3. RESEARCH DESIGN AND PROTOCOLS

3.1. INITIATION AND APPROVALS:

The joint bilateral Indo-US research study entitled “Study of Lead Exposure and Outcomes Amongst Children in Chennai, India” was technically approved for funding by the Fogarty International Center of the National Institutes of Health (NIH) and was launched in 2003.

This joint research study encompassed three components on the whole:

1. Neuropsychological and Behavioral Component;
2. Environmental Component and

This current thesis focuses on the research work related to the first component, conducted on the neuropsychological and behavioral aspects of the above-mentioned study.

Since this study involves invasive procedures, proper proposals along with detailed explanation of the various protocols were submitted to the respective authorities and approvals were obtained from the parent technical agency – Indian Council of Medical Research (ICMR), Inter-Ministerial and Health Ministry Screening Committee and the Medical Ethics Committee of Sri Ramachandra University. Informed written consents were also obtained from the participating subjects’ parents or guardians. Parent and Teacher Questionnaires, batteries of neuropsychological tests were translated into Tamil and back-translations were also approved by the respective authorities. The questionnaires and the neuropsychological testes were validated under large-scale field conditions with the assistance of trained clinical neuropsychologists.
3.2. RESEARCH DESIGN:

The design of this study was a cross-sectional epidemiological study of children aged 3-7 years attending specific public schools in the area of Chennai. These schools were chosen to represent areas of the city that are low traffic/low industry; low traffic/high industry; high traffic/low industry and high traffic/high industry. The sample size was calculated to be 750 subjects. This sample size calculation was based on other international studies that used similar design, in terms of measures of exposure, measures of outcomes, covariates to be controlled for, socioeconomic status of children to be studied, selection criteria in relation to neurobehavior in early school age children and which found significant negative relationship despite having sample sizes lower than or similar to the one employed in this investigation (Needleman et al., 1979 (n=158); Lyngbye et al., 1990 (n=400), Rabinowitz et al., 1991 (n=744)).

3.3. MODE OF SELECTION OF SCHOOLS:

The distribution of industries in Chennai city was obtained from the respective Government Authorities. The industries were classified into the following three types based on the type of industrial processes carried out in the industries and the nature of the industrial/hazardous wastes:

1. Red category industries
2. Orange category industries and
3. Green category industries.
Fig. 3: Industrial and Traffic Density Zones in Chennai, India

<table>
<thead>
<tr>
<th>Place</th>
<th>Area in sq. kms</th>
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<tr>
<td>Tondiarpet</td>
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<tr>
<td>Kodungaiyur</td>
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<td>Perambur</td>
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<td>Sembiyam</td>
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<td>Purasawalkam</td>
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<td>Arumbakkam</td>
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<td>Kodambakkam</td>
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<td>T. Nagar</td>
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<td>K. K. Nagar &amp; Ashok Nagar</td>
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<tr>
<td>Guindy</td>
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</tr>
<tr>
<td>Velacheri</td>
<td>1.5</td>
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<tr>
<td>Taramani</td>
<td>1.5</td>
</tr>
<tr>
<td>Besant Nagar &amp; surroundings</td>
<td>9</td>
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<tr>
<td>Chennai City</td>
<td>174</td>
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Traffic density throughout Chennai city was obtained from the various traffic units found in the city. Based on the distribution of different category industries and the traffic density in Chennai city, the localities that fall under the following zones were earmarked:

1. Low industry, low traffic zone;
2. Low industry, high traffic zone;
3. High industry, low traffic zone;
4. High industry, high traffic zone.

The surface areas of all such earmarked localities were found out from the Corporation of Chennai and were mapped on the map of Chennai city. (Fig. 3)

A survey of schools in Chennai city was done and a list of the following primary, middle, high and higher secondary schools was prepared:

1. Government Schools
2. Corporation Schools
3. Adi-Dravidar Welfare Schools
4. Aided Schools
5. Unaided Schools
6. Matriculation Schools
7. Anglo-Indian Schools
8. Kendriya Vidyalaya Schools
9. Central Board of Senior Schools
10. Special Schools

Out of the above-mentioned list of schools, the matriculation schools were selected for the study as the matriculation schools had the following advantages over the other schools:
1. The matriculation schools serve children who are likely to be in relatively stable households from a range of socioeconomic backgrounds but centered mainly on the middle-class.

2. The number of matriculation schools was more than the number of any other category of schools and the locations of these schools were widespread, spanning the entire area of Chennai city.

3. All the matriculation schools had a common Head, the Director of Matriculation Schools. Hence it was easy to approach and get prior permission for the study.

4. There was a good distribution of matriculation schools in the localities of interest for the study.

5. Most of the matriculation schools supported a good strength of students in the primary classes.

3.4. IDENTIFICATION OF STUDY SUBJECTS:

A detailed list of matriculation schools in the selected localities was prepared and the Principal and/or the Management of the schools were approached to seek permission for the study.

The School Managements that accepted to participate in the study were mapped on the map of Chennai city to describe the spread of schools chosen. (Fig. 4)

Single-page fliers (Annexure-I) entitled “Lead exposure poses a major environmental health problem worldwide” in both English and the vernacular language Tamil were distributed to all the schools that have agreed to co-operate for the study.
Fig. 4: Location of schools that accepted to participate in the study
The fliers were in turn, sent to the parents of children studying in primary classes in those schools. The fliers outlined the importance of lead poisoning awareness and invited the parents for a PTA meeting on a specific day within the school premises.

On the day of PTA meeting, detailed explanation about the entire study was given to both the parents and the teachers in English and Tamil. The purpose of the study, existing conditions in Chennai, the various modes of exposure to lead, susceptibility of children to lead poisoning, the effects of lead poisoning on children, the various methods employed to determine the IQ and visual-motor abilities of the children, aseptic conditions and the mode of blood draw and other minor details were clearly elucidated. All the queries were clarified and finally the “Parental Consent Form” (Annexure-II) entitled “Lead exposure, nutrition, genetics, child development and dental caries study” was read out in both English and Tamil to the parents. Then, informed consent was obtained from each child’s primary caregiver and the child was enrolled as a study subject (Fig. 4).

3.5. COLLECTION OF PRIMARY SOCIODEMOGRAPHIC DATA:

Data was then collected from each child’s parent or primary caregiver (mother of the child in most cases) using an extensive questionnaire (Annexure-III) covering topics related to the child’s birth history, birth rank and infant-feeding history, child’s age at testing, gender and standard in school, parents’ status, education and occupation, socio-economic background of the family (assessed with the Kuppuswamy’s Socio-Economic Status Scale (Urban), revised in 2001 to reflect current wage scales), maternal age at delivery, family size, living conditions (overall health, food habits, intake of alcohol, passive smoking), nutritional and dietary habits of the child (intake of milk, red meat, fish, eggs, ragi,
jaggery, green vegetables and fruits), use of dietary supplements (calcium and iron), medical details (use of ayurvedic, herbal, homeopathic and other alternative medicines) and environmental surroundings of the child (industrial exposure, traffic exposure, hobbies and residential exposure from paints and play toys).

Fig. 5: Some of the children enrolled as study subjects

3.6. MEASUREMENT OF VISUAL-MOTOR ABILITIES AND I.Q.:

The Wide Range Assessment of Visual Motor Abilities (WRAVMA) Test and the Binet-Kamath I.Q. Test were implemented on each study subject individually in a small, quiet, separate and comfortable room within the school premises. The examiners who conducted the assessments were blind to all aspects of the children’s lead exposure and developmental histories.
At the end of the testing all children were given a small and attractive safe case filled with stationary and other useful items as a souvenir (Fig. 5).

