:232-41.

[39] Charach A, Yeung E, Climans T. Lithe E. Childhood Attention-deficit/hyperactivity disorder and future substance use disorders: comparative meta-analyses'. J Am Academy Child Adol Psychiatry 2001;50:9-21.

[40] Ridgers ND, Stratton G, Clark E, Fairclough SJ, Richardson DJ. Day-to-day and seasonal variability of physical activity during school recess. Preventive Med 2006;42:372-4.

Chapter IV – Conclusions and Recommendations

Introduction

Key objectives of the study were 1) to investigate the effects of Fe and n-3 FA on spontaneous motor activity and ADHD-related behaviour in ID children, 2) to assess school time spontaneous motor activity of primary school children over time, and 3) to evaluate the Actical accelerometer as a tool for assessing break and class time motor activity in a behavioural context. To achieve this, physical activity measurements of school children were recorded during school time using an accelerometer (A*ctical*). ADHD-related behaviour of children was assessed using a generalised teachers' questionnaire. Time effects were calculated using repeated measures and t-tests. Associations between supplementation, activity and ADHD-related behavioural were assessed.

The purpose of this chapter is to draw general conclusions and provide recommendations for future investigations involving Fe or n-3 FA supplements and/or accelerometers in a clinical, setting and behavioural context.

Main findings

Spontaneous motor activity of primary school children during class time was lower in subjects supplemented with long-chain polyunsaturated FAs, DHA and EPA than in subjects who were not. Additionally, DHA and EPA were negatively associated with motor activity during class time, and n-6 to n-3 FA ratio was positively associated with classroom motor activity. Since activity was also positively associated with ADHD-related behaviour during class time, the results support evidence for a relationship between n-3 FAs status and ADHD.

Fe status was found to have an impact on spontaneous motor activity by positive association between Fe status and activity during school break time. Break time was a time during which subjects were allowed to move around freely as they wished and thus if a child was moving around less it may be due to reduced aerobic or endurance capacity, or lower energetic efficiency – all which have often been associated with poor Fe status (Haas and Brownlie, 2001).

The *Actical* accelerometer was found to be a good complementary tool for use in detecting activity patterns over time and for assessing of activity in a behavioural sense. Accelerometer readings also showed ability to pick up gender differences and seasonal changes in activity. The accelerometer was found to be a helpful tool for detecting classroom and break time activity.

Conclusions

Fe and FA status both influence child behaviour. Children with poor n-3 FA status may be at risk for ADHD and supplementation with DHA/EPA may improve ADHD symptoms. The *Actical* accelerometer can be used as a tool for measuring both activity and behavioural outcomes in school children. Further research in larger trails is needed to establish a relationship between iron and spontaneous motor activity.

General Recommendations

In views of the above-summarized results and in line with current evidence from previous studies, the following recommendations are therefore made:

1. **Investigate effects of n-3 FA supplementation on ADHD in larger trails**
   In the present study a main effect of DHA/EPA supplementation was found for lower ADHD-related behaviour in a population with unknown ADHD prevalence. Previous observational studies have confirmed the coexistence of ADHD symptoms and poor n-3 FA status (Schuchard *et al*., 2010). A few intervention trails have found some positive effects for n-3 FA treatment on ADHD-diagnosed children or children with high frequency of ADHD-symptoms prevalence, but sample size of trails have been small, and thus further investigation in larger trails is necessary.

2. **Guidance for use of the Conners' Rating Scales**
   The CRS include two parts – the CPRS (parent) and CTRS (teacher) rating scales. For the present study, due to logistical limitations, only the CTRS was used. The CPRS and CTRS should ideally be used together for a more comprehensive assessment of child behaviour (Sinn and Bryan, 2007). Using the combination of the two scales together with measurement of spontaneous motor activity would provide a better overview of child behaviour related to ADHD.

3. **Assess all day spontaneous motor activity**
   Spontaneous motor activity of school children was a useful measure in assessing child activity behaviour in the study. The only time during which voluntary or free-living activity was measured in the present study was during the 30 minutes in which school break time took place. This short time period of measurement was already sufficient to pick up certain notions in voluntary activity. In the current study statistical analyses were done using activity counts per minute. However, future studies should obtain information on specific activity levels within this population group to additionally perform stratification of physical activity intensity.

Extending the recording time of activity measurement beyond school time could provide additional benefits, stronger results and a more comprehensive assessment of voluntary activity in subjects.

