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A sibling-paired observational study investigating the accumulation and deficiency of toxic and essential elements in children with autism spectrum disorders

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A SIBLING-PAIRED OBSERVATIONAL STUDY INVESTIGATING THE ACCUMULATION AND DEFICIENCY OF TOXIC AND ESSENTIAL ELEMENTS IN CHILDREN WITH AUTISM SPECTRUM DISORDERS

by

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ABSTRACT

This study evaluated if elemental concentrations differed in children with an autism spectrum disorder (ASD) versus normally developing children, at the pre-natal level of development, by means of a sibling-paired design, using deciduous teeth as biomarkers of pre-natal exposure to the elements. A total of 22 families were sampled from the Southern Ontario region (London, Hamilton, Orangeville, Newmarket, Toronto, Kingston and Ottawa) in which the full set of deciduous teeth from all children, where only one child was diagnosed with an ASD, was acquired. An analogous sample was collected from 7 control families. The concentrations of K, Mn, Na, Pb and Sr in the deciduous teeth of children with an ASD were found not to differ from that of their normally developing siblings and the control group ($\alpha = 0.01$). The concentrations of Mg, Ni, Cu, Fe and Zn were found to be lower in the deciduous teeth of children with an ASD versus their normally developing siblings, while Cr concentrations were found to be elevated ($p < 0.001$). The differences were correlated to a general trend by which the concentrations decreased (or increase, in the case of Cr) in children as the mother conceived more children within a family. This may indicate that the trends and differences observed may be a secondary effect to another underlying condition, presumably at the level of the mother.

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Meu velho pai

Preste atenção no que eu lhe digo

Meu pobre papai querido

Enxugue as lágrimas do rosto

Porque papai que voc chora tão sozinho

Me conta meu papaizinho

O que lhe causa desgosto

...

Você sofreu quando eu era ainda criança

Não me sai mais da lembrança

Seus carinhos, seus cuidados

Eu fiquei grande, estou seguindo meu caminho

E você ficou velhinho

Mas estou sempre ao seu lado

...

Meu papaizinho não precisa mais chorar

Saiba que não vou deixar

Você sozinho, abandonado

Eu sou seu guia, eu sou seu tempo e sou seus passos

Sou [a] sua luz e sou seus braços

Seu filho abençoado

—Carmem Silva

To Justin

This one's for you, little man

"Il sait tout, mais il manque d'inexpérience "

-Hector Berlioz

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Chapter 1

Introduction

1.1 Autism and Pervasive Developmental Disorders

The pervasive developmental disorders (PDDs) consist of five disorders, as detailed by the *International Statistical Classification of Diseases and Related Health Problems*, tenth revision (ICD-10) (World Health Organization, 1992) and the *Diagnostic and Statistical Manual of Mental Disorders*, fourth revision (DSM-IV)(American Psychiatric Association, 2000):

1. Autistic disorder (autism, AD),
2. Asperger's disorder (AS),
3. Retts disorder (RD),
4. Childhood disintegrative disorder (CCD),
5. Pervasive developmental disorder not otherwise specified/atypical autism (PDD-NOS).

The PDDs are generally characterized by the presence of characteristics/traits that are similar to those of the first described PDD, autistic disorder (Kanner, 1943), making them known alternatively (and referred to interchangeably) as autism spectrum disorders (ASD)[1] as each exhibits

[1]The PDDs are often referred to as ASDs interchangeably; however, due to the rarity of both Retts disorder and CCD, at times, only autistic disorder, Asperger's disorder and PDD-NOS are encompassed in the term ASD. This

traits and characteristic symptoms, albeit of varying severity, generally presenting themselves within the first two years of childhood (World Health Organization, 1992; American Psychiatric Association, 2000). Although the concept of whether autism truly does classify as a spectrum disorder is still in debate, it is clinically accepted, and diagnosis and treatment provided, based on classifying each disorder in regards to symptom/trait severity and the overall morbidity presented in the child (Green *et al.*, 2003; Benaron, 2009).

Although ASDs/PDDs (referred to from this point forward as ASDs), are now understood to be childhood developmental disorders of their own class and have been given a significant amount of attention both clinically and academically, they are still a poorly understood and a highly complex class of behavioural disorders that are distinguished by their ability to significantly disturb an individual's ability to interact socially.

Autism's complexity has historically led to a confusion in its identification and has also left the academic and clinical worlds bewildered as to its ætiology as the degree of morbidity between the ASDs varies substantially: One child with an ASD can be fully functioning and of normal intelligence or showing advanced and highly outstanding skills (*i.e.* savants[2]), while others are confined to wheelchairs, show signs of clear mental retardation, need care-givers to accomplish their daily needs and are left in what has become the stereotypical state of hand-flapping and mumbling of completely incoherent phrases as their only means of communication (Benaron, 2009). It is not

differentiation is research area specific (generally in regards to genetic studies) and the differentiation is made usually to limit the scope of a study based on the severity of symptoms, degree of morbidity and ætiology. For the purpose of this study, and in particular, this introductory discussion, all of the PDDs will be considered interchangeably as an ASD.

[2]Savant syndrome, generally referred to as savantism (savants being individuals with the condition first referred to as *"idiot savants"* by John Langdon Down) is a condition presented by an outstanding brilliance, ability or adherence to a given area of expertise that is well outside of the usual abilities of the average population (Treffet, 2009). Savantism is considered a rare condition, generally limited to individuals with behavioural disorders (Treffet, 2009), although some individuals with no apparent mental or behavioural handicap do occasionally present some extraordinary skills. It has been documented that 50% of savants are autistic, while the rest show some signs of different mental disabilities, mental retardation or have undergone brain trauma (Treffet, 2009). Approximately 28% of individuals with autism seem to present some extraordinary ability, that is, the ability to perform some task, such as memorize large volumes of data or perform difficult calculations with no aid (Treffet, 2009; Howlin, 2009). The mechanism of the condition is not understood and the reader is referred to the works of Snyder (2009), Pring (2005), Happé and Vital (2009), Baron-Cohen *et al.* (2009) and Mottron *et al.* (2009) for more information on the condition and for a review of current understanding on savantism mechanisms and proposed ætiology.

surprising, that prior to World War II, children who presented the now known symptoms/traits of autism, including a complete perceived aloneness and isolation from society (including from their families), the amazement with simple objects and stereotypical behaviours as well as lack of communication including muteness and mumbling/grunting, were considered to be mentally retarded individuals with severe emotional disturbances and were treated much like the "insane" (*i.e.* schizophrenic[3] individuals) (Green *et al.*, 2003) which most likely resulted in institutionalization.

1.1.1 Autistic Disorder

The first description of autism (as its own disorder) was made by Kanner (1943) and he described the condition as "infantile autism" or "autistic disorder" due primarily to the children's intensive "aloneness"[4] and inability to act socially. Kanner's (1943) work was based on a case study of a group of eleven children (eight boys and three girls) who presented odd behaviour(s). Kanner's observations were made on a subset of children which are all now considered to have been high-functioning autistics (in regards to their level of intelligence) and did not encompass the spread of symptoms and behavioural profiles seen today in children with ASD; yet, Kanner's observed set of traits are still considered the nuclear core set of autistic traits (referred to as "Kanner autism") (Green *et al.*, 2003; Gillberg, 1992; Benaron, 2009). Kanner (1943) identified three common characteristics to the group of children in his 1943 study:

1. an extreme autistic aloneness and a tendency to be drawn inward into themselves with an inability to enter into affective contact with others,

2. a failure to use language in a communicative fashion which included issues with muteness, literalness, echolalia and/or pronoun reversal,

[3]The term *"autism"* was borrowed by Kanner (1943) from Bleuler in 1910/1911 (Bleuler, 1984) (from the latin *autismus*) who in fact used the term to describe one of his observed behavioural traits in schizophrenic individuals. Kanner (1943) seems to have adopted this word, as autism was perceived prior to his discovery as a childhood form of schizophrenia/dementia (Green *et al.*, 2003; Benaron, 2009).

[4]The term autism itself stems from the root word *"autos"*, Greek for *"self"*.

3. an anxious obsessive desire for the maintenance of sameness, with the child showing distress at any changes in their usual routines.

Kanner (1943) also made note that in relation to these factors the children possessed a severely limited repertoire of spontaneous activities and an associated tendency to repeat the same actions in a ritualized manner, which was argued by Kanner to be due to their extreme need for constancy which ultimately led to an enjoyment of inanimate physical objects. These traits, in which children present severe social impairment, severe language and communication problems and a tendency towards sameness, routine and repetitive stereotypical behaviours have become the defining traits of autism and the ASDs in general. Kanner (1943) also made two more important observations which was an initial step in separating autism from what was until then believed to be a form of childhood dementia/schizophrenia and mental retardation (Green *et al.*, 2003; Benaron, 2009). Kanner noted that the children all possessed normal intellectual potential and were all in good physical health and concluded that,

> "these [autistic] children have come into the world with innate inability to form the usual, biologically provided affective contact with people, just as other children come into the world with innate physical or intellectual handicaps"

labelling "infantile autism" as its own childhood disability/disorder, separate from dementia/schizophren (Green *et al.*, 2003; Benaron, 2009).

1.1.2 Asperger's Disorder

At approximately the same time, Asperger (1944) described a similar study and disorder (albeit calling it "autistic psychopathy"); however, all of the children had completely normal communication skills giving this deviant of Kanner's described disorder the name of its discoverer (Asperger's disorder/syndrome) as it differed only in regards to communication skills, one of Kanner's main traits defining his disorder. Given the similar pervasive nature of the social deficient

with the exception of communication deficiencies, AS is considered to fall on the autism spectrum and is classified as an ASD (World Health Organization, 1992; American Psychiatric Association, 2000; Attwood, 2007). Asperger's disorder presents itself in a similar manner to the previously discussed AD (section 1.1.1), only that children with AS nearly always demonstrate average or very good IQs (versus children with other ASDs possibly presenting mild to severe mental retardation) and have either completely normal or very good language skills (Green *et al.*, 2003; Attwood, 2007)[5]. Asperger's disorder, with the requisit of normal development of communication skills and the presented intelligence level in children, is often regarded as being equivalent to high-function autism, although high-functioning autistics (with AD) may still present some issues in communication skills and poorer IQs (Attwood, 2007; Benaron, 2009). Children with issues focusing, that is with attention-deficit hyperactive disorder (ADHD), who may demonstrate some intellectual deficit but do not present classic traits of the ASDs have, at times, been mistakenly diagnosed with AS (Green *et al.*, 2003; Attwood, 2007; Benaron, 2009). Also, as described by Asperger (1944) in passing, child with AS generally present a marked clumsiness and a gait when walking which is characteristic to the disorder (Green *et al.*, 2003; Asperger, 1944; Attwood, 2007; Benaron, 2009).

1.1.3 Childhood Disintegrative Disorder

Childhood disintegrative disorder (CDD) and Retts disorder (RD, section 1.1.4) differ from the other ASDs as they often develop after a period of normal development and are thus regressive disorders; they are also the two disorders that generally present the highest level of morbidity in children with an ASD.

Childhood disintegrative disorder (also known as Heller's disorder, childhood dementia or childhood psychosis) was first described several years prior to the observation and definition of AD by Kanner (1943) (Heller (1908) *in* Green *et al.*, 2003; Benaron, 2009). The disorder is quite

[5]As noted by James (2006) several very well distinguished and accomplished individuals have been diagnosed (some retroactively) with AS including: Sir Issac Newton, Béla Bartók, Alfred Kinsley, Vincent van Gogh and Henry Cavendish to name only a few. The ability to communicate well and the very good level of intelligence in individuals with AS seems to allow for an outward expression of their savant skills if present (see footnote 2, pg. 2)

rare, in which a child, after approximately 6-18 months, or as many as 3 years, of normal development, regresses and losses their already acquired social and linguistic capabilities and presents the standard symptoms of AD (section 1.1.1) along with symptoms of psychosis, loss of some motor abilities resulting in loss of control of their bowels and bladder and is left in a state of a complete stupor (Green *et al.*, 2003; Benaron, 2009). Due to the morbidity and near complete loss of all social, communication and to some degree, motor function, CDD is often regarded as one of the lowest functioning of the ASDs along with RD (section 1.1.4). Childhood disintegrative disorder, although a regressive condition, is regarded by the DSM-IV (American Psychiatric Association, 2000) and the ICD-10 (World Health Organization, 1992) as a PDD due to the similarity and ultimate pervasive nature of the disorder to AD (section 1.1.1).

1.1.4 Retts Disorder

Retts disorder, like CDD, generally presents itself after a short period of normal development of 6-18 months. At this point, an enormous regression in early childhood development is observed, which includes the loss of communication and social skills (presentation of the classic autistic traits), and unlike CDD (section 1.1.3), cerebral atrophy is also a presented symptom which results in shrinking of the head and loss of motor skills. The condition presents itself in four distinct stages (Green *et al.*, 2003; Benaron, 2009):

1. **The Early Stagnation Stage:** lasts several months at the onset and generally presents itself by the child's loss of interest in playing, showing of odd hand-waving behaviours, head growth deceleration, and possible reduction in the child's ability to communicate and to perform simple eye contact with others;

2. **The Rapid Destructive Phase:** may last a few weeks or several months at which point hand wrangling, hand clapping and hand washing behaviours appear (the classic Rett stereotype) and all purposeful hand-using is lost. The child will begin to demonstrate substantial clumsiness including ataxia and apraxia, will develop irregular breathing including the potential

for occasional hyperventilation while the child's cognitive function mimics that of dementia and the behavioural stereotypes of classical autism (section 1.1.1) are present;

3. **The Pseudostationary Phase:** may last several years at which time there is some cognitive recovery away from the presented dementia-like behaviour and autism like symptoms nearly disappear and the child presents symptoms of severe mental retardation versus those of autism as emotional contact with others improves. At this stage, there is substantial motor function decay and the development of seizure disorders is common.

4. **The Late Motor Deterioration Phase:** occurs usually between age five and late adolescence in which the child's emotional contact with others improves further and any seizure disorders (namely epilepsy) diminish. The individual will present significant wasting away of their motor function including muscle weakness, severe scoliosis and trophic foot syndromes generally requiring the individual to be wheelchair bound. Symptoms of severe mental retardation persist.

Like CDD (section 1.1.3), RD is severe and also leaves a child in a stupor with little to no ability to act independently. The shrinking of the head (cerebral atrophy) is also one of the stereotypical anatomical morphologies and deviations most associated with autism (Benaron, 2009).

1.1.5 Pervasive Developmental Disorder Not Otherwise Specified

The similarly in the overall traits an individual with Retts (section 1.1.4) and CDD (section 1.1.3) presents is what causes them to be considers as PDDs/ASDs and not as distinct disorders (Tsai, 1992) regardless of the fact that they differ in the time of onset and in being regressive conditions. Under this same theme, any disorder that presents these classic autistic traits (section 1.1.1), yet does not fit the criteria of any given ASD (sections 1.1.1-1.1.4) is diagnosed a pervasive developmental disorder not otherwise specific/ atypical autism (PDD-NOS) (World Health Organization, 1992; American Psychiatric Association, 2000).

Diagnosis of any one of the ASDs (except for CDD and RD) is also required to fit the condition that initial diagnosis be made before the age of two (World Health Organization, 1992; American Psychiatric Association, 2000) in which children prior to age two can already be shown to demonstrate odd behaviours like lack of smiling, lack of acknowledging their parents and other children, repetitive behaviours as well as issues in communication. After this age (2), some individuals show *broader autistic traits* which may include mild issues in socializing and communicating (Ronald *et al.*, 2008). These individuals are not considered autistic but these broader autistic traits that are observed in individuals—which usually have some family connection to an autistic individual—can be considered to fall on the autism spectrum given the very large range of morbidities. The concept and definition of the actual spectrum is however a complex matter and the reader is referred to the works of Green *et al.* (2003), Benaron (2009) and Baron (2008) for a more thorough discussion of the conceptualization of the actual spectrum, which is far outside of the scope of this immediate work.