**Fig. 5: Souvenir being presented to a child**

**3.6.1. WIDE RANGE ASSESSMENT OF VISUAL MOTOR ABILITIES TEST:**

The 1995 Wide Range Assessment of Visual Motor Abilities (WRAVMA) Test provides psychometrically sound and quick assessments of visual-motor, visual-spatial and fine motor skills, with the norms for each measure derived from the same standardization sample. This creates a test battery for clinicians and researchers that will permit a psychometrically sound comparison of a given child’s overall visual-motor ability. The multiple tests of the WRAVMA also broaden the conceptualization of visual-motor ability by providing measurement of three moderately related components involved in most visual-motor tasks.
As far as WRAVMA’s relationship with cognitive ability is concerned, its visual-motor skills are positively associated with general cognitive ability and the matching sub-test is likely to correlate more highly with general cognitive ability than the other two sub-tests. WRAVMA also correlates moderately with reading, spelling and arithmetic abilities. All WRAVMA standard scores adhere to traditional psychometric properties, with a mean of 100 and standard deviation of 15. No statistical differences were found between male and female performance on any of the three WRAVMA tests and there were also no ethnic differences on item difficulty.

The WRAVMA assessed three important aspects of visual-motor functioning using three tests. These three aspects have been selected because of their relevance to school-related activities:

1. The Drawing Test measures integrated visual-motor ability;
2. The Matching Test measures visual-spatial ability and
3. The Pegboard Test measures fine motor ability.

Each of these subtests has been extensively validated and co-normed so that a child’s performance on one subtest can be compared directly with the other subtests. For each of these subtests, brief instructions were given to the child in Tamil.

3.6.1.1. DRAWING (VISUAL-MOTOR) TEST:

In the Drawing Test, the child was made to copy designs which were developmentally arranged in order of increasing difficulty. Starting at an age-appropriate item, the child simply copied a standard design and proceeded until three consecutive items were failed. Items were scored by criteria conveniently
located on the Examiner Form. The Drawing Test yielded a visual-motor standard score.

3.6.1.2. MATCHING (VISUAL-SPATIAL) TEST:

The Matching Test provided a measure of spatial skill by presenting visual-spatial tasks developmentally arranged in order of increasing difficulty. Starting at an age-appropriate item, the child simply marked which of the four options “goes best” with the standard item. Making the correct choice in each test item was heavily dependent upon various visual-spatial skills such as perspective, orientation, rotation, size discrimination, etc. The child was allowed to continue until he/she made six errors within a series of eight consecutive items. The Matching Test yielded a visual-spatial standard score.

Numerous tasks that are involved within and/or parallel to the visual-spatial analysis step include:

- Visual recognition,
- Visual discrimination,
- Figure-ground determination,
- Spatial symmetry detection,
- Executive planning and monitoring and
- Sustained attention.

3.6.1.3. PEGBOARD (FINE MOTOR) TEST:

In the Pegboard Test, the child inserted as many pegs as possible within 90 seconds using a nearly square pegboard. The pegboard was waffled to add to its fine motor demands as well as to increase its aesthetic appeal. Basically the fine motor task involves sensory feedback through muscles and tendons
(kinesthetic feedback) and inhibiting as well as initiating movements including ocular-motor interactions.

First, the dominant hand (defined as the hand the child uses to write or draw) was used, and then a trial was given using the non-dominant hand. The dominant hand score was used to determine the Test’s Fine Motor standard score, since it was the hand involved with written tasks, usually the area of diagnostic interest.

3.6.1.4. VISUAL-MOTOR ABILITIES COMPOSITE:

A composite standard score was calculated as an overall estimate of visual-motor ability and as such can serve as a summary statistic. The composite standard score was derived from performance on the visual-motor, visual-spatial and fine motor areas assessed on the WRAVMA. The composite standard score was then converted into a percentile scale demonstrating how the child’s performance compares to other children of his/her age.

While each WRAVMA test can be used individually, all three tests can be efficiently administered, yielding a comparison of a child’s integrated visual-motor ability with the skill areas of visual-spatial and fine motor abilities. Difficulty performing visual-motor tasks, such as copying from the chalkboard, drawing or handwriting, most logically can be linked to either fine motor deficits, spatial deficits, and/or to an “integration” deficit when motor and spatial “systems” are combined.

For example, to copy words or a design, a child must:

❖ Look at and perceive what is on a page, performing a spatial analysis;
- 66 -

- Organize his/her motor system to execute successive coordinated movements with the appropriate fingers and thumb of his/her dominant hand;
- Check whether the production being created is similar to the original spatial analysis and make necessary adjustments as the motor activity proceeds, integrating the visual with the fine motor aspects.

Thus the process of copying is a simplified version of the likely series of highly complicated neurological processes that underlie visual-motor behavior (Williams, 1983).

3.6.2. BINET-KAMATH I.Q. TEST:

Intelligence or I.Q. tests are intended to measure aptitude and intellectual capacities and provide an estimate of a child’s mental abilities. The standardized tamil translation of the Binet-Kamath I.Q. Test was used to determine the intelligence quotient (I.Q.) of the child. It is basically the modified Indian version of the Stanford-Binet I.Q. Test. It contained items that test various aspects of a child’s language and thinking skills appropriate to various age levels.

This test measures the child’s scholastic abilities like verbal reasoning, comprehension, quantitative reasoning, vocabulary, abstract reasoning, similarities, differences, visual reasoning and short-term memory. The child’s mental age (in months) was calculated from his/her responses to the questions given in the Binet-Kamath I.Q. Test Procedure. The mental age was then divided by the chronological age (in months) and multiplied by 100 to obtain the child’s I.Q.
3.7. MEASUREMENT OF BEHAVIORAL OUTCOMES:

Behavioral outcomes were assessed through the administration of three forms/questionnaires to the teacher of each child. Teachers were asked to fill out the questionnaires translated into Tamil. The questionnaires that were distributed to the teachers are explained below:

3.7.1. BEHAVIOR RATING SCALE BY CONNER: (Annexure-IV)

The behavioral outcomes of the child were assessed through the administration of this Conners' Rating Scales – Revised (CRS-R) to the teacher of each child. This scale consisted of 39 questions that were used to gauge five subscales of each child’s behavior:

1. Aggressiveness;
2. Inattentiveness;
3. Anxiety;
4. Hyperactivity and
5. Sociability.

Certain questions corresponded with each subscale. Each question was rated on a scale of 1 to 4 based on the following aspects:

1. Whether the behavior does not apply to the child at all;
2. Whether it applies just a little;
3. Whether it applies quite a bit;
4. Whether it applies very often.

For each subscale, the numeric value of each pertinent question was averaged to arrive at a mean behavioral value for the child and the child’s
behavior was qualitatively assessed based on the range of normal values provided for each of the subscales.

3.7.2. CADS – TEACHER VERSION: (Annexure-V)

This version consisted of 27 questions that were used to determine the following:

1. Conners’ Attention Deficit-Hyperactivity Disorder (ADHD) Index;
2. Diagnostic and Statistical Manual (DSM)-IV Inattentive;
3. Diagnostic and Statistical Manual-IV Hyperactive-Impulsive;

Certain questions corresponded with each of these subscales. This questionnaire addressed the wide variety of problems that children commonly have in school and the teacher was asked to rate the items according to how much of a problem it has been in the last month and circle the best answer for each item. Each question was rated on a scale of 1 to 4:

1. Not true at all (Never, Seldom)
2. Just a little true (Occasionally)
3. Pretty much true (Often, Quite a bit)
4. Very much true (Very often, very frequent)

For each subscale, the numeric value of each pertinent question was added and matched to the respective profile depending on the age and gender of the child to obtain the CADS-T-Score from the CADS-T Male/Female Profile.

Conners’ ADHD Index identifies children “at risk” for ADHD. High scores of DSM-IV Inattentive indicate an above average correspondence with the DSM-
IV diagnostic criteria for Inattentive type of ADHD. High scores of DSM-IV Hyperactive-Impulsive indicate an above average correspondence with the DSM-IV diagnostic criteria for Hyperactive-Impulsive type of ADHD. High scores of DSM-IV Total indicate an above average correspondence to DSM-IV criteria for combined Inattentive and Hyperactive-Impulsive type of ADHD.

3.7.3. BEHAVIOR RATING INVENTORY OF EXECUTIVE FUNCTION – TEACHER FORM: (Annexure-VI)

This form consisted of 86 statements that were used to gauge eight subscales of each child's behavior over the past six months:

1. Inhibit;
2. Shift;
3. Emotional Control;
4. Initiate;
5. Working memory;
6. Plan/Organize;
7. Organization of materials and
8. Monitor.

Certain questions corresponded with each subscale. Each question was rated on a scale of 1 to 3:

1. Never;
2. Sometimes or
3. Often.

For each subscale, the numeric value of each pertinent question was added to obtain the total scale raw scores. The total scale raw scores were used
to find out the T scores from the normative tables given in the BRIEF Manual. The T scores were basically linear transformations of the raw scale scores. The T scores were used to interpret the child’s level of executive functioning as reported by the teachers on the BRIEF rating form. The corresponding percentiles were also obtained from the tables. The exact percentile for each raw score varied slightly for each scale, as the scores were not normally distributed. Higher raw scores, percentiles and T scores indicate greater degrees of executive dysfunction.

The inhibit scale assessed inhibitory control (i.e., the ability to inhibit, resist, or not act on an impulse) and the ability to stop one’s own behavior at the appropriate time. This is a well-studied behavioral regulation function that is described as constituting the core deficit in ADHD, Predominantly Hyperactive-Impulsive Type, as described in the fourth edition of the Diagnostic and Statistical Manual (DSM-IV; American Psychiatric Association, 1994).

The shift scale assessed the ability to move freely from one situation, activity, or aspect of a problem to another as the circumstances demand. Key aspects of shifting include the ability to make transitions, problem-solving flexibility, switch or alternate attention and change focus from one mindset or topic to another. The DSM-IV diagnostic criteria for the Pervasive Developmental Disorders (PDD) include poor shifting ability.

The emotional control scale addressed the manifestation of executive functions within the emotional realm and assesses a child’s ability to modulate emotional responses. Poor emotional control can be expressed as emotional liability or emotional explosiveness. Children with difficulties in this domain may have overblown emotional reactions to seemingly minor events.
The initiate scale contained items relating to beginning a task or activity, as well as independently generating ideas, responses, or problem-solving strategies. Poor initiation typically does not reflect non-compliance or disinterest in a specific task. Children with initiation problems typically want to succeed at a task, but they cannot get started.

Items from the working memory scale measured the capacity to hold information in mind for the purpose of completing a task. Working memory is essential to carry out multi-step activities, complete mental arithmetic, or follow complex instructions. Integral to working memory is the ability to sustain performance and attention. Given the posited relationship between working memory as an executive function and the diagnostic criteria for ADHD, Predominantly Inattentive Type (ADHD-I), the BRIEF Working Memory scale can be clinically useful in assessing the presence or absence of ADHD-I.

The Plan/Organize scale measured the child’s ability to manage current and future-oriented task demands. The plan component of this scale related to the ability to anticipate future events, set goals and develop appropriate steps ahead of time to carry out a task or activity. Planning involved imagining or developing a goal or end state and then strategically determining the most effective method or steps to attain that goal. It often requires sequencing or stringing together a series of steps. The organizing component of this scale related to the ability to bring order to information and to appreciate main ideas or key concepts when learning or communicating information. This involves the ability to organize oral and written expression, as well as to understand main points expressed in presentations or written material.

The organization of materials scale measured orderliness of work, play and personal storage spaces. The organization of materials scale assessed the manner in which children order or organize their world and belongings. Children
who have difficulties in this area often cannot function efficiently in school or at home because they do not have their belongings readily available for their use.

The monitor scale assessed work-checking habits (i.e., whether a child assessed his or her own performance during or shortly after finishing a task to ensure appropriate attainment of a goal). This scale also evaluated a personal monitoring function (i.e., whether a child keeps track of the effect of his or her behavior has on others).

Based on theoretical and empirical factor analytic findings, the clinical scales were combined to form two indexes, the Behavioral Regulation Index (BRI) and the Metacognition Index (MI) and one composite summary score, the Global Executive Composite (GEC).

The Behavioral Regulation Index represented the child’s ability to shift cognitive set and modulate emotions and behavior via appropriate inhibitory control. It is comprised of the Inhibit, Shift and Emotional Control scales. Behavioral regulation enables the metacognitive processes to successfully guide active, systematic problem solving, and more generally, supports appropriate self-regulation.

The Metacognition Index represented the child’s ability to initiate, plan, organize and sustain future-oriented problem solving in working memory. This index was interpreted as the ability to cognitively self-manage tasks and reflects the child’s ability to monitor his or her performance. The MI related directly to a child’s ability to actively problem solve in a variety of contexts.

The Global Executive Composite was a summary score that incorporated all eight clinical scales of the BRIEF. This summary score would be an accurate reflection of the child’s executive dysfunction level.
3.8. ANTHROPOMETRIC MEASUREMENTS:

Nutritional status of individuals and populations can be assessed by anthropometry and by biomedical methods.

Anthropometry involves measurement of body dimensions, that is, weight and height and comparing them with reference standards. It is simple, safe, easy to perform, relatively inexpensive, non-invasive, portable and requires minimal training.

During the evaluation conducted in an enclosed room at the school, the children were first measured for their height (in centimeters) (Fig. 6) weight (in kilograms) (Fig. 7) and arm circumference (in centimeters) (Fig. 8).

Fig. 6: Photograph showing the measurement of height in centimeters
Fig. 7: Photograph showing the measurement of weight in kilograms

Fig. 8: Photograph showing the measurement of mid-arm circumference in centimeters
Fig. 9: Photograph showing the overall general examination by a pediatrician

The three anthropometric indices, namely height for age z-score, weight for age z-score and weight for height z-score were calculated using the US NCHS (National Center for Health Statistics) reference values.

3.9. ASSESSMENT OF EXPOSURE:

Then, they were subjected to overall general examination by a pediatrician who also paid attention to mild ataxia, bilateral wrist drop and a peculiar blue line at the base of the gums (Fig. 9).

Blood samples were then drawn from each child from the antecubital vein after carefully washing the puncture site with 100% isopropanol (Fig. 10, 11 and 12). Refreshments were provided to the children soon after blood draw (Fig. 13).
Fig. 10: Photograph taken at the time of blood draw

Fig. 11: Photograph taken at the time of blood draw
Ph.D. Thesis entitled “Study of Lead Exposure and Outcomes amongst Children in Chennai, India”
The blood samples were collected into lead-free tubes (Becton-Dickinson lead-free vacutainers) during the above standard venous phlebotomy. The fresh whole blood samples were then aliquoted as follows:

1. 3 ml for blood lead analysis and complete blood count was aliquoted into lead-free tubes coated with dipotassium ethylene diamine tetra acetic acid (K₂EDTA) as anti-coagulant (concentration of EDTA in the tube was 1.8 mg/ml of blood);
2. 3 ml for the measurement of serum ferritin aliquoted into serum tube coated with increased Silica Act Clot Activator;
3. 4 ml for genotyping aliquoted into lead-free plastic whole blood tube spray-coated with K₂EDTA;
4. The remaining sample as a duplicate aliquoted into lead-free tubes coated with K₂EDTA.

The last two aliquots were stored in the deep-freezer (-76 degree Celsius) for future use.

3.9.1. BLOOD LEAD MEASUREMENT:

Quantitative measurement of lead was performed in fresh whole blood using the LeadCare Analyzer instrument (ESA Laboratories, Chelmsford (formerly Bedford), MA, USA), which is a well-validated field instrument with sensitivity to as little as 1 microgram/dL blood lead (Fig. 14). Lead levels were measured in batches of 20 samples interspersed with calibration checks using LeadCare Blood Lead Controls provided by the manufacturer.

LeadCare Blood Lead Controls were intended for use in quality control. They were intended to monitor the accuracy and precision of blood lead testing using the LeadCare Blood Lead Testing System. The correlation coefficient
between LeadCare System and Atomic Absorption Spectroscopy was calculated to be 0.97 with about 112 samples at a major lead outreach and referral hospital.

Fig. 14: LeadCare Analyzer Instrument

3.9.1.1. PRINCIPLE AND METHOD OF LEADCARE SYSTEM:

The LeadCare System relies on electrochemistry and a unique sensor to detect lead in whole blood. Most of the lead is carried in red blood cells. When a sample of whole blood (50 microliters) is mixed with the Treatment Reagent (250 microliters) of a dilute hydrochloric acid solution in water (0.1 mol/L), the lead in red blood cells is removed and made available for detection. When a test is run,
the analyzer causes the lead to collect on the LeadCare sensor (The active electrode area in each sensor contains a small amount of gold particles in an inert matrix). After a period of time, the analyzer removes this lead and measures it and converts the result into a displayed blood lead result. The result is in micrograms of lead per deciliter (dL) of whole blood. The range of the test is 1 to 65 microgram/dL.

The LeadCare Blood Lead Controls are prepared from bovine blood containing metabolized lead. The LeadCare Controls consist of a low-level blood lead control (Level 1) and a high-level blood lead control (Level 2). Each control provides a range of acceptable results. The 2 ml of bovine blood based controls have been lyophilized (freeze dried) to obtain a long shelf life. Reconstitution of the lyophilized controls with LeadCare Water (2 ml of purified water with isothiozolones (<0.002%) as preservative) produces the blood lead control samples. These were the controls used in internal quality control (QC) program.

3.9.2. MEASUREMENT OF COMPLETE BLOOD COUNT:

On completion of the measurement of blood lead levels, the remaining blood samples in that vacutainer was subjected to complete blood count at the Pathology and Hematology laboratory of Sri Ramachandra University using the BC-3000 plus MINDRAY Auto hematology analyzer. The results obtained from the complete blood count included the hemoglobin content in grams per deciliter, packed cell volume in percentage, total white blood cell count in WBCs per microliter, polymorphic, lymphocyte, eosinophilic, monocyte and basophilic counts in percentage, red blood cell count in million RBCs per microliter, mean corpuscular volume in femtoliters, mean corpuscular hemoglobin in picograms, mean corpuscular hemoglobin concentration in grams per deciliter, platelet count in platelets per microliter, blood group and rhesus factor of the subject under study.
3.9.3. MEASUREMENT OF SERUM FERRITIN:

The blood sample collected into the serum tube was subjected to the measurement of serum ferritin at the Biochemistry laboratory of Sri Ramachandra University using the Automated Chemiluminescence System (ACS):180. The serum ferritin of the subject under study was obtained in nanograms per milliliter.

The ACS:180 Ferritin assay is a two-site sandwich immunoassay using direct, chemiluminometric technology, which uses constant amounts of two anti-ferritin antibodies. The first antibody, in the Lite Reagent, is a polyclonal goat anti-ferritin antibody labeled with acridinium ester. The second antibody, in the Solid Phase, is a monoclonal mouse anti-ferritin antibody, which is covalently coupled to paramagnetic particles.

The system automatically performs the following steps:

- Dispenses 25μL of sample into a cuvette;
- Dispenses 100μL of Lite Reagent and 450μL of Solid Phase and incubates for 7.5 minutes at 37ºC;
- Separates, aspirates and washes the cuvettes with reagent water;
- Dispenses 300μL each of Reagent 1 and Reagent 2 to initiate the chemiluminescent reaction;
- Reports results according to the selected option.

The ACS:180 Ferritin assay measures ferritin concentrations up to 1650 ng/mL with a minimum detectable concentration of 0.5 ng/mL.
3.10. CLASSIFICATION OF THE STUDY GROUP:

The study subjects were classified into 5 groups, namely, the iron depletion group, iron deficient erythropoiesis group, iron deficiency anemia group, iron deficiency anemia with lead poisoning group and healthy group. Subjects with a normal hemoglobin concentration according to the World Health Organization’s criteria of ≥11 g/dL and a normal serum ferritin concentration of ≥12 ng/mL, but with a decreased mean corpuscular volume of less than 73 fL were classified into the iron depletion group according to the criteria of American Academy of Pediatrics (Bessman et al., 1983; Oski, 1993).

The iron deficient erythropoiesis group was formed with subjects whose mean corpuscular volume and serum ferritin concentration measures were below 73 fL and 12 ng/mL respectively. The subjects who showed a decreased mean corpuscular volume of less than 73 fL, decreased serum ferritin concentration of less than 12 ng/mL and a decreased hemoglobin concentration of less than 11g/dL, were included in the iron deficiency anemia group. In the above group, subjects who had blood lead levels above 10 µg/dL were included in the iron deficiency anemia with lead poisoning group. All the subjects who had normal mean corpuscular volume (≥ 73 fL), serum ferritin concentration (≥ 12 ng/mL), hemoglobin concentration (≥ 11 g/dL) and blood lead levels (< 10 µg/dL) constituted the healthy group.

The iron deficiency anemia group was further classified according to the World Health Organization’s criteria, into categories of mild anemia (hemoglobin level 10 – 10.9 g/dL), moderate anemia (hemoglobin level 8 – 9.9 g/dL) and severe anemia (hemoglobin level < 8 g/dL).
3.11. STATISTICAL ANALYSIS METHODS:

The software SPSS version 13.0 was used to derive the following results:

- A basic description of the study population was prepared by means of frequency distributions and the basic statistics like mean, median, mode, standard deviation, variance, etc;
- Correlation coefficients and their significance between the important parameters under study;
- Mean blood lead levels were calculated across variables of interest;
- Analysis of variance tests were performed to assess the statistical significance of these variables in predicting blood lead levels after a logarithmic scale conversion as the distribution of blood lead levels was skewed;
- Variables significant at the 0.1 level were assessed in multivariate linear regression models, after including age, sex, mother’s education level and the family’s average monthly income in the base model;
- Final models were obtained by applying a backwards elimination procedure that kept variables with p-values less than 0.05.
- Chi-square tests were performed to compare population characteristics, including socio-demographic and economic variables, with the varying blood lead levels.
- The effect of increase in blood lead levels on IQ, Visual-Motor function and neurobehavioral factors were assessed using multivariate linear regression models after including gender, age, hemoglobin, average monthly income of the family and the mother’s education level in the base model;
- Both log-transformed and untransformed blood lead concentrations were tested in the statistical models and the findings were similar.
The software R version 2.5.1 was used for the following purposes:

- The end-points in each domain that were most strongly related to blood lead level in the multiple regression analyses were then examined in two sets of analyses focusing on dose-response and threshold.
- Dose-response was examined initially by nonparametric regression.
- In nonparametric regression, regression lines were fitted locally to each region of the data. Then, a smoothing spline was used to produce a smooth curve from these short lines to identify the shape of the distribution.
- The generated GAM (Generalized Additive Models) were fed excluding the blood lead levels to obtain the residuals of the model; the residuals were then smoothed over blood lead level and/or log transformed blood lead levels using penalized spline to see the non-linear effect of blood lead levels on the various parameters of concern.
- Care was taken to ensure that the results were not unduly influenced by the data of individual children.
- The cases that are influential with respect to any one of DFBETAS (the change in the regression coefficients resulting from the deletion of a particular child), DFFITS (the change in the predicted score for a child resulting from the deletion of that child from the data set used to calculate coefficients), covariance rations, Cook’s distances and/or the diagonal elements of the hat matrix measures were excluded from the study to obtain the final estimates and their significance.
- Models were refitted after deletion of highly influential observations: $\text{DFBETAS} \geq 0.161$ and $\text{DFFITS} \geq 0.622$.
- Scatter plots that gave a general description of the study population characteristics and dose response curves were generated.
- The individual parameters of concern, that is IQ, visual-motor abilities and neurobehavioral factors were assessed within the various quintiles and
deciles of blood lead levels and were compared to arrive at the concentration of blood lead level that has a more prominent effect on the parameters.