4. **Investigate effects of n-3 FA and iron supplementation on ADHD-diagnosed children; treat with of multi-micronutrients**

   Subjects used in this study were iron-deficient, non ADHD-diagnosed primary school children. Scores from the ADHD behaviour-assessing tool were simply used for comparisons, and not for diagnosis of ADHD. Previous studies assessing the effects of iron and n-3 FA supplements were conducted using ADHD-diagnosed subjects/children with high scores for prevalence of ADHD symptoms. This makes it difficult for comparison of our results to previous studies, and future studies should consider using ADHD-diagnosed subjects – particularly for investigation of the effects of iron supplementation, since this is an area in the research which is lacking.

   It is well-known that micronutrient deficiencies co-exist. A limitation of this study may have been that the iron was the only micronutrient treatment which children received after detection of ID. Other micronutrient deficiencies may have been existing and not treated by the intervention of the study, which may have confounded the results. Thus, it may be recommended that future studies incorporate multi-micronutrient treatment.

References

HAAS, J.D. & BROWNLIE IV, T. 2001. Iron deficiency and reduced work capacity: A critical review of the research to determine a causal relationship. *Journal of nutrition,* 131(2):676S.

SCHUCHARDT, J.P., HUSS, M., STAUSS-GRABO, M. & HAHN, A. 2010. Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. *European journal of pediatrics,* 169(2):149-164.

SINN, N. & BRYAN, J. 2007. Effect of supplementation with polyunsaturated fatty acids and micronutrients on learning and behavior problems associated with child ADHD. *Journal of developmental and behavioral pediatrics,* 28(2):82-91.

ADDENDUM

Appendix A

Example of the Conners' Teacher Rating Scale-Revised: Short Form

Figure 4.1
Sample Response Sheet for the CTRS–R:S

Conners' Teacher Rating Scale - Revised (S)

by C. Keith Conners, Ph.D.

Child's Name: John Smith **Gender:** (M) F

Birthdate: 08 / 17 / 86 (Month / Day / Year) **Age:** 10 **School Grade:** 4

Teacher's Name: Mary Adams **Today's Date:** 11 / 03 / 96 (Month / Day / Year)

Instructions: Below are a number of common problems that children have in school. Please rate each item according to how much of a problem it has been in the last month. For each item, ask yourself, "How much of a problem has this been in the last month?", and circle the best answer for each one. If none, not at all, seldom, or very infrequently, you would circle 0. If very much true, or it occurs very often or frequently, you would circle 3. You would circle 1 or 2 for ratings in between. Please respond to each item.

	NOT TRUE AT ALL (Never, Seldom)	JUST A LITTLE TRUE (Occasionally)	PRETTY MUCH TRUE (Often, Quite a Bit)	VERY MUCH TRUE (Very Often, Very Frequent)
1. Inattentive, easily distracted	0	(1)	2	3
2. Defiant	0	1	(2)	3
3. Restless in the "squirmy" sense	0	(1)	2	3
4. Forgets things he/she has already learned	(0)	1	2	3
5. Disturbs other children	0	1	(2)	3
6. Actively defies or refuses to comply with adults' requests	0	1	(2)	3
7. Is always "on the go" or acts as if driven by a motor	0	(1)	2	3
8. Poor in spelling	(0)	1	2	3
9. Cannot remain still	(0)	1	2	3
10. Spiteful or vindictive	0	(1)	2	3
11. Leaves seat in classroom or in other situations in which remaining seated is expected	0	(1)	2	3
12. Fidgets with hands or feet or squirms in seat	0	(1)	2	3
13. Not reading up to par	(0)	1	2	3
14. Short attention span	(0)	1	2	3
15. Argues with adults	0	(1)	2	3
16. Only pays attention to things he/she is really interested in	0	(1)	2	3
17. Has difficulty waiting his/her turn	(0)	1	2	3
18. Lacks interest in schoolwork	0	(1)	2	3
19. Distractibility or attention span a problem	0	(1)	2	3
20. Temper outbursts; explosive, unpredictable behavior	0	1	2	(3)
21. Runs about or climbs excessively in situations where it is inappropriate	0	(1)	2	3
22. Poor in arithmetic	(0)	1	2	3
23. Interrupts or intrudes on others (e.g., butts into others' conversations or games)	0	(1)	2	3
24. Has difficulty playing or engaging in leisure activities quietly	0	(1)	2	3
25. Fails to finish things he/she starts	0	(1)	2	3
26. Does not follow through on instructions and fails to finish schoolwork (not due to oppositional behavior or failure to understand instructions)	0	(1)	2	3
27. Excitable, impulsive	(0)	1	2	3
28. Restless, always up and on the go	(0)	1	2	3

Appendix B

Ethical Approval from the NWU

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: (018) 299-4900
Faks: (018) 299-4910
Web: http://www.nwu.ac.za

Ethics Committee
Tel +27 18 299 4850
Fax +27 18 293 5329
Email Ethics@nwu.ac.za

Prof CM Smuts

Dear Prof Smuts

21 Oktober 2008

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: The effect of iron and DHA supplementation, alone and in combination, on cognition: a radomized, double-blind, 2x2 intervention trial in SA children

Ethics number:

N	W	U	-	0	0	6	1		-	0	8	-	A	1
Institution				Project Number					Year				Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 10 September 2008 **Expiry date**: 09 September 2013

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof MMJ Lowes
(chair NWU Ethics Committee)

Prof HH Vorster
(Chairman: NWU Ethics Committee: Author)

Appendix C

Authors guidelines: Physiology and Behaviour

Guide for Authors

Page charges

This journal has no page charges.

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal offers you the option of making your article freely available to all via the ScienceDirect platform. To prevent any conflict of interest, you can only make this choice after receiving notification that your article has been accepted for publication. The fee of $3,000 excludes taxes and other potential author fees such as color charges. In some cases,

institutions and funding bodies have entered into agreement with Elsevier to meet these fees on behalf of their authors. Details of these agreements are available at http://www.elsevier.com/fundingbodies. Authors of accepted articles, who wish to take advantage of this option, should complete and submit the order form (available at http://www.elsevier.com/locate/openaccessform.pdf). Whatever access option you choose, you retain many rights as an author, including the right to post a revised personal version of your article on your own website. More information can be found here: http://www.elsevier.com/authorsrights.

Language and language services

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who require information about language editing and copyediting services pre- and post-submission please visit http://webshop.elsevier.com/languageservices or our customer support site at http://support.elsevier.com for more information.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

As part of the submission process your paper may be screened for English language usage and conformity to the guide for authors before it reaches the review stage. This is to ensure the journal's high standards are maintained and the review process is kept to a minimum. Passing this check is not a guarantee that your submission will subsequently proceed to the peer review process, which is a decision to be made at the sole discretion of the journal editor.

If you are asked to revise your manuscript during the review stage please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. The standard deadline for authors to submit the revision is 5 weeks. If you require a time extension to return the revision you should state the reasons for this extension and advise on a more feasible date for returning it.

Submission Address

http://ees.elsevier.com/phb/

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

- Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
- Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be

avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Drugs

Proprietary (trademarked) names should be capitalized. The chemical name should precede the trade, popular name, or abbreviation of a drug the first time it occurs.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.). Please seek permission from individuals before acknowledging them in your article.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Database linking and Accession numbers

Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.

Authors should explicitly mention the database abbreviation (as mentioned below) together with the actual database number, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.

Please use the following format: Database ID: xxxx

Links can be provided in your online article to the following databases (examples of citations are given in parentheses):

- GenBank: Genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: BA123456)
- PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)
- OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Anesthesia

In describing surgical procedures on animals, the type and dosage of the anesthetic agent should be specified. Curarizing agents are not anesthetics; if these were used, evidence must be provided that anesthesia of suitable grade and duration was employed.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as 'graphics' or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.
Please do not:

- Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive

information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Text graphics

Text graphics may be embedded in the text at the appropriate position. Further, high-resolution graphics files must be provided separately whether or not the graphics are embedded. See further under Electronic artwork.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9.
Reference to a book:
[2] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:
[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also http://www.nlm.nih.gov/bsd/uniform_requirements.html).

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Submission checklist

- The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
- Ensure that the following items are present:
- One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address
- Telephone and fax numbers

- All necessary files have been uploaded, and contain:
- Keywords
- All figure captions
- All tables (including title, description, footnotes)
- Further considerations
- Manuscript has been 'spell-checked' and 'grammar-checked'
- References are in the correct format for this journal
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web)
- Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
- If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes
- For any further information please visit our customer support site at http://support.elsevier.com.

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. The correct format for citing a DOI is shown as follows (example taken from a document in the journal Physics Letters B):

doi:10.1016/j.physletb.2010.09.059

When you use the DOI to create URL hyperlinks to documents on the web, the DOIs are guaranteed never to change.

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html.

If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately – please let us have all your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use.

For inquiries relating to the submission of articles (including electronic submission) please visit this journal's homepage. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher. You can track accepted articles at http://www.elsevier.com/trackarticle. You can also check our Author FAQs (http://www.elsevier.com/authorFAQ) and/or contact Customer Support via http://support.elsevier.com.