Although exact and rigorous diagnostic criteria for these disorders can be found elsewhere within the DSM-IV (American Psychiatric Association, 2000) and the ICD-10 (World Health Organization, 1992), the large variation in symptoms and overall morbidity is apparent, and further, the exact cause of these disorders is very much unknown (with the exception of RD, section 1.2).

1.2 The Genetics of the ASDs

As the psychiatric community tried to explain the underlying caused of the ASDs (section 1.3) several individuals immediately recognized that the ASDs may have an underlying cause which may be explained by genetics (Sauna, 1986; Smalley *et al.*, 1988). Retts disorder (section 1.1.4), is the only disorder with a clear genetic basis. It is now well understood that RD effects females nearly exclusively, as affected males die *in utero*. The disorder is directly related to mutations in the *MCEP2* gene (location at *Xq28* on the *X* chromosome). These mutations are almost entirely found on the paternal copy of the gene/chromosome (Trappe *et al.*, 2001). The mutation has been

linked to the encoding of the methyl-CpG-binding protein 2 and the mutation itself generally being created *de novo* in 95% of the cases of RD (Amir *et al.*, 1999). Germ-line mutations have also been shown, thus illustrating possible hereditability from the mother (Amir *et al.*, 1999).

Given that RD presents similar pervasive symptoms to other PDDs has led to the search for possible roles that genetics may play in the ætiology of the other ASDs; however, genetically, the ASDs are still very poorly understood with no specific gene or series of genes being held responsible for the actual ætiology (other than RD). It is however clear that genetics must play a role as it has been shown through monozygotic twin studies that there is an approximately 90% concordance rate (Trottier *et al.*, 1999; Folstein and Rosen-Shidley, 2001), although Freitag (2007) suggests that this may be an overestimate but provides no real estimate of an actual concordance. Probabilities of non-twin siblings in families with a child with an ASD presenting broader autistic traits and other behavioural, learning or social disabilities have been estimated at 30% (Folstein and Rosen-Shidley, 2001) which does demonstrate some genetic linkage. Although autism seems to have a clear genetic component, the actual role genetics plays in the ætiology of the ASDs (barred RD) is still very much not understood (Freitag, 2007; Sykes and Lamb, 2007). Recent work has demonstrated that families with a child with AD have mutations on the *HTC3R* gene which codes for the serotonin receptor (Rehnstrom *et al.*, 2009). Although such a finding is significant as serotonin receptor malfunction has been correlated to cases of mental retardation and many disorders including sleep disturbances (which is prevalent in children with ASD (Malow and McGrew, 2008)) (Rehnstrom *et al.*, 2009), the findings are rather inconsistent and no real gene has been identified as being responsible for the entire series of symptoms and presented traits observed in children with an ASD. Given that children with certain ASDs generally present co-morbidity with other disorders, mainly gastrointestinal disorders, obsessive compulsive disorders and attention deficient/hyperactivity (Green *et al.*, 2003; Benaron , 2009), it may indicate that several genes are involved in the ultimate ætiology. These co-morbidities are in themselves the difficulty in performing molecular genetics studies as definition and classification of sub-groups with certain domains of symptoms is lacking (Mazefsky *et al.*, 2008). For this reason, epigenetic

and environmental factors are most strongly implicated in the ætiology of the ASDs (section 1.3) and the genetic component is believed to be a highly complex matter.

1.3 Proposed Environmental Factors in the Ætiology of the ASDs

1.3.1 Factors Related to Childhood Psychology

At the onset of autism's definition as its own disorder, environmental factors were immediately implicated as being a possible cause, mostly related to the child's developmental psychology. Prior to the development of theories and hypotheses based on genetics (section 1.2) and possible epigenetic and organic environmental factors, the psychiatric community was the first to develop possible models to explain the development of autism in children. In general, the psychiatric community seemed to focus largely on psychodynamic theory with the major theory being focused largely on blaming bad parenting and insufficient care by the mother in particular (Kanner, 1949; Bene, 1958; Despart, 1951; Eisenberg and Kanner, 1956; Mahler, 1952; Mahler and Gosliner, 1955; Hobson, 1990)[6]. Throughout the development of these psychodynamic theories, which to a certain extent, would hinder treatment due to the possibility of parents not wanting to explore therapy as an option in fear that they would be blamed for their children's condition, the progression into the shift in ideology from psychodynamics to true biological causes. A more rigorous discussion on this progression of ideologies is presented by Sanua (1986) and in general, followed the belief that if a biological/organic cause is determined then rehabilitation, not only treatment of the ASDs may be possible (Helt *et al.*, 2008). Although psychology cannot be completely ignored as a possible factor, organic environmental factors have been implicated heavily over the last several years due to claims of increased incidences of the ASDs and due to the possibility of linking these organic factors epigenetically (Deth *et al.*, 2008).

[6]Kanner himself (Kanner, 1949; Eisenberg and Kanner, 1956) and Bettelheim (1967) were individuals with a strong belief, given the psychiatry of the time, that autism was caused directly "due to a lack of genuine warmth from the mother". This phrase has resulted in the famous hypothesis of the *"refrigerator mother"* as it relates to childhood [developmental] disorders.

1.3.2 Organic Environmental Factors

Environmental factors on all three developmental time frames (pre-, peri- and post-natal) have been correlated to increased outcomes of ASDs in children—the list being exhaustive and cannot be completely covered in this section as they are to the most part, outside of the scope of this work (Waterhouse, 2008; Matson and LoVullo, 2009). Pre-natal factors that have been implicated in the ætiology of the ASDs include mostly factors that are related to the mother including: maternal infections (Mendelsohn and Schaefer, 2008; Meyer *et al.*, 2007; Dalton *et al.*, 2003; Braunschweig *et al.*, 2008; Martin *et al.*, 2008), gestational diabetes (Gardener *et al.*, 2009), pesticide exposures (Singh *et al.*, 2007; D'Amelio *et al.*, 2005; Karr *et al.*, 2007), thyroid disease (related to iodine deficiency or excess) (Román, 2007; Sullivan, 2008), hormonal imbalances at the time of pregnancy (Manson, 2008; Spelke, 2005), excessive stress at the time of pregnancy (Kinney *et al.*, 2008), folic acid deficiency (Muskiet and Kemperman, 2006) and the use of ultrasound as an imaging modality during pregnancy (Caviness and Grant, 2006; Ang *et al.*, 2006; Abramowicz, 2007) have been correlated to increased rates and probabilities of autism. Peri-natal factors have also been strongly correlated to the prevalence of autism and include low-birth weights, hypoxia and premature births as well as mode of deliver (*i.e.* caesarean versus natural birth) (Bilder *et al.*, 2009).

The most studied developmental period has been the post-natal period, presumably due to the ease in obtaining data. Some groups have implicated factors such as early childhood development during periods of excessive rain (with associated increased television watching) as a factor in the prevalence and development of the ASDs (Waldman *et al.*, 2008; Weiss *et al.*, 2008; Guidotti, 2005). This age range has seen a substantial amount of work and theories include those regarding autoimmunity (Ashwood *et al.*, 2004; Ashwood *et al.*, 2006; Wills *et al.*, 2007; Schmitz and Rezaie, 2008), viral infections (Libbey *et al.*, 2005), vitamin D deficiency (Cannell, 2008), use of measles-mumps-rubella (MMR) vaccines (Hilton *et al.*, 2006; Gerber and Offit, 2009; Doja, 2006; Wakefield *et al.*, 1998)[7], leaky gut syndromes (Johnson, 2006; MacDonald *et al.*, 2007; Christison and Ivany,

[7]The fear surrounding MMR vaccines as a causitive agent of autism was based on Wakefield *et al.*'s (1998) paper in the *Lancet* which was later retracted due to some significant issues relating to academic and professional misconduct

2006), oxidative stress due to reduced anti-oxidant capacity and activities (Ng *et al.*, 2008; Kern and Jones, 2006) and as related to this study, metal exposure (section 1.3.3). The epidemiological data on autism's prevalence has indicated that diagnoses of ASDs has been increasing over the years (Fombonne, 1999; Fombonne, 2002; Lotter, 1966; van den Hazel, 2008); although, this phenomenon is generally regarded as due to better and more rigorous diagnostic criteria (thus indicating a potential for more diagnosis of the condition) (Green *et al.*, 2003; Benaron , 2009). Some workers have interpreted this phenomenon to correlations in the increased industrial activity and air pollution and general routine use of certain metals including, most notably, mercury (Baker, 2008). Metal exposure and deficiency have thus been highly correlated to being a factor in the ætiology of the ASDs.

1.3.3 Exposure and Deficiency to the Elements: An Ætiology of the ASDs?

1.3.3.1 Post-Natal Toxic Metal Accumulation

Post-natal exposures and deficiencies of elements have been implicated quite strongly in the ætiology of the ASDs, with some associated grave consequences resulting in some cases.

The first such correlation, and the most significant, impacting society quite significantly, was the postulation that the ASDs are an alternate form[8] of mercury poisoning.

One of the most controversial and socially impacting issues surrounding autism research and treatment has been statements linking mercury, particular ethylmercury (thimerosal) as a preservative in vaccines (namely for measles-mumps-rubella) (Fitzpatrick, 2004; Kirby, 2005), to the prevalence and apparent epidemic of ASD, while in the United Kingdom, the measles-mumps-rubella (MMR) vaccine itself has been implicated in the ætiology of ASD (Baker, 2008)[9].

(see Murch *et al.*, 2004).

[8]It is important to stress that mercury poisoning does not present the symptoms of autism (section 1.1.1) (Kirby, 2005). Thus, the theory is that the ASDs are in fact an alternate form, with different symptoms, from classical heavy mercury poisoning.

[9]The issue in the UK steamed from the work of Wakefield *et al.* (1998). Wakefield's worked claimed that the actual vaccine itself was responsible for the progression of the ASDs and may be responsible for such gastrointestinal problems as observed in children with ASD.

Mercury has been traditionally used as an antiseptic from the time of Koch who introduced mercury chloride as an alternative and more effective agent to phenol (Baker, 2008). The Lily Company was responsible for the introduction of ethylmercury as a preservative to vaccines (multi-dose vaccines, such as the MMR) as it was found to be at least forty times more effective as an antiseptic in comparison to phenol while showing limited toxicity in rats at the dosages that would be ultimately administered (Powell and Jamieson, 1931). Although it was apparent that large dosages of thimerosal would be toxic, the minuscule amounts any child would be exposed to through a series of vaccinations was largely regarded as safe up until the late 1990s with the only concerns being those of bacteriologists who remained studying its efficiency and dermatologists demonstrating rare instances of skin hypersensitivity to mercury (Cox and Forsyth, 1988; Moller, 1994; Department of Health, Education, and Welfare, Food and Drug Administration, 1977).

Over time, data on ethylmercury exposure at the levels introduced in vaccines demonstrated little to no toxicity; however, the parallel interest in methylmercury toxicity (which due to its similar sounding name to the layman may have sparked the confusion) did fuel some controversy, where this historical progression is best reported by Baker (2008) with further commentary by Silbergeld (2008).

Methylmercury was used for a period as an anti-fungal agent in agricultural crops and was accumulated environmentally (*i.e.* in fish); thus, was brought to the public attention after a series of highly significant environmental disasters. As discussed by Baker (2008) and Silbergeld (2008), several environmental disasters were responsible for making the public aware of the dangers of mercury including the 1950s incident in Minamata Bay, Japan in which inhabitants presented substantial neurological symptoms after exposure to methylmercury through the company's effluent (Harada, 1995; Hunter *et al.*, 1940) and the 1970s case in Iraq where a substantial number of individuals were hospitalized after eating bread made from wheat contaminated with methylmercury-based fungicide. These environmental exposures and the resultant longitudinal studies that determined that mercury is transmitted *in utero* resulting in crippling mental retardation in children (more so than in adults) was the first time in history in which the dangers of mercury were made

clear.

The hypothesis regarding thimerosal containing vaccines and the prevalence of autism was made not by scientists, but rather, by parents and government officials taking care of autistic children, who were in desperation, and by chance, correlated the symptoms of ASD with those being rather recently reported for mercury exposure/toxicities; thus, attempting to correlate the increased incidence of autism with the increase exposures to mercury both environmentally and through routine use in vaccines (Baker, 2008). With the evidence that mercury (albeit methyl- not ethyl-mercury) can cause *in utero* neurological damage to a child and that post-natal exposure and toxicity can also cause neurological damage, a group of parents set out and hypothesized that the mercury in vaccines was causing their child's ASD (Bernard *et al.*, 2001). This claim caused great consequences, in particular, left several hospitals and clinics in the United States, as well as parents, with the opinion not to vaccinate their children with the MMR vaccine. This scare has according to some, resulted in the increased prevalence of measles in North America. Sadly, measles which was previously an abolished disease due to vaccination (Baker, 2008). Several safety reviews (see Baker, 2008) have confirmed that thimerosal is a safe preservative, however, it was fully removed as a preservative in vaccines and to date, no mercury is being administered to children in any form as controllable as medication and vaccination (Baker, 2008).

Although mercury from vaccines has become an obsolete factor in the discussion of ASD ætiology due to 1) clear evidence showing no correlation to vaccine exposure and ASD prevalence and 2) clearance from several safety reviews (Baker, 2008), following the work of Holmes *et al.*, (2003) it has been postulated that ASD may still be the result of exposure to mercury (and other metals) manifesting itself as a alternate form of metal (*i.e.* mercury, arsenic, cadmium) poisoning due to the inability of children with ASD to excrete metals in comparison to the normal population—what has seemed to be a necessary shift in paradigm to maintain the idea of metal poisoning as an ætiology for ASD. This theory, known as the *poor excretion theory*, largely became popular amongst alternative medicine practioners after Holmes *et al.* (2003) determined that children with ASD presented substantially lower levels of hair mercury in comparison to their non-ASD coun-

terparts. Holmes *et al.* (2003) also found a direct correlation between fish consumption, exposure to mercury containing vaccines and the number of amalgam fillings in the mother to the hair mercury levels in normal children, but no such correlation in children with ASD, thus hypothesizing that children with ASD are not excreting mercury equivalently to the normal population (through hair), and therefore must be storing the metals. This hypothesis, based on work by Quig (1998), also a practioner of alternative medicine, who postulated that stored mercury sequesters in the brain and other tissues, has sparked the hypothesis that ASD is directly caused by the toxic accumulation of mercury and other trace elements such as lead (Zafeiriou *et al.*, 2007) due to the children's (with ASD) inability to excrete the metal and that ASD is in fact an alternate form of mercury poisoning.

Further work by groups such as James *et al.* (2004) has fuelled this hypothesis as they have found that children with ASD have a markedly lower blood concentration of glutathione which they correlate to a poorer ability to excrete mercury. Other groups have postulated that children with ASD are generally prescribed much heavier regimes of oral antibiotics in early childhood than normally developing children which results in an unbalanced intestinal flora (Konstantareas and Homatidis, 1987) which has been correlated in rats to the near total loss of the ability to excrete mercury (Rowland *et al.*, 1975; 1980; 1984). This loss in the ability to excrete mercury is postulated to be due to the loss of certain anaerobes in the gut which normally are able to convert methylmercury (easily absorbed by the intestine) to inorganic mercury (excreted from the intestine and not absorbed) (Rowland et al., 1975). Bradstreet *et al.* (2003) have attempted to verify the poor excretion theory by treating children with ASD and controls with the chelating agent 2,3-dimercaptosuccinic acid (DMSA)[10] finding that in general children with ASD excrete up to six-times the amount of mercury in comparison to normally developing controls thus indicating

[10](*meso*)-2,3-dimercaptosuccinic acid is an Food and Drug Administration approved chelating agent approved mostly for the treatment of acute toxic metal (including lead) poisioning, but which has been extended to the routine use as a chelating agent for the treatment of autism within the alternative medicine community. This chelating agent, as well as 2,3-dimercapto-propane sulfonate (DMPS), lipoic acid, dimercaprol, allithiamine as well as high doses of natural compounds such as vitamin C, selenium, glutathione, garlic, NDF and EDTA including natural "recipes" for these agents including mixing garlic and apple derived malic acid to form EDTA are all recommended through the Autism Canada Foundation as a potential form of treatment (Autism Canada Foundation, 2009).

an accumulation. These studies are however highly susceptible to other variations that would cause mercury accumulation, including familial history and geographical location, and cannot be necessarily regarded as true in all cases. Further, most of this work has come under some harsh scrutiny as conflicts of interest have not been disclosed in some cases/publications and retractions have become quite common, particularly recently (*i.e.* see Martin, 2009 in regards to Geier *et al.*, 2009).

Many of the proponents of the poor excretion theory have based their hypotheses on other studies simply postulating and correlating environmental load of mercury to the prevalence of ASD (Palmer *et al.*, 2006; Windham *et al.*, 2006; Williams *et al.*, 2008). In general, these theories are largely extrapolated to include toxicity of most sulfhydryl-reactive metals (*i.e.* mercury, arsenic, cadmium) which have been implicated most strongly with the poor excretion theory as it would directly be related to deficiency in glutathione. Several works have previously demonstrated abnormal levels of mercury, lead, bismuth, cadmium, and arsenic in autistic children versus normal populations (Lonsdale, 2002; Filipek *et al.*, 1999; Eppright *et al.*, 1996; Fido and Al-Saad, 2005; Wecker *et al.*,1985; Shearer *et al.*, 1982; Accardo *et al.*, 1988; Kern *et al.*, 2007), for example, correlating this to the potential of different sulfation chemistry or low levels of glutathione. In general these findings compliment the poor excretion theory as they are backed by evidence of abnormal sulfation chemistry in children with autism (Kern *et al.*, 2007; Kern et al., 2004; Waring and O'Reilly, 1990; Waring and Klovrza, 2000).

1.3.3.2 Essential Element Imbalances

More recently, essential element imbalances, mainly those related to copper and zinc have also been implicated in the ætiology of autism as they regulate the metallothionein protein system which regulates trace element content and detoxification and is a critical protein in neurodevelopment. It has been shown by Faber *et al.*, (2009) that the serum Zn/Cu ratio in children with ASD is significantly lower that the 0.7 ratio observed in normally developing health children. This theory that Zn/Cu regulation is an issue in autistic children is analogous to the idea of poor excretion as

it would imply that trace elements accumulate more readily in children with ASD than their normally developing counter parts. Zinc deficiency in particular has been implicated in the ætiology of mental retardation as zinc is an essential element necessary for several co-factors and enzymes, in particular metallothionein.

In regards to metal/element deficiencies at the post-natal level, this is a common finding in children with ASD. Children with ASD are known to be susceptible to digestive problems (Kerwin *et al.*, 2005; Liu *et al.*, 2005; Levy *et al.*, 2007; Nikolov *et al.*, 2009) and several alternative therapies center around the exclusion of foods that contain such ingredients as gluten and casein (in particular to combination of the two exclusions resulting in a generally prescribed gluten-casein free diet) which follow largely the issues with the intestinal tract and possible leaky gut observed in children with ASD (Johnson, 2006; MacDonald *et al.*, 2007; Christison and Ivany, 2006). This issue has been evaluated by several groups who have noted that several factors influence the ultimate dietary status of autistic children. Eating habits have been agreed upon to differ from normally developing children where children with ASD are known to demonstrate a high level of pickiness, refusal of foods and high selectiveness to shapes, textures, packaging and even brand names and introduction to new foods (Kerwin *et al.*, 2005; Cornish, 1998; Cornish, 2002; Martins *et al.*, 2008; Lockner *et al.*, 2008; Kalyva, 2009), which may simply be a manifestation of the known repetitive behaviours known as a classic symptom/trait of ASD with several individuals specifically working at the development of behavioural therapies to combat these issues in regards to food and nutrition (Kodak and Piazza, 2008; Matson and Fodstad, 2009). It is however well agreed upon that children with ASD, either due to their digestive issues and/or due to behavioural issues that lead to improper nutrition generally present significant deficiencies in vitamins such as vitamin C, vitamin D, niacin, riboflavin, vitamin B6 and vitamin E as well as essential elements such as iron, calcium and zinc, (Cornish, 1998; Martins *et al.*, 2008; Lockner *et al.*, 2008; Herndon *et al.*, 2009) and these deficiencies linked to certain specialized diets have been relatively recently linked to bone disorders in children with ASD (Hediger *et al.*, 2008) and in some cases issues as severe as becoming completely blind (McAbee *et al.*, 2009).

1.4 Aim of This Work

Trace, toxic and essential elements have all been implicated in the ætiology of the ASDs, yet, studies which investigate environmental factors such as metal exposure have come under some controversy and their reliability is in question due to the fact that most ignore factors such as dietary restrictions which may impact the deficiencies observed in autistic children (Rutter, 2005; Szpir, 2006). Metals in general are known to impact human health as they are needed as co-factors for several enzymes and in the case of toxic elements, can replace essential elements in enzymes and are known to cause cellular oxidative damage (Friberg, 1986). Trace and essential elements, *e.g.* manganese and lead, have been implicated in the possible ætiology of other behavioural disorders, mainly attention deficit hyper active disorder (ADHD) and general behavioural problems in children (Bouchard *et al.*, 2007; Bellinger *et al.*, 1994) and recent work has demonstrated that at the post-natal level, zinc deficiency may be a factor in the ætiology of the ASDs (Faber *et al.*, 2009). Environmental exposures to metals such as manganese in areas high in industrial activity which have also been shown to have poor air quality, high indices of exposure for elements such as manganese, and an associated elevated prevalence of neurological disorders such as Parkinson's disease in adults (*i.e.* Hamilton, Ontario, Canada) (Finkelstein and Jerrett, 2007). The study of pre-natal exposures does however require the identification of a proper and suitable biomarker of the exposures.

Deciduous teeth are often used as the biomarker of pre-natal metal accumulation or deficiency since biomarkers such as hair, blood, serum, urine and faeces generally present information regarding recent exposure (Smith *et al.*, 2007), which for the purposes of this work, to evaluate metal accumulation of deficiency at the pre-natal level would not be valid. For this reason, to evaluate the issue, deciduous teeth were selected as biomarkers as they mineralize nearly completely during the gestational period (complete crown formation between 9-11 months after conception, Table 1.1). Deciduous teeth are also relatively easy to collect in studies of this nature, particular when the necessity arises in which pre-natal events need to be monitored. That is, blood and other

fluids are not generally obtainable from the developing foetus and meconium and placental blood require specialized permission to obtain. Furthermore, in regards to metal concentrations, these tissues are not generally necessary at the observational level of a study as in this case.

The hydroxyaptite (HAp) mineral matrix of teeth (as well as bone) is of interest in regards to monitoring the trace and minor elemental content as measured by calcified tissues. During the mineralization process it is assumed that within the same fluids transporting calcium and phosphate ions, if present, other cations and anions are able to integrate within the HAp lattice (Rabinowitz *et al.*, 1993; Ash and Nelson, 2004). Once trapped, elements such as lead have been reported to present a half-life of *ca.* 24 years (Rabinowitz *et al.*, 1993) making primary teeth suitable markers of pre-natal exposures to elements. Deciduous teeth have traditionally been used as biomarkers of environmental exposures to several elements mainly for studies involving the mobility of toxic anthropogenic elements in areas near industrially busy areas (Stack *et al.*, 1976; Fischer *et al.*, 2008; Wilhelm *et al.*, 2007; Youravong *et al.*, 2008; van Wyk and Grobler, 1983; Rabinowitz *et al.*, 1993; Hernández-Guerrero *et al.*, 2004; Haavikko *et al.*, 1984; Gomes *et al.*, 2004; Frank *et al.*, 1999; Fosse and Justesen, 1978; Costa de Almeida *et al.*, 2007; Cleymaet *et al.*, 1991; Bud *et al.*, 2000) and to evaluate pre-natal exposures (Arora *et al.*, 2006; Ericson *et al.*, 2001).

The aim of this work, was to evaluate, using deciduous teeth as markers of pre-natal metal exposure, if children with an ASD are deficient or present a toxic accumulation of various elements in comparison to normally developing children. Given the lack of information regarding this topic at the pre-natal level, this study was designed as an observational study, intended to evaluate the issue at a preliminary level. To date, no work has been available (to the author's knowledge) that would implicate metal accumulation or deficiency as a factor in the ASD's ætiology at the prenatal level—a critical time frame in neurodevelopment (Green *et al.*, 2003; Benaron , 2009). Further, studies such as those regarding deficiency (Faber *et al.*, 2009) fail to control for the known nutritional deficits in these children. This study was thus aimed at evaluating possible metal accumulations and deficiencies, at a multi-elemental scale, using deciduous teeth as biomarkers, by a sibling-paired design to compensate for factors such as maternal diets, possible

Table 1.1: Developmental time-frames of deciduous teeth in humans as adapated from Ash and Nelson (2004). Abbreviations: *wk* = weeks, *mo*=months, *yr*=years. Notice that crown formation is generally regarded as complete during the pre-natal period of development, that is, prior to birth of the child.

	Maxillary teeth				
	Central Incisor	**Lateral Incisor**	**Canine**	**Primary Molar**	**Secondary Molar**
Initial calcification	14 wk	16 wk	17 wk	15.5 wk	19 wk
Completed crown	1.5 mo	2.5 mo	9 mo	6 mo	11 mo
Root completed	1.5 yr	2 yr	3.25 yr	2.5 yr	3 yr

	Mandibular teeth				
	Central Incisor	**Lateral Incisor**	**Canine**	**Primary Molar**	**Secondary Molar**
Initial calcification	14 wk	16 wk	17 wk	15.5 wk	18 wk
Completed crown	2.5 mo	3 mo	9 mo	5.5 mo	10 mo
Root completed	1.5 yr	1.5 yr	3.25 yr	2.5 yr	3 yr

genetic influences on the metal uptake and geographical location. This study is however, highly observational in nature, simply evaluating where these deficiencies or accumulations exist.

Chapter 2

Materials and Methods

2.1 Sampling of Teeth for the Purpose of System Optimization

Adult teeth collected from local dentists' offices were stored by the dental professionals in a 0.5% solution of Cetylcide II® (Cetylite Industries Inc., New Jersey, USA) which is a mixture of disinfectant quaternary ammonium chlorides effective as an anti-bacterial, anti-viral and anti-fungal agent, including as an anti-viral agent against Human Immunodeficiency Virus (HIV). These teeth were not used for the study of metal exposure/deficiency but were used initially as a means of optimization of the analytical systems when needed. Teeth were also provided to the laboratory on a voluntary basis after extraction of wisdom teeth. These teeth, were prepared by the dental surgeon, sealed in an appropriate sealed package and surface sterilized by exposure of the package including the tooth to UV radiation. Once these teeth were acquired by the laboratory, they were allowed to soak in a 70% solution of denatured ethanol for a period of approximately 30 days prior to being prepared as per section 2.3.1 as a precautionary step to ensure the teeth were free of any pathogens.

2.2 Sampling of Deciduous Teeth within Ontario

Deciduous teeth were collected after receiving ethics approval from the Office of Research Services, Ryerson University, to proceed with the sampling/project (file #: REB 2008-106) and the project was advertised under the title *"The Canadian Toothfairy Project"*. Sampling was performed by presenting the project to several daycare centers and primary schools throughout the Toronto, Ontario region and by advertising through a website which was circulated through the use of generally available media. In particular, several on-line forums targeting the group of interest, parents of autistic children, which allowed for a geographically farther reaching project. A substantial effort was made to pass along information regarding the project through word-of-mouth while remaining within the bounds of the research ethics condition of privacy. For this reason, sampling was performed with no interaction between the donors and the research group. This anonymity was achievable *via* the internet and by recruiting third parties at institutions interested in aiding in the sampling. When institutions—in particular primary schools and daycare centers—were involved in the sampling, the highest authority (*i.e.* school board or regional daycare director) was contacted for permission prior to contacting any specific individual to aid in sampling. This was done in order to maintain the condition that the research group would have no interaction with the children and families. As an incentive, with the *a priori* knowledge that most parents of autistic children are interested in their children's metal burden, compensation for the donated teeth was offered in the form of certificate of analysis of the donated teeth.

Parents were asked to mail their children's primary teeth directly to the laboratory with no return address and to provide a unique Tooth ID number by which to identify their tooth/teeth when attempting to access their results. The teeth were to be sent to the laboratory with a short questionnaire which provided:

1. the child's date of birth,

2. the child's place of birth,

3. all places in which the child lived (with dates),
4. all places in which the mother lived before and during pregnancy (with dates),
5. smoking habits of the parents including potential exposure to second hand smoke by the parents' tolerance to anyone smoking indoors during pregnancy and the first year of the child's life,
6. the mother's occupation before, during and after pregnancy,
7. voluntary inclusion of the child and mother's medical history including any medications taking before, during and after pregnancy (mother) and any complications at birth, diagnosed disorders (*i.e.* autistic status, diagnosed with attention-deficit hyperactive disorder *etc.*), medications taken by the child (*i.e.* peri-natal use of antibiotics), special diets.

The child's sex was determined by asking the parents to use complete sentences when completing the survey. This allowed for the parent's to decide if they preferred to use neutral pronouns if they had issues in disclosing the sex of the child (*i.e.* had issues with the differentiation of gender versus sex). As such, phrases such as "he/she was diagnosed with..." were used to extract sex.

This information was also provided in sealed envelopes at the daycare centers and primary schools selected for sampling.

The full set of exfoliated deciduous teeth ($n = 20$) were collected, by donation, from all children within 22 families in which at least one child was diagnosed with a known ASD. The full set of deciduous teeth was also collected from 7 families with no known history or diagnosis of an ASD in any of their children which acted as a control group. The sample is summarized in Table 3.1 (section 3.1). The teeth were collected from families in which all children were accounted for, which allowed for a sibling-paired study of exposures and deficiencies. In all cases, the sample of families with autistic children presented the phenomenon in which the youngest child was the only one diagnosed with an ASD and this fact used as a normalizing condition and should be considered when evaluating all figures, tables and data from this point onward.

2.3 Preparation and Categorization of Teeth After Collection

2.3.1 Permanent teeth for the purpose of system optimizations

Teeth were prepared differently depending on the category in which they were placed (section 2.1 and 2.2). Adult teeth collected from dental practices were prepared by thoroughly rinsing them free of Cetylcide II® in fresh distilled deionised water for a period of 24 hours in a contained environment under light sonication and with the water changed approximately every 2 hours (equivalent preparation for wisdom teeth soaked in ethanol). Teeth were sorted and all teeth containing more than approximately 10 % of the tooth's surface area in restorative fillings or decay were discarded by dissolution in a mixture of 3:1 HCl/HNO_3 (to dissolve all fillings) and disposed of as chemical waste (as per Research Ethics Board file #: REB 2008-106). Teeth that were deemed to be in acceptable condition were then stripped, by scraping using a standard dental pick, of any significant organic residue, including parts of the gum-line and periodontal ligament, presumed to be left as a by-product of the extraction procedures. The teeth were then treated with a 5 % solution of hydrogen peroxide for a period of 72 hours, with one change of solution at 30 hours, to remove any remaining organic debris followed by washings in distilled deionised water and drying under desiccation.

2.3.2 Deciduous teeth

Teeth which were sent directly to the laboratory by mail, or collected in person if sampling was *via* an institute, were prepared immediately upon arrival. All of the teeth were clean and there were no signs of any significant organic matter on any of the teeth. This is presumed to be due to the fact that all teeth were naturally exfoliated and not extracted as there was no evidence of any fracture from extraction at the base of the tooth and no teeth presented a root which indicates complete absorption and natural exfoliation (Ash and Nelson, 2002). As such, to avoid any contamination from washings or metal migration under high temperatures and pressures (*i.e.* if autoclaved), the teeth were not cleaned further. Further, hydrogen peroxide, being an oxidizing agent, may oxidize

a small portion of the organic matrix in dentin and cementum, and as a result, decrease the total mass of the deciduous tooth. As such, no further cleanings were performed.

All teeth were weighed to the nearest 0.1 mg after drying at 105 °C for a period of 48 hours (to constant weight). Any decay was removed by careful picking and scraping of the decayed area with a platinum coated dental pick to avoid contamination from the use of burrs or sanding papers. Picking was done carefully after optimization of the technique on decayed adult teeth (sections 2.1 & 2.3.1). After removal of the decay, the teeth were weighed again after drying for a period of 48 hours at 105 °C.

Teeth were milled in a Retsch tungsten/carbide ball mill (Retsch GmbH, Haan, Germany) at a frequency of $7\,s^{-1}$ for 1 minute and the powders transferred to an Eppendorf vial acid washed in a 10% solution of HNO_3 and dried under clean conditions in a sealed desiccator and stored until needed. The mill was cleaned by grinding a small portion of quartz sand in the mill, followed by three millings of high purity cellulose (99.999+%, Sigma-Aldrich, Oakville, Ontario, Canada), the last of which was checked for contamination by wavelength-dispersive X-ray fluorescence spectrometry (as calcium and phosphorous) using an S4 Explorer spectrometer (Bruker-AXS, Madison, WI, USA) and the associated standardless algorithm (SpectroPlus® v.1.7.2).

2.4 Elemental Analysis

2.4.1 Preparation of Samples

Digestion of the tooth samples was performed in acid washed (section 2.3.2) polypropylene tubes (5 mL maximum capacity). The tooth powders were weighed to the nearest 0.1 mg and placed in the tube, with a second weighing of the weighing paper to account for any potential loss of sample. The tooth powders were treated with 1 mL of a 3:1 HCl:HNO_3 mixture (*aqua regia*) (HCl, 37.0–38.0%; HNO_3, 69.0-70.0%, EMD Biosciences,Darmstadt, Germany, used throughout this study) which was found to digest the sample nearly completely upon contact in comparison to pure HNO_3. The solution was then allowed to stand at 50 °C for a period of approximately 4 hours

prior to being quantitatively transferred to an acid washed Pyrex® volumetric flask. No sign of incomplete digestion was observed with a resultant clear solution. The solution was brought to a final volume of 5 or 10 mL depending on the mass of the tooth powder used (5 mL dilution of *ca.* less than 0.17 g, 10 mL for *ca.* equal to or greater than 0.17 g) using distilled deionised water (18.2 $M\Omega \cdot cm$) drawn from a MilliQ® purification system (used throughout this study). Standard reference materials were also prepared in an identical matter for system checks and validation throughout the analysis using a *ca.* 0.1 g of SRM bone ash (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA) and a final volume of 10 mL. The SRM was prepared after drying at 105 °C for a period of 48 hours and storing under desiccation until needed. Solutions were quantified within 1 hour of preparing of the final dilution.

2.4.2 Quantitative Analysis

Quantitative analysis was performed by inductively-coupled plasma atomic emission spectrometry (ICP-AES) using a similar system and method as described by Webb *et al.* (2005). The ICP-AES system consisted of a SpectroFlame Compact E spectrometer (SPECTRO Analytical Instruments, Kleve, Germany) equipped with an axial view torch and a concentric glass nebulizer. The system operating conditions consisted of a argon plasma power of 1450 W, a coolant flow rate of 15.0 L/min (Ar), an auxiliary flow rate of 0.3 L/min (Ar), a nebulizer flow rate of 1.0 L/min (Ar) and a sample aspiration rate of 0.7 mL/min. Integration times of 800 ms were used over 1 measurement due to a lack of sample volume. Measurements were made using the analytical lines shown in Table 2.1 all within the simultaneous mode of detection.

Standard curves were prepared using 1,000 $\mu g \cdot mL^{-1}$ solutions of the analyte (ULTRA Scientific, USA). Each calibrator was made to a final concentration of 1000 $\mu g \cdot mL^{-1}$ Ca^{2+} to compensate for matrix issues (10,000 $\mu g \cdot mL^{-1}$ stock; ULTRA Scientific, USA) (Webb *et al.*, 2005).

Cleaning of the aspirator line was performed using a 2% HNO_3 solution using a cleaning/flush time of 45 seconds. Cleaning was considered complete when a sample of water was quantified with no signal above background.

Table 2.1: Analytical lines used for the quantitative analysis of deciduous teeth digests by ICP-AES. The analyses were made using simultaneous detection due to the rather small sample volumes necessary for analysis. Limits of detection (LOD) are listed at the 3σ level.

Element	Line (nm)	LOD ($\mu g \cdot L^{-1}$)
Al	167.080	14
Cr	267.716	8
Cu	654.792	8
Fe	259.940	3
Hg	184.950	8
K	766.491	12
Mg	279.079	17
Mn	257.610	4
Na	589.592	15
Ni	231.604	8
Pb	168.215	3
Sr	407.771	11
Zn	213.856	7

Calibrations were checked after every five samples by using the highest and lowest concentration calibrators as monitors. At this time, a solution of SRM was quantified to validate the calibration.

Quantiative analysis was performed directly on the SmartAnalyzer software associated with the spectrometer in which a digital smoothing algorithm was employed for elements at concentrations in the lower $\mu g \cdot L^{-1}$ concentration range.

2.5 Statistical Treatment of Data

All statistical analyses were performed in MatLab version 7.0. The data were found not to follow a normal distribution in all cases by means of a Lillifore test of normality and a Shapiro-Wilk test of normality ($\alpha = 0.05$), regardless of pairing, and the data was non-responsive to transformation. Non-parametric statistics were thus performed to estimate the level of significance of our conclusions. The Kruskal-Wallis test was used as an alternative to the one-way ANOVA (analysis of variance) for the initial comparison of multiple populations (based on the evaluation of equality

of medians) (section 3.2). Following the Kruskal-Wallis test if differences were detected (if $p < \alpha$) then a multi-comparison was performed based on the mean ranks using the Scheffé procedure at $\alpha = 0.05$ (section 3.2). Comparison of the sibling-paired differences were made to zero using the Wilcoxon signed-rank test which is the non-parametric equivalent to a paired t-test (section 3.3).

Chapter 3

Results and Discussion

3.1 General Sample Description and Statistics

The sample was acquired from the southern Ontario region and is summarized in Table 3.1. The sample presented 22 families in which one child in each family was diagnosed with an ASD. The most prevalent ASD in the sample was non-verbal autistics followed by functioning autistics, high-functioning autistics and children with Asperger's syndrome representing the lowest proportion of the sample (Table 3.1). The sample presented only two of the known ASDs: autistic disorder and Asperger's disorder (Table 3.1). Autistic disorder was also classified by the parents based on the level of functioning (Table 3.1). The absence of any children with Retts disorder and childhood disintegrative disorder is likely to be due to the rarity of these disorders, (section 1.1) (Green *et al.*, 2003; Benaron, 2009) and thus the low probability of observing them in a small sample size ($n = 22$ children with an ASD, Table 3.1). This study was thus limited to evaluating the two major types of ASD: autistic disorder and Asperger's disorder. A total of 31.8% of the families with a child diagnosed with an ASD also presented at least one other child with attention-deficit hyperactive disorder (ADHD). Out of the seven control families, no children presented any history of ADHD. Co-morbidity of ASD with ADHD is known (Green *et al.*, 2003), however actual proportions within families is not presented elsewhere.

Table 3.1: General description of the sample collected throughout the duration of the *"Toothfairy Project"*. Proportions are listed in percentages with the corresponding ratio presented in parentheses.

	Autistic Group	**Control Group**
Numer of Families	22	7
Total Number of Children	62	20
Average Number of Children per Family	2.9 ± 0.8	2.9 ± 0.9
Range of Children per Family	2-5	2-4
Families allowing smoking indoors and during pregancy (%)	13.6 (3/22)	0
Families presenting children with ASD and ADHD	31.8 (7/22)	0
male:female (total)	1:1.6	1:1.5
male:female (ASD)	6.3:1	N/A
male:female (ADHD)	1.3:1	N/A
% ASD type		
% Asperger's	18.2 (4/22)	0
% High-functioning autistics	4.5 (1/22)	0
% Functioning autistics	36.4 (8/22)	0
% Non-verbal autistics	40.9 (9/22)	0
Families from a given location (%)		
Toronto	18.2 (4/22)	85.7 (6/7)
Hamilton	22.7 (5/22)	14.3 (1/7)
Burlington	9.1 (2/22)	0
Ottawa	22.7 (5/22)	0
Kingston	4.5 (1/22)	0
Newmarket	9.1 (2/22)	0
Orangeville	9.1 (2/22)	0
London	4.5 (1/22)	0

The male-to-female ratio of children with ASD was found to be 6.3:1 which is slightly higher than the 4:1 expected for ASD (Green *et al.*, 2003). The male-to-female ratio of children with ADHD was found to be 1.3:1 which is lower than the ratio expected for ADHD of 9:1 (Biederman *et al.*, 2002). This discrepancy is however attributed to the relatively small sample size in this study in comparison to much larger epidemiological works evaluating this phenomenon specifically looking at this sex difference ($n = 520$ in Biederman *et al.*, 2002).

All teeth in this study were provided with no intact root, which allowed for a study of metal exposure during the pre-natal and peri-natal period(s) only (Table 1.1), in which the child is largely dependant on the mother for any possible exposure or deficiency. The root continues to mineralize up until 3.25 years of life (Ash and Nelson, 2004; Table1.1) and thus would provide information regarding exposures at the post-natal level which is not the intention of this study. The root undergoes demineralization and re-absorption prior to natural exfoliation; thus, teeth that are provided with no root or sign of significant fracture would indicate natural exfoliation (Ash and Nelson, 2004)(as in this case). Since the crown of the tooth (used for analysis in this work) mineralizes nearly completely during the gestational period (Table1.1), it was used as a means of estimating pre-natal elemental exposure and deficiencies.

Diagnosis of the listed ASD was not performed by the research group and the accuracy was dependant on the diagnosis known to the parent(s) who donated the teeth. Diagnosis of the ASDs follow strict criteria, one of the most significant being diagnosis, in its final version, prior to the age of two (World Health Organization, 1992; American Psychiatric Association, 2000; Green *et al.*, 2003; Benaron, 2009). Since deciduous teeth generally shed/exfoliate several years after this period (between ages 6-12) (Ash and Nelson, 2004; Brand and Isselhard, 1974), then by definition, parents must know of the autistic status of the child prior to donating teeth to this study. Diagnosis of PDD-NOS is also rare in older children and is often diagnosed at a preliminary level (before the age of two) prior to any firm diagnosis after the observation of the child's progression (World Health Organization, 1992; American Psychiatric Association, 2000; Green *et al.*, 2003; Benaron, 2009). Given the age in which all teeth are expected to exfoliate, the present evidence that all

Table 3.2: The relative proportions of families with a child diagnosed with ADHD correlated to the type of ASD one of the children within the family was diagnosed with.

ASD	$(ADHD_{ASD}/ADHD_{total})$
ASP	1/7
HFA	2/7
FA	3/7
NVA	1/7

the sampled teeth were exfoliated naturally and the average of 4 years after the last tooth was exfoliated to the time of donation, diagnosis is thus assumed to be accurate in regards to the listed ASD.

The diagnosis criteria for ADHD seems, however, not to be as stringent as that for the ASDs (World Health Organization, 1992; American Psychiatric Association, 2000) and in some cases, high-functioning autistics (including those with Asperger's) are mistakenly diagnosed with ADHD as it is a co-morbid disorder (Green *et al.*, 2003; Attwood, 2006; Benaron, 2009). Attention-deficient hyperactive disorders have been shown to be co-morbid with ASDs and also follow genetic hereditability (Green *et al.*, 2003). The sample had families families in which some children were diagnosed with ADHD and in all cases, had siblings with a higher-functioning form of ASD (Table 3.2). This could indicate that ADHD and ASD may have some similar pre-disposition. It was also apparent that the sample presented families in which only the youngest child was diagnosed with an ASD. This may indicate that the children presenting ADHD may in fact have a case of a higher-function ASD or be presenting broader autistic traits which were not disclosed to the research group, may not be known to the parents and may be presenting themselves as hyperactivity/attention deficit. There may also be a bias in that hyperactivity may have been perceived by the parents and not officially diagnosed. These possible issues were not differentiable in our sample and by the allowable sampling procedure, thus, in this work, ADHD is largely ignored in the analysis as its own disorder since firm diagnosis cannot be assured.

3.2 Influence of Geographical Location of Deciduous Tooth Elemental Content

Prior to comparing elemental differences between children with ASD to normally developing siblings, other factors were evaluated in regards to their influence on the metal concentrations in deciduous teeth. Factors such as smoking could not be evaluated due to the small sample size and the fact that parents who smoked or allowed smoking indoors all came from the Hamilton region. The largest influence on the elemental content in deciduous teeth was identified as being the geographical location in which the teeth were sampled/provided. In all cases, the median of the elemental contents were found to differ significantly between at least one of the geographical locations for all elements under study ($p < 0.001$, Kruskal-Wallis, Figures 3.1-3.11).

Under the statistical restrictions of a very small sample size (when considering families/number of children per location, see Table 3.1) a non-parametric view (from the box-whisker plots) of the geographical distribution of elements such as manganese (Figure 3.1), lead (Figure 3.2), copper (Figure 3.3), iron (Figure 3.4), nickel (Figure 3.5), chromium (Figure 3.6) and zinc (Figure 3.7) indicated that these elements seemed to be highest in the deciduous teeth of children from the Hamilton/Burlington area of Ontario where these concentrations differed statistically from the surrounding areas ($p < 0.05$, Scheffé).

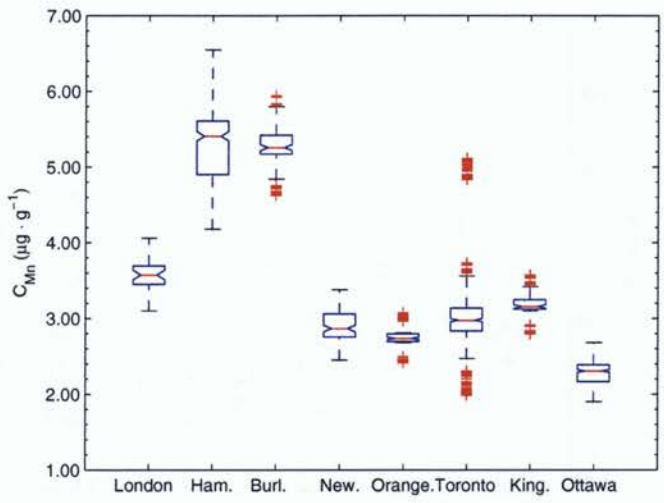
The Hamilton region has been speculation to be burdened by a high manganese air concentration which has further been implicated in a higher prevalence of Parkinson's disease in adults (Finkelstein and Jerrett, 2007). The differences observed in this study may be an indication of a highly polluted area in regards to both air and water that may be reflected in the deciduous teeth of the children living in that area. The elements listed previously are generally of interest in regards to anthropogenic activity and its effects on humans as well as in mobility studies (Stack *et al.*, 1976; Fischer *et al.*, 2008; Wilhelm *et al.*, 2007). Due to the very small sample size however, statistical analysis is hindered and this observation/trend should be investigated further with a much larger and randomized sample set. In regards to the known neurotoxins manganese and

lead, it has been shown that children exposed to high levels of these elements, through water and air, present behavioural problems, particularly attention deficits (Bouchard *et al.*, 2007; Bellinger *et al.*, 1994). Since the sample presented in this study is biased in that only children with a known behavioural problem were sampled, this observation should be evaluated as the results indicate, at a preliminary level, that the Hamilton/Burlington area presents children with a heavier burden of these anthropogenic and known toxic elements. This preliminary observation should be evaluated further both at the pre- and post-natal levels in a similar fashion to that of Bouchard *et al.* (2007).

Aluminium was found to be below the detection limits of *ca.* 0.80-1.85 $\mu g \cdot g^{-1}$ in all cases, which did not allow for an analysis of this anthropogenic element in a similar fashion to those previously discussed.

Variations in the essential element content—that is sodium (Figure 3.8), magnesium (Figure 3.9) and potassium (Figure 3.10)—as well as that of strontium (Figure 3.11) (a non-essential element), may be artifacts of a small sample size and random variable blocking which may have registered a difference by Scheffé's procedure. These changes and differences may also be effects of variations in the water supply, or in the dietary habits of the sample. Given that the sample size was small, this registered difference may in fact be differences observed due to dietary differences and choice of water supply between families, rather than in the general population.

The fact that deciduous teeth are able to register differences that would be expected for the Hamilton/Burlington region (based on Finkelstein and Jerrett, 2007), does indicate the usefulness of deciduous teeth as biomarkers for the main aim of this work: that is, if differences can be observed based on geography, they would be expected to be observed if present as a function of autistic status.



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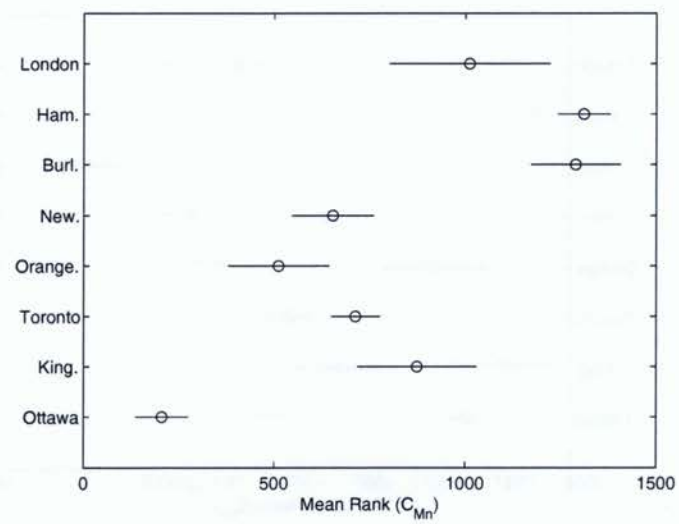
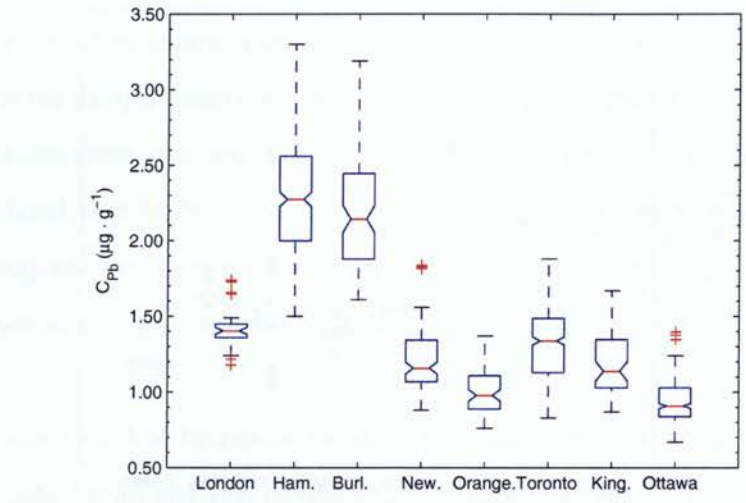


(b)

Figure 3.1: (a) Box-and-whisker plot describing the differences in **manganese** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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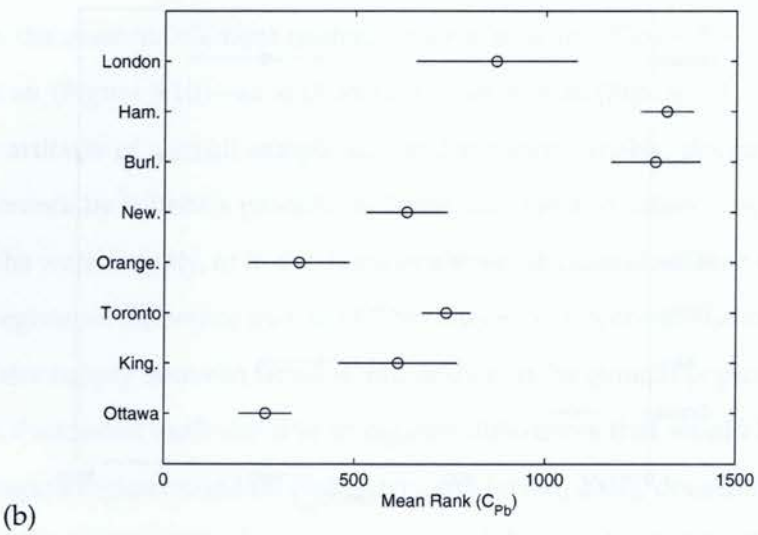
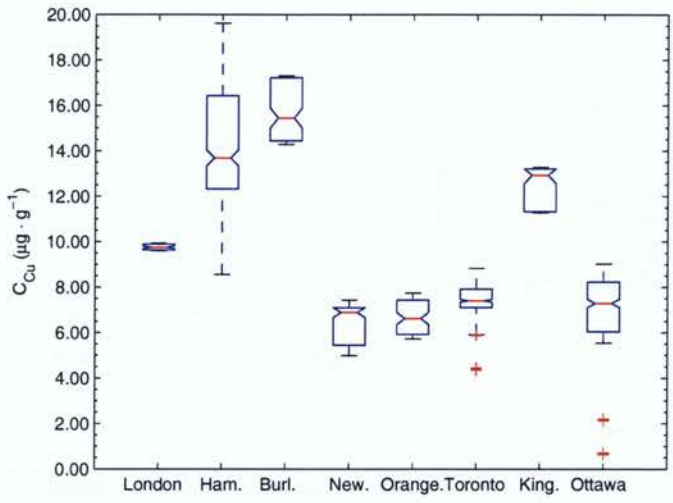


(b)

Figure 3.2: (a) Box-and-whisker plot describing the differences in **lead** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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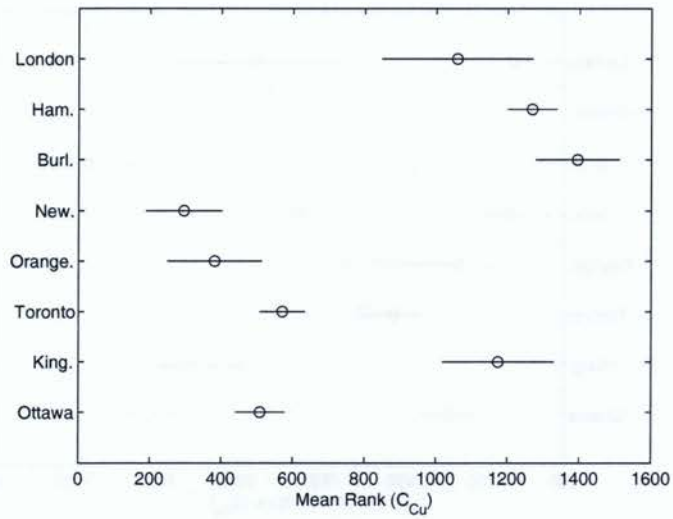
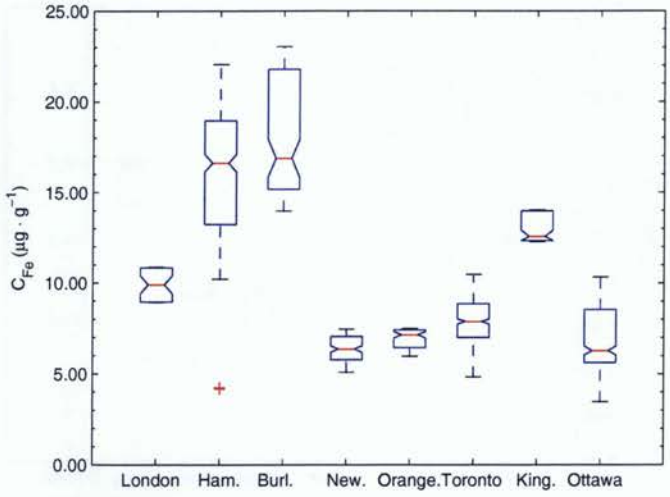


(b)

Figure 3.3: (a) Box-and-whisker plot describing the differences in **copper** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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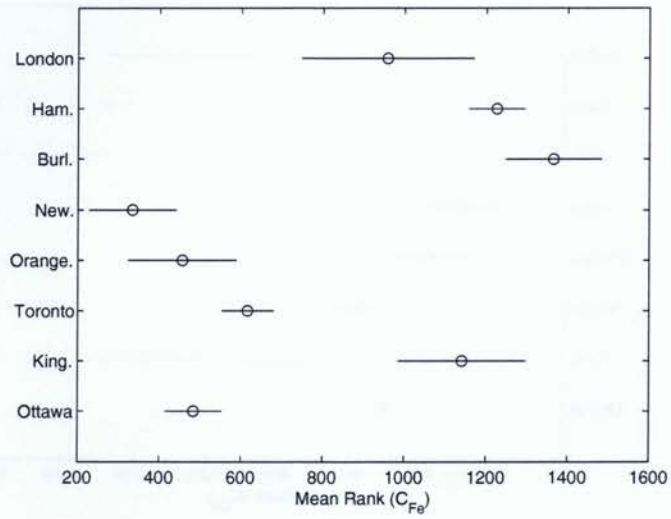
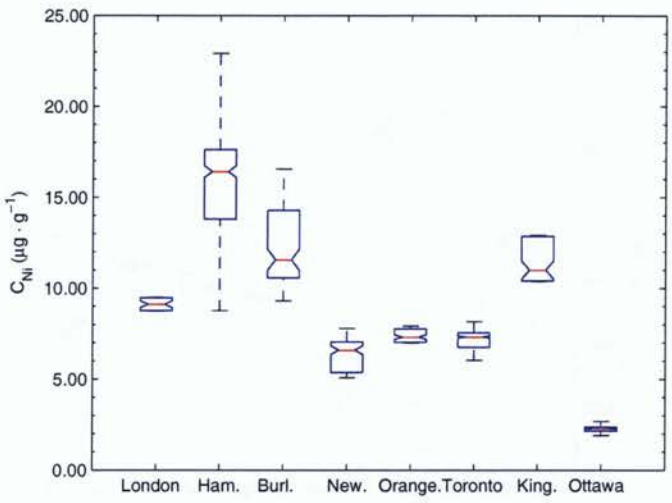


(b)

Figure 3.4: (a) Box-and-whisker plot describing the differences in **iron** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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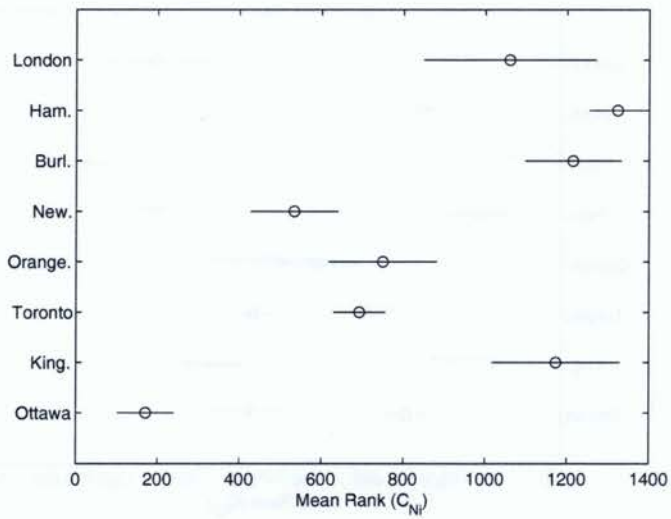
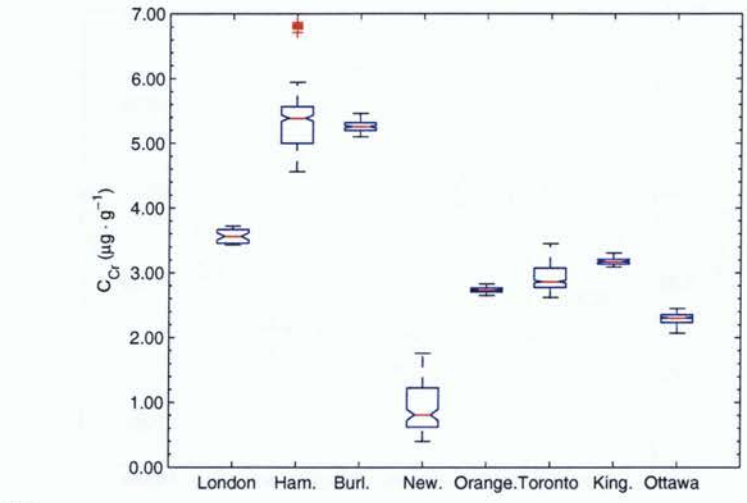


(b)

Figure 3.5: (a) Box-and-whisker plot describing the differences in **nickel** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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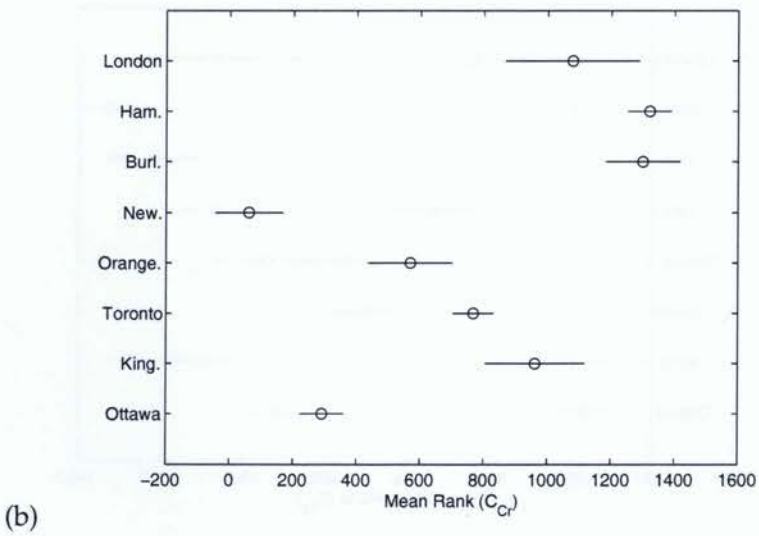
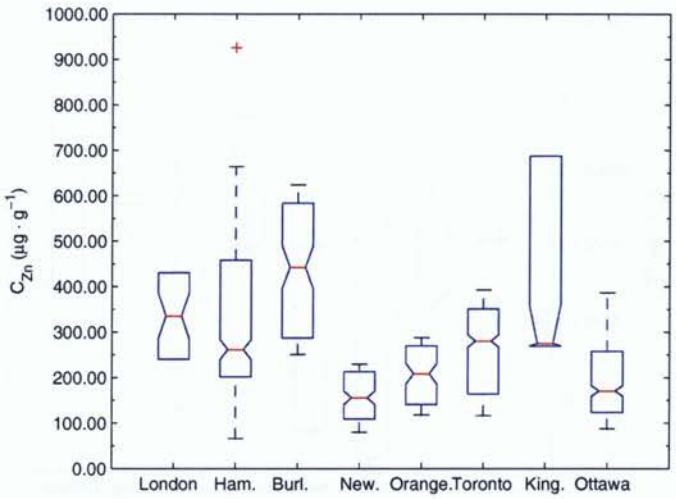


(b)

Figure 3.6: (a) Box-and-whisker plot describing the differences in **chromium** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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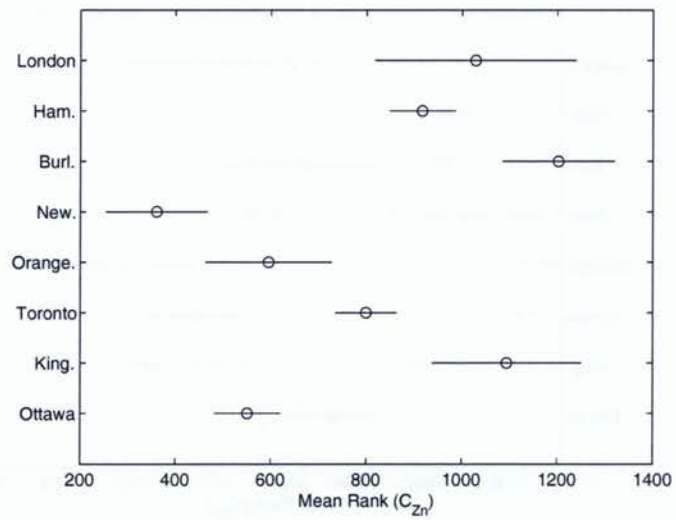
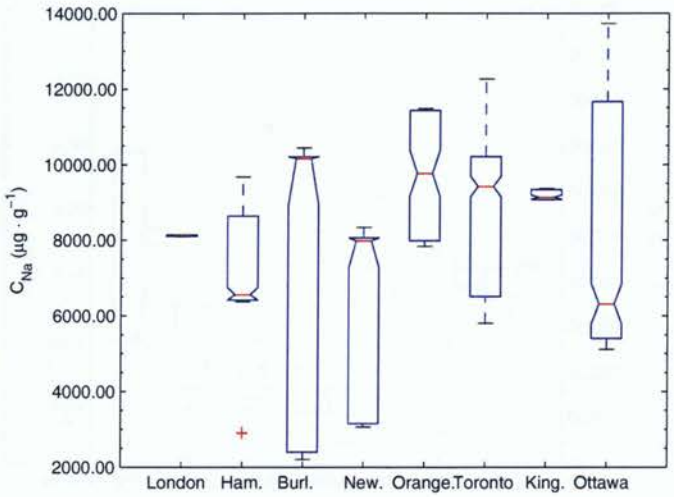


(b)

Figure 3.7: (a) Box-and-whisker plot describing the differences in **zinc** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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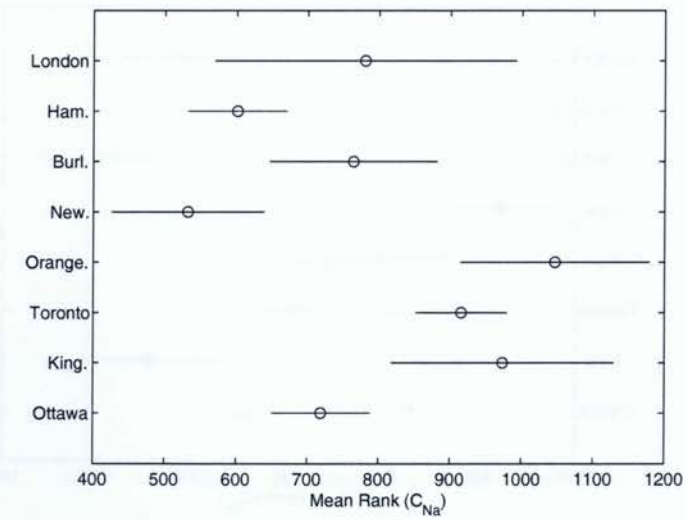
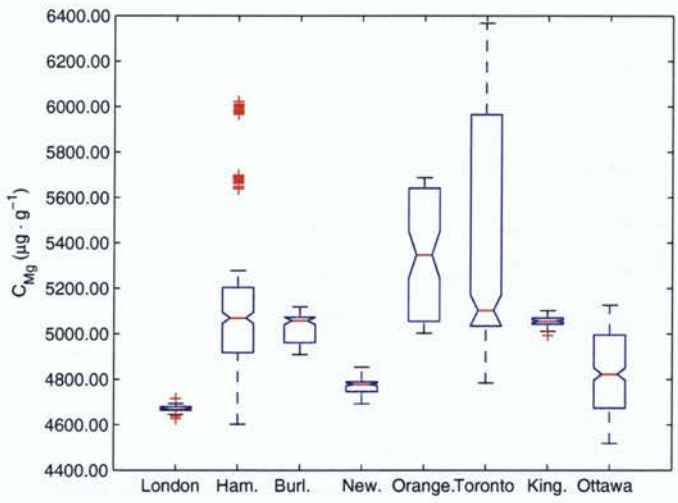


(b)

Figure 3.8: (a) Box-and-whisker plot describing the differences in **sodium** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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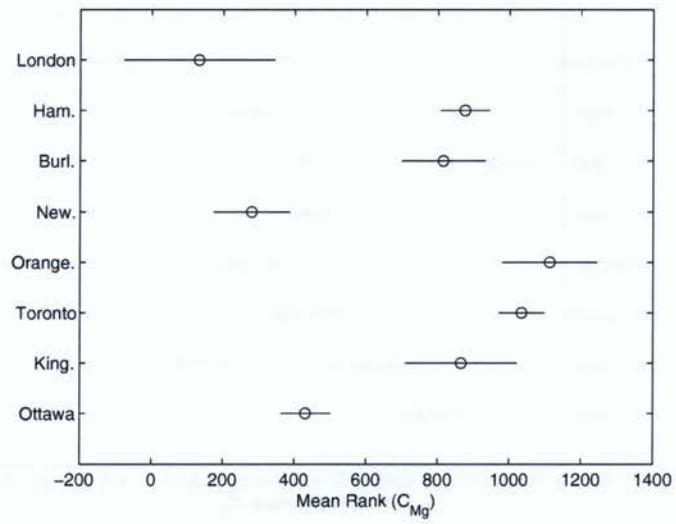
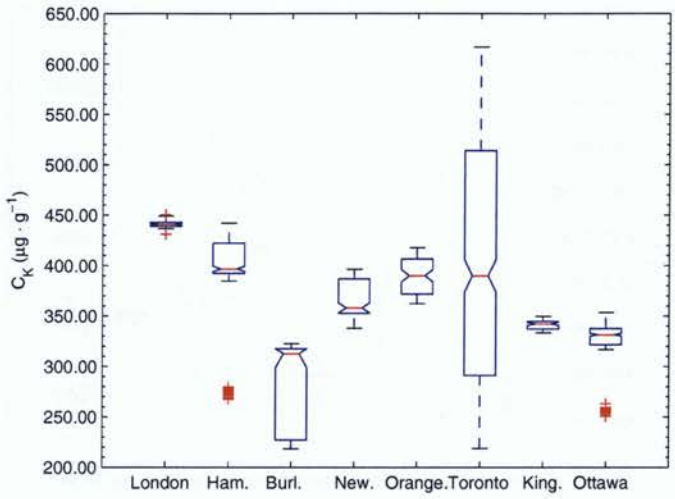


(b)

Figure 3.9: (a) Box-and-whisker plot describing the differences in **magnesium** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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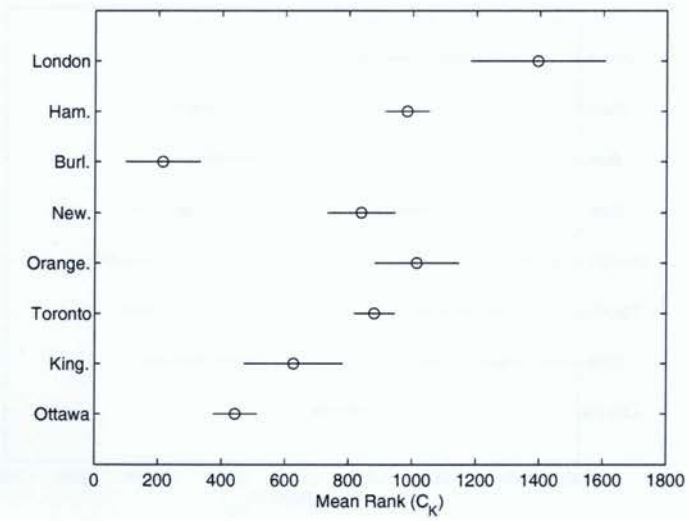
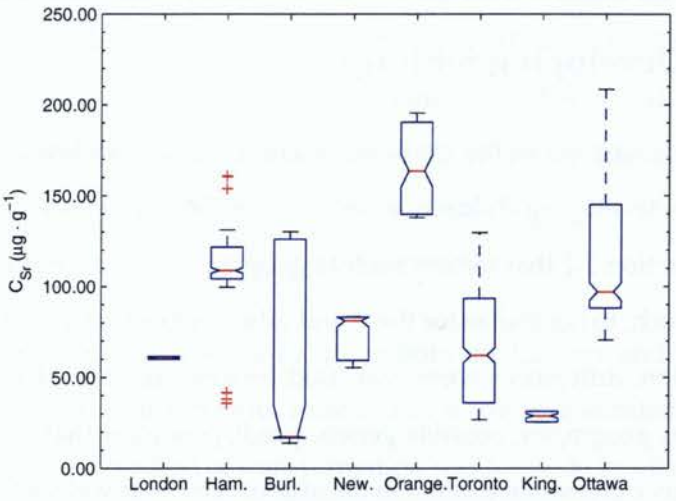


(b)

Figure 3.10: (a) Box-and-whisker plot describing the differences in **potassium** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).



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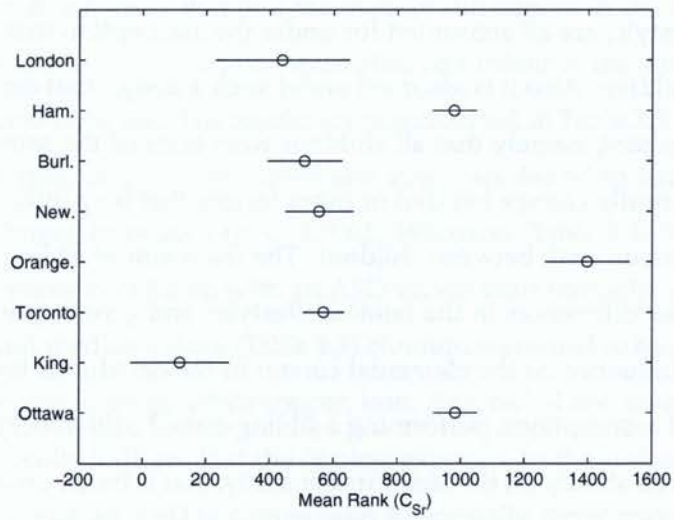


(b)

Figure 3.11: (a) Box-and-whisker plot describing the differences in **strontium** concentrations in the deciduous teeth of children organized by the geographical location in which they were sampled. The upper and lower limits of the box represent the 25th and 75th percentiles while the total distance within the box is the interquartile range (IQR). The whiskers represent the maximum and minimum of the data for data points not considered outliers (represented by the red crosses which are data with values greater than 1.5IQR). Notches in the box represent graphical confidence intervals to the median. (b) A multi-comparison plot produced from the mean ranks after an initial Kruskal-Wallis comparison of multiple means ($\alpha = 0.05$) by Scheffé's procedure. The figure is read by comparing for overlapping intervals between populations, in which, overlap represents no difference between population medians ($\alpha = 0.05$). In all cases, the signal was above the detection limit of the spectrometer at the time of measurement (Table 2.1).

3.3 Difference in Elemental Content in Children with ASD versus Their Normally Developing Siblings

To evaluate possible differences in the elemental accumulation or deficiency in children with an ASD versus normally developing children, a sibling-paired design was employed. It has already been established in section 3.2 that factors such as geography influence the elemental content in deciduous teeth. As such, to normalize for these and other factors that may influence the observed elemental concentration, differences were evaluated between siblings. Under this experimental design, factors such as geography, possible genetic predisposition—that is unknown genetic factors that would influence elemental content in deciduous teeth—as well as dietary considerations, that is, diets based on ethnic background that may include different types of food or the choice to live a vegetarian lifestyle, are all accounted for under the assumption that they remain relatively constant between children. Also it is assumed under such a design that certain aspects within the family remained constant, namely that all children were born of the same parents and that the mother did not drastically change her diet or other factors that may influence elemental concentrations in the deciduous teeth between children. The main aim of sibling-pairing however, was to normalize for gross differences in the familial lifestyles and genetic pre-dispositions that may ultimately have an influence on the elemental conent in the deciduous teeth of children. Under these conditions and assumptions, performing a sibling-paired (difference) analysis should allow for a comparison made strictly on the factor under study, that is based on autistic status.

Comparison was thus made on the paired differences of mean elemental concentrations in the deciduous teeth from the given children to compensate for any variations within the teeth (these variations were found to be less than 10% and showed no specific trend). Using the mean concentration between $n = 20$ teeth for each child allows for an overall evaluation of total gestational exposures or deficiencies (Table 1.1). The sample presented families in which the youngest child was always the one diagnosed with an ASD (section 3.1). This allowed for normalization based on the autistic child in which the difference under consideration (and used for evaluation purposes)

was the difference between the concentrations of a given element in the deciduous teeth of the autistic child ($\bar{C}_x^{ASD}$) from that of the normal child ($\bar{C}_x^{norm}$) (Eqn. 3.1). The control group was also treated in an identical fashion, in which the difference was taken based on the youngest child (where $\bar{C}_x^{ASD}$ is replaced by the mean concentration in the youngest child's teeth).

$$\delta\bar{C}_x = \bar{C}_x^{ASD} - \bar{C}_x^{norm} \tag{3.1}$$

The differences were evaluated for normality by both the Lillifore and Shapiro-Wilk tests of normality. All differences for all elements were found to deviate substantially from a normal distribution, and no standard method of transformation was found to be effective at transforming the differences into normally distributing random variables ($p < 0.05$). Since the differences were being compared to zero in all cases, that is if the median differences in the sampled population were significantly different from zero, a non-parametric equivalent to the usual paired *t*-test was used, the Wilcoxon signed-rank test. The results are summarized in Table 3.3.

Chromium, copper, iron, magnesium, nickel and zinc were found to have a median paired-difference that was different from zero ($p < 0.0001$, Wilcoxon, Table 3.3) indicating that these elements showed differences in children with an ASD versus their normally-developing counterparts. Based on mean and median values (Table 3.3) chromium seemed to be in excess in comparison to normally developing siblings, while copper, iron, zinc, nickel and magnesium were found to be in deficit. These results indicate that the *in utero* exposure to these elements was generally different in the children with an ASD in comparison to normally developing siblings. No effect was, however, observed for the toxic and non-essential elements, manganese, lead and strontium (Table 3.3).

Although manganese and lead have been shown to be correlated to behavioural disorders at the post-natal level (Bouchard *et al.*, 2007; Bellinger *et al.*, 1994), this observation suggests that there is no difference in levels between children with ASD and their normally developing siblings at the pre-natal time of neurodevelopment. The concentration of these toxic/trace elements

Table 3.3: Results of the Wilcoxon signed-rank test to compare if the sibling-paired differences were in fact different from zero. The null hypothesis in this case is that the difference is equal to zero, thus a p-value less than $\alpha = 0.01$ indicates that there is a difference at the 99% level of confidence. All units of concentration are in $\mu g \cdot g^{-1}$.

Element	Group	Mean (±SD)	Range	Median	p	
Cr	ASD	0.1 ± 0.1	0.50–(-0.21)	0.08	1.44×10^{-6}	$\delta\bar{C}_x \neq 0$
	Control	-0.2 ± 0.5	0.42–(-1.75)	-0.14	0.0742	$\delta\bar{C}_x = 0$
Cu	ASD	-2 ± 2	-0.16–(-10.32)	-1.80	3.57×10^{-8}	$\delta\bar{C}_x \neq 0$
	Control	-0.3 ± 0.5	0.64–(-1.52)	-0.17	0.0449	$\delta\bar{C}_x = 0$
Fe	ASD	-3 ± 3	1.88–(-11.77)	-2.00	4.98×10^{-7}	$\delta\bar{C}_x \neq 0$
	Control	-0.2 ± 0.5	0.42–(-1.75)	-0.14	0.90967	$\delta\bar{C}_x = 0$
K	ASD	0 ± 5	11.21–(-11.55)	-1.15	0.76745	$\delta\bar{C}_x = 0$
	Control	-4 ± 7	9.09–(-16.60)	-5.72	0.063965	$\delta\bar{C}_x = 0$
Mg	ASD	-8 ± 8	10–(-24)	-5	3×10^{-7}	$\delta\bar{C}_x \neq 0$
	Control	-94 ± 272	316–(-570)	-5	0.67725	$\delta\bar{C}_x = 0$
Mn	ASD	0.1 ± 0.2	0.50–(-0.35)	0.02	0.56774	$\delta\bar{C}_x = 0$
	Control	0.02 ± 0.15	0.21–(-0.33)	0.02	0.53418	$\delta\bar{C}_x = 0$
Na	ASD	-56 ± 293	956–(-1092)	-1092	0.10387	$\delta\bar{C}_x = 0$
	Control	-180 ± 738	-111–(-1050)	-1160	0.33936	$\delta\bar{C}_x = 0$
Ni	ASD	-3 ± 4	1.36–(-14.10)	-14.10	2.40×10^{-6}	$\delta\bar{C}_x \neq 0$
	Control	-0.4 ± 0.7	1.01–(-1.75)	-1.75	0.074219	$\delta\bar{C}_x = 0$
Pb	ASD	0.1 ± 0.2	0.55–(-0.39)	0.08	0.077116	$\delta\bar{C}_x = 0$
	Control	0.01 ± 0.19	0.44–(-0.23)	-0.01	0.96973	$\delta\bar{C}_x = 0$
Sr	ASD	-1 ± 9	20.20–(-29.75)	-1.78	0.19692	$\delta\bar{C}_x = 0$
	Control	-2 ± 7	11.44–(-16.33)	-1.53	0.46973	$\delta\bar{C}_x = 0$
Zn	ASD	-159 ± 146	18–(-682)	-123	5.22×10^{-8}	$\delta\bar{C}_x \neq 0$
	Control	8 ± 20	59–(-16)	-9	0.26611	$\delta\bar{C}_x = 0$

Table 3.4: Basic statistics on the number of days between births/conception of normal and autistic children. There is no difference between the ranges ($p > 0.04$).

	Range	Mean $\pm SD_{(0.05)}$	Median
Autism	467–2005	898 ± 922	766
Normal	441–1492	711 ± 532	606

was also found to be statistically equivalent to the control group within the same geographical region (comparison only possible within the Hamilton and Toronto regions, Table 3.1) ($p < 0.001$, Wilcoxon). This would indicate that although a large variation is observed between the regions, these metals may be producing no effect during gestation that would indicate a concern in regards to the ætiology of ASDs. As discussed previously (section 3.1), the sample did contain families which presented children with ADHD along with children with an ASD. The fact that the metals do not vary in concentration between the sub-populations may indicate there is no real effect, at least within this sample, for both disorders at the pre-natal level although the authenticity of the ADHD diagnosis is under question (section 3.1). Other groups have demonstrated that children with an ASD may be presenting symptoms related to toxic metal accumulation, in particular suggesting that children may be poor excretors of metals (James *et al.*, 2004; Konstantareas and Homatidis, 1987; Bradstreet *et al.*, 2003; Geier *et al.*, 2009; Palmer *et al.*, 2006; Windham *et al.*, 2006; Williams *et al.*, 2008; Lonsdale, 2002; Filipek *et al.*, 1999; Eppright *et al.*, 1996; Fido and Al-Saad, 2005; Wecker *et al.*,1985; Shearer *et al.*, 1982; Accardo *et al.*, 1988; Kern *et al.*, 2007; Kern et al., 2004; Owens 1998, 2001; Waring and O'Reilly, 1990; Waring and Klovrza, 2000). If this is in fact an effect, it would most likely be due to post-natal exposures or deficiencies and not due to pre-natal exposures/deficiencies based on the observation of no statistical difference by this sibling-paired design.

Further comparison was made as to the actual rate of deficiency or accumulation that was observed as the mother produced more children. In cases of families with children with an ASD, it was found that the elemental content was either decreasing or increasing as the mother had more children which was not a consistent trend in control families (Figures 3.12-3.17, Table 3.5).

Table 3.5: Rates of decline or increase as a function of the mother's age in relation to the birthdate of the first child. The units are in $\mu g \cdot g^{-1} \cdot day^{-1}$.

Element	Group	Mean (±SD)	Range	Median	p
Cr	ASD	9 ± 7	1.59–23	6	0.00004
	Control	-56 ± 146	-378–58	-23	0.38
Cu	ASD	-160 ± 143	-618–(-13)	-116	0.00004
	Control	-16 ± 85	-154–138	-23	0.22
Fe	ASD	-261 ± 307	-1223–271	-160	0.00043
	Control	-41 ± 165	-296–146	25	0.94
Mg	ASD	-571 ± 676	-2186–410	-374	0.00029
	Control	7474 ± 28313	-20969–68324	986	0.58
Ni	ASD	-99 ± 224	-772–257	-84	0.05750
	Control	-59 ± 101	-157–141	-103	0.22
Zn	ASD	-18337 ± 12820	-59373–(-6113)	-15378	0.00004
	Control	418 ± 2081	-3564–2863	637	0.58

Although the mother's age was not provided in this study, a rough estimate of the rate of deficiency/accumulation based on the number of days between children's births was performed for these elements and is summarized in Table 3.5. In all cases, the median rate of decline (used as a means to determine directionality of the trend) was found to statistically differ from zero, which indicates a general trend of a steadily changing difference in the concentration of these elements as the mother has more children. In general, the range of days between children did not differ between autistic children and normally-developing siblings which would indicate that the number of days between births/conception does not have any effect (Table 3.4, $p > 0.04$) (that is that these deficiencies/accumulation are not due to having children too close after previous conception). Whether this effect is age related cannot, however, be evaluated but would be a factor to include in any further work.

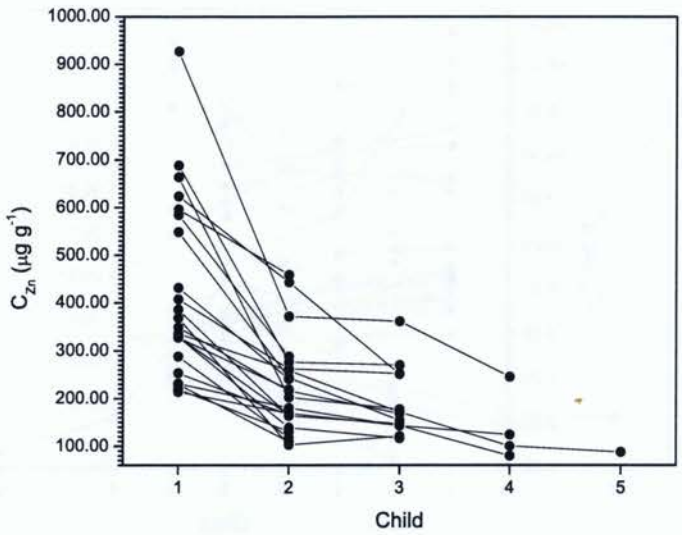
Although differences were observed, as was a general rate of decline in these elemental contents (Figures 3.12-3.16), with the exception of chromium (Figure 3.17), an absolute concentration that would group the autistic children could not be identified based on the observed deficien-

cies and accumulation. That is, that the ultimate concentration of these elements in any given child within a family with an ASD was roughly equivalent to a concentration for the same element present in at least one normal child or a control child (Figures 3.12-3.17). These elemental ranges observed in children with an ASD roughly fall within the range expected and observed for geographical locations (section 3.2).

Zinc and copper in particular (and exclusively) have been quite recently implicated as elements that are responsible for ASDs at the post-natal level (Faber *et al.*, 2009). Faber *et al.* (2009) have demonstrated that children with an ASD have lower zinc levels and higher copper levels in their blood in comparison to the normal population. They propose the possibility that this is due to a malfunctioning methallothionein system, implicated in the ætiology of mental retardation, and in trace element accumulation. Supporters of the poor excretion theory also argue that the zinc– and copper–mediated glutathione is also responsible for the ætiology of the ASDs, as low levels of these elements would reduce the production of the protein, which is argued to be responsible for the detoxification of toxic elements (Kern *et al.*, 2007; Kern et al., 2004; Owens 1998, 2001; Waring and O'Reilly, 1990; Waring and Klovrza, 2000). In regards to this study, although a deficiency was observed in comparison to siblings, the fact that no absolute range of deficiency was observed would indicate that these hypotheses are invalid at the pre-natal level, as this associated deficiency would result in marked differences in the trace/toxic element content in children with ASD, which was not observed here (Table 3.3). This indicates, along with the fact that no particular level of deficiency specific to ASD children could be identified, that the deficiency may be a secondary effect of another problem.

This observed trend is elusive and the ultimate meaning inconclusive and outside of the scope of this work; however, a genetic understanding of the ASDs has been hindered the significant issues with co-morbidity and the associated issues in identifying specific genes responsible for the ASDs (section 1.2). The most significant co-morbid factor that is nearly exclusive to children with ASD is digestive tract problems (Green *et al.*, 2003; Benaron , 2009). In fact, these issues and the presented symptoms are so unique that they have been hypothesized to be their own disorder

known as autistic enteritis. These conditions are well documented to produce nutritional deficiencies in children with ASD. The marked decrease that is observed in zinc, copper, iron and magnesium concentrations (most pronounced for copper, zinc and iron) may in fact be the manifestation of problems in the mother, potentially at the level of the gut. This could be manifesting from a genetic predisposition to similar intestinal and absorption issues as to children with ASD. These health conditions may be influencing the absorption and transfer of essential elements to the children as the disorder progresses, and the consistent trend over time would indicate that if such a condition is present, that it may be progressive. These conditions, whatever they may be, may therefore cause other stressors that may be the actual trigger for the development of ASD in the children, rather than the presented deficiency or accumulation of the said elements. Such an explanation is in accordance with groups investigating peri-natal and maternal factors associated with possible epigenetics of ASDs (Bilder *et al.*, 2009; Kolevzon *et al.*, 2007; Green *et al.*, 2003).



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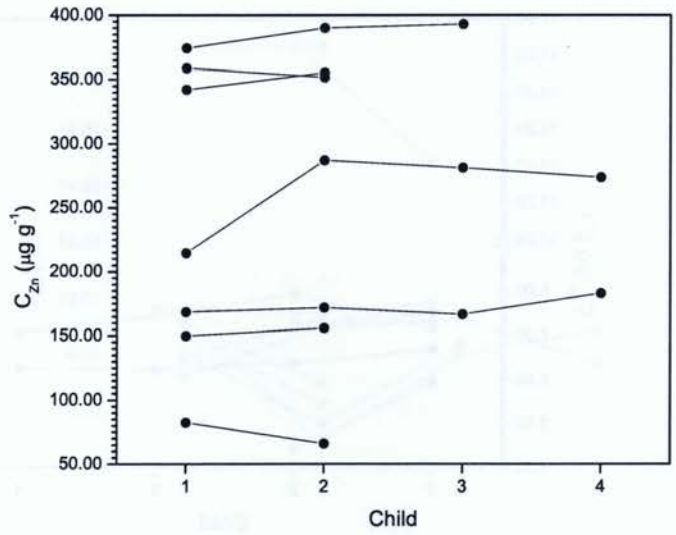
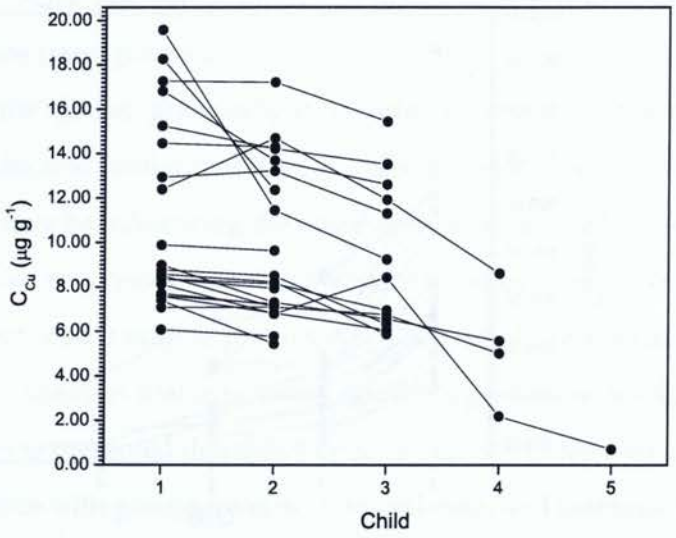


(b)

Figure 3.12: Zinc content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 zinc is shown to decrease over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*). This decrease is relatively consistant over the number of children.



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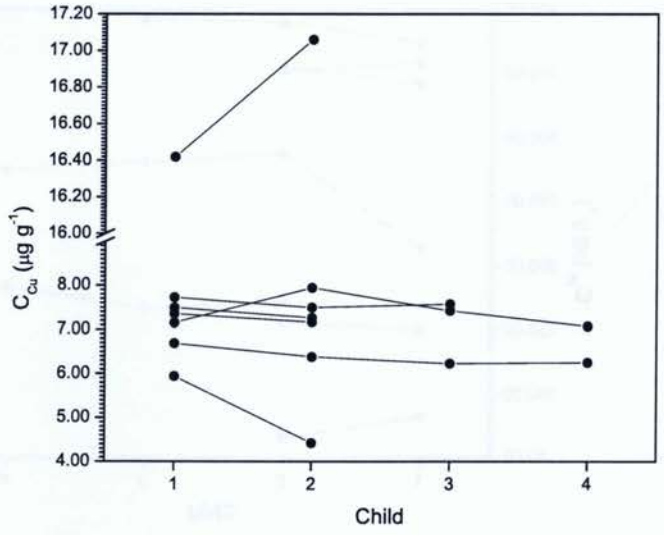
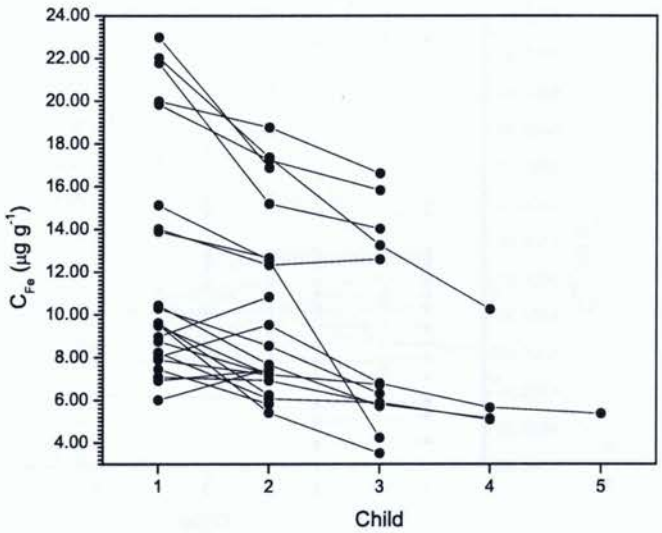


(b)

Figure 3.13: Copper content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 copper is shown to decrease over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*). This decrease is relatively consistant over the number of children.



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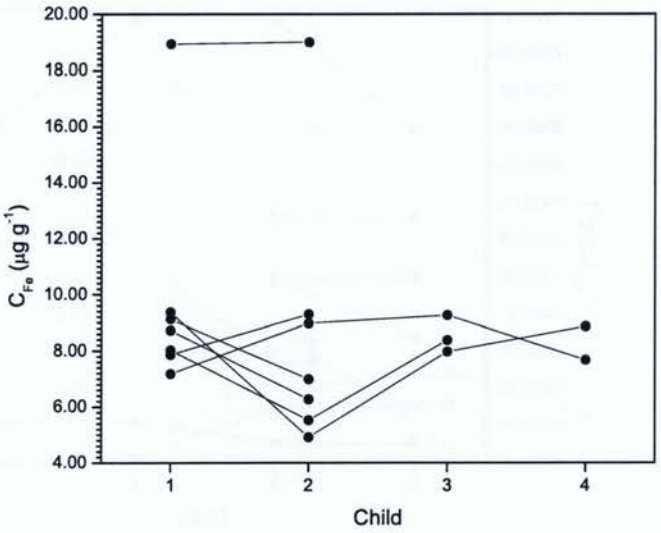
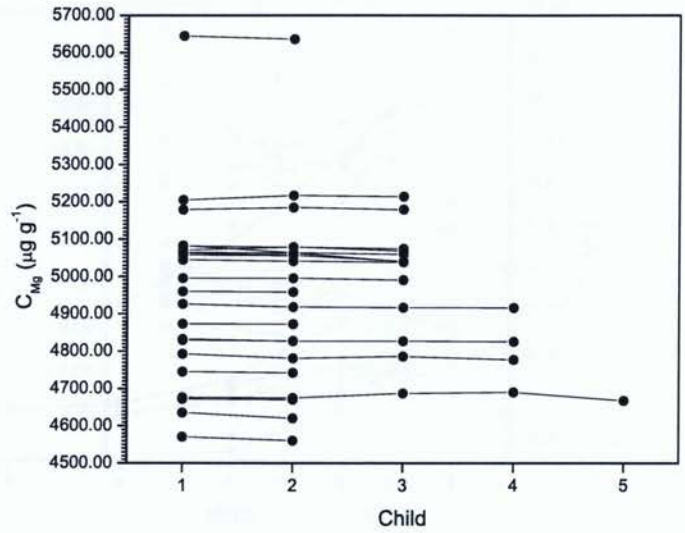


(b)

Figure 3.14: Iron content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 Iron is shown to decrease over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*). This decrease is relatively consistant over the number of children.



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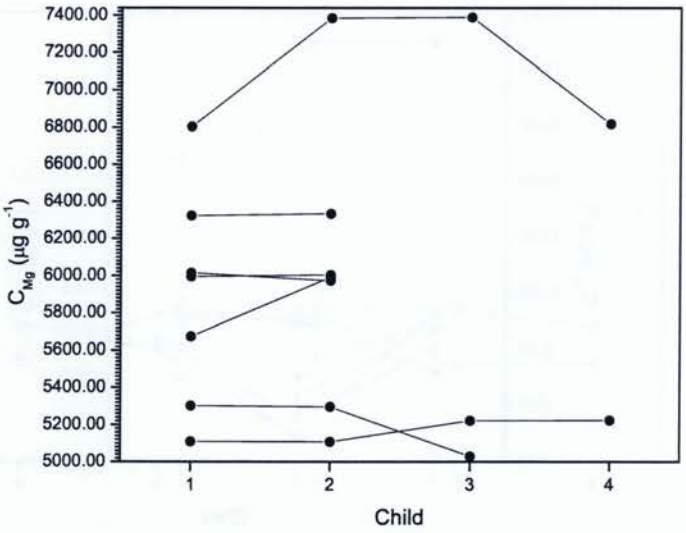
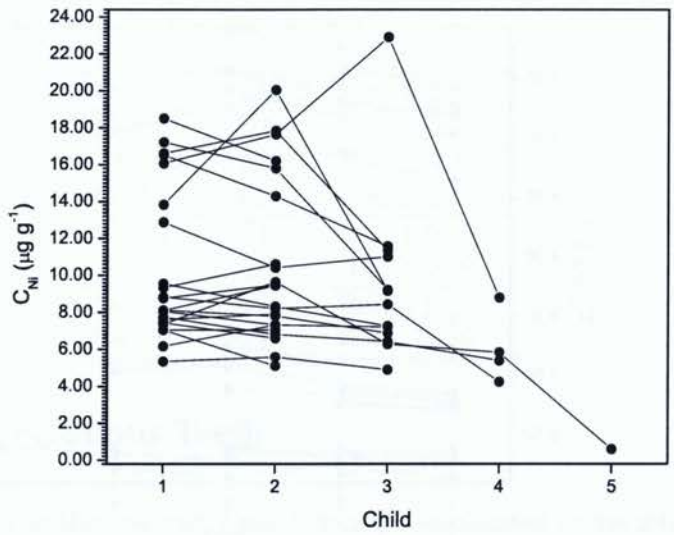


(b)

Figure 3.15: Magnesium content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 magnesium is shown to decrease over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*). This decrease is relatively consistant over the number of children.



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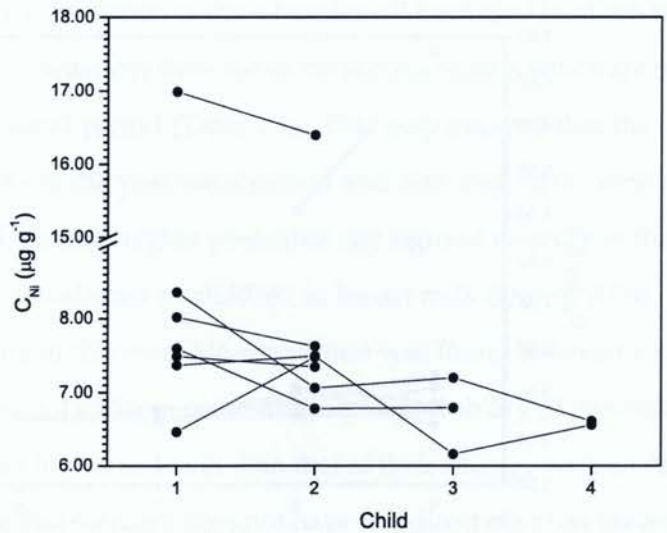
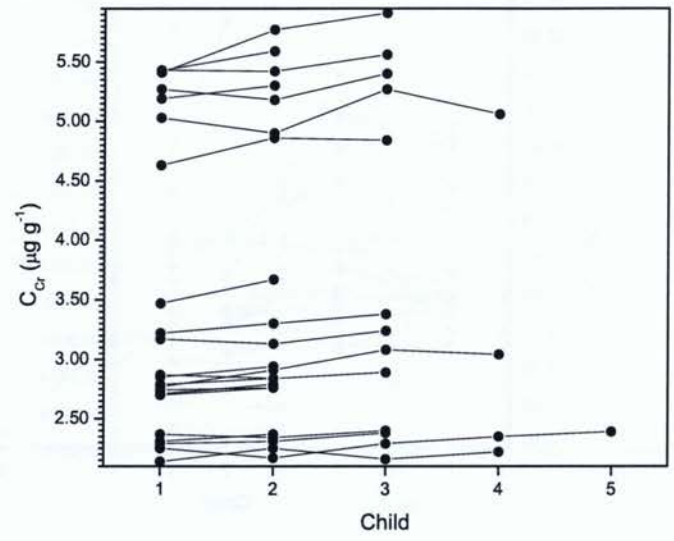


(b)

Figure 3.16: Nickel content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 nickel is shown to decrease over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*). This decrease is relatively consistant over the number of children.



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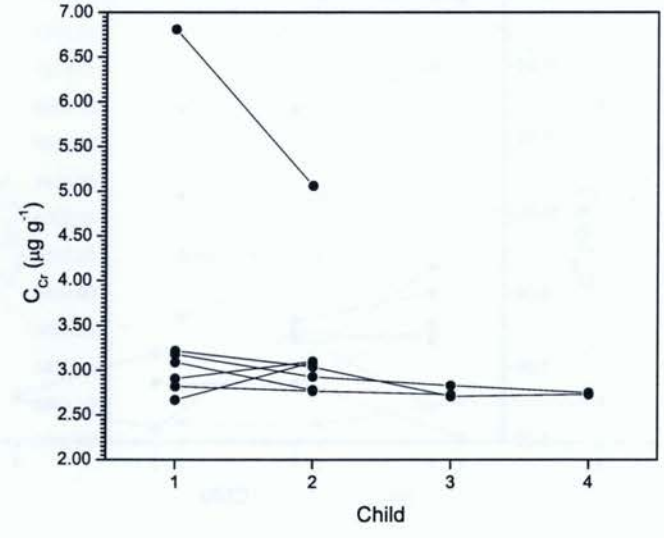

(b)

Figure 3.17: Chromium content in the deciduous teeth of children from the sampled families represented as a function of their birth order. With reference to Table 3.5 chromium is shown to increase over time (over the number of children the mother produces) (*a*) which is not a clear trend observed in families with no history of ASD (*b*).

Table 3.6: Mercury content in the secondary molars of the only three children presenting any mercury in their deciduous teeth above the LOD. All children were from the Hamilton. The mercury concentration is listed with its associated 95 % confidence interval. In no case was the difference between the children found to be significant ($p > 0.6$).

Disorder	Child	C_{Hg} ($\mu g \cdot g^{-1}$)
Norm	C1	0.71 ± 0.17
ADHD	C2	0.62 ± 0.20
NVA	C3	0.55 ± 0.18
Norm	C1	0.41 ± 0.10
ASP	C2	0.57 ± 0.09
Norm	C1	0.68 ± 0.15
FA	C2	0.40 ± 0.10

3.4 Mercury in Deciduous Teeth

Although mercury has been the one metal most strongly implicated in the ætiology of the ASDs (section 1.3), this work was unable to detect any mercury above the detection limit (Table 2.1) in all deciduous teeth with the exception of three families all from the Hamilton region (Table 3.6). In these children, the mercury was only detected in the second molars which are the only crowns that mineralize into the post-natal period (Table 1.1). This may indicate that the exposures occurred within the first few weeks of the post-natal period and also may have come from the mother as no other children in the Hamilton region presented any signs of mercury in their deciduous teeth. Mercury is known to be transferred to children in breast milk (Dorea, 2004), and this may have been the route of exposure in this case. No correlation was found between autistic status and the mercury content, with normal children presenting equal probability of presenting a tooth mercury concentration that is either higher or lower than that of their siblings with an ASD (Table 3.6). This observation may indicate that mercury does not have any direct effect on the ætiology of the ASDs and that it is excreted equivalently in deciduous teeth for both normal and autistic populations (section 1.3).

Chapter 4

Conclusions and Future Recommendations

The ætiology of the autism spectrum disorders is still largely unknown. Genetically, the disorders are not yet fully understood and several theories as to the possible role that several environmental factors may have on the ultimate cause and progression of the ASDs have been proposed (section 1.3). Metal exposure and deficiency, of both the trace/toxic and essential elements have been postulated as possible factors, either due to the children's inability to detoxify, due to the direct oxidative stress caused by metal accumulation or due to the inability of certain protein systems (*i.e.* metallothonein, glutothoinein) to act efficiency due to metal deficiency (namely Zn); yet, these studies focus on the post-natal time frame of development. Although it is clear that metals may have a role in the ætiology of behavioural disorders in children (Bouchard *et al.*, 2007; Bellinger *et al.*, 1994)—namely attention-deficit hyperactive disorders—the role metals actually play in the ætiology of the ASDs is still rather unclear on all three developmental time-frames (pre-, peri- and post-natal). This study was thus intended at evaluating this issue, at the pre-natal level, by monitoring the metal content in the deciduous teeth of children with ASD, using deciduous teeth as a biomarker of exposure or deficiency at the pre-natal level, while using normally developing siblings as controls. Given the lack of information in this area, this study was observational in

nature.

This study proceeded by collecting the full set of deciduous teeth from families in which one child was diagnosed with an ASD. A total of 22 families with one child affected with an ASD were sampled in which all siblings were accounted for. The sample presented only two of the known ASDs, autistic disorder and Asperger's disorder and 31.8% of the families also presented children with ADHD. Seven families with no known history of ASD, and no children diagnosed, were also sampled as complete controls. The study proceeded by a sibling-paired design to evaluate elemental differences in order to normalize out factors that may influence the metal concentrations in the deciduous teeth of children, including the geographical location in which they lived, possible, yet unknown genetic factors that may influence the ultimate elemental content as observed in the deciduous teeth and other familial factors such as general diet. Inductively-coupled plasma atomic emission spectrometry was used for elemental analysis of the teeth.

The children were all sampled from various regions within southern Ontario including: London, Hamilton, Burlington, Newmarket, Orangeville, Toronto, Kingston and Ottawa, which allowed for an analysis of possible differences in the elemental content in the deciduous teeth of children as a function of where they lived and were raised. The dental concentrations of all elements present above the detection limit of the spectrometer were found to differ between at least one geographical regions ($p < 0.001$). The anthropogenic elements Cr, Cu, Fe, Mn, Ni, Pb and Zn were found to consistently be highest in the deciduous teeth of children from the Hamilton/Burlington region in comparison to the surrounding areas ($p < 0.05$). This observed trend may be due to the high industrial activity that is present in this area of Ontario.

The sibling-paired/difference experiment determined that children with ASD do not present any different concentration for the elements K, Mn, Na, Pb, Sr and Hg in comparison to their normally developing siblings but did have statistically different levels of Cr, Cu, Fe, Mg, Ni and Zn in their deciduous teeth in comparison to their siblings ($p < 0.01$). This difference observed between children with an ASD and their associated normal siblings was not an effect observed for families with no known history of ASD. It was observed that as the mother had more children,

that the dental concentrations for Cu, Fe, Mg, Ni and Zn were found to be generally decreasing while Cr concentrations seemed to gradual increase. The concentrations did however not cluster into a concentration range that would indicate an absolute range in which such a deficiency or excess would be correlated just to children with ASD, but rather, children with ASD, even though deficient or in excess of the said metal(s), presented absolute concentrations of these metals that were equivalent in range to that expected between geographical location. The observed trend in which the concentrations generally decreased (or increased, for Cr) over time (with subsequent pregnancies) would indicate the possibility that the mother may be becoming deficient, or accumulating, these elements due to other conditions that may possibly be triggering (epigenetically) the progression of ASD and that the observed effect in regards to metal content in deciduous teeth may be secondary to other underlying conditions.

In regards to future work, several areas should be evaluated as this study was observational. In regards to the trends observed in deciduous tooth elemental content as a function of geographical location, the sample size was small and these effects should be evaluated further in a larger sample set and with a more randomized sample. The fact that each geographical region only presented between 1-10 families does leave the possibility that the registered differences were in fact familial and not necessarily true of the populations. A proper study of this effect should be evaluated using a much large sample size and sampling using a more randomized design as these differences may also be due to the rather biased nature of this sample focusing on a specialized subset of the population.

The observed trends as a function of ASD status should also be evaluated further under a design that is better controlled to account to known co-morbidities which may have influenced the observed differences (Green *et al.*, 2003). This study was based on diagnosis of ASD as known by the parents. Although the diagnosis criteria, being dependant on diagnosis by the age of two (World Health Organization, 1992; American Psychiatric Association, 2002), allowed for a good probability that the parent(s) knew of the autistic status at the time of donating the deciduous teeth, this factor should be included and controlled in any future work. Also, sibling-pairing was

employed and is assumed to normalize for familial factors. In regards to the assumption that normalization by difference is able to normalize any genetic predispositions, this study was only able to ensure that the children within a family had the same mother, yet no information was obtainable regarding the possibility of a different paternal unit. Diet is known to alter metal concentrations in the system and as shown in this work, the area in which individuals may live. The geographical locations presented in this work were representative of those in which the mother lived during all pregnancies and all children lived during their early development until the time of the last exfoliation of their teeth. Dietary concerns are not necessarily important in regards to the child as any specialized diet at the post-natal level will not be reflected in the deciduous teeth as they mineralize nearly completely during the pre-natal period of development. The mother's diet should however be controlled for. Under the assumptions that these normalizing conditions hold, then it can be presumed that this observed decrease in metal content as the mother has more children may be due to an underlying condition; however, without knowledge of any possible dietary changes, possible medications due to unknown medical histories *etc.* it is uncertain if the sampled population had similar changes which may be reflected in the deciduous teeth of her children and may in fact be a possible factor in ASDs ætiology. Since this study, being observation, was originally designed with the intention of evaluating the child at the pre-natal level, it seems clear that future work should focus on a highly controlled study of the mothers.

This study was also focused on using total tooth concentrations and that of the total concentration of the given element, regardless of species. Recent work has however postulated the exsistance of the prenatal line in deciduous teeth (Ericson *et al.*, 2001) which can thus be used to determine pre- versus post-natal exposures on the same tooth. This concept is however elusive, as deciduous teeth are known to mineralize nearly completely during the gestational period (Ash and Nelson, 2004, Table 1.1). Future work should however focus on evaluating the actual exsistance of this line from a physiological stand point and then evaluate suitable methodologies to probe it at the micro-scale, as to date time-of-flight secondary ion mass spectrometry (ToF-SIMS) and laser-ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) are used with as-

sociated issues in calibration (Ericson, 2001). Synchrotron-based X-ray fluorescence analysis (SR-XRF) (Van Grieken and Markowicz, 2002) of teeth would allow for evaluating these differences as the physical process leading to emission are well characterized and calibration regimes simpler to produce. Also, since total element concentrations were used, it would be a next step to evaluate whether there is a different exposure and accumulation of different elemental species of these metals (namely, Zn, Cu, Fe and Cr) which may ultimately provide information as to whether children with ASD present different relative concentrations of different species of the metals and thus exposed to the potential of greater oxidative stress. Such a study, to evaluate species and the possible exsistance of the pre-natal line, can be done nearly simultaneous using combined SR-XRF and synchrotron-based X-ray absorption near edge spectrometry (XANES).

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