School of Public Health

Factors affecting skeletal integrity in an Australia Rett syndrome cohort and best practice guidelines for prevention and management of low bone density in Rett syndrome

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics (For projects involving human participants/tissue, etc): The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Numbers HR51/2010 and HR44/2010.

Signature:

Date: 4th March 2016

Abstract

Mutations on the X-chromosome in the gene encoding methyl-CpG binding protein 2 (*MECP2*) have been identified as the principal genetic cause of Rett syndrome, which is a neuro-developmental disorder predominantly occurring in females. The skeletal health of individuals with Rett syndrome has been shown to be poor, with studies demonstrating low bone density and increased risk of fracture compared with the general population. The aim of this study was to investigate the longitudinal bone accrual in Rett syndrome and develop clinical guidelines for the management of bone health in this condition.

The first study conducted collected cross sectional data using dual energy X-ray absorptiometry in 97 participants with Rett syndrome sourced from the population-based Australian Rett Syndrome Database. Data were analysed and z-scores calculated and compared with age (as well as height, weight and lean tissue mass adjusted) norms. Most z-score values were low, although once adjusted for bone area and lean tissue mass the mean was above zero, suggesting that low bone mineral content can be explained by narrow bones and decreased muscle mass. Multivariate linear regression identified the p.Arg168* and p.Try158Met mutations as more likely to have poorer bone outcomes.

Seventy-four of these participants were reassessed after three to four years. Bone as well as body composition data of fat mass and lean tissue mass was measured. Annual changes in z-scores were calculated, and the effects of age, genotype, mobility, menstrual status and epilepsy diagnosis were investigated. Overall, bone area and lean tissue mass z-scores declined over time. Changes were positive in the lumbar spine, particularly for those who could walk unaided. Changes for areal bone mineral density measurements were negative for both the lumbar spine and total body, however considerably more so for the total body. Menses appeared to have a protective effect on total body z-score changes with those achieving menses having more positive changes. Total body bone mineral content showed the most negative change, however this normalized once adjusted for lean tissue mass and bone area.

The findings from the cross-sectional and longitudinal studies, together with a literature review, were used to formulate draft clinical guidelines for the management of bone health in Rett syndrome. An international expert panel was

recruited to review the draft guidelines iteratively in a two-stage Delphi process until consensus was reached. Items described the clinical assessment of bone health, bone mineral density assessment and technique, and pharmacological and non-pharmacological interventions.

Bone mineral density and bone mineral content in Rett syndrome decreased with age but muscle mass and menses were protective. The consensus-based guidelines have the potential to improve bone health in those with Rett syndrome, reduce the frequency of fractures, and stimulate further research that aims to ameliorate the impact of this serious comorbidity.

Dedication

I would like to dedicate this thesis to several important people in my life.

First and foremost, I dedicate this thesis to my daughter Amelia. Although we can never get back the lost time together due to my research commitments, I am hoping that when you see the final result you will be proud of me. Whilst I regard this thesis as one of my most memorable and satisfying achievements, you will always remain my most treasured.

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Those who contributed to the development of the guidelines

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Publications

The studies conducted during my PhD candidacy are published in the following journals:

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Acronyms and Abbreviations

Areal bone mineral density	aBMD
Australian Rett Syndrome Database	ARSD
Body mass index	BMI
Bone area	BA
Bone mineral apparent density	BMAD
Bone mineral content	BMC
Bone mineral density	BMD
95% Confidence interval	CI
Cerebral palsy	CP
Consumer reference group	CRG
Dual energy X-ray absorptiometry	DXA
Effective dose	ED
Femoral neck	FN
Growth hormone	GH
Height	HT
Insulin like growth factor 1	IGF-1
International Society for Clinical Densitometry	ISCD
Large deletion	LD
Late carboxyl-terminal truncation	CT
Lean tissue mass	LTM
Lumbar spine	LS
Methyl-CpG binding protein 2	*MECP2*
Peak bone mass	PBM
Peripheral Quantitative Computed Tomography	pQCT
Quantitative ultrasound	QUS
Region of interest	ROI
Standard deviation	SD
Total body	TB

Chapter One

Introduction

Rett syndrome is a rare neurodevelopmental disorder principally caused by a X-linked mutation in the transcription gene encoding methyl-CpG binding protein 2 (Amir et al., 1999). Over 200 mutations in this gene have been identified, with some mutations appearing more frequently (Bebbington et al., 2008). Mutations in this neuromodulatory gene influence the function of the Mecp2 protein in different ways ranging from partial loss of function to total loss (Nan, Meehan, & Bird, 1993). The type of mutation, coupled with the unique pattern of X-chromosome inactivation, leads to a disorder with a wide variety of phenotypic severity (Archer et al., 2007).

Rett syndrome is typically characterized by normal infant development, followed by a regression period between six to 18 months of age (Neul et al., 2010). During this period, acquired language and hand skills are lost, hand stereotypies appear and there may be difficulties in ambulation (Neul et al., 2010). Other clinical manifestations of Rett syndrome are poor growth, scoliosis, respiratory and gastrointestinal disturbances, epilepsy and motor impairments, including abnormal muscle tone (Neul et al., 2010).

An additional clinical manifestation of Rett syndrome is poor skeletal health. The fracture incidence in this disorder has been found to be four times greater than that of the general population (Downs, Bebbington, Woodhead et al., 2008). Cross-sectional studies investigating bone density using dual energy x-ray absorptiometry (DXA) in populations of females with Rett syndrome have shown low bone mineral content (BMC) and areal bone mineral density (aBMD) in children, however greater deficits were seen in older participants (Jefferson et al., 2011; Motil, Ellis, Barrish, Caeg, & Glaze, 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen et al., 2011; Zysman, Lotan, & Ben-Zeev, 2006).

At the commencement of this thesis, there had only been one longitudinal bone study in Rett syndrome. The study obtained quantitative ultrasound (QUS) measurements in the diaphysis of the proximal phalanx of digits two to five over a three year period in 109 females with Rett syndrome, aged between 3-25 years. Comparisons were then made using measurements from 101 age and gender matched controls (Gonnelli et al., 2008). Those who were non-ambulant at baseline

showed decreasing QUS measurements over time compared to controls, although no change was demonstrated in ambulant subjects (Gonnelli et al., 2008). However, assessing the acquisition of bone mass and density longitudinally using DXA, the gold standard for assessing bone density, in the femur, lumbar spine and total body would provide a more accurate depiction of bone changes overtime in weight-bearing bones at risk of fracture. An understanding of accrual of bone in Rett syndrome and the risk factors for low bone density is clinically important and may have a bearing on this markedly increased risk of fracture (Downs, Bebbington, Woodhead et al., 2008).

Currently, there is a limited evidence base for management of bone health in Rett syndrome. Due to the rarity of Rett syndrome, clinicians may have limited exposure to patients with this disorder. Therefore, a set of clinical guidelines for the diagnosis, management and prevention of low bone density would support clinicians by providing a clear and comprehensive approach to improving bone health in Rett syndrome.

1.1 Overall aim

The first aim of this study is to investigate bone accrual in Rett syndrome and identify factors that may negatively impact bone development in this disorder. The study also aims to provide recommendations for assessment of bone density and treatment and prevention protocols, in the hopes of reducing the incidence of fracture in Rett syndrome.

1.2 Specific objectives

1. To determine baseline and annual changes in bone mass and density in a Rett syndrome population.

2. To investigate the influence of age, genotype, mobility, epilepsy, body mass index, fracture history, pubertal status, anticonvulsant use and lean tissue mass on bone mass and density, and their change over time in Rett syndrome.

3. To define best practice for the prevention, screening, monitoring and management of osteoporosis and fracture in Rett syndrome.

1.3 Significance

This study is the first to assess bone density in Rett syndrome longitudinally using DXA and the first to perform this analysis with adjustments for bone size and lean tissue mass, which are important considerations in bone assessment. It will provide a greater understanding of bone accrual in Rett syndrome and determine the degree of annual attenuation in accrual in these individuals. Identification of factors that influence bone mass and density will highlight those individuals at greater risk of low peak bone mass and therefore low bone strength which is a risk factor for fractures. This will be the first study to investigate the effect of the *MECP2* mutations, which are associated with a range of phenotypic severities, on bone density in Rett syndrome.

To date there have been no intervention studies on osteoporosis and fractures in Rett syndrome. Combining information gained from the DXA studies related to risk factors associated with low bone density in Rett syndrome, with the views of experts, will allow for the development of clinical guidelines for preventing, screening and treating low bone density and in turn fractures in individuals with Rett syndrome. These guidelines are an initial step to make use of current knowledge and best practice to facilitate more widespread use of best management and support for those with Rett syndrome and their families, which in the long term could improve the quality of life for individuals with Rett syndrome and reduce the health costs associated with this disorder. The findings of this research may also be used as the impetus for more informed intervention studies on treatment and prevention of low bone density in Rett syndrome.

1.4 Thesis outline

1.4.1 Chapter Two

This chapter describes the characteristics of Rett syndrome and the clinical outcomes of this disorder. The chapter then presents an overview of the literature on skeletal health in Rett syndrome. Following is a description of bone tissue, bone acquisition and factors that influence accrual of bone in the general population. The final section of this chapter describes management of bone health, which leads to the principles of guideline development.

1.4.2 Chapter Three

This methodological chapter firstly focuses on the principles of dual energy x-ray absorptiometry (DXA) as a tool for assessing bone density. The chapter then details the methods used for the baseline (cross-sectional) and follow-up (longitudinal) DXA assessments to supplement the methods section of chapters four and five. The chapter concludes with a description of methods related to guideline development to discuss additional information to that which is presented in chapter six.

1.4.3 Chapter Four

This chapter presents the population based cross-sectional study investigating bone density, bone area and lean tissue mass in Rett syndrome measured with DXA. The specific objective of this study was to gain an understanding of areal bone mineral density and bone mineral composition in the femoral neck, lumbar spine and total body in Rett syndrome and identify factors that influence these parameters. This study was published in *Pediatric Research*.

1.4.4 Chapter Five

This chapter investigated the annual changes in areal bone mineral density and bone mineral composition in the lumbar spine and total body in Rett syndrome. A follow-up DXA assessment allowed the calculation of annual changes in z-scores to be made by comparing baseline and follow-up z-scores. Multivariate regression analysis was then used to identify predictors of decreased bone accrual. This study was published in *Bone*.

1.4.5 Chapter Six

The sixth chapter presents the final study in this thesis, whereby clinical guidelines for the diagnosis, management and prevention of low bone density are presented. The guidelines provide recommendations in Rett syndrome for bone health assessment, techniques related to measuring bone density and intervention protocols of a non-pharmacological and pharmacological nature. This study was published in *PLOS ONE*.

1.4.6 Chapter Seven

A discussion of the key findings and their significance are presented in chapter seven, along with the limitations of the studies and recommendations of areas for future research related to improving bone health in Rett syndrome.

Chapter Two

Literature Review

2.1 Rett syndrome

Rett syndrome is a progressive, severe neuro-developmental disorder with a diagnosis incidence of one in 8,905 females by the age of 32 years (Fehr, Bebbington, Nassar et al., 2011). Rett syndrome predominantly occurs in females, who can be categorised as having either a typical (classic) or an atypical (variant) form (Hagberg & Skjeldal, 1994). Atypical Rett syndrome is classified as the form of the disease in which the individual has the preserved speech, early seizure or congenital form (Neul et al., 2010). There are varying levels of severity in those with Rett syndrome, especially in females who exhibit atypical forms (Hagberg & Skjeldal, 1994) but the disability is nevertheless severe.

In 1999, the principal genetic cause of Rett syndrome was identified as a result of mutations on the Xq28 chromosome in the gene encoding methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999). Approximately 95-97% of individuals with typical Rett syndrome (Neul et al., 2008) and approximately 75% of those with atypical Rett syndrome have a mutation in the *MECP2* gene (Percy et al., 2007). Mutations in *MECP2* can result in a total loss of function of this gene or partial inactivation, which may explain the phenotypic severity observed in Rett syndrome. In addition, the degree of X chromosome inactivation can influence the expression of mutated *MECP2* genes. As females have two X chromosomes, activation of the functional (wild type) *MECP2* allele and inactivation of the mutant *MECP2* allele can decrease the severity of the Rett syndrome neurological phenotype (Archer et al., 2007).

There are three functional domains on the *MECP2* gene; the methyl binding domain which allows for DNA binding (Nan et al, 1993), a nuclear localisation sequence which plays a role in coordinating the movement of the MeCP2 protein to the nucleus (Nan, Tate, Li, & Bird, 1996) and a transcriptional repression domain controlling gene transcription (Nan et al., 1993). Although there are over 240 types of pathogenic nucleotide changes, eight common missense and nonsense mutations have been identified including p.Arg106Trp, p.Arg133Cys, p.Thr158Met, p.Arg168*, p.Arg255*, p.Arg270*, p.Arg294* and p.Arg306Cys, which affect more than 60% of Typical Rett syndrome cases (Bebbington et al., 2008; Christodoulou &

Weaving, 2003). Two additional common mutation groups that have been identified are small deletions or insertions which lead to late carboxy-terminal truncations (C-terminal truncations) (Amir et al., 1999) and larger deletions (Bebbington et al., 2008).

Clinical severity of Rett syndrome which includes aspects of phenotype such as the level of ambulation, hand use and speech, has been shown to be linked with specific *MECP2* mutations (Bebbington et al., 2008; Cuddapah et al., 2014; Neul et al., 2008). The mutations shown to have the least severe phenotype are the p.Arg133Cys and the p.Arg294*. The mutation Arg306Cys has also been shown to be less severe, however the use of words in this group is often limited (Neul et al., 2008). In one study, large deletions and p.Arg168* mutations had the highest clinical severity score, meaning their functional losses were more severe (Neul et al., 2008). In a separate study, p.Arg255*, p.Arg270* and p.Try158Met mutations were seen to be more severe (Bebbington et al., 2008). A more recent study which investigated the relationship between mutation type and clinical severity using a cohort of 152 females with Rett syndrome, found similar results with the large deletions, p.Arg168*, p.Arg255*, p.Arg270* and p.Arg106Trp mutations having a more severe disease course (Cuddapah et al., 2014).

The clinical presentation in Rett syndrome is not only influenced by the specific mutation type, but may also be influenced by the degree and direction of X-inactivation (Archer et al., 2007), thus leading to a wide range of phenotypes. It is generally accepted that the p.Arg133Cys, p.Arg294*, p.Arg306Cys and C-terminal deletion mutations are less severe than the p.Arg270*, p.Arg168* and large deletion mutations, with differences noted in level of mobility, hand use and language (Bebbington et al., 2008).

The typical form of Rett syndrome was first described in German by Andreas Rett in 1966 (Rett, 1966) but diagnostic criteria for Rett syndrome were not established until 1988 by Hagberg and colleagues (Hagberg, Goutieres, Hanefield, Rett, & Wilson, 1985). The most recent update to the diagnostic criteria was published by Neul et al in 2010, who described four main criteria and 11 supportive criteria as listed in Table 2.1. The four main criteria which are observed in individuals with typical Rett syndrome are: 1) partial or complete loss of hand skills, 2) loss of spoken language, 3) gait abnormalities and 4) stereotypic hand movements.

Two of the four main criteria and five of the supporting criteria are considered to be required to make a diagnosis of atypical Rett syndrome. The 11 supportive criteria include breathing disturbance or bruxism when awake, impaired sleeping, abnormal muscle tone, peripheral vasomotor disturbances, scoliosis/kyphosis, growth retardation, small cold hands and feet, inappropriate laughing or screaming, decreased pain sensitivity and intense eye communication (Neul et al., 2010). Although post-natal deceleration of head growth is no longer part of the diagnostic criteria, as it is not experienced in all individuals with Rett syndrome, it is highlighted as a clinical finding that may prompt clinicians to investigate further (Neul et al., 2010).

Table 2.1: Diagnostic criteria for Rett syndrome

	Diagnostic Criteria
4 Main criteria	• Partial or complete loss of hand skills • Loss of spoken language • Gait abnormalities • Stereotypic hand movements
11 Supporting criteria	• Breathing disturbance when awake • Bruxism when awake • Impaired sleeping • Abnormal muscle tone • Peripheral vasomotor disturbances • Scoliosis/kyphosis • Growth retardation • Small cold hands and feet • Inappropriate laughing or screaming • Decreased pain sensitivity • Intense eye communication

Note: Adapted from "Rett Syndrome: Revised Diagnostic Criteria and Nomenclature" by J. L. Neul, W. E. Kaufmann, D. G. Glaze, J. Christodoulou, A. J. Clarke, N. Bahi-Buisson, H. Leonard, M. E. S. Bailey, N. C. Schanen, M. Zapella, A. Renieri, P. Huppke, A. K. Percy & for the RettSearch Consortium, 2010, *Annals of Neurology*, 68(6), 944-950. Copyright 2010 by the American Neurology Association.

The natural progression of typical Rett syndrome is variable, however a period of regression followed by recovery is generally observed (Neul et al., 2010). Most infants experience a period of regression between six to 18 months of age, where development begins to stagnate then regress (Neul et al., 2010). Purposeful hand use diminishes and a characteristic pattern of hand stereotypies emerges (Neul et al., 2010). Speech and acquired motor skills decline with some losses more profound than others (Neul et al., 2010). Parents have also reported abnormalities in their child's behaviour or development prior to this regression period, including excessive calmness and placidity, feeding problems, lack of muscle tone and delayed crawling and sitting (Fehr, Bebbington, Ellaway et al., 2011). These abnormalities are generally too subtle to warrant investigation prior to regression.

Diagnosis of Rett syndrome has occurred at a younger age since the year 2000 than it was prior to this date, most likely due to the availability of genetic testing (Fehr, Bebbington, Ellaway, et al., 2011). The average age of diagnosis of Rett syndrome in an Australian Rett syndrome cohort of 293 females was 33.1 months, with nearly 89% diagnosed after the onset of developmental regression (Fehr, Bebbington, Ellaway, et al., 2011). The age of diagnosis was seen in this study to be related to the level of developmental milestones achieved, including gross motor skills such as crawling and sitting and speech milestones. Those whose skill loss was more profound, tended to be diagnosed at an earlier age. Furthermore, those who achieved gross motor and speech milestones, irrespective of the age of regression, were diagnosed later. A strong relationship was also seen in this cohort between genotype and age of diagnosis, whereby those with the less severe mutations such as p.Arg133Cys, p.Arg306Cys and p.Arg294* who were more likely to have achieved more milestones, were diagnosed at a later age (Fehr, Bebbington, Ellaway, et al., 2011).

Investigation of survival in an Australian Rett syndrome cohort of 396 females, showed just less than 60% reached 38 years of age (Anderson, Wong, Jacoby, Downs, & Leonard, 2014). This large population based study followed the health and well being of individuals with Rett syndrome over a 20 year period. The most common causes of mortality in this cohort, in decreasing order, were lower respiratory infections, aspiration/asphyxiation and lower respiratory or seizure related complications (Anderson et al., 2014). Those individuals with the phenotypically more severe mutations such as the large deletion, were at three

times greater risk of mortality than the milder p.Arg133Cys mutation (Anderson et al., 2014).

Anderson and colleagues (2014) also investigated various aspects of health in the adult Australian population based cohort with Rett syndrome and combined this with data from the international database InterRett. Seizures were found in 45.3% (n=411) of females in this combined data set. Over 60% of 422 adult women were able to walk either independently or with varying degrees of assistance. Mutation type appeared to influence the ability to walk with those with the large deletion more immobile than those with the less severe p.Arg133Cys mutation. Ability to walk was also negatively impacted by the presence of scoliosis, which was present in 85.5% (n=420) of this combined data set, with those with a large deletion having a greater chance of having this orthopedic problem (Anderson et al., 2014).

Additional comorbidities have been observed in Rett syndrome which affect the clinical course of this disorder including, growth abnormalities, irregular breathing patterns, seizures, gastrointestinal problems and scoliosis (Anderson et al., 2014). Seizures occur commonly in Rett syndrome with one study reporting the presence of seizures in 60% of 602 individuals with Rett syndrome (Glaze et al., 2010). Furthermore, impairment of ambulation, hand use, and communication was greater in those with seizures (Glaze et al., 2010). A greater proportion of participants experiencing seizures was seen in an Australian population based study of which 81% of the 275 cohort of females with Rett syndrome had reported seizures, with the median onset occurring at four years old (Jian et al., 2006). Scoliosis is another common comorbidity of Rett syndrome, where studies have shown between 75% (Ager et al., 2006) and 85% of individuals over the age of 16 years suffer with the condition (Percy et al., 2010). Scoliosis can affect the ability to walk and sit, cause pain and lead to restrictive lung disease (Berven & Bradford, 2002). Guidelines for management of scoliosis in Rett syndrome were developed in 2009, providing clinicians with intervention strategies, guides to monitoring scoliosis progression, in addition to assessment and treatment protocols (Downs et al., 2009).

Short stature, with declining height and weight for age, has consistently been reported in Rett syndrome (Motil et al., 2012; Oddy et al., 2007). This poor growth may be, in part, causally related to nutritional deficits identified in this disorder (Motil et al., 2012). A decline in oromotor function and the presence of digestive tract dysfunction such as gastroesophageal reflux, gastroparesis and constipation all

contribute to decreased food intake in Rett syndrome (Leonard et al., 2013; Motil et al., 2012). In 2013, clinical guidelines for the management of growth and nutrition in Rett syndrome were developed which aim to improve energy intake, decrease feeding problems and provide recommendations on gastrostomy use in extreme cases (Leonard et al., 2013).

2.2 Skeletal health in Rett syndrome

In addition to the comorbidities previously mentioned, a number of skeletal abnormalities, which prompted the investigation of skeletal integrity, have been observed in Rett syndrome. Radiogrammetry studies of the metacarpal and phalangeal bones in the left or right hands of 100 girls and young women with Rett syndrome in the Australian Rett Syndrome Database (ARSD) showed bone age was more advanced in younger children than in the general population, but tended to normalise with age (Leonard et al., 1999; Leonard, Thomson, Bower, Fyfe, & Constantinou, 1995) The underlying mechanism for advanced bone age was not identified in this study, however given that precocious puberty has been observed in Rett syndrome (Killian et al., 2014; Knight et al., 2013), hormonal factors may play a role. Additionally, cortical thickness in the second metacarpal bone was lower than in controls and it was estimated that one third would have sustained a fracture by the age of 15 years (Leonard et al., 1999).

Fracture incidence in Rett syndrome was later further investigated in 234 cases within the ARSD, where the incidence was nearly four times greater than that in the population and risk of fracture was increased for individuals with epilepsy and in those with the phenotypically more severe mutation types (Downs, Bebbington, Woodhead et al., 2008). Fractures are common in healthy children with estimations that the accumulated risk of sustaining a fracture between birth and 16 years is 34% (Hedstrom, Svensson, Bergstrom, & Michno, 2010). However, this figure does vary amongst studies. Fractures most commonly occur in the upper limb in healthy children during sporting activities, particularly within the radius (Hedstrom et al., 2010; Naranje, Erali, Warner, Sawyer, & Kelly, 2016). This pattern is in contrast with that observed in Rett syndrome where fractures in the lower limb, particularly the femur and tibia, were more common, with most being caused by low energy mechanisms (Downs, Bebbington, Woodhead et al., 2008; Roende, Ravn, Fuglsang, Andersen, Vestergaard, et al., 2011).

Abnormal bone structure has also been shown at the calcaneus and phalanges in 84 females with Rett syndrome using quantitative ultrasound (QUS) as have a higher bone turnover and lower levels of 25 hydroxyvitamin D (25OHD) (Cepollaro et al., 2001). Quantitative ultrasound values were significantly greater in those that were ambulant, but still less than normal controls. Those taking anticonvulsants had lower QUS parameters than those not taking this medication (Cepollaro et al., 2001; Leonard et al., 1999). Subsequent to these findings, Gonnelli and colleagues (2008) used QUS to assess the bone transmission time (BTT) in the phalanx of 109 individuals with Rett syndrome. The mean BTT z-score was low, especially in those who had low mobility levels or vitamin D deficiency (serum 25OHD levels <9pg/ml or 0.0532nmol/L) (Gonnelli et al., 2008). Quantitative ultrasound was also used in another study to assess the bone strength in the radius and tibia of 35 individuals with Rett syndrome. Results from this study showed bone strength was increased with a greater calcium and caloric intake and higher mobility level. Bone strength was reduced by a history of fracture, scoliosis, epilepsy and abnormal breathing, although these findings were only seen in univariate analysis (Zysman et al., 2006). Unfortunately, this study calculated T-scores for pediatric data, thus inappropriately comparing measurements to adult reference data.

Dual energy X-ray absorptiometry (DXA) was originally used in Rett syndrome in 1997, to measure areal bone mineral density (aBMD) and bone mineral content (BMC). The aBMD and BMC in 20 girls with Rett syndrome were compared with values in 11 individuals with cerebral palsy (CP), with similar mobility levels, and 25 age and gender matched controls (Haas, Dixon, Sartoris, & Hennessy, 1997). Both mean aBMD and BMC in the lumbar spine (LS) and total body (TB) were lower in the Rett syndrome group compared to both the CP and control groups (Haas et al., 1997). As weight and age increased in the CP and control groups, so too did bone mineralisation. This trend was not seen in the Rett syndrome participants (Haas et al., 1997). In addition to the use of QUS, Cepollaro and colleagues (2001) also used DXA to assess the aBMD in the mid and ultradistal radius. Results showed that aBMD was lower than that in the age and gender matched controls and those who were non ambulant or on anticonvulsant medication had lower bone outcome measures than their counterparts (Cepollaro et al., 2001). Bone mineral deficits using DXA were further observed in 50 females with Rett syndrome aged between two to 38 years (Motil et al., 2008). This study assessed the aBMD and BMC at the hip, LS and TB (Motil et al., 2008). Both aBMD and BMC in all sites were lower than in the reference population and decreased with age. The presence of seizures,

previous anticonvulsant use, previous fractures and scoliosis had a negative impact on these bone measurements (Motil et al., 2008). Lean tissue mass (LTM), was also lower when compared to the reference group. No relationship between genotype and bone density was seen in this study. This may be due to the small sample size, which would only allow mutation categorisation into severe, moderate, mild, C-terminal and large deletions mutation groups as opposed to more specific mutational types (Motil et al., 2008).

Subsequent studies support these earlier findings that individuals with Rett syndrome have low bone mineral density and composition (Caffarelli et al., 2012; Jefferson et al., 2011; Motil et al., 2014; Roende, Raven, Fuglsang, Andersen, Nielsen et al., 2011; Shapiro, Bibat et al., 2010). Lumbar spine aBMD and BMC has been shown to be reduced in almost 50% of 54 individuals with Rett syndrome between the ages of 1.9-33 years, as early as three years old (Shapiro, Bibat et al., 2010). Similar results were also observed in 61 females with Rett syndrome, where aBMD and BMC were significantly lower compared to an age and gender matched control group (Roende, Raven, Fuglsang, Andersen, Nielsen et al., 2011). However this difference did not remain after calculation of volumetric LS BMD (bone mineral apparent density, BMAD). This result correlated with the study's finding of a 10% decrease in lumbar vertebrae size. Hip aBMD and BMC analysis performed during this study, showed lower bone outcome measures compared to controls, that persisted even after calculation of hip BMAD (Roende, Ravn, Fuglsang, Andersen, Nielsen et al., 2011). Total body aBMD and BMC assessment in Rett syndrome was significantly lower in 123 females with Rett syndrome compared to a normal age and gender matched control group of 55 individuals (Caffarelli et al., 2012), findings also observed in a separate study of 50 females with Rett syndrome (Motil et al., 2014). This most recent study, calculated total body aBMD and BMC z-scores using an algorithm which accommodated for age, gender, ethnicity, race and the reduced height observed in Rett syndrome, in addition to comparing their results to a large control group (>2200).

2.3 Bone tissue

Bone is a connective tissue which is composed of minerals, an organic matrix and water. The mineral component of bone is predominantly a mineralised substance called hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which is coupled to small amounts of magnesium carbonate, sodium and potassium ions (Young & Heath, 2000). The amount of hydroxyapatite equates to approximately 65% of the weight of bone

(Chen, Toroian, Price, & McKittrick, 2011). The organic material termed osteoid, contributes to approximately 25% of bone's weight, and consists of 90% type I collagen and 5% of non-collagenous proteins (Chen, Toroian et al., 2011) and ground substance (Young & Heath, 2000). Ground substance consists of chondroitin sulphate and hyaluronic acid in the form of proteoglycans. In addition to controlling water content, ground substance contributes to the regulation of collagen formation (Young & Heath, 2000). Around 10% of bone's weight is water and less than 3% is lipid (Chen, Toroian et al., 2011). The organic substance of bone also includes osteocalcin, osteonectin and sialoproteins, which play roles related to the binding of calcium during mineralisation and collagen and mineral interactions (Young & Heath, 2000). However, the proportion of the organic and inorganic matrix of bone can vary between skeletal sites and are influenced by age, gender, ethnicity and health status (Boskey, 2013). The quantity and quality of the mineral and organic matrix of bone determines the property of bone. The propensity for bone to fracture is influenced by the amount of mineral present, its crystallite size, distribution and orientation, the collagen content and crosslinks formed, in addition to the shape of bones, its microarchitecture and the existence of micro-cracks (Boskey, 2013).

Type I collagen triple helix fibrils in bone are staggered throughout the tissue with gaps between each fibril. Hydroxyapetite crystals are then organised into layers within these gaps and span the longitudinal length of the collagen fibrils so that they are parallel to one another (Chen, Toroian et al., 2011). Further expansion of hydroxyapatite results in crystals within the extra-fibrillar space and surrounding the fibrils (Chen, Toroian et al., 2011). Microscopically, this produces a layered appearance termed lamellae (Weatherholt, Fuchs, & Warden, 2012). Hydroxyapatite allows bone to be stiff and rigid, whereas, collagen provides flexibility and elasticity to the tissue, stabilises extracellular matrix and provides a scaffold for mineral deposition and other bone matrix proteins (Boskey, 2013). It is the interplay between these two which allows bone to resist deformation yet be flexible enough to absorb energy (Weatherholt et al., 2012).

2.4 Cortical and trabecular bone

Macroscopically bone can be divided into two types of tissue, cortical (compact) and trabecular (cancellous) bone. Approximately 80% of the skeleton is cortical bone (Weatherholt et al., 2012). It is considerably less porous than trabecular bone and contains a high matrix mass per unit volume, allowing it to withstand compressive

forces (Weatherholt et al., 2012). The lamellae in cortical bone form a structural unit called an osteon. A cement line forms the outer boundary of each osteon, with layers of bone formed closest to the cement line moving inwards, with a centralised canal for blood vessels, lymphatic drainage and nerve innervation (Weatherholt et al., 2012). Numerous spaces referred to as lacunae, exist within the lamellae to house resident bone cells, with small channels carved throughout the bone matrix forming interconnecting tunnels termed canaliculae (Weatherholt et al., 2012). Compact bone is distributed within the shafts of long bones (diaphysis) forming an outer column of bone surrounding a medullary cavity, which provides strength and rigidity (Boskey, 2013). Cortical bone thickness decreases towards the ends (epiphyses) of long bones where trabecular bone dominates (Weatherholt et al., 2012).

Trabecular bone is significantly more porous than compact bone with between 50-90% of trabecular bone volume consisting of pores rather than bone tissue (Weatherholt et al., 2012). Collagen fibrils are less organised in trabecular bone, which leads to the formation of struts of tissue between pores (Weatherholt et al., 2012). These struts are arranged in vertical and longitudinal planes (Weatherholt et al., 2012). Trabecular bone's compression strength is only one-tenth of that of cortical bone, however the orientation of the struts allows energy absorption and even force distribution (Weatherholt et al., 2012). This is an important property given that trabecular bone is found proximal to joints of long bones (Weatherholt et al., 2012).

All other appendicular and axial bones, excluding long bones, have a layer of compact bone on the external surface, with trabecular bone internally (Weatherholt et al., 2012). The outer surface of long bones is covered by the periosteum and the internal lining, adjacent to the medullary cavity, the endosteum (Weatherholt et al., 2012). A periosteal outer cover is still present in all other bones, however the endosteum covers the trabecular struts internally (Weatherholt et al., 2012).

2.5 Bone cells and bone modeling and remodeling

There are three main bone cells; osteoclasts, osteoblasts and osteocytes. Osteoclasts are large multinucleated cells that are formed from the fusion of several mononuclear preosteoclast cells, hence their large size (Weatherholt et al., 2012). They attach using integrins and isolate that area of bone from its surrounding environment. Hydrogen ions and proteolytic enzymes are secreted by osteoclasts

causing the inorganic bone matrix to acidify, thereby releasing its bone mineral and proteases degrade the organic matrix (Binkley, Berry, & Specker, 2008; Weatherholt et al., 2012). This leads to resorption of bone leaving a cavity in its place (Weatherholt et al., 2012). Osteoclasts are important for bone remodeling, whereby bone is removed from the surface of the trabeculae or endosteal surface of cortical tissue, so that new bone may be formed (Binkley et al., 2008). Bone resorption, which is stimulated by parathyroid hormone, releases calcium and phosphate ions, leading to an increase in blood calcium levels (Binkley et al., 2008). Conversely, calcitonin secreted by C-cells in the thyroid gland and to a lesser extent the parathyroid gland and other tissues, decreases osteocyte activity and bone remodeling, resulting in reduced blood calcium levels. This occurs throughout one's life and in response to fractures (Binkley et al., 2008).

Bone deposition occurs due to the activity of osteoblasts, which are large cells active in protein and proteoglycan synthesis. These cells produce type I collagen and osteocalcin proteins, in addition to alkaline phosphatase used during the bone mineralisation process (Binkley et al., 2008). Osteoblasts may become cells lining bone or those that are trapped within the lacunae of bone where they mature into osteocytes, the most abundant bone cells (Binkley et al., 2008). Osteocytes are believed to influence mineral metabolism (Binkley et al., 2008), have an endocrine role and sense mechanical loading (Dallas, Prideaux, & Bonewald, 2013). Osteocytes secrete several proteins which influence phosphate homeostasis, supporting the notion that these are important endocrine cells (Dallas et al., 2013). In addition, osteocytes are mechanically sensitive cells, which are stimulated by deformation of the matrix surrounding the cells and changes in fluid flowing within the surrounding canaliculi, producing fluid flow shear stress (Dallas et al., 2013). Various complex signaling pathways are activated when osteocytes are exposed to mechanical stress (Dallas et al., 2013). Once activated, these pathways lead to increased bone formation due to their positive effect on osteoblast differentiation and proliferation and matrix production (Dallas et al., 2013).

Bone modeling occurs during childhood in response to mechanical stimuli exerted upon them (Binkley et al., 2008). This relates to Wolfe’s Law whereby bones, in response to loads placed on them, achieve a shape and volume which allows them to best perform their function (Binkley et al., 2008). During bone modeling, osteoblasts produce new bone on the outer (periosteal) surface and osteoclasts

remove bone on the inner endosteal surface (Binkley et al., 2008). This appositional bone growth changes the shape and diameter of bone (Weatherholt et al., 2012).

The process of bone modeling has been shown to be reduced in Rett syndrome (Budden & Gunness, 2001, 2003). Two studies have used bone quantitative histomorphometric techniques to investigate osteoblast and osteoclast numbers and activity in a total of eight females with Rett syndrome (Budden & Gunness, 2001, 2003). Both studies found a decrease in bone volume compared to age matched controls and a decrease in bone formation, reflecting abnormal numbers or activity of osteoblasts. Changes in osteoclast numbers were not identified in the study participants. A decrease in the rate of mineralisation on the periosteal surface leading to reduced bone modeling may, in part, explain the lower than expect bone volume for age seen in the study participants (Budden & Gunness, 2001, 2003). This finding is further supported by a study which investigated biomarkers of bone turnover and dietary intake in a cohort of 50 females with Rett syndrome (Motil et al., 2014). Findings from this study showed decreased osteocalcin (secreted by osteoblasts) levels in the presence of normal C-telopeptide, which may signify a reduction in bone formation as opposed to resorption (Motil et al., 2014). The study by Motil and colleagues (2014), also found dietary intake of protein, calcium and phosphorus were inadequate and did not meet local recommended dietary intakes. This was coupled with the finding that urinary calcium loss may be occurring due to the inverse relationship between ionised calcium and TB bone density measurements (Motil et al., 2014). A separate study which investigated markers of bone formation and resorption in 61 females with Rett syndrome, found reduced levels of N-terminal propeptides of collagen type I, C-terminal telopeptide cross links, osteocalcin and bone specific alkaline phosphotase, compared to 122 to healthy controls (Roende et al., 2014). Furthermore, the mesenchymal stem cells (MSCs), which are progenitor cells to osteocytes, of a child with Rett syndrome, were shown to be aged compared to two normal age matched controls (four to six years old). This may be due to the observed reduction in apoptosis of MSCs in the child with Rett syndrome compared to the two healthy control samples. Therefore cells, which perhaps are no longer functioning optimally and should be destroyed, are remaining within bone tissue for longer periods of time (Squillaro et al., 2008).

2.6 Bone mass acquisition

Adult bone mass is the strongest predictor of osteoporotic fracture risk in the normal population (Bailey, Martin, McKay, Whiting, & Mirwald, 2000). Adult bone mass is

dependent on the peak bone mass (PBM) achieved in early adulthood, minus the subsequent bone loss (Bailey et al, 2000). The terms osteopenia and osteoporosis are commonly used terms when adult bone BMD T scores are between -1 to -2.5 or less than -2.5, respectively (Bachrach, 2005). However, as recommended by the International Society for Clinical densitometry (ISCD), osteopenia should not be used when reporting pediatric bone mineral mass and density (Schousboe, Shepherd, Bilezikian, & Baim, 2013). The preferred term in pediatrics when z-scores for bone outcome measures are less than or equal to -2.0 standard deviations, is "low bone mineral content" or "low bone mineral density" (Schousboe et al., 2013). Furthermore, a diagnosis of osteoporosis cannot be made on z-score results alone in children but must be accompanied by a clinically significant fracture history (Schousboe et al., 2013) as described in Section 6.5. Most fracture prevention strategies aim at reducing or reversing the loss through drugs, exercise and dietary supplementation (Bailey et al, 2000). An alternative and potentially more effective approach would be enhancing the attainment of PBM during the growth years of adolescence (Bailey et al, 2000; Whiting et al., 2004). Critical factors needed for optimal bone growth are adequate nutritional intake for formation of bone matrix, normal puberty and sufficient physical activity for the mechanical stimulation of bone development (Whiting et al., 2004).

2.6.1 Genetic influences on bone mass acquisition

There are many factors that are known to influence bone mass in an individual. It has been suggested that 80% of peak bone mass is determined by genetic predisposition, with the remaining 20% influenced by environmental factors and sex hormones (Davies, Evans, & Gregory, 2005).

It is possible that the low bone density seen in Rett syndrome may be directly associated with the type of *MECP2* mutation. It has been proposed, as osteoporosis has been seen in girls as young as three years old (Budden & Gunness, 2003) and due to the links between genotype and fracture previously described, that *MECP2* may play a role in the skeletal health of individuals with Rett syndrome. In 2009 a study compared the ultrastructure and density of bone in 21 and 60 day old mice with and without the MeCP2 protein (O'Connor, Zayzafoon, Farach-Carson, & Schanen, 2009). Those without the functional protein showed growth retardation, abnormal growth plates in the tibia and femur, with irregularly shaped chondrocytes and decreased cortical, trabecular and calvarial bone. As proposed by this study, the reduced bone density may be caused by the mutation in the *MECP2* gene

causing osteoblastic dysfunction reducing the inorganic matrix of bone (O'Connor et al., 2009). Furthermore, cortical bone stiffness, microhardess and tensile modulus has been shown to be reduced in mice where 100% of the *MECP2* gene in male mice and 50% in female mice, had been silenced, compared to wild-type (normal) mice (Kamal et al., 2015). The *MECP2* deficient mice also had a decrease in collagen concentration within bone and smaller, wide spaced trabecular struts, which may relate to reduced osteoblast function. Interestingly, these returned to normal after the *MECP2* gene was un-silenced (Kamal et al., 2015).

The ability to walk is an important skill when considering bone health given the influence that mechanical strain has on stimulating bone modeling (Branca & Valtuena, 2001). The mutations in Rett syndrome which have been seen to be the least ambulant are the p.Arg168* (Bebbington et al., 2008; Neul et al., 2008), the p.Arg270, p.Arg255* and large deletion mutations (Bebbington et al., 2008; Cuddapah et al., 2014), which may highlight these groups as being at greater risk of low bone density or fractures. An understanding of the relationship between mutation type and clinical severity may allow clinicians to predict clinical features in Rett syndrome, which can be used to formulate intervention programs and guide future care.

2.6.2 Nutritional influences on bone mass acquisition

Principally bone consists of collagen, an organic extracellular matrix impregnated with crystallized calcium phosphate (hydroxyapetite). Calcium is either retained as bone mineral or excreted via renal, faecal or dermal loss (Bailey, Martin, McKay, Whiting, & Mirwald, 2000). Approximately 99% of the body's calcium is found within the skeletal system (Sherwood, 2009). Other bone-related nutrients associated with bone are vitamin D, phosphorus, magnesium and fluoride, which influence development and maintenance of bones (Bergman, Gray-Scott, Chen, & Meacham, 2009). Adequate nutrition, particularly in adolescence, is essential to provide the building blocks for bone and maintenance of normal bone metabolism (Whiting et al., 2004). Under-nourishment, such as that seen in individuals with anorexia nervosa, has been linked with reduced bone formation subsequently leading to osteopenia and an increased fracture risk (Soyka, Grinspoon, Levitsky, Herzog, & Klibanski, 1999). This effect has been shown to be reversed when weight gain and thus nutritional status have improved (Bolton, Patel, Lacey, & White, 2005). Conversely, overweight or obese children have also been shown to have low bone mass and decreased bone area, relative to their body weight. Without adequate

bone mass to support their weight, falls are more likely to result in fractures (Goulding et al., 2000).

Several biochemical markers can be measured in urine or serum to assess both bone resorption and bone formation and hence metabolism. These markers represent enzymes derived from osteoblasts during bone formation or osteoclasts involved in bone resorption, or components of bone matrix (Seibel, 2005; Seibel, 2006). Short-term changes in these markers have not been shown to directly relate to long-term fracture risk and extreme values may be normal for that individual, an outlier or represent the dynamic nature of bone (Seibel, 2005; Seibel, 2006). Serum total alkaline phosphatase is the most commonly used marker of bone formation but does not have the specificity and sensitivity in patients with osteoporosis (Seibel, 2005; Seibel, 2006). Assessment of collagen synthesis also provides information about bone formation (Seibel, 2005; Seibel, 2006). Measurement of collagen cross-links, which occurs when intermolecular links between adjacent collagen molecules occurs, is another measurable marker of bone formation as they form during the mineralisation phase of bone development (Saito & Marumo, 2010). The degree of collagen cross-links influences the tensile strength of bone, whereby decreases in these links leads to reductions in the bending strength and elastic modulus of bone (Saito & Marumo, 2010). Significant decreases in the enzymatic collagen cross links have been observed in osteoporotic individuals, where collagen fibres are generally thinner producing defective bone matrix mineralisation (Saito & Marumo, 2010). Bone turnover can also be assessed by measuring the concentration of urinary or blood N-terminal telopeptide of collagen type I. When bone is resorbed, there is an increase in the concentration of N-terminal telopeptide, with high levels indicating a high rate of bone turnover and therefore bone loss (Dalskov et al., 2014).

Lack of adequate nutrition is of concern in Rett syndrome due to feeding related problems and lack of mobility, leading to reduced sun exposure, which may affect concentrations of 25(OH) vitamin D (Reilly & Cass, 2001). Studies have shown that difficulties with swallowing, gastro-oesophageal reflux and constipation have serious consequences on the nutritional intake of individuals with Rett syndrome (Morton, Bonas, Minford, Tarrant, & Ellis, 1997). In a study assessing the feeding experiences in 201 girls with Rett syndrome, 20% had enteral nutritional support and 87.4% moderate to severe feeding difficulties, with the potential to compromise nutritional intake (Oddy et al., 2007). Motil and colleagues (Motil, Schultz, Abrams, Ellis, & Glaze, 2006), also found in 13 girls with Rett syndrome, that total energy and

calcium intake were lower than those in the height and age matched reference data. In view of the growth and nutritional deficits identified in Rett syndrome, guidelines were developed in 2013, which provide recommendations relating to the assessment and treatment protocols for issues relating to nutrition assessment and management (Leonard et al., 2013). Furthermore, positive associations between dietary protein, calcium and phosphorus intakes and total body BMD z-scores have been observed in 50 females with Rett syndrome. These findings support the notion that adequate nutrition provides opportunity for improving bone health in this disorder (Motil et al., 2014).

2.6.3 Calcium

Calcium has a known anabolic effect on growing bones and thus adequate calcium intake is essential for appropriate bone deposition (Davies et al., 2005). Calcium estimates in the body typically measure the balance between absorption (ingested-faecal) and retained (absorption-secretion in faeces, renal, dermal) (Branca & Valtuena, 2001). Fractional calcium absorption is highest in infants (60%), declines in childhood and adolescence (34%) and young adulthood (25%), with further decreases throughout life (Branca & Valtuena, 2001). Nevertheless, approximately 26% of adult skeletal calcium is obtained during the two years of peak skeletal growth (Bailey et al., 2000). Calcium retention usually occurs when dietary intake in adolescence is above 1500mg/day (Bailey et al., 2000). Calcium supplementation has been shown to improve skeletal mineralisation in pre-pubertal children, and in post pubertal adolescents, but this positive effect declines after cessation of the supplements (Bailey et al., 2000). A lower calcium absorption in the intestines and increased calcium excretion by the kidneys was hypothesised by Motil et al (2006), as the possible cause of lower bone density in Rett syndrome. When analysing these rates in ten girls with Rett syndrome aged 2.9-8.5 years, fractional calcium absorption was higher than in controls (fractional calcium absorption typically increases when calcium intake is low) and mild hypercalcuria was observed (Motil et al., 2006).

2.6.4 Vitamin D

Vitamin D is essential for the maintenance of the skeleton and calcium homeostasis in the body. Vitamin D is predominantly obtained via cutaneous production upon exposure to ultraviolet B light, which subsequently leads to the synthesis of pre-vitamin D3 from 7-dehydrocholesterol (Cranney et al., 2007). During winter months, when sunlight is decreased, dietary sources and stored vitamin D help maintain

serum vitamin D concentrations (Cranney et al., 2007; Holick et al., 2011; Wagner & Greer, 2008). Factors such as, latitude, season, time of day, degree of sun exposure, use of sunscreen, skin pigmentation and race, affect the amount of vitamin D synthesis in the skin (Cranney et al., 2007).

Vitamin D is essential for calcium absorption in the small intestines, but also induces osteoblast activity and the differentiation and activation of osteoclasts (Cranney et al., 2007). When calcium intake is high, more calcium is absorbed passively across the intestinal wall and therefore there is less reliance on vitamin D (Cranney et al., 2007; Holick et al., 2011). When vitamin D levels are low, bone remodeling increases, leading to decreased structural integrity and formation of porous bone, and the mobilisation of calcium (Cranney et al., 2007). When Vitamin D deficiency takes place during skeletal development, rickets occurs, particularly affecting the growth plates. Severe deficiencies later in life impair new bone mineralisation, leading to osteomalacia (Holick et al., 2011). Less severe vitamin D deficiencies lead to an increase in parathyroid hormone, which is stimulated to maintain blood calcium levels (Wagner & Greer, 2008). Subsequently, bone turnover and hence bone loss increases, resulting in osteoporosis (Cranney et al., 2007; Holick et al., 2011; Wagner & Greer, 2008).

Individuals with inadequate sunlight exposure are at risk of vitamin D deficiency. Circulating 25(OH)D levels exhibit seasonal fluctuations where summer sunlight exposure may not maintain adequate vitamin D levels throughout winter months (Holick et al., 2011). The most widely accepted indicator of vitamin D status is the serum 25(OH)D, which is the more abundant precursor of active vitamin D than 1,25-dihydroxyvitamin D (Holick et al., 2011). 25(OH)D is the biologically active form of vitamin D and has a greater half-life of between two to three weeks compared to the half life of 1,25-dihydroxyvitamin D (Cranney et al., 2007). This precursor reflects the combined levels achieved by cutaneous synthesis and dietary intake. The cut-off points for adequate vitamin D levels vary from between 40-120nmol/L due to differences in functional end points such as fractures or serum parathyroid hormone levels, and the non standardisation of assay methods used to measure vitamin levels (Cranney et al., 2007; Holick et al., 2011; Wagner & Greer, 2008).

Serum 25(OH)D levels have been shown to be low in Rett syndrome with 20% of a cohort of 157 having levels less than 50nmol/L (Motil et al., 2011). This study also found that individuals whose daily consumption did not include vitamin D-fortified

milk, formula, or supplements had quantitatively lower 25(OH)D concentrations than those who consumed more than one dietary source of vitamin D per day (Motil et al., 2011). This study provides support to the monitoring of vitamin D levels in individuals with Rett syndrome and the use of supplementation or consumption of fortified formulas or foods to improve skeletal mineralisation in this disorder (Motil et al., 2011).

2.6.5 Physical activity and bone mass acquisition

Although the degree of bone modeling is determined partly by genetic factors, physical activity and gains in body weight (and thus magnitude of loading) also greatly influence this process. Modeling allows bones to increase in width by formation of bone on the periosteal surface, causing an increase in bone size (Binkley at al, 2008). Loads and strain placed on bone from physical activity cause bone to ultimately achieve a shape, size and orientation to best fit its function (Binkley et al, 2008). It has been shown that exercise in the pre-pubertal and pubertal years has the greatest impact on improving bone mass acquisition in an individual (Davies et al., 2005). Prospective longitudinal studies following individuals from childhood to adolescence to young adulthood, have shown that these positive results are maintained into young adulthood (Baxter-Jones, Kontulainen, Faulkner, & Bailey, 2008).

Adolescence represents a period when substantial changes in body composition are occurring. Skeletal muscle mass (measured as lean tissue mass) and bone mass are closely associated, as the physiological load and stress placed on bones is predominately due to muscle contraction (Branca & Valtuena, 2001). The stress placed on bone affects its geometry, microarchitecture and matrix (Branca & Valtuena, 2001; Rauch, 2012). In order to prevent unsafe deformation, the skeleton continually adapts in response to loads and force placed on bone. Therefore the increasing muscle mass and subsequent force, stimulates an increase in bone mass and hence strength (Baxter-Jones, Eisenmann, Mirwald, Faulkner, & Bailey, 2008; Janz et al., 2006).

Weight and body mass index (BMI) are positive predictors of bone mass in adults as they exert a constant load on bone, although this relationship does not identify whether it is due to fat mass, LTM or total weight (Pietrobelli et al., 2002; Wang et al., 2005). Studies have shown that from childhood to early adulthood, LTM is a stronger predictor of bone density than fat mass, although decreases in either tissue

type are associated with decreased bone density (Pietrobelli et al., 2002; Wang et al., 2005). Consequently, reducing the amount of time spent weight-bearing can lead to a decreased bone cross section, especially at the hip and lumbar spine (Pietrobelli et al., 2002; Wang et al., 2005). To date no studies have investigated the LTM in Rett syndrome and relationships with activity levels. In view of the strong associations seen between LTM and bone mass in the general population, these analyses are necessary in populations such as Rett syndrome that are vulnerable to poor bone health.

Physical activity is progressively limited in Rett syndrome, correlating with declining motor skills, increasing muscle contracture, scoliosis and rigidity (Budden, 1997). The level of physical activity in 11 females with Rett syndrome, who also had the ability to walk either independently or with assistance, was investigated using a StepWatch activity monitor, which when attached to the ankle, counts the number of steps taken (Downs, Leonard, & Hill, 2012). On average the total daily steps in this Rett syndrome group was 5652, with participants sedentary during more than two thirds of their awake hours (Downs et al., 2012). This result is almost half that of the average 11,738 steps taken per day by the normative comparison group of Western Australian children and adolescents aged between 7-16 years (Martin et al., 2008).

Using video analysis, the motor skills of 255 females with Rett syndrome were rated on ability to perform three groups of motor tasks including "Sitting", "Standing and Walking" which required participants to move from sitting to standing to walking and lastly more complex "Challenge" tasks such as walking on a slope, stepping over an obstacle and standing up from the floor and running (Downs et al., 2016). Individuals were scored on their ability to perform these movements, whereby higher scores represented better movement capabilities. The highest median score was achieved for the sitting task with a score of six out of a possible nine points, although teenagers and adults achieved scores on average three points lower than those less than eight years old. Out of a total of 27 points the median score for standing and walking was three. The lowest motor score was achieved in the challenge task with a median score of zero out of nine. Motor scores declined with age and were significantly lower in those older than 19 years. Some participants in the study retained the ability to walk, however this was generally in the milder mutation groups, including those with the p.Arg133Cys, p.Arg306Cys and p.Arg294* mutations (Downs et al., 2016).

Retaining the ability to walk over time in Rett syndrome was observed by Anderson et al (2014) who investigated the health and wellbeing in a large cohort of females with Rett syndrome over a 20 year period. Observations from this study support the view that women who could walk, generally retained this ability either walking independently or with a little or moderate amount of assistance later in life (Anderson et al., 2014). In addition, those with the more severe large deletion mutation were more severely affected than those with the mild p.Arg133Cys mutation (Anderson et al., 2014). The progression of motor skills in individuals with Rett syndrome over the course of a four year period, was shown to be reasonably stable with only small gains or losses in general motor skills including tasks such as sitting, sit to stand, standing and walking (Foley et al., 2011). Maintaining mobility in Rett syndrome is important for bone health given the strong association between LTM and bone density, whereby decreases in LTM reduce the loads placed on bone and bone modeling rates (Pietrobelli et al., 2002; Wang et al., 2005).

2.6.6 Hormonal influences on bone mass acquisition

Growth hormone (GH) and thyroid hormone during childhood are major supporters of bone mass accrual, even after peak height has been reached (Davies et al., 2005). During the pre-pubertal phase, an increase in adrenal androgens leads to an increase in bone accretion (Binkley et al., 2008). Subsequently, during puberty, in response to the oestrogen surge, there is an increase in both bone length at the growth plate and cortical thickness, especially at the endosteal surface (Binkley et al., 2008). The oestrogen surge also results in an increase in GH and its downstream hormone, insulin-like growth factor 1 (IGF-1) (Binkley et al., 2008). The rise in GH and IGF-1 increases osteoblast proliferation and differentiation and hence bone turnover (Binkley et al., 2008). A further increase in muscularity associated with increases in these hormones also has a positive effect on bone accretion (Gilsanz & Wren, 2007; Huppke, Roth, Christen, Brockmann, & Hanefeld, 2001).

Two studies have shown that pubertal development differs in Rett syndrome compared to the general population (Killian et al., 2014; Knight et al., 2013). A study, which investigated the pubertal trajectory in an Australian cohort of 213 females with Rett syndrome, found the median age of menarche was slightly later, at 14 years, compared to the normal population (Knight et al., 2013). Premature adrenarche and menarche were also observed in eight and six percent of girls respectively (Knight et al., 2013). Body mass index and the type of *MECP2* mutation was shown to influence the age of onset of menarche in this group. Early menarche

occurred in those who had normal or high weight to height ratios compared to underweight females with Rett syndrome. (Knight et al., 2013). Mutations which are regarded as having a less severe phenotype such as the C-terminal and early truncating mutations, had an earlier age of onset of menarche, while a later onset was seen in those with a severe mutation such as p.Arg168* (Knight et al., 2013). This is an important finding as delays in menarche in Rett syndrome may place those at risk of low bone density (Marchand, 2009). A larger study including 318 females with typical Rett syndrome, found approximately one quarter reached puberty early and nearly a fifth experienced delayed menarche (Killian et al., 2014). Further supporting the genetic effect of mutation type on the pubertal trajectory in Rett syndrome (Knight et al., 2013), this study identified a trend whereby the more severe mutations as a group which included p.Arg168* , p.Arg106Trp, p.Arg255*, p.Arg270*, early truncations and large deletions, experienced a later onset of menarche (Killian et al., 2014). Although the mechanisms of abnormal pubertal development in Rett syndrome are not clear, given the influence hormones exert on bone development during puberty, the assessment and monitoring of pubertal development in Rett syndrome may highlight those at risk of decreased bone accrual during adolescence.

2.7 Management of bone health

An understanding of the factors needed for optimal bone growth, in combination with factors which reduce bone loss, explain many of the current management strategies. Osteoporosis is characterised by a deterioration of bone architecture, porous bones and low bone mass, which leads to bone fragility and an increased risk of fracture (Kling, Clarke, & Sandhu, 2014).

As most of one's adult bone mass is obtained during childhood and adolescence, optimising bone accrual during this period may reduce the risk of fractures during childhood and prevent or delay the development of osteoporosis later in life (Binkley et al., 2008). Both males and females begin to lose bone mass from the age of approximately 35 years, however women's decrease in bone mass accelerates after menopause due to a decline in oestrogen and progesterone secretion, placing women at greater risk of fracture (Osteoporosis Society of Canada, 1996). Men's risk of developing osteoporosis increases after the age of 65 years, which may be due to decreases in gonadal hormone secretion (Osteoporosis Society of Canada, 1996). Osteoporosis has also been observed in childhood where a diagnosis can only be made when both a significant fracture history and low bone density and

mass are present, as described in Section 2.6 (Schousboe et al., 2013). Children who are particularly at risk are those who have an intrinsic genetic bone abnormality (primary osteoporosis) or a medical condition, where the condition on its own negatively impacts bone or its treatment is the cause (secondary osteoporosis) (Munns & Cowell, 2005).

Strategies that aim to optimise skeletal development, maintain the structural integrity of bone and prevent causes of bone fracture, center around two general principles. The first principle relates to maintenance of adequate intake of calcium and vitamin D and the second focuses on adequate weight-bearing physical activity (American Association of Clinical Endocrinologists, 2003).

Consumption of a diet with adequate calcium concentration is recommended (American Association of Clinical Endocrinologists, 2003; Kling et al., 2014; Osteoporosis Society of Canada, 1996; Sambrook, Seeman, Phillips, & Ebeling, 2002) including dairy products, sardines, tinned salmon, broccoli, kale, corn, eggs and calcium fortified foods (bread, cereals) (American Association of Clinical Endocrinologists, 2003; Kling et al., 2014). Maintenance of adequate vitamin D levels is also important whether by consuming fish oils, vegetables and fortified foods or by exposing the skin to sunlight causing vitamin D synthesis (American Association of Clinical Endocrinologists, 2003; Kling et al., 2014). As previously mentioned in Section 2.6.4, vitamin D also helps maintain adequate calcium levels as it enhances calcium's absorption in the digestive tract. In addition to dietary approaches, calcium and vitamin D levels can be improved by supplementation (American Association of Clinical Endocrinologists, 2003; Kling et al., 2014). Children with a chronic illness or a disability may have suboptimal nutrition or malabsorption problems placing them at greater risk of calcium and vitamin D deficiency (Zacharin, 2004). There may be a reduced opportunity for time outdoors and with decreased fat stores, additional clothing may be required when outside, reducing skin's exposure to the sun. Therefore the need to monitor calcium and vitamin D levels in this group may be required on a more regular basis than in the general population (Zacharin, 2004).

Engaging in physical activity has been shown to increase muscle strength, improve coordination and balance and reduce the risk of falls, which may decrease fracture incidence, especially in the elderly (Kling et al., 2014; Sambrook et al., 2002). Physical activity has also been shown to positively influence bone mass, reduce

bone loss and increase functional ability in menopausal and postmenopausal women (Osteoporosis Society of Canada, 1996). Positive responses to pre-pubertal bone mass in response to exercise have also been observed in healthy ambulant children (Zacharin, 2004). Furthermore, physical activity has been shown to increase bone strength at trabecular bone sites in children (Macdonald et al., 2008). A novel school based physical activity program in Canada, which incorporated exercises such as skipping, dancing, circuits and resistance bands for 15 minutes each day, has shown increases in bone mineral accrual in boys and bone mass and strength in girls aged nine to eleven years (Macdonald et al., 2008). However, few studies have investigated the responses to physical activity in children with a disability. Chronic immobilisation, often seen in those with chronic illness or a disability, reduces the muscle load placed on bones (Munns & Cowell, 2005; Zacharin, 2004). When the magnitude of muscle force on bone decreases, its microarchitecture and mineral content is negatively affected (Zacharin, 2004). These changes are more frequently experienced in those with neuromuscular disorders such as cerebral palsy or spinal cord lesions (Munns & Cowell, 2005). Studies have shown improvements in bone density in ambulant and non-ambulant children with spastic cerebral palsy after weight-bearing activity, with the changes being proportional to the amount of time weight-bearing (Munns & Cowell, 2005). To prevent bone loss due to immobilisation, weight-bearing activity is suggested, although there is a paucity of large studies which have investigated therapies that best prevent or treat low bone density in immobile children with a disability (Munns & Cowell, 2005).

2.8 Principles of guidelines development

Successful guidelines should be multidisciplinary, valid, reliable, clinically relevant, flexible and clear (Royal College of Paediatrics and Child Health, 2006). As in most clinical conditions, there is a variation in practice for treating and preventing osteoporosis. Nonetheless, the purpose of clinical practice guidelines for osteoporosis is to assist practitioners assimilate, evaluate and implement the broad range of evidence and opinion on best current practice.

A number of clinical guidelines exists for the diagnosis, prevention and treatment of osteoporosis in the general population over the age of 50 years, yet limited guides exist for children and adolescents with chronic illness or a disability, (American Association of Clinical Endocrinologists, 2003; Kling et al., 2014; Lentle et al., 2011; Osteoporosis Society of Canada, 1996; Sambrook et al., 2002). However, treatment

and prevention strategies for low bone density are available for conditions such as osteogenesis imperfecta (Antoniazzi, Mottes, Fraschini, Brunelli, & Tato, 2000), cerebral palsy (Fehlings et al., 2012) and glucocorticord induced low bone density in children and adults (Pereira et al., 2012). In spite of this, clinically based guidelines are limited and strategies are either directed to groups with specific conditions or the general population.

In 2014, the International Society for Clinical Densitometry (ISCD) published an updated official position titled "Bone health in children and adolescents with chronic diseases that may affect the skeleton" (Bianchi et al., 2014). The position statements address the use of DXA for assessing bone density in children and adolescence with primary or secondary bone disease, however, only in conditions where sufficient literature exists to support each statement. The document is solely focused on assessment of bone density in the young using DXA and does not discuss prevention and treatment of low bone density in chronic disease. Furthermore, due to the large number of diseases, their range of severity and varied therapeutic approaches, the official positions cannot be regarded as standardised recommendations (Bianchi et al., 2014). Therefore, development of clinical guidelines for managing bone health for rare disorders such as Rett syndrome, are needed so that the characteristics of the condition and risk factors can be addressed.

2.9 Conclusion

Rett syndrome is a rare disorder with a wide range of comorbidities, placing a significant burden upon those affected and their families. Several studies have shown that individuals with Rett syndrome are at greater risk of having low bone mineral density and content (Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011; Shapiro et al., 2010). However, it is not clear whether these parameters improve, remain the same or decline with age. Investigating bone density and body composition longitudinally, in a complete and genetically characterised population based cohort of females with Rett syndrome, provides a greater understanding of bone accrual in Rett syndrome and determines the degree of attenuation in accrual over time. Using DXA, the gold standard for assessing bone density, provides accurate measurement of bone density and content in skeletal sites, in addition to body composition data. Identification of factors that influence bone mass and density highlights those individuals at greater risk of low peak bone mass and thus low bone strength, which is likely to result in fractures.

With access to a database containing comprehensive information on participants such as genotype, epilepsy and use of anticonvulsant medications, anthropometry, pubertal stage, fracture history and mobility, factors that predict low bone density or influence bone strength in Rett syndrome could be identified.

To date there have been no intervention studies testing treatments for osteoporosis or fractures in Rett syndrome. Using guidelines for managing bone health which are directed to the general population, may not be useful in Rett syndrome as they do not take in to consideration the complex clinical course of the disorder and range of comorbidities. This is compounded by the fact that many clinicians would have very limited experience treating patients with Rett syndrome throughout their careers. Thus widely available information developed by experts within the field, provides an opportunity to inform clinicians monitoring the health of individuals with Rett syndrome throughout the world. Identification of factors that have the strongest influence on bone density in Rett syndrome highlights areas of importance in the development of guidelines to prevent, manage, monitor and treat low bone density in Rett syndrome. With the development of these guidelines the onset or progression of low bone density and the burden associated with this disorder may be reduced and may lead to (later) more informed intervention studies.

Chapter Three

Methodological Background

3.1 Introduction

The cross sectional and longitudinal studies which formed part of this thesis involved assessing bone and body composition parameters in Rett syndrome participants using dual energy X-ray absorptiometry (DXA). This chapter provides a description of the principles of DXA including, the physics of absorptiometry, the bone outcome measures and the skeletal sites measured by this apparatus. Furthermore, recommendations for DXA use in adults and children, precision, accuracy and radiation exposure in DXA and the limitations associated with its use in children are discussed. Following on from this, the chapter then provides a description of how DXA was used in the cross-sectional and longitudinal studies to assess bone mineral density, bone mineral content and body composition parameters in Rett syndrome. The final part of this chapter provides background on the methods of guidelines development.

3.2 Assessing bone mass and density Using dual energy X-ray absorptiometry

Bone densitometry is a quantitative technique using DXA for the early detection and treatment of osteoporosis (Webber, 2006). Densitometry machines are able to assess the mass of bone mineral per unit of the projected area, due to the measurement of differential attenuation (reduction in strength) of high and low energy X-rays, as they pass through tissues within the body. The greater the density of a tissue, the greater the attenuation of X-ray beams (Chun, 2011). As the X-ray beams pass through the area scanned, an attenuation or density map is produced using an imaging processing technique (Crabtree & Ward, 2009).

3.3 Physics of absorptiometry

In absorptiometry, the electromagnetic radiation in X-ray beams contains photons, which have a zero charge (Hobbie & Roth, 2007). This allows them to penetrate material to a greater degree than charged particles. X-rays have distinct photon energies, which were created after excitation of the atom by an electron beam (Hobbie & Roth, 2007). As photons pass through tissue they lose some of their energy due to their interaction with the electrons in tissues (Hobbie & Roth, 2007). Tissues, which have a greater density, generally contain a higher amount of electron

particles, leading to the partial or total transfer of photon energy to electron energy (Hobbie & Roth, 2007). It is the attenuation of photon energy that is detected by the DXA machine, which allows for the calculation of various tissue densities (Hobbie & Roth, 2007).

As the photons in X-rays used in DXA pass through atoms within tissues they can interact in three ways: photoelectric absorption, compton scattering and coherent scattering (International Atomic Energy Agency, 2013). Photoelectric absorption occurs when an atom absorbs a photon and an excited electron is ejected (Webber, 2006). This excited photoelectron will eventually lose its kinetic energy by obtaining another electron, thereby returning to its resting state (Hobbie & Roth, 2007). Compton scattering occurs when a photon disappears as it passes through an atom and a lower energy photon and an electron are ejected (Hobbie & Roth, 2007; Webber, 2006). The number of photons that emerge and the angles at which they scatter, in addition to the kinetic energy of the ejected electron, are all measureable (Hobbie & Roth, 2007). This type of scattering can reduce the contrast of the image created and increases radiation exposure in technicians. The amount of compton scatter in DXA machines is reduced due to the placement of a collimator, a device which causes the direction of motion of the photons to be more aligned and less dispersed, proximal to the X-ray tube. Furthermore, as the collimator controls the size of the beam exposed to the patient, it also reduces energy absorption (Hobbie & Roth, 2007).

The last photon interaction which affects attenuation is coherent scattering, however it accounts for less than 10% of the total photon interaction (International Atomic Energy Agency, 2013). In this instance the photon, which passes in close proximity to the atom, becomes elastically scattered from the atom due to the vibration of its electrons (Hobbie & Roth, 2007). The photon is not absorbed and the projection of the electrons' energy, which radiates in all directions, is the same frequency as the energy of the initial photon (Hobbie & Roth, 2007). These three absorption processes explain the total attenuation of the X-ray flux as it passes through tissue, whereby attenuation is increased when the density of material for a given thickness increases.

3.4 Densitometry and tissue type differentiation

As X-rays pass through the body they are attenuated by two distinct tissue types; bone mineral and soft tissue (Heymsfield, Wang, Baumgartner, & Ross, 1997). Soft

tissue consists of both lipid and lipid free soft tissue (Heymsfield et al., 1997). Lipid tissue is mostly composed of fat, more specifically triglycerides, with only 10% constituting water, connective tissue and cellular membranes (Heymsfield et al., 1997). Lipid free soft tissue includes protein, glycogen, extraosseus mineral and water and is often used as a surrogate for the estimation of muscle mass, as approximately three quarters of this tissue contains skeletal muscle (Chen, Toroian, et al., 2011; Heymsfield et al., 1997).

Densitometry measures the composition of tissues in the body at the molecular level including the chemical compounds lipids, water, proteins, glycogen and minerals (Heymsfield et al., 1997). Each of the tissues in the body have known amounts of these chemical compounds, which when measured by DXA, allows the apparatus to differentiate between tissues (Heymsfield et al., 1997). In order to differentiate between the tissue types the X-ray beam contains high and low energy photons, whereby a mathematical equation separates attenuation caused by the presence of soft tissue from that of bone (Hawkinson et al., 2007; International Atomic Energy Agency, 2013).

Bone, lipid and lean tissues all have an experimentally determined attenuation of photons, expressed as their mass attenuation coefficient at particular photon energy levels (Heymsfield et al., 1997). A ratio called the *R* value, is then determined, which represents the ratio of attenuation of photons as it passes through different tissues at the low and high energy peak levels (Blake & Fogelman, 1997). At energies within the range of 30-50 keV, attenuation occurs to a greater degree by bone as opposed to soft tissue. At energy levels greater than 70keV, attenuation of the X-ray beams by bone and soft tissue are similar (Blake & Fogelman, 1997).

3.5 Densitometry measurements

There are three main bone values reported by DXA systems: bone mineral content (BMC), bone area (BA) and areal bone mineral density (aBMD). The BMC is the mass in grams (g) of the mineral hydroxyapetite ($Ca_{10}(PO_4)_6(OH)_2$) (International Atomic Energy Agency, 2013). This measurement does not assess the amount of organic matrix such as collagen and bone marrow in bone (International Atomic Energy Agency, 2013). Bone area is the region of bone that is captured by the DXA and projected onto the image plane. This is a two dimensional area and is thus reported in cm^2 (Binkovitz & Henwood, 2007; International Atomic Energy Agency, 2013). The last bone measurement provided by DXA analysis is aBMD, which is

calculated by taking in to consideration the BMC and BA, as it represents the mass of hydroxyapetite within that region of bone (Binkovitz & Henwood, 2007; International Atomic Energy Agency, 2013). The formula used to calculate aBMD is:

aBMD = BMC/BA (g/cm^2) (Binkovitz & Henwood, 2007; International Atomic Energy Agency, 2013)

As aBMD measures the amount of bone within an area, it can be influenced by the size of that area. The problems related to this relationship are described in section 3.11. Body composition measurements, including fat mass (FM) and lean tissue mass (LTM), can only be measured during a whole body DXA scan. Fat mass, which is measured in grams or kilograms (kg), includes the sum of all adipose tissue within the body, including phospholipids, organ, marrow and subcutaneous adipose. Lean tissue mass, also known as fat free soft tissue, includes all the tissue in the body excluding bone and fat and is mostly used to measure skeletal muscle mass (Heymsfield et al., 1997; International Atomic Energy Agency, 2013).

3.6 Densitometry systems

There are several DXA systems currently available, however all contain an X-ray source, source collimator, patient table, detector collimator and an X-ray detector (Binkovitz & Henwood, 2007). All these components have a fixed geometry supported by a gantry, which moves across the area being scanned termed the region of interest (ROI) (Binkovitz & Henwood, 2007). Attenuation measurements from the detector are then forwarded to a computer, where the DXA software applies calibration factors and generates images of areal density of bone and soft tissue. The images are then analysed in order to calculate bone and body composition values (Binkovitz & Henwood, 2007).

The main difference between DXA systems is the geometry of the X-ray detector element, of which there are two types. The pencil beam scanner contains a single X-ray path where the detector is positioned opposite the X-ray tube (International Atomic Energy Agency, 2013; Lang, 2010). The detector and tube move in tandem, scanning from point to point, collecting one pixel at a time (Lang, 2010). The time taken to complete a scan using the pencil beam DXA is extensive, taking between three to five minutes for the hip and spine and 20 minutes for the whole body (International Atomic Energy Agency, 2013). Although the acquisition is slower in

pencil beam scanners, their cost is considerably less than other DXA systems (International Atomic Energy Agency, 2013).

The other type of DXA system termed fan-beam (or array) scanners contains an array of X-rays and detectors, which scan the ROI line by line (Lang, 2010). These detectors are able to collect 10 or more pixels at a time decreasing scanning times to 30 seconds for the hip and spine and three minutes for the total body (International Atomic Energy Agency, 2013). The fan beams may be wide angled where the beams are oriented transverse to the longitudinal axis of the body, or narrow-angled where the beams are parallel (International Atomic Energy Agency, 2013). Section 3.10 provides further details about DXA machines and radiation exposure.

3.7 Densitometry skeletal measurement sites

There are four regions of interest that are assessed by DXA; the spine, proximal femur, forearm and total body. The spine and hip are regarded as clinically important sites for bone density assessment in adults, as fractures in these regions are associated with a high rate of morbidity and mortality (Blake & Fogelman, 2001). The most common site of fracture in healthy children is the forearm, however distal femoral fractures are more frequent in children with a physical disability such as cerebral palsy (Mergler et al., 2009). Although measurement of the distal femur would have been useful in the Rett syndrome cohort, the Lunar DXA did not provide this ROI at the time of this study.

3.7.1 Spine

The most commonly scanned region by the DXA machine is the spine. Scanning occurs between the fifth lumbar vertebrae and the twelve thoracic vertebrae, although bone density measurements usually focus on lumbar vertebrae one to four (Lang, 2010). Measurements can be obtained from an anteroposterior projection that incorporates the vertebral bodies and transverse processes (International Atomic Energy Agency, 2013). In addition, the vertebrae may be scanned laterally showing the vertebral bodies, laminae and spinous processes. This type of scan is referred to as the vertebral fracture assessment or instant vertebral analysis and allows for improved visualisation of vertebral fractures (International Atomic Energy Agency, 2013), although there have been no studies using this technique in Rett syndrome. In comparison to anterior posterior DXA scans, which incorporates the dense cortical bone of the spinous process, lateral vertebral scans are less

influenced by anterior vertebral wedge compression fractures and spinal degenerative diseases (Wong & McGirt, 2013).

Lumbar spine scans can often be difficult to obtain in Rett syndrome due to the high prevalence of scoliosis, with studies suggesting between 75-85% of individuals with Rett syndrome over the age of 16 years suffer from this condition (Ager et al., 2006; Percy et al., 2010). The progression of spinal curvature has been shown to be quite considerable averaging between 14 to 21 degrees of curvature per year (Downs et al., 2015; Harrison & Webb, 1990; Keret, Bassett, Bunnell, & Marks, 1988; Lidstrom, Stokland, & Hagberg, 1994). Accurate identification of the ROI in the lumbar spine may not be possible when curvature is severe. With spinal curvature progression, as stated in the guideline for management of scoliosis in Rett syndrome, spinal fusion surgery is often performed once the Cobb angle has reached between 40-50 degrees (Downs et al., 2009). It is not possible to use DXA scans of the lumbar spine when metal rods and plates are present as they occlude bone and are labeled as artifact by DXA software.

3.7.2 Proximal femur

For proximal femur scans, accurate positioning is critically important in order to maximise the femoral neck projection. The scanning positioning protocol includes slight abduction and internal rotation of the femur at the hip joint (International Atomic Energy Agency, 2013). Each DXA system employs their specific guidelines for achieving this position and maintaining it during the scan. The regions of interest in the proximal femur are the femoral neck, greater and lesser trochanters, intertrochanteric region and Ward's triangle, which is the femoral neck region with the lowest bone density (Blake & Fogelman, 1997; International Atomic Energy Agency, 2013). In a clinical setting, the femoral neck and total hip measurements are the most commonly used as they have been shown to be the most accurate predictors of hip fracture risk (Bachrach & Sills, 2011). However, achieving appropriate positioning of the femur of Rett syndrome patients during a DXA scan may present a problem due to their general neuromuscular weakness and dystonic rigid stiffness (Hagberg, 2005). Furthermore, as motor skills have been shown to decrease with age in Rett syndrome (Downs, Bebbington, Jacoby, et al., 2008), analysis of bone density using DXA may become more difficult overtime.

3.7.3 Forearm

Peripheral DXA scanners can be used to measure aBMD in the ultradistal, distal (mid-radius) and shaft of the radius (International Atomic Energy Agency, 2013). The DXA scanner is smaller and more portable than a central scanner, allowing assessments to be made with the patient seated and the forearm placed on a table (International Atomic Energy Agency, 2013). Ultradistal radial measurements are useful as this area contains a significant amount of trabecular (cancellous) bone, whereas the shaft contains only cortical bone (Blake & Fogelman, 1997).

3.7.4 Total body

Total body scans assess the mineral content in the entire skeletal system, which may be a more precise measurement in Rett syndrome, given that it includes limb bones which are most influenced by muscle strains and loads. Patients lie on a scanning table in a supine position, with all body regions included in the scanning field (International Atomic Energy Agency, 2013). Careful placement of the arms and legs need to be considered in preparation for this scan, so that all bones are incorporated in the scan and no overlap occurs (Binkovitz & Henwood, 2007). In addition to calculating the total BMC and aBMD, sub-regional measurements are also calculated including the left and right upper and lower limbs, the head, ribs, thoracic and lumbar spine and the pelvis (International Atomic Energy Agency, 2013). Furthermore, total body scans provide body composition data, estimating the LTM and FM in regions absent of bone (Blake & Fogelman, 1997). When bone is present, the DXA machine is only able to differentiate between bone and soft tissue as a whole (Blake & Fogelman, 1997). Achieving a total body DXA scan without movement or overlap may be difficult in Rett syndrome patients due to their declining motor skills and rigidity (Hagberg, 2005). In addition, given that total body scans require the patient to remain still for approximately three minutes (International Atomic Energy Agency, 2013), movement artifact may occur when scanning individuals with Rett syndrome.

3.8 ISCD recommendation for adults and pediatrics

The International Society for Clinical Densitometry (ISCD) has developed official positions for adults and pediatric use of densitometry (Schousboe et al., 2013; Gordon, Leonard, Zemel, & International Society for Clinical Densitometry, 2014). These positions offer a guide as to what indications there are in a patient that warrants bone mineral density testing. In addition, recommendations for appropriate reference data and calculation of T-scores or z-scores are included in the positions,

including their cut off points for the diagnosis of osteoporosis and low bone density (previously referred to as osteopenia) (Gordon et al., 2014; Schousboe et al., 2013). As shown in the equation below, the T-score is primarily referred to for post-menopausal women and men over 50 years. This score is the difference between the patient's measurement and a young adult reference measurement in units of the population standard deviation (SD) (International Atomic Energy Agency, 2013).

$$\text{T-score} = \frac{aBMD_{patient} - aBMD_{young\ adult\ mean}}{SD_{young\ adult\ mean}}$$

The z-score is the difference between the patient's measurement and an age and usually ethnicity and gender matched reference (International Atomic Energy Agency, 2013). This score is more appropriately used in children, given that they are yet to achieve peak bone mass (Binkovitz & Henwood, 2007).

$$\text{z-score} = \frac{aBMD_{patient} - aBMD_{age,\ ethnicity,\ gender\ matched\ mean}}{SD_{age,\ ethnicity,\ gender\ matched\ mean}}$$

The remainder of the ISCD positions provide a guide to DXA use, including suggested ROI, serial measurements, fracture risk assessment, calibration and cross-calibration of DXA machines, precision assessment, reporting results and body composition analysis (Schousboe et al., 2013; Gordon et al., 2014). It should be noted that the ISCD protocols relate to the general adult and pediatric population and do not discuss DXA use in those with a disability, whose physical limitations may influence scanning procedures and data analysis. The official positions are developed by an international panel of experts within the field after considerable review of the current literature and clinical practice. Once the ISCD Board of Directors have approved the official positions, they are then made accessible to the public via the ISCD website. The adult official positions, was recently updated in 2015 (International Society of Clinical Densitometry [ISCD], 2015) from its previously published version in 2008 (Baim et al., 2008), whereas the current pediatric official position was published in 2013 (Schousboe et al.,, 2013).

3.8.1 ISCD adult official skeletal site recommendations for DXA assessment

There are two skeletal sites currently recommended for adult DXA scans in at-risk patients; the posteroanterior lumbar spine at levels one to four and the hip. The hip ROI includes the left or right femoral neck or the left or right total proximal femur, with the decision based on the ROI with the lowest aBMD. If a DXA scan of the

lumbar vertebrae and hip is not possible, the ISCD recommends assessing aBMD in the proximal one third of the radius (ISCD, 2015).

3.8.2 ISCD pediatric official skeletal site recommendations for DXA assessment

The ISCD recommends the aBMD and BMC in pediatric patients should be assessed using DXA in the posteroanterior lumbar spine, inclusive of levels one to four. In addition, aBMD should be measured in the total body with the cranial bones removed (headless aBMD) (Schousboe et al., 2013).

3.9 DXA precision and accuracy

Precision and accuracy of DXA scans needs to be considered and evaluated when interpreting serial quantitative clinical assessments. Precision relates to the reproducibility of the test to obtain the same result when repeated on an individual using the same technique (Webber, 2006). Without adequate precision, it is not possible in serial measurements, to differentiate between a change in aBMD due to random error and a statistically significant change in bone density (Webber, 2006). Precision testing involves measuring aBMD in a skeletal site such as the femoral neck or lumbar spine, two to three times in 30 or more patients (Hawkinson et al, 2007). These scans can be repeated at the same time or within a two-four week period (Engelke & Gluer, 2006; Hawkinson et al., 2007). Precision is calculated in DXA systems by finding the root mean square standard deviation in grams per centimeter squared of aBMD and dividing this by the mean. This calculation produces a percentage called the precision error. Published DXA precision errors exist for all skeletal sites measured. When precision error is poor (percentage increase), changes in aBMD need to be greater in order for these changes to be identified as clinically important (Baim et al., 2005). As changes in bone density over time are generally small, a low precision error is optimal so that significant changes are highlighted (Baim et al., 2005).

There are several strategies that can be applied to minimise precision error in longitudinal bone density analysis, of which most rely on the skill of the DXA technician (Engelke & Gluer, 2006). Precision error is reduced when technicians receive adequate training so that standard ROI, positioning and scan analysis adhere to manufacturer protocol. In addition, maintaining the same scan mode for longitudinal scans as that used in the baseline scan, also improves precision (Baim et al., 2005). Although challenging in a clinical setting, use of the same technician

for baseline and follow-up scans is also advantageous (Baim et al., 2005). Furthermore, repeated scans should occur using the same DXA model (International Atomic Energy Agency, 2013). Variation in bone outcome measures between DXA models of the same manufacturer and between the major manufacturers has been shown to be as high as 10-15% in some skeletal sites and are thus not useful for comparative bone density changes over time (Baim et al., 2005).

Accuracy of DXA technique reflects the closeness of the measured value by the DXA machine to the actual known value of the measured object. The difference between these values is referred to as the accuracy error, which is typically better than 10% in DXA studies (Baim et al., 2005). According to the World Health Organisation, the accuracy error percentage is an acceptable level for DXA to be used as a tool to assess fracture risk and diagnose osteoporosis (Baim et al., 2005). Densitometry manufacturers provide quality control procedures in order to monitor scanner performance over time. As accuracy varies between DXA models, in addition to within the same machine if upgrades or maintenance has occurred, quality control and calibration are performed. A phantom is used which consists of either aluminum or hydroxyapatite bone imitation of known density (Emaus, Berntsen, Joakimsen, & Fonnebo, 2005). Semi-anthropometric phantoms such as the one consisting of hydroxyapatite are considered the better phantom as they mimic measurements of bone more closely than aluminum phantoms (Emaus et al., 2005). As recommended by Hologic and GE Lunar manufacturers, scanning of these phantoms should occur each morning before patients are scanned (Baim et al., 2005).

3.10 Densitometry radiation dosage

During a DXA scan both the patient and the operator are exposed to ionizing radiation, albeit to a lesser degree compared to other medical imaging techniques using ionising radiation (Njeh, Fuerst, Hans, Blake, & Genant, 1999). The radiation hazard is typically expressed as the effective dose (ED) and reported in sieverts (Sv) or microsieverts (μSv). Natural background radiation to which we are exposed within a 12 month period is approximately 2400μSv, however this can vary by a factor of up to four due to changes within the environment, altitude and geological substrate (Baim et al., 2005). The ED is the total absorbed radiation dose to each irradiated organ, which has been weighted for the type of radiation and the organ's susceptibility to it (Njeh et al., 1999). Publication of the weighting factors of all

organs and tissues and their radiogenic risk, have been provided by the International Commission on Radiological Protection (ICRP, 2007).

Several key parameters influence the ED received by adults and children during a DXA scan. The greater the number of scans a patient receives the higher the ED due to summation of radiation absorption, with most individuals receiving between two to three scans per clinic visit (Larkin et al., 2008). Effective dose is also influenced by acquisition techniques such as imaging length, speed and width settings, which are generally determined by the dimensions of the patient (Larkin et al., 2008). Furthermore, ED varies between DXA models due to differences in the focal spot size (fan beam or pencil beam), type of source collimation, X-ray tube filtration, tube current and tube potential used by each model (Larkin et al., 2008). As acquisition mode is the main factor that can be controlled by the operator in reducing ED, imaging protocols to minimise risk have been developed for both adult and pediatric scans (Damilakis, Solomou, Manios, & Karantanas, 2013; Larkin et al., 2008). Changes in acquisition mode can influence ED by a factor one and a half to three times the radiation dose when using the highest to lowest modes (Blake, Naeem, & Boutros, 2006). Modes are generally set by the size of the individual being scanned with higher modes needed for larger individuals. When comparing the ED in pediatric and adult patients, the exposure is similar as long as appropriate acquisition modes are applied. When children are scanned in adult modes, the ED can be significantly higher (International Atomic Energy Agency, 2013; Njeh, Samat, Nightingale, McNeil, & Boivin, 1997).

The ranges of ED for the following DXA scans are as follows; posteroanterior lumbar spine one to five is 0.5-0.21uSv, proximal femur is 0.15-5.4uSv and the total body 0.6-4.6uSv (Hawkinson et al., 2007). These values are influenced by the speed of the scan and whether the DXA is a fan beam or pencil beam model (Blake & Fogelman, 1997). Scan times are considerably shorter using a fan beam DXA, which is advantageous in children who may have difficulty remaining still for the length of the scan (Blake & Fogelman, 1997). However, a disadvantage of this DXA model is an increase in radiation exposure to operators and patients (Blake & Fogelman, 1997). Nonetheless, doses associated with use of the fan beam model are still low compared to other radiological procedures (Blake & Fogelman, 1997). Of the two main DXA manufacturers, GE Lunar and Hologic, the GE Lunar has been shown to have lower ED values for the same scan sites (International Atomic Energy Agency, 2013). Hologic systems use voltage switching between high and

low energies, with short bursts between them (International Atomic Energy Agency, 2013). Whereas the GE Lunar system uses a K-edge filtration system, whereby the X-ray tube delivers a steady current and K absorption splits the X-ray spectrum in to the two energy levels (International Atomic Energy Agency, 2013).

3.11 Densitometry in children

Densitometry was first used in the 1980's to track osteoporosis progression in post-menopausal woman (Fewtrell, 2003). It was designed to assess bone density and bone mineral in adults who had already achieved peak bone mass and predict their fracture risk (Van Rijn & Van Kuijk, 2009). Densitometry was not used in pediatrics until the 1990's at which time improvements in the algorithms which detect bone edges, was developed (Binkley et al., 2008). As children grow, the size of their bones, geometry and mineral content are changing, with appendicular bone growth preceding axial bones (Bachrach, 2005). The timing of this growth is more dependent on pubertal and skeletal maturation than chronological age and varies between gender and ethnicity. Therefore the stage of growth, height and pubertal development need to be taken in to consideration when measuring pediatric aBMD (Zacharin, 2009).

Densitometry presents a bone-size related problem when assessing aBMD in pediatrics as it measures the amount of bone within a given area. As calculation of aBMD is obtained by dividing the BMC by the bone area, it is not a true density measure but rather a two dimensional measurement of a three dimensional structure (Bachrach, 2005; Binkley et al., 2008; Binkovitz & Henwood, 2007; Van Rijn & Van Kuijk, 2009; Zacharin, 2009). Therefore the size of the area being scanned will influence the aBMD results, such that individuals who have small bones will appear to have a lower aBMD than those who have larger bones, even if their volumetric BMD is the same (Binkovitz & Henwood, 2007). Longitudinal studies are also affected by DXA's inability to measure volumetric bone density as changes in aBMD may represent a change in bone size or mineral composition as opposed to density (Gilsanz & Wren, 2007). The bone-size related problem with DXA is particularly important in children and adolescents with disabilities or with chronic disease, as bones may be short and narrow for their age leading to an underestimation of their true volumetric bone density (Van Rijn & Van Kuijk, 2009; Zacharin, 2009).

Various approaches have been used to address this bone size related problem. Mathematical calculation of a volumetric bone density (bone mineral apparent density, BMAD) may be performed by dividing the BMC by the projected bone area to the power of two for the femoral neck and one and a half for the lumbar spine (Binkley et al., 2008). This approach is based on the premise that bones scale proportionately in all directions (Gilsanz & Wren, 2007) and can be viewed as a cube or a cylinder (Zacharin, 2009). Routinely, DXA compares results with subjects of a similar age and gender and calculates an age z-score. Alternatively, comparing measurements with individuals of the same height or weight, as opposed to age, will more accurately determine if aBMD and BMC are normal for their size (Fewtrell, 2003). The limitation of this approach is the lack of normative pediatric data available for height and weight bone outcome measures, as most DXA manufacturers only provide age related normative reference data (Fewtrell, 2003). Furthermore, multiple regression models can be used to adjust BMC for bone area (BA), weight, height, age and pubertal status (Fewtrell, 2003). Various parameters can also be calculated that provide information on bone parameters including assessing height for age, which determines if bones are short, BA for height which identifies narrow bones and BMC for BA which identifies if bone have reduced mineralisation and are therefore thin (Fewtrell, 2003).

Interpretation of bone growth using DXA in children with disabilities is not only influenced by stature, problems may arise due to the presence of bone deformities or muscular contractures which limit appropriate positioning and view of the ROI (Zacharin, 2009). Positioning may also be a problem due to poor motor control preventing the child or adolescent from remaining still during the scan. In addition, the presence of fixation devices, hip surgery, hip dislocations or hip dysplasia may make the ROI unable to be scanned (Zacharin, 2009).

3.12 Overview of densitometry measurement methods in the cross-sectional and longitudinal studies

A cross-sectional bone density and body composition assessment, described in Chapter Four, was performed on female participants with Rett syndrome using the GE Lunar Prodigy DXA scanner. Areal bone mass density (aBMD) was measured in the lumbar spine (LS), right and left femoral neck (FN) and total body (TB). Bone mineral content (BMC) was assessed in the LS and TB. Body composition assessments included lean tissue mass (LTM) and fat mass (FM). Following on from the cross-sectional scans, all participants were invited to have a follow-up DXA

scan of the LS and TB, allowing longitudinal changes in the bone and body composition outcome measures to be investigated. The results comparing the cross-sectional and follow-up scan are described in Chapter Five.

3.13 Densitometer model

The Prodigy DXA system was used in the cross-sectional and longitudinal (follow-up) studies in preference to its largest competitor, the Hologic DXA (Blake et al., 2006), as the Prodigy has been shown to deliver a much lower radiation dose (Damilakis et al., 2013). The effective radiation dose for spine and hip scans in a five year old using the Prodigy is approximately 0.65 and 0.36 microsievert (uSv) respectively, compared to the Express Hologic scanner dose of 16.1 and 10uSv, with similar differences seen across ages (Blake et al., 2006).

3.14 Participant source

Participants in this study were sourced from the Australian Rett Syndrome Database (ARSD), which is a population-based registry established between 1993-1995 (Leonard, Bower, & English, 1997). Cases in this registry are ascertained from the Australian Paediatric Surveillance Unit, which is a unit of the Australian College of Physicians focused on supporting research into rare pediatric disorders (Gazarian et al., 1999; He, Zurynski, & Elliot, 2010). Cases are also obtained from the Rett Syndrome Association of Australia, which is a parent support group. Once an individual is enrolled in the database, their clinician and family are asked to complete a comprehensive questionnaire encompassing various aspect of functioning, daily living activities, health status, the impact the condition has on families and the types of medical or therapeutic interventions used and their benefits (Laurvick, de Klerk, et al., 2006). Follow-up questionnaires were first completed by families in 1996, and subsequently completed approximately every two years since 2000 (Laurvick, de Klerk, et al., 2006). Video analysis is also performed approximately every four years, since 2004. Follow-up data collection has been achieved in over 80% of those enrolled in the ARSD. With identification of the *MECP2* mutation being the principal cause of Rett syndrome in 1999 (Amir et al., 1999), all cases who were enrolled in 2000 were offered genetic testing for the mutation, with those enrolled after this date able to have genetic testing organised by their clinician. Collection of genetic data enabled investigations in to the relationship between genotype and phenotype in Rett syndrome. In both the cross-sectional and longitudinal DXA studies forming part of this thesis, only those in the

ARSD who were four years or older were invited to participate, as this was the youngest age in the comparative normative dataset (Lu et al., 1994).

3.15 Identification of scanning locations within Australia

In light of the geographical spread of participants throughout Australia, scanning locations were identified online including private Radiology Clinics and Radiology Departments in hospitals throughout Australia. Locations that used the Hologic DXA were excluded from both studies, whereas those using the Lunar prodigy were marked as potential scanning sites. The Radiologist or Radiographer was contacted by phone or email and informed of the study's objectives and methods and participation was sought.

Clinics which agreed to participate were Perth Radiological Clinic, Perth, WA; North Coast Radiology in Lismore, NSW; Newcastle Radiology in Charlestown, NSW; the Royal Brisbane Women's and Children's Hospital in Brisbane, Queensland; Westmead Children's Hospital, Sydney, NSW; Monash Medical Center, Melbourne, Victoria; and Benson Radiology, Adelaide. All clinics contacted in the Northern territory used a Hologic DXA and therefore were not included in both studies.

Although the overall study had ethics approval from Curtin University, additional ethics applications were submitted and approved at Westmead Children's Hospital, Monash Medical Centre, Brisbane Women's and Children's Hospital and Benson Radiology. The study had not applied for ethics approval for sedation during the DXA scans.

3.16 Instructions provided to radiology clinics

To maintain precision and assure optimal scan quality across scanning locations throughout Australia, densitometrists were provided with a scanning protocol specific to the study (Appendix A). This explained which bone outcomes were to be measured, the DXA scanning depth mode based on the size of the area measured, participant positioning during the scans, and recommendations to reduce participant stress and movement. The document also stated the means by which clinics were to forward the results to the Telethon Kids Institute in Perth.

3.16.1 Scanning protocol

The scanning protocol was developed in consultation with Ms Julie Briody, the Chief Radiographer at Westmead Children's Hospital. Ms Briody has over 10 years of

experience performing densitometry assessments using a Prodigy DXA on individuals with Rett syndrome and was therefore aware of their physical and intellectual limitations.

3.16.2 Skeletal sites measured

The following requests were made to each clinic regarding the skeletal sites measured and their order:

- First scan: scan the lumbar spine, L1-L4, enabling individuals to accommodate to the noise of the machine, unless a gastric button or lumbar rods were present.
- Second scan: scan the left femoral neck and if possible also the right.
- Third scan: scan the total body.

3.16.3 Scan positioning and DXA depth mode

Radiographers were requested to use the standard Lunar Prodigy manufacturer positioning, although strict adherence to these position guides may be challenging in individuals with Rett syndrome. For lumbar and femoral neck scans the GE Prodigy guidelines stated that the participant lies supine on the marked centerline of the table, with their legs internally rotated and their arms placed on their chest away from each hip. The guideline further stated that femoral neck scans should include a minimum of three centimeters of tissue superior to the greater trochanter and three centimeters inferior to the ischium. The lesser trochanter should be predominantly hidden in femoral neck scans due to the internal rotation of the femur. The only deviation from standard Prodigy positioning request was for the total body scans in pediatric patients (up to and including 20 years) as radiographers were asked to place the participant's hands in a loose fist, where possible, as this was the method by which the comparative normative data used in the study was obtained (Lu et al., 1994).

The recommended depth setting during the scan, which is influenced by the area measured, was based on Lunar Prodigy normals, whereby depths less than 13cm were scanned on thin mode, 13-25cm depth scanned in standard mode and greater than 25cm the thick mode. The scanning protocol recommended that radiographers use the larger scan mode if the patient was on the cusp of the depth cut-off on the first scan due to expected growth occurring between the baseline and follow-up scans. The radiographer at each scanning location was asked to use the standard

Prodigy quality assurance procedures and document machine changes on the Shewart charts in thin and standard depth settings.

3.16.4 Further recommendations during the scanning procedure

In order to obtain an accurate scan, no movement should occur during the procedure. Due to the movement difficulties experienced by those with Rett syndrome (Downs, Bebbington, Jacoby, et al., 2008), recommendations were given to help minimize movement during the procedure. The radiographer was asked to wrap a sheet around the participant during the extended period taken to complete the total body scan. The participant's parent was asked to be present in the room, close to their child's head, to reduce the level of patient anxiety. In addition, clinics were asked to request parents bring items such as favourite toys, pacifiers, music or videos that were known to promote relaxation in their child. To prevent attenuation of X-ray beams during the scan, clinics were also asked to advise parents that their child should not to be wearing any metal on their clothing such as zips, press-studs, earrings, hooks and eyes or similar objects.

3.16.5 Data transmission and reporting

The process of transmission of data was negotiated in each study location. The means of transmission included emailing data to the aussierett@ichr.edu.au address or sending scans to the Telethon Kids Institute on a CD or disc. Radiology clinics were asked to send a hard copy of their standard densitometry patient report to the Telethon Kids Institute and the participant's nominated clinician for follow-up. The consent form was also forwarded to the Institute. In instances where parents had not returned to their daughter's clinician to receive DXA results, they were sent a letter stating the findings of each scan as a z-score, which was then further described as being above normal, normal, low or very low.

3.16.6 Densitometry scan re-analysis

The Lunar Prodigy enCORE software automatically determines the ROI in each scan and differentiates between the type of body tissue, termed tissue type, labeling it as bone, fat or lean tissue. To maintain consistency and accuracy of scan analysis between clinics, each scan was reviewed and re-analysed using the GE Healthcare enCORE Lunar Prodigy software version 10.50.085.

The imaging tool in the enCORE software was used to check accurate contrast of each image in order to improve the bone edging and magnification of bone. This

was followed by a review of the ROI for lumbar spine, femoral neck and total body scans.

3.16.7 Femoral neck positioning

Correct positioning of the femoral neck ROI, as shown in Figure 3.1, included the following criteria:

- The femoral neck axis was perpendicular to the femoral neck ROI box.
- No portion of the greater or lesser trochanter was captured in the ROI.
- Soft tissue was contained at either ends of the ROI.
- No portion of the ischium was included in the ROI.
- Width of the ROI box, which was perpendicular to the axis of the neck, was 1.0cms for those whose weight was 34kgs or less and 1.5cms for those greater than 34kgs.

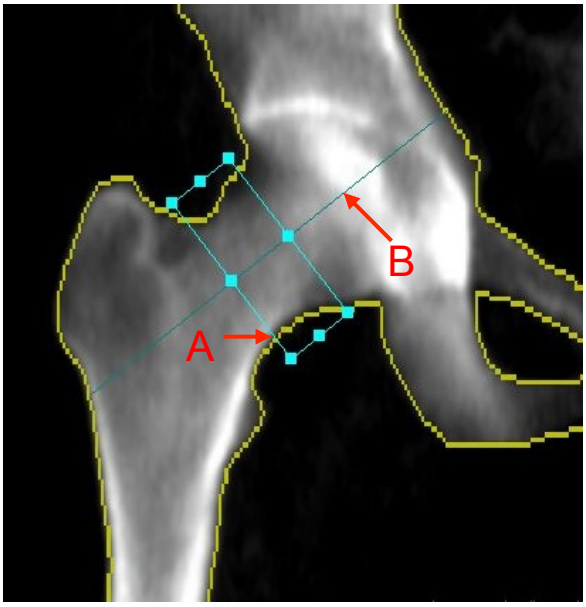

Figure 3.1: Femoral neck box (A) was perpendicular to the femoral neck axis (B) and excluded the trochanters and ischium

3.16.8 Lumbar spine positioning

Correct positioning of the lumbar spine ROI, as shown in Figure 3.2, included the following criteria:

- Lumbar vertebrae 1-4 were clearly identified.
- Positioning of the intervertebral markers were guided by the bone profile which highlighted the lowest points of bone density
- Intervertebral markers were positioned between the vertebral bodies.

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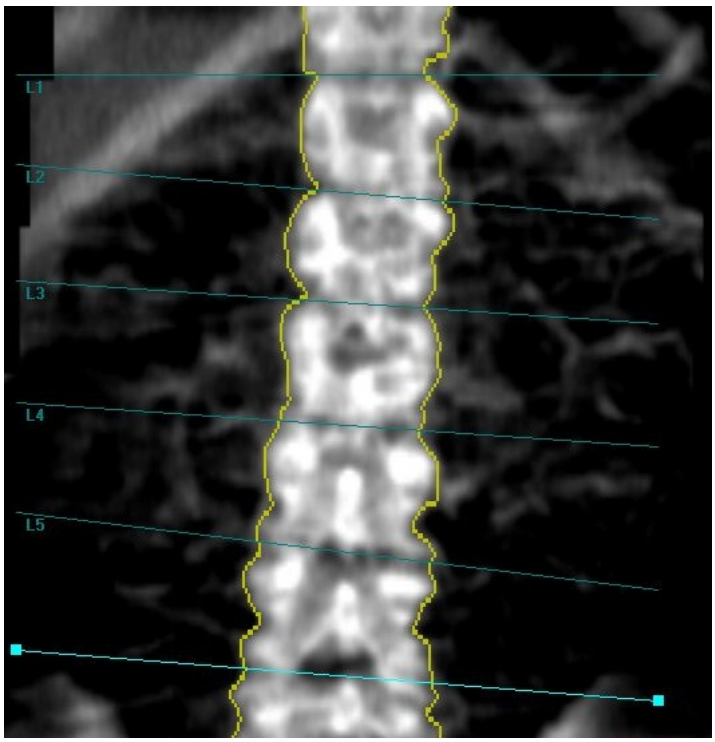

Figure 3.2: Lumbar spine ROI with intervertebral markers passing between vertebral bodies

3.16.9 Total body positioning

Correct positioning of the total body cut points, as shown in Figure 3.3, included the following criteria:

- The cranial cut was positioned immediately inferior to the mandible.
- Left and right arm: the vertical cut passed through the glenohumeral joint, ensuring the arms and hands were separated from the body.
- Left and right forearm: the vertical cut separated the elbow and forearm from the body.
- Left and right spine: two vertical cuts were immediately lateral to the vertebrae, avoiding inclusion of the ribs.
- Left and right pelvis: both pelvis cuts passed through the femoral necks and did not touch the pelvis.
- Pelvic upper margin: positioned immediately superior to the iliac crests.
- Left and right leg: both vertical leg cuts separated the hands and forearm from the legs.
- Center leg: the vertical cut separated the right and left leg.

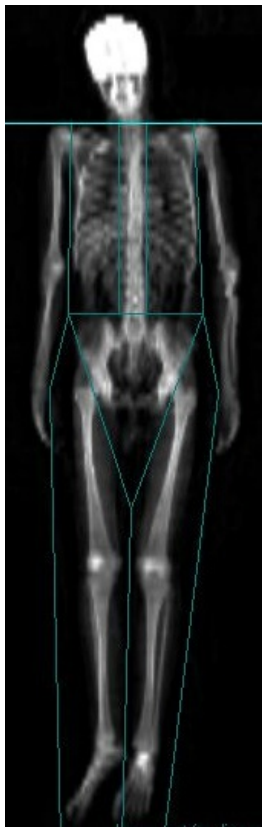

Figure 3.3: Total body ROI separating the head, arms, legs, vertebrae, thoracic cage and pelvis

In addition to the review of the ROI in each scan, point typing, which is the enCORE tool used to differentiate tissue types, was also analysed. The enCORE software contains a brush tool, which allows manual alterations to be made to the areas labeled as bone, artifact and the border surrounding bone. Using this tool, alterations were made to scans where bone was labeled incorrectly in the lumbar spine (Figure 3.4) and femoral neck (Figure 3.5) scans. Additional changes were made to the brush type termed the neutral border, which forms a thin layer surrounding bones. The composition of this neutral border does not contribute to bone or tissue measurements in DXA scans. The artifact brush type, which is the only point typing which can be altered in total body scans, was used to exclude orthopedic implants such as spinal or femoral neck rods.

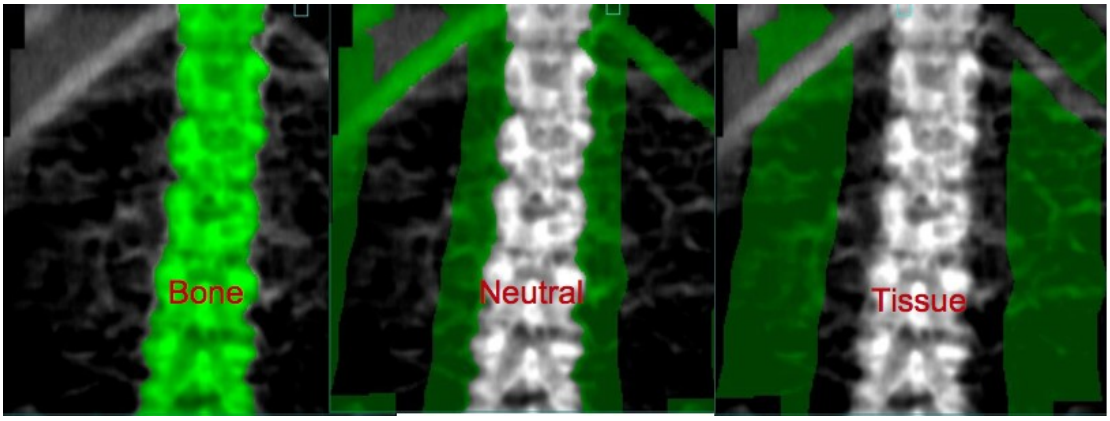

Figure 3.4: Lumbar spine point typing scan

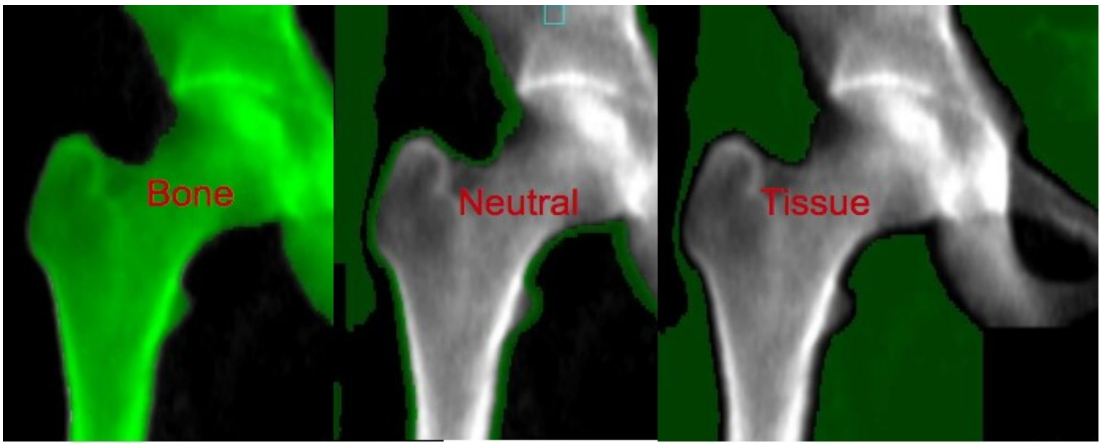

Figure 3.5: Femoral neck point typing scan

3.17 Australian normative data

Once scans were re-analysed, the data were then sent to Radiographer Julie Briody at Westmead Children's Hospital for calculation of z-scores. The normative data set used to calculate z-scores consisted of healthy adults and children as young as four years from the Australian population. In 1994 (Lu et al., 1994), the normative data set initially consisted of 136 males and 130 females who participated in cross-sectional and longitudinal bone density and body composition studies. Having grown since its inception, the database now contains information from over 800 children and adults. All individuals in this data set were outpatients at Westmead Children's Hospital, staff member's children and their friends and students studying medicine. None of the participants had a known medical condition.

The normative data consisted of areal bone mass density and bone mineral content of the LS, FN and TB, which were measured using a Lunar Prodigy DXA. Individuals who contributed to this normative dataset also had their LTM and FM measured and height and weight were recorded prior to each scan. Fifty-three out of the original 130 participants, had these parameters measured at baseline and at follow-up (Lu et al., 1994).

The data collected during these normative studies allowed the development of linear curves for the mean prediction of aBMD and BMC in the LS, femur and TB. Linear curves for lean tissue and fat mass for age, weight and height were also obtained. These predictive models could then be used to calculate age, height and weight related z-scores for bone and body composition outcome measures (Lu et al., 1994).

3.18 Delphi technique

When a draft guideline has been formulated, it is often reviewed following the Delphi process where panel members provide their opinion on draft statements or questions, without meeting any other panel members or guideline developers (Hsu & Sandford, 2007; Murphy et al., 1998). The Delphi process was used in this thesis when formulating the clinical guidelines for managing bone density in Rett syndrome, as it has been shown to create anonymity, decrease expert panel bias and allow participants to be geographical dispersed (Murphy et al., 1998). The number of rounds of guideline review by panel members is determined by the degree of consensus sought by developers and typically ranges between three to five (Hsu & Sandford, 2007). However, in this study, once responses from panel

members were statistically analysed in order to objectively summate the data and determine if consensus had been reached (Hsu & Sandford, 2007), only two rounds were required. As is often the case, the resulting guidelines were a mixture of research based and consensus based recommendations, where lack of supporting scientific data was used to identify key areas for future research (Hsu & Sandford, 2007)

3.19 Guideline formulation

Formulation of the guideline began with identifying key questions relating to the topic of interest and a literature search to identify quality evidence to address these questions (Scottish Intercollegiate Guideline Network [SIGN], 2011). Publications identified during the literature search were then graded based on their strength of evidence related to the quality of studies, which support the guideline or recommendation. High quality meta-analysis, systematic reviews of randomised controlled trials or randomized control trials with minimal bias were considered the best level of evidence, whereas case reports, case series and expert opinion, where literature was lacking, received the weakest grading (SIGN, 2011).

To form the literature review the following databases were searched; EMBASE, Medline, PubMed, ScienceDirect, SpringerLink, Web of Science, ProQuest Health and Medical Complete, PsychINFO, The Cochrane Library and EMBASE. Online libraries reviewed were those of the World Health Organisation, Canadian Medical Association Infobase, Clinical Practice Guidelines, Geneva Foundation for Medical Education and Research, the National Guidelines Clearinghouse, National Institutes for Health and Care Excellence, Scottish Intercollegiate Guidelines and the Trip search engine. Keywords used in the search were a combination of Rett syndrome with, epilepsy, diagnosis, genotype, phenotype, *MECP2*, mutation, clinical severity, scoliosis, epilepsy, growth, nutrition, diet, mobility, ambulance, survival, puberty, bone, bone density, osteoporosis, osteopenia, fracture, vitamin D, calcium and exercise. Furthermore, keywords associated with bone included, matrix, osteoblast, osteoclast, accrual, development, modeling, remodeling, acquisition, puberty, vitamin D, calcium, nutrition, physical activity, exercise, mineral density, mineral composition, hormones, osteoporosis, osteopenia, disability, cerebral palsy. Lastly the terms used to research the literature relating to the clinical guidelines were, consensus, expert panel and Delphi technique/model

A draft guideline was then formulated, including a list of the identified literature (Appendix B) and subsequently reviewed by an expert panel (Hsu & Sandford, 2007).

Expert panel members were highly trained and experienced in all or specific areas of the topic being addressed and were willing and available to participate in the review process (Hsu & Sandford, 2007; Murphy et al., 1998).

Chapter Four

Bone mineral content and density in Rett syndrome and their contributing factors

Study publication:

Jefferson, A. L., Woodhead, H. J., Fyfe, S., Briody, J., Bebbington, A., Strauss, B. J., Jacoby, P., Leonard, H. (2011) Bone mineral content and density in Rett syndrome and their contributing factors. *Pediatric Research*, 69(4), 293-8.

4.1 Abstract

This study used densitometry to investigate the areal bone mineral density (aBMD) and bone mineral content (BMC) in an Australian Rett syndrome cohort and assess how factors such as genotype, epilepsy, BMI and mobility affect these parameters. The influence of lean tissue mass (LTM) and bone area (BA) on total body BMC (TBBMC) was also investigated.

Participants, recruited from the Australian Rett Syndrome Database (ARSD), had TBBMC and lumbar spine (LS) and femoral neck (FN) aBMD measured using dual energy X-ray absorptiometry. Mean height standardised z-scores and confidence intervals for the bone outcomes were obtained from multiple regression models. The mean height z-score for the FN aBMD was low at -2.20, while the LS aBMD was -0.72. The TBBMC mean height z-score was -0.62, although once adjusted for BA and LTM the mean was above zero, suggesting that low BMC can be explained by narrow bones and decreased muscle mass, likely secondary to decreased mobility. Multiple linear regression identified the p.Arg168X* and p.Try158Met mutations as the strongest predictors of low BMC and aBMD for all bone outcomes. The strong relation between genotype, BMC and aBMD, is likely underpinned by the strong relation between LTM, mobility and bone outcome measures.

4.2 Introduction

Rett syndrome is a progressive neuro-developmental disorder, with a diagnosis incidence of 1.09 per 10,000 females by the age of 12 years (Laurvick, de Klerk, et al., 2006). Rett syndrome predominantly occurs in females, who can be categorised as having either a typical (classic) or an atypical (variant) form (Hagberg & Skjeldal, 1994). Severity varies, but the motor and cognitive disability is nevertheless severe (Hagberg & Skjeldal, 1994). In 1999, mutations on the X-chromosome in the gene encoding methyl-CpG binding protein 2 (*MECP2*) were identified as the principal

genetic cause of Rett syndrome (Colvin et al., 2004; Renieri et al., 2003). Although there are over 240 types of pathogenic nucleotide changes, eight common missense and nonsense mutations have been identified (Christodoulou & Weaving, 2003).

In a population-based study the fracture risk in Rett syndrome has been found to be nearly four times the general population rate (Downs, Bebbington, Woodhead, et al., 2008). The presence of epilepsy almost doubled the risk of fracture as did the use of two or more antiepileptic drugs (Downs, Bebbington, Woodhead, et al., 2008). In another investigation, valproate was shown specifically to increase the fracture rate three fold (Leonard et al., 2010).

Bone mass has been shown to be a strong predictor of fracture risk in adults (Flynn, Foley, & Jones, 2007) and children (Flynn et al., 2007; Hui, Slemenda, & Johnston, 1988). Radiographic and ultrasound studies have demonstrated osteopenia and reduced cortical thickness in Rett syndrome (Leonard et al., 1999; Leonard et al., 1995). Additionally, using dual energy X-ray absorptiometry (DXA), the areal bone mineral density (aBMD) and bone mineral content (BMC) have been shown to be reduced in studies involving 20 (Haas et al., 1997) and 50 females with Rett syndrome (Motil et al., 2008).

Multiple factors are thought to influence bone mass and fracture risk. Eighty percent of peak bone mass may be determined by genetic predisposition (Davies et al., 2005). It is feasible that the reduced bone density seen in Rett syndrome may be directly associated with the type of *MECP2* mutation. The ultrastructure and density of bone in mice with and without the *MeCP2* protein, have recently been examined (O'Connor et al., 2009). Those without the functional protein showed growth retardation, abnormal growth plates with irregularly shaped chondrocytes and decreased cortical, trabecular and calvarial bone (O'Connor et al., 2009). Thus, lack of *MeCP2* may reduce bone density through osteoblastic dysfunction (O'Connor et al., 2009).

Other critical factors for optimal bone acquisition are sufficient weight-bearing activity and adequate nutrition (Davies et al., 2005). Physical activity is often limited in Rett syndrome with individuals having difficulty in standing and walking, which declines further with age (Downs, Bebbington, Jacoby, et al., 2008). Feeding related problems are also observed in Rett syndrome, with possible consequences for

nutritional intake and growth (Oddy et al., 2007; Reilly & Cass, 2001). Growth retardation is one of the supportive criteria for Rett syndrome (Hagberg, 2002) and may occur independently of nutritional intake.

The main aims of this study were to determine bone mass and density in a national Rett syndrome population and investigate the influence of genotype, epilepsy, body mass index (BMI) and mobility on these measures. To aid in the interpretation of bone outcomes, the influence of height z-score, bone area (BA) and lean tissue mass (LTM) on BMC at the total body (TB) was also assessed.

4.3 Methods

The Australian Rett Syndrome Database (ARSD) was established in 1993 and is a population-based register of Rett syndrome cases born in 1976 and subsequently (Laurvick, de Klerk, et al., 2006). Individuals four years and older were eligible for participation in this study. Ethics approvals were provided by Princess Margaret Hospital in Western Australia and the major Australian hospitals involved. The family of each eligible person was contacted by phone, invited to participate and informed consent obtained. Appointments were organised at the nearest location for the family with the appropriate DXA equipment.

4.3.1 Densitometry scan protocol

A scanning protocol was developed by an experienced densitometrist based on standard manufacturer positioning for pediatric and adult patients. Lumbar spine (LS) (L2-L4), left and right femoral neck (FN) and TB scans were obtained. Height and weight were measured immediately before the scan and used to determine the appropriate acquisition mode. The width of the femoral neck region of interest was set at 1.50cm for participants with weight greater than 34kg and 1.00cm for participants 34kg or less, as this procedure was used by Lu and colleagues in the analysis of population controls (Lu et al., 1994).

4.3.2 Densitometry measurements

Areal BMD was measured by DXA, using GE-LUNAR Prodigy Densitometers (GE Medical Systems – LUNAR, Madison WI) and analysed using proprietary software version 10.50. Outcome measures were aBMD (g/cm^2) at the LS and FN, BMC (grams) at the TB, BA (cm^2) and LTM (grams) (Fewtrell, 2003). To better reflect the volumetric density at the LS, bone mineral apparent density (BMAD) was also

calculated (g/cm^3)(L1-L4) (Fewtrell, 2003). Scans were carried out in seven locations throughout Australia, between August 2005 and February 2008.

4.3.3 Densitometry analysis

All scans were sent electronically to the Telethon Kids Institute in Perth, Western Australia in de-identified format. Scans were then re-analysed in a standardised format, by one trained operator. For patients with spinal rods, only FN scans were included. Scans with movement artifact were also excluded. Normative Australian data for children and adults (n>800) were used to calculate sex-matched height standardised z-scores for aBMD, BMAD and BMC (Lu et al., 1994). In our study, an aBMD or BMC height standardised z-score greater than one standard deviation (SD) below zero was classed as low bone density for height and z-scores greater than two SD below zero was classed as very low bone density for height (Baim et al., 2008).

Using the 'Molgaard approach' the TBBMC was further evaluated (Molgaard, Thomsen, Prentice, Cole, & Michaelsen, 1997), including analysis of z-scores for height for age (short or long bones), BA adjusted for height (narrow or wide bones) and TBBMC adjusted for BA. Due to the close relation between muscle and bone, LTM adjusted for height and TBBMC adjusted for LTM, were also investigated (Hogler, Briody, Woodhead, Chan, & Cowell, 2003).

4.3.4 Australian Rett Syndrome Database questionnaires

Questionnaires are completed by caregivers and/or clinicians for all participants upon registration into the ARSD. Follow-up questionnaires have been administered every two years, since 2000. Data were obtained from the most recently completed questionnaire, on presence of epilepsy, mobility level, fracture history and pubertal stage. Mobility was classified into three levels, of “walks unaided” (level 1) or “with a degree of unsteadiness” (level 2), “walks with assistance” and “exhibits minimal movement/wheelchair bound” (level 3). Pubertal stage was based on the five Tanner stages of breast development and pubic hair. Mutation data were categorised into 13 groups as follows: each of the eight most common individual *MECP2* gene point mutations, large deletions (LD), late carboxyl-terminal truncation (CT) mutations and finally the less common *MECP2* gene mutations referred to as the “Other” group (p.R306H, p.S134C, p.P152R, p.P255R, early truncating and exon one). Some participants did not have a *MECP2* mutation elicited, or had not

been tested. Age was expressed categorically, in four groups (4-8, >8-14.5, >14.5-20 and >20 years).

4.3.5 Statistical analysis

Height, weight and BMI standardised z-scores were calculated using the US Centers for Disease Control and Prevention online data files (Centers for Disease Control and Prevention [CDC], 2005). The Pearson Chi-square test and Fisher exact test were used to compare age distribution, epilepsy diagnosis and mutation type between the study group and the remaining Australian Rett syndrome population. The effects of age, epilepsy, BMI, mobility and mutation type on the TBBMC, LS and FN aBMD height standardised z-scores, were investigated using multivariate linear regression. Tanner stage, previous fracture and anticonvulsant use were also investigated, using univariate regression models, but found not to be associated with bone outcomes and therefore not included in subsequent models. The mean height standardised z-scores and 95% confidence intervals (CI) for the bone outcomes were obtained from the multiple regression models. All statistical analyses were performed using the STATA software version 9 (STATA, College Station, TX).

4.4 Results

In August 2005, there were 274 girls and women with Rett syndrome aged over four years living across Australia who were theoretically eligible for this study. However, organising a DXA scan was only feasible in those who lived within reasonable proximity to one of the major densitometer locations. Nearly half of the group (n=130) had scans arranged by the study team or by their own clinician within the timeframe of the study. Scans in 26/130 (20%) were performed on densitometers other than Lunar and therefore could not be incorporated into the study. Scans were received at the Telethon Kids Institute for 97 of the 104 and thus were available for analysis. For the remaining 144 of the girls and women, arranging a scan was not possible due to lack of contact, geographical location or difficulty for the child/family.

The mean age of the 97 females on whom a scan was received was 15.0 years (SD=7.1, range=4-30.5 years). Fifty-three participants had all bone outcomes assessed, including the TB, LS and left and right FN. TBBMC was assessed in 83 participants and 73 (64 without spinal rods) had LS aBMD measured. The LS BMAD was subsequently calculated for 62 individuals. Scans at the FN each totaled 73.

Comparisons of age, mutation type distribution and the percentage with epilepsy between the study group and the remaining Rett syndrome population are shown in Table 4.1. When compared to the remaining Rett syndrome population, the mean age of the study group was higher (15.0 vs 10.1 years, p=0.001) and the proportion of individuals with epilepsy lower (75% vs 84%, p=0.063). Genetic testing had been performed in 95 (98.0%) of study group participants and the distribution of mutation types was similar to the remaining Rett syndrome population (p=0.363). Thus the distribution of the most common mutation types in the study group was an adequate representation of that seen within the Australian Rett syndrome population.

Table 4.1: Characteristics of the bone study group and the Australian Rett syndrome population greater then 4 years who did not participate in the study

	Bone study group (n=97)	**Remainder of Rett syndrome (n=177)**
***Age** mean±SD	15.00 ± 7.16	10.09 ± 7.10
****Epilepsy n(%)**		
No	24(24.7%)	28(16.1%)
Yes	73(75.3%)	146(83.9%)
§**Mutation** n(%)		
C-Terminal	12(12.4%)	10(5.9%)
Large Deletion	7(7.2%)	7(4.1%)
No Mutation	20(20.6%)	37(21.9%)
Other	9(9.3%)	20(11.8%)
p.Arg106Trp	3(3.1%)	5(3.0%)
p. Arg133C	6(6.2%)	12(7.16%)
p. Arg168*	10(10.3%)	9(5.3%)
p. Arg255*	5(5.2%)	9(5.3%)
p. Arg270*	3(3.1%)	12(7.1%)
p. Arg294*	7(7.2%)	11(6.5%)
p. Arg306Cys	6(6.2%)	7(4.1%)
p.Try158Met	7(7.2%)	13(7.7%)
Not tested	2(2.1%)	14(8.3%)

* p=0.001

**p=0.363

§ p=0.0833

The mean height and weight were reduced in the study group relative to the general normative population (Lu et al., 1994), with the height range being 97 to 167cm (mean age standardised z-score =-1.10, SD=2.42) and the weight range from 11 to 77kg (mean age standardised z-score =-1.24, SD=3.16). The mean BMI z-score was -1.00 (SD=2.59). Mobility level and frequency of those with epilepsy in each mutation group are shown in Table 4.2. Overall, 29 (32.2%) participants could walk unaided, 15 (16.7%) could walk with assistance and 46 (51.1%) were wheelchair bound, or had minimal movement.

Table 4.2: Mobility level and epilepsy diagnosis by mutation group

Mutation	**Mobility level n(%)**			**Epilepsy n(%)**	
	Walks unaided	**Walks with assistance**	**Doesn't walk/ wheelchair bound**	**Yes**	**No**
Carboxyl-terminal truncation	4(33)	3(25)	5(42)	9(75)	3(25)
Large deletion	0(0)	1(14)	6(86)	6(86)	1(14)
No mutation	3(15)	4(20)	13(65)	18(90)	2(10)
Other	1(12)	4(50)	3(38)	7(78)	2(22)
p. Arg106Trp	0(0)	0(0)	3(100)	2(67)	1(33)
p. Arg133Cys	2(50)	1(25)	1(25)	5(83)	1(17)
p. Arg168*	1(11)	3(33)	5(56)	7(70)	3(30)
p. Arg255*	0(0)	1(20)	4(80)	3(60)	2(40)
p. Arg270*	0(0)	1(33)	2(67)	3(100)	0(0)
p. Arg294*	4(67)	2(33)	0(0)	5(71)	2(29)
p. Arg306Cys	3(50)	2(33)	1(17)	2(33)	4(67)
p.Try158Met	0(0)	2(40)	3(60)	4(57)	3(43)
TOTAL	18(20)	24(27)	46(53)	71(75)	24(25)

Twenty-six (31.7%) individuals had previously had one or more fractures. Nineteen (73.1%) of the 26 had one or more clinically significant fractures of the lower limb or vertebrae, or two or more long-bone fractures in the upper limb, which are consistent with the International Society for Clinical Densitometry fracture criteria for osteoporosis in children and adolescents (Baim et al., 2008).

The TBBMC mean height standardised z-score was -0.62 (SD=1.52). The LS mean height standardised z-score for aBMD was -0.72 (SD=2.01) and LS BMAD was -0.60 (SD=1.68). The mean aBMD height standardised z-scores for the left and right FN were very low, at -2.15 (SD=1.84) and -2.20 (SD=1.68), respectively. Thirty-one (41.3%) of the LS aBMD, 37 (44.6%) of the TBBMC and 57 (78%) of the right FN aBMD height standardised z-scores were greater than one standard deviation below the population mean.

Table 4.3 shows the mean z-score for body composition data for all participants by mutation group. The mean BA adjusted for height was -1.02 (SD=1.97) and mean LTM adjusted for height was -1.10 (SD=1.61). After adjusting for either LTM (mean TBBMC=0.32, SD=1.78) or BA (mean TBBMC=0.28, SD=1.48), the mean TBBMC z-score was above zero.

Table 4.3: Mean body composition measures (SD) by mutation group

Mutation	Height z-score	BA/Height	LTM/Height	TBBMC/ LTM	TBBMC/ BA
CT truncation	-0.88(2.58)	-0.58(2.05)	-1.13(1.65)	1.16(1.98)	0.67(1.78)
Large deletion	0.64(2.89)	-2.21(1.30)	-1.40(1.15)	-0.40(1.94)	0.69(1.56)
No mutation	-1.14(1.80)	-1.28(1.81)	-1.34(1.51)	0.07(1.24)	0.03(1.21)
Other	-1.66(2.53)	-0.71(1.53)	-1.28(1.14)	-0.04(1.55)	-1.10(1.01)
p. Arg106Trp	-1.87(2.05)	-2.89(2.05)	-1.90(1.43)	0.27(0.67)	2.21(1.03)
p. Arg133Cys	-1.67(3.34)	0.65(1.53)	-0.45(1.18)	2.16(1.44)	0.72(1.19)
p. Arg168*	-2.17(2.40)	-1.78(2.85)	-0.87(2.49)	-0.84(1.85)	0.37(1.29)
p. Arg255*	-0.80(2.26)	-1.91(1.09)	-0.87(0.75)	-0.66(0.79)	1.06(1.74)
p. Arg270*	-3.55(1.41)	-0.06(1.16)	-0.18(0.47)	0.38(0.41)	1.02(3.11)
p. Arg 294*	-1.09(1.58)	0.60(1.16)	-0.14(2.25)	0.58(1.40)	-0.30(0.86)
p. Arg306Cys	1.19(2.73)	-0.33(1.97)	-0.16(1.40)	-0.11(1.26)	0.00(0.50)
p.Try158Met	-1.00(2.38)	-1.94(1.53)	-2.52(1.48)	1.47(3.02)	0.32(1.68)
TOTAL	-1.10(2.43)	-1.02(1.97)	-1.10(1.62)	0.32(1.78)	0.28(1.48)

Multivariate linear regression models for each bone outcome (height standardised), included mutation type, age group, epilepsy diagnosis, BMI and mobility level. Table 4.4 shows the mean height standardised z-score and CI obtained from the regression models for the LSaBMD, right FN and TBBMC by age group, epilepsy

diagnosis and mobility. There were contrasting results between the effects of age on the LS aBMD and FN. When compared to the four to eight year old group, the LS aBMD was higher in individuals in age groups >14.5 to 20 and >20 years (mean z-scores >14-20=0.46 and 1.11 respectively, p=0.009), whereas the FN aBMD was substantially lower in those greater than 20 years (mean z-score=-3.07) compared to the younger age groups (mean z-scores >14-20=-1.31, >8-14.5=-1.63, 4-8=-1.00, p=0.026). Although the mean height standardised z-scores for the three bone outcomes were lower for those with epilepsy, only the FN z-scores were significantly different (mean z-scores=-1.00 without and -2.32 with epilepsy, p=0.005). The aBMD and BMC mean height standardised z-scores were lower in the "walks with assistance" and "exhibits minimal movement/wheelchair bound" mobility groups.

Table 4.4: Mean height standardised z-score and confidence interval for each bone outcome by age group, epilepsy diagnosis and mobility level

	LSaBMD	Right FN aBMD	TBBMC
Age group (yrs)			
4-8	-1.40 [-3.14, 0.33]	-1.00 [-3.09, 1.10]	0.26 [-1.10, 1.62]
>8-14.5	-1.21 [-2.73, 0.31]	-1.63 [-3.23,-0.04]	-0.39 [1.57, 0.79]
>14.5-20	0.46 [-1.24, 2.15]	-1.31 [-3.11, 0.47]	0.19 [-1.10, 1.49]
>20-31	1.11 [-0.84, 3.06]	-3.07 [-5.02,-1.12]	0.08 [-1.27, 1.44]
Epilepsy			
No	-1.40 [-3.14, 0.33]	-1.00 [-3.09, 1.10]	0.26 [-1.10, 1.62]
Yes	-1.73 [-3.37,-0.09]	2.32 [-2.64,-2.00]	-0.01 [-1.31, 1.30]
Mobility			
Unaided	-0.30 [-1.75, 1.14]	0.01 [-1.98, 2.00]	0.63 [-0.61, 1.87]
Assistance	-0.59 [-2.11, 0.92]	-1.95 [-3.07, 0.81]	-1.71 [-0.28, 2.04]
Wheelchair	-1.40 [-3.14, 0.33]	-1.00 [-3.09, 1.10]	0.26 [-1.10, 1.62]

The reference groups used in the models were the "CT" mutation group, the "walks unaided" mobility group and the "no epilepsy" group.

Figure 4.1, Figure 4.2 and Figure 4.3 show the adjusted mean height standardised z-scores and CI for the TBBMC, LSaBMD and right FN by mutation group, from the multivariate regression models. The p.Arg168* (mean=-1.20) and p.Try158Met (mean=-1.54) mutation groups had significantly lower height adjusted TBBMC than the CT reference group (mean=0.26). The negative impact of the presence of these mutations can also be seen for LS aBMD height standardized z-score (p.Arg168*

mean=-2.85, p.T158M mean=-3.47, CT mean=-1.40). The mean height standardised z-score for both the p.Arg168* and p.Try158Met mutation groups was also low at the FN, but not appreciably different to the CT group.

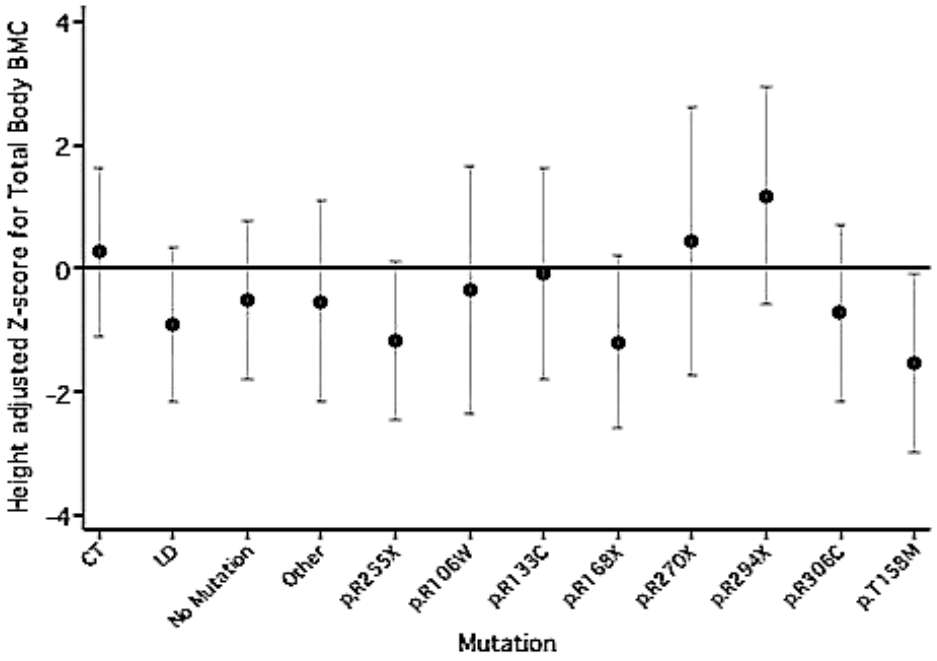

Figure 4.1: Mean height standardised z-score and confidence interval for Total Body BMC in each mutation group

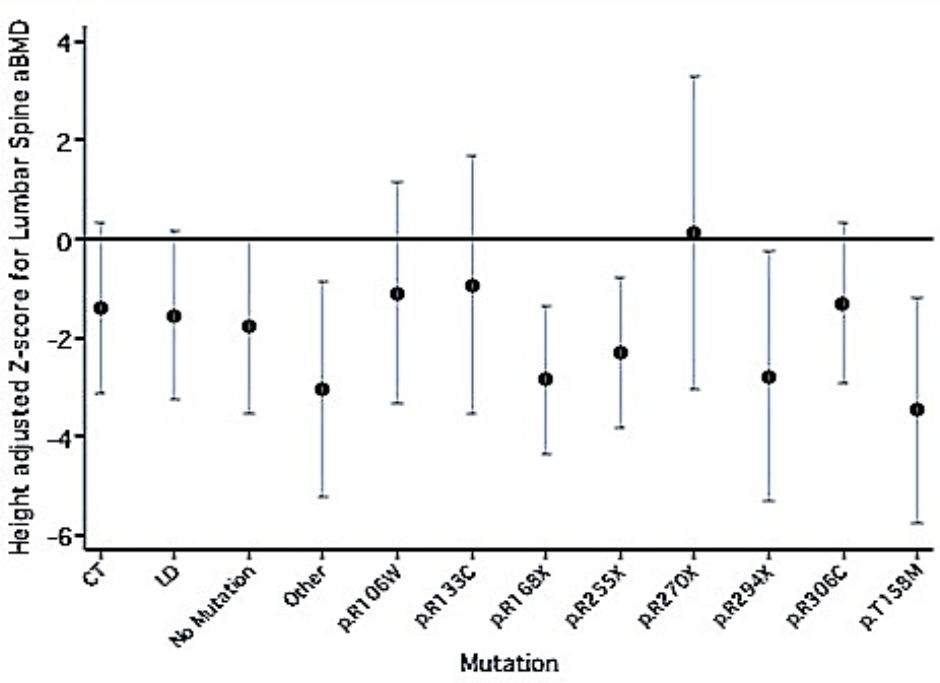

Figure 4.2: Mean height standardised z-score and confidence interval for Lumbar Spine aBMD in each mutation group

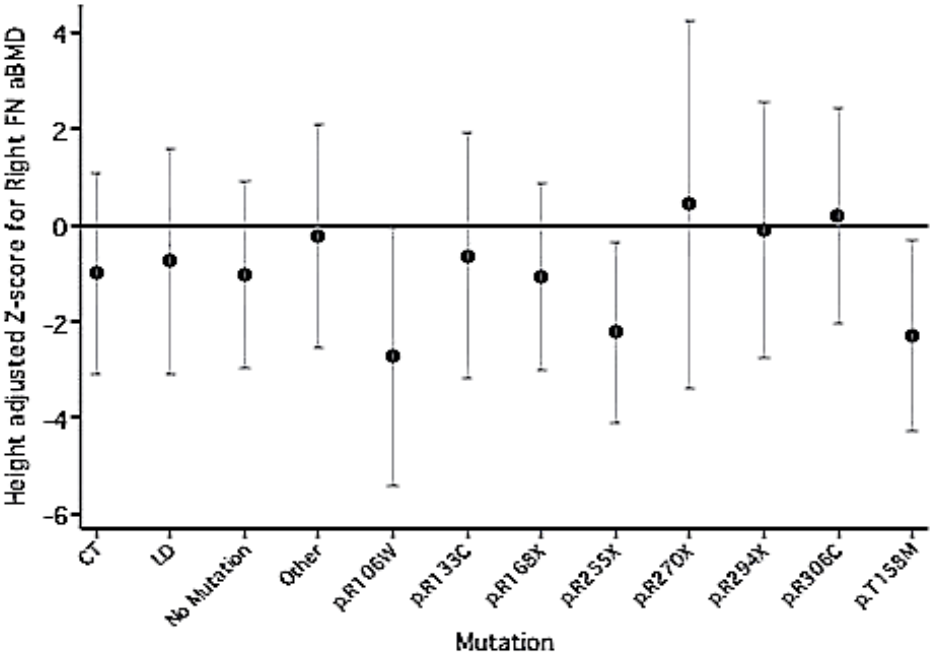

Figure 4.3: Mean height standardised z-score and confidence interval for Right Femoral neck aBMD in each mutation group

4.5 Discussion

This study showed that individuals with Rett syndrome have low aBMD and BMC, particularly at the FN. Bone area was also reduced for height. The mean aBMD height standardised z-score at the FN decreased with age and presence of epilepsy. Those with less mobility also had lower mean values for all bone outcomes. Regression models showed that the strongest predictors for low aBMD and BMC were the p.Arg168* and p.Try158Met mutation types. Body composition analysis showed that height, weight, BMI and LTM adjusted for height were lower than that expected for age. However, when TBBMC was adjusted for either LTM or BA, results approximated the population norm.

A major strength of the study was that the participants were a close representation of the population based Australian Rett syndrome cohort, thus supporting the generalisation of findings to all individuals with Rett syndrome. Comprehensive data were available on each participant, increasing the number of factors that could be included in the analysis. A detailed universal scanning protocol was developed and adopted by all centres. All scans were re-analyzed by the same operator, thus maintaining consistency in highlighting regions of interest. Internal calibrations were performed within each scan location and each centre used the same type of densitometer. Results were calibrated against Australian normative data.

In order to accommodate the decreased stature seen in Rett syndrome (Oddy et al., 2007; Reilly & Cass, 2001), calculation of height standardized z-scores and BMAD were made. Using the 'Molgaard approach' (Molgaard et al., 1997) and another similar (Hogler et al., 2003) approach, a more in depth interpretation of TBBMC was made by adjusting the bone outcome for LTM and BA. Thus we examined bone mineralization taking into account the effects of short stature, narrow bones and reduced muscle mass.

Although DXA is the commonly used method for evaluating pediatric bone mass and density, its bone outcomes are strongly influenced by bone size and are thus highly growth dependent (Fewtrell, 2003). LTM, as measured by DXA, is a major predictor of BMC and accords with other methods of measuring muscle mass (Hogler et al., 2003). As muscle action delivers the largest loads and bone strains (Frost & Schnau, 2000), participants with low muscle mass for height should have a proportionately low BMC.

Performing this analysis on the study group, with a mean TBBMC height standardised z-score of -0.62, bones were short (as indicated by a low mean height z-score of -1.10), narrow (low mean BA adjusted for height z-score of -1.02), but with normal mineralization for BA (normal mean TBBMC adjusted for BA of 0.28). Notably, there was low LTM for height (mean z-score=-1.10) indicating low muscle mass, however when bone mineralization was adjusted for muscle mass, the result was normal (TBBMC adjusted for LTM of 0.32). These findings indicate only a slightly reduced bone mineralization, due largely to low BA (narrow bones) and low muscle mass (low muscle pull) on slightly short bones. It is interesting that the mutations associated with the lowest height adjusted BMC/aBMD (at all sites), had the lowest bone length (p.Arg168* height z-score=-2.17), narrowest bones (BA height adjusted z scores -1.78 and -1.94 for p.Arg168* and p.Try158Met, respectively) and lowest muscle mass (LTM/height adjusted z-score=-2.52 for p.Try158Met). It is illustrative that individuals with these two mutations, with the greatest clinical severity, including lower mobility and a high prevalence of epilepsy (Jian et al., 2005), have the most severe bone mineral phenotype.

Downs et al previously found fracture rate in this Rett syndrome population to be four times that of the general population and those with the p.Arg168* and p.Arg270* mutations at particularly increased risk (Downs, Bebbington, Woodhead, et al., 2008). Unexpectedly in the present study, which unlike the fracture study is by its nature limited to live cases, the p.Arg270* mutation was not a strong predictor of bone outcome measures. The three participants with this mutation, aged between 18-30 years, may represent a survivor bias and may be functionally more able than typically seen with this mutation (Jian et al., 2005). Downs and colleagues found the most common fracture site to be the femur (Downs, Bebbington, Woodhead, et al., 2008), which correlates with the findings in this study, where the femoral neck region (and the legs, data not shown) had the lowest aBMD values. Whilst the difficulties associated with FN positioning are acknowledged, a likely clinical explanation for the very low FN measurements may be that over half of the participants were predominantly wheelchair bound. However, as scans for participants with spinal rods could only be analysed at the FN, femoral neck results may be more representative of the total Rett syndrome population, with LS and TB results biased towards those with less severe phenotypes, with less scoliosis and more mobility.

The reduced height and BA for age found in this study suggests that girls with Rett syndrome have bones both shorter and more narrow than expected. Reduced height, weight and BMI have already been well documented in Rett syndrome (Oddy et al., 2007; Reilly & Cass, 2001). Reassuringly, evidence of a low calcium intake in females with Rett syndrome has not been identified, either in an Australian (n=84) (Leonard et al., 1999) or US study (n=10) (Motil et al., 2006). However, another study did identify reduced serum 25 hydroxyvitamin D levels (Cepollaro et al., 2001). It may well be beneficial to monitor vitamin D levels in Rett syndrome and supplement as necessary. On the other hand, biochemical analysis of serum calcium, phosphate, alkaline phosphatase and parathyroid hormone levels in Rett syndrome were not shown to differ from controls in a sample of 82 individuals (Cepollaro et al., 2001). These normal biochemical findings suggest that vitamin D deficiency, if present, is not severe.

However, the major explanation for the reduced BA and BMD observed in this study is the decreased LTM. The major physiological loads placed on bone originate from the pull of muscles, as they contract (Janz et al., 2006). This in turn causes remodeling of the bone, to alter (in particular) the bone geometry, thus increasing the strength of bone, such that it is appropriate to the loads placed upon it (Janz et al., 2006). As LTM largely represents skeletal muscle mass, a surrogate of muscle action, any reduction in LTM will influence bone size and geometry (Wang et al., 2005). Reduced LTM in this cohort was not surprising, as the majority (80%) of participants were unable to walk independently or were wheelchair bound. Lean tissue mass has been found to be a strong predictor of BMD and BMC in adults and in normally developing children and adolescents (Hogler et al., 2003; Wang et al., 2005). The dual findings in this study, that TBBMC was normalised after adjustment for BA and LTM and that those who were less mobile had lower aBMD and BMC values together, support the contention that mobility is a major factor in the low bone density observed in Rett syndrome.

In this study aBMD at the FN decreased with age and all bone outcomes were lower in those diagnosed with epilepsy. Our findings are in agreement with a US study performed in 50 females with Rett syndrome, aged between two to 38 years (Motil et al., 2008). This US study also found that previous anticonvulsant use, previous fractures and the presence of scoliosis all had negative associations with LS, FN and TB bone measurements (Motil et al., 2008). In our study, these factors were not significant predictors of aBMD or BMC. On the other hand, the US study was unable

to demonstrate any relationship between genotype and bone density, potentially because of the small sample size, which only allowed mutation categorisation into five broad groups (Motil et al., 2008). Our study included access to a larger cohort of individuals, allowing more refined categorisation of mutations. Furthermore, our study also investigated the impact of BA and LTM on TBBMC measurements.

Necessary limitations to our study included reduction in the sample size by exclusion of scans where spinal rods were present or from movement artifact. Furthermore, height may have been underestimated in those (more severely affected) individuals with scoliosis, leading to an overestimation of their aBMD or BMC height standardised z-scores.

In conclusion, individuals with Rett syndrome have low aBMD, BMC, BA and LTM measures, which is only partially accounted for by their decreased height. Bone outcome measures were particularly low at the FN, the most common site of fracture, and decreased at this site with age. Those with the more severe p.Arg168* and p.Try158Met mutations were at greatest risk of low bone density. Wheelchair bound individuals and those with epilepsy had lower bone outcome measures. Whilst we identified a strong relationship between genotype and BMC and aBMD, there was also a clear relationship between LTM, mobility and bone outcome measures. Thus, maintaining mobility, particularly in those with high-risk mutations, has potential as an environmental modification to optimize bone health. In future research use of a 3D technology, such as high resolution Peripheral Quantitative Computed Tomography, could give additional insights into compartmental mineralization, microstructure and geometry and their interaction with muscle. It is our recommendation that guidelines for the management of bone health in individuals with Rett syndrome should be developed and implemented, early in life.

Chapter Five

Longitudinal bone mineral content and density in Rett syndrome and their contributing factors

Study publication:

Jefferson, A., Fyfe, S. D., Downs, J. A., Woodhead, H., Jacoby, P., & Leonard, H. (2015). Longitudinal bone mineral content and density in Rett syndrome and their contributing factors. *Bone,* 74(1), 191-198.

5.1 Abstract

Bone mass and density are low in females with Rett syndrome. This study used dual energy X-ray absorptiometry to measure annual changes in z-scores for areal bone mineral density (aBMD) and bone mineral content (BMC) in the lumbar spine and total body in an Australian Rett syndrome cohort at baseline and then after three to four years. Bone mineral apparent density (BMAD) was calculated in the lumbar spine. Annual changes in lean tissue mass (LTM) and bone area (BA) were also assessed. The effects of age, genotype, mobility, menstrual status and epilepsy diagnosis on these parameters were also investigated.

The baseline sample included 97 individuals who were representative of the total live Australian Rett syndrome population under 30 years in 2005 (n=274). Of these 74 had a follow-up scan. Less than a quarter of females were able to walk on their own at follow-up. Bone area and LTM z-scores declined over the time between the baseline and follow-up scan. Mean height-standardized z-scores for the bone outcomes were obtained from multiple regression models. The lumbar spine showed a positive mean annual BMAD z-score change (0.08) and a marginal decrease in aBMD (-0.04). The mean z-score change per annum for those 'who could walk unaided' was more positive for LS BMAD (p=0.040). Total body BMD mean annual z-score change from baseline to follow-up was negative (-0.03). However this change was positive in those who had achieved menses prior to the study (0.03, p=0,040). Total body BMC showed the most negative change (-0.60), representing a decrease in bone mineral content over time. This normalised to a z-score change of 0.21 once adjusted for the reduced lean tissue mass mean z-score change (-0.21) and bone area mean z-score change (-0.14).

Overall, the bone mineral content, bone mineral density, bone area and lean tissue mass z-scores for all outcome measures declined, with the TB BMC showing significant decreases.

Weight, height and muscle mass appear to have impacts on bone formation and we recommend that nutritional intake should be closely monitored and a physical activity plan developed to optimise bone health. Pubertal progression should also be assessed in conjunction with serial densitometry assessments to track bone mass and density changes over time.

5.2 Introduction

Rett syndrome a rare genetic disorder mainly affecting females, results in marked impairments in the development of neurological function including communication, gross motor function and hand use (Bebbington et al., 2008; Neul et al., 2008). The disability is complicated by the development of comorbidities such as scoliosis (Downs et al., 2009), epilepsy (Jian et al., 2006), impaired growth (Oddy et al., 2007), autonomic disturbances (Julu et al., 2001), decreased bone density and skeletal abnormalities (Gonnelli et al., 2008; Jefferson et al., 2011; Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011; Shapiro et al., 2010; Zysman et al., 2006). More than 200 mutations in the *MECP2* gene have been shown to be the main cause of Rett syndrome, however eight occur more commonly (Amir et al., 1999; Christodoulou & Weaving, 2003; Dragich et al., 2000). The wide range of phenotypic severity observed can be explained in part by the type of mutation (Bebbington et al., 2008; Neul et al., 2008) and the degree of skewing of X-chromosome inactivation (Archer et al., 2007).

Cross-sectional densitometry studies in Rett syndrome have shown low bone mineral content (BMC) and areal bone mineral density (aBMD) in children as young as three or four years old (Jefferson et al., 2011; Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011; Zysman et al., 2006). Even greater reductions in BMC and aBMD z-scores have been observed in older age groups (Jefferson et al., 2011; Motil et al., 2008). To date, only one study (Gonnelli et al., 2008), using quantitative ultrasound (QUS), has investigated the changes in bone status in Rett syndrome longitudinally. That study assessed bone status in the diaphysis of the proximal phalanx of digits two to five, over a three year period in 109 females with Rett syndrome aged between 3-25 years and 101 age and gender matched controls (Gonnelli et al., 2008). Those who were non-ambulant at baseline

showed decreasing QUS measurements over time compared to controls, although no change was demonstrated in ambulant subjects (Gonnelli et al., 2008).

It is well documented that individuals with Rett syndrome often have restricted mobility. This was observed in a study of 84 females with Rett syndrome, showing just under half (48%) were immobile or required considerable assistance and almost three quarters (71.5%) either could not stand or needed support to stand (Cass et al., 2003). These findings were echoed in our video analysis of the gross motor profile in 99 Australian females with Rett syndrome whereby just over half (54.2%) either required assistance to stand or could not stand (Downs, Bebbington, Jacoby, et al., 2008). Broad relationships between genotype and mobility were also found, with the less severe phenotypic mutations (p.Arg133Cys, p.Arg294*) showing better mobility levels than those with the more severe p.Arg270* mutation or a large deletion (Downs, Bebbington, Jacoby, et al., 2008). Subsequently, we found reductions in mobility to be associated with a decrease in lean tissue mass potentially affecting bone mass acquisition (Davies et al., 2005; Jefferson et al., 2011). However, we also found that low total body (TB) BMC z-scores in Rett syndrome normalised when they were adjusted for lean tissue mass (Jefferson et al., 2011), suggesting low muscle volume lean tissue mass may be the mechanism by which low mobility levels precipitated poor bone mineral acquisition.

We know that accretion of bone during childhood is also influenced by hormonal factors including pubertal increases in oestrogen and growth hormone secretion, leading to increased bone mass and cortical thickness (Davies et al., 2005) and increased bone strength in healthy children (Remer et al., 2003). In females both axial and appendicular skeletal growth rapidly increase during early and mid-puberty, followed by a slower period of growth in late puberty (Clark et al., 2006). At the onset of puberty, higher levels of oestrogen cause an increase in the secretion of growth hormone (GH) by the anterior pituitary gland, which leads in turn to the generation of insulin like growth factor 1 (IGF-1). Oestrogen decreases bone remodeling (resorption) by reducing osteoclast numbers, whilst having an anabolic effect on osteoblasts leading to net bone modeling at the endosteum and inhibition at the periosteum (Eastell, 2005). The actions of GH and IGF-1 lead to linear growth of bone by stimulation at the growth plate and also to increased periosteal apposition. During the latter stages of puberty, high oestrogen levels close the growth plate by causing apoptosis of chondrocytes, thereby halting linear growth. At

this stage, oestrogen also increases the activity of osteoblasts, having a positive influence on trabecular bone formation (Eastell, 2005).

The normal progression of puberty is an essential component of bone development in childhood and adolescence, with an estimated 40% of total bone mass accumulating during the pubertal years, leading to increased BMD and BMC values (Cromer & Harel, 2000). Between the ages of 6-16 years, total body BMC increases by a factor of two and a half and aBMD doubles mostly from increases in bone size (Clark et al., 2006). In Rett syndrome, our recent study showed that the median age of onset of menarche was slightly delayed compared to available normative data (Knight et al., 2013).

The acquisition of bone mass and density is clinically important and in Rett syndrome, may have a bearing on increased risk of fracture (Downs, Bebbington, Woodhead, et al., 2008). This study investigated longitudinal changes in bone mass and density in individuals with Rett syndrome over a three to four year period and the influence of lean tissue mass and menstrual status on these parameters.

5.3 Methods

5.3.1 Study population

Female participants were sourced from the population based Australian Rett Syndrome Database (ARSD), established in 1993 (Downs et al., 2009). For both baseline (Jefferson et al., 2011) and follow-up densitometry measurements, appointments were organised at the most convenient location following informed consent procedures. The follow-up scans were performed at the same location as the baseline bone measurements. Ethics approvals were provided by Princess Margaret Hospital in Western Australia, the Children's Hospital at Westmead in New South Wales, Monash Medical Centre in Victoria, Royal Brisbane and Women's Hospital in Queensland and Benson Radiology in South Australia.

5.3.2 Densitometry scans

Densitometry was performed and analysed as in our initial cross-sectional study (Jefferson et al., 2011). Outcome measures were areal bone mass density (aBMD) (g/cm^2) at the lumbar spine (LS) (L2-L4) and total body (TB), and bone mineral composition (BMC) (grams) at the TB. The TB BMC was further analysed after adjustments for height and lean tissue mass (LTM) (grams) were made (Fewtrell, 2003). Height adjusted LTM and bone area (BA) were also assessed. Bone mineral apparent density (BMAD) (g/cm^3) was calculated, as a measure of volumetric density at the LS (Fewtrell, 2003). All scans affected by movement were excluded from the analysis, as were lumbar bone measures in the presence of spinal rods. Densitometry analyses at baseline and follow-up were performed by one trained operator as previously described (Jefferson et al., 2011) and z-scores calculated using Australian normative data (Lu et al., 1994).

5.3.3 Australian Rett Syndrome Database questionnaire data

Information on each individual with Rett syndrome was sourced from questionnaires completed by their caregivers and/or clinicians, upon registration into the ARSD and at two to three year intervals thereafter (Downs et al., 2009). Mutation type, diagnosis of epilepsy, mobility level, fracture history and menstrual stage were extracted from questionnaires completed nearest to the date of baseline and follow-up scans. Mobility level was classified as “walks unaided” (level 1), "walks with a degree of unsteadiness or with assistance” (level 2) or "does not walk/wheelchair dependent” (level 3). Cases were categorised into one of three menstrual groups; those who had achieved menses more than six months prior to their baseline scan (post-menarcheal), within six months of their baseline scan or between their first and second scan (menarcheal) and those who had not achieved menses during the study period (pre-menarcheal). *MECP2* mutation type was categorised into five groups. The "Mild", "Mixed and "Severe" groups were based on general severity of phenotype (Bebbington et al., 2008). The mild mutation group included late carboxyl-terminal truncations (CT), p.Arg133Cys, p.Arg294* and the p.Arg306Cys mutation types. The mixed mutation group consisted of p.Arg106Trp, p.Arg168* and p.Try158Met mutations. Large deletions (LD), p.Arg255* and p.Arg270* were placed in the most severe mutation group. The fourth category, referred to as the “Other” group, included the less common *MECP2* gene mutations (p.R306H, p.S134C, p.P152R, p.P255R, early truncating and exon one). The fifth group contained those without a M*ECP2* mutation.

Age in years was calculated at the time midway between the baseline and follow-up scan, and then classified into four age groups (4-<10, 10-<15, 15-20 and >20 years). The frequency of fracture was categorised as no fracture history, one fracture, or more than one fracture from birth until the time of the follow-up scan.

5.3.4 Statistical analysis

Body mass index (BMI) standardised z-scores were calculated using the US Center for Disease Control and Prevention online data files. Univariate regression models were used to investigate the associations of age, menstrual status, previous fracture, epilepsy diagnosis, BMI, mobility, height adjusted LTM z-score at baseline and mutation groups with bone outcomes, calculated as the annualised change for TB BMC, TB aBMD, LS aBMD and LS BMAD. Variables in the univariate regression models, which were shown to be significant predictors of bone outcomes, were then included in the multivariate linear regression models. A p value <0.05 was considered to be statistically significant. The mean annual change in height standardised z-scores and 95% confidence intervals (CI) for the bone outcomes for different levels of the predictor variables, were obtained from the multiple regression models. All statistical analyses were performed using the STATA software version 12.1 (STATA, College Station, TX).

5.4 Results

5.4.1 Characteristics of the Rett syndrome cohort

At the commencement of this study there were 274 females with Rett syndrome in Australia who were eligible for participation (Jefferson et al., 2011). Ninety-seven (35.4%) of the 274, who were shown to be an adequate representation of age distribution, mutation type and proportion with epilepsy of the whole Rett syndrome population, had baseline DXA measurements (Jefferson et al., 2011). Seventy-four (76.3%) also had a follow-up scan. Twenty-three of the original 97 were unable to complete the follow up scan due to poor health (n=5), family difficulties or time constraints (n=15) or death (n=3). Therefore, 74 with both baseline and follow-up scans were included in the current analysis.

The mean age at baseline was 15.2 years (4.4-30.5 years) and at follow-up 18.6 years (9.6-34 years). Midway between scans there were 16 (21.6%) in both the 4-<10 and 10-<15 year old age groups, 17 (23%) in the 15<20 age group and a slightly larger proportion in the oldest age group >20 years, where there were 25

(33.8%) individuals. At the follow-up scan, 37 (50%) were post-menarcheal, 10 (13.5%) menarcheal and 27 (36.5%) pre-menarcheal.

Distribution of the eight common *MECP2* mutations is shown in Table 5.1. There were 22 (29.7%) in the "mild" group, 14 (18.9%) in the "mixed" group and 11 (14.9%) within the "severe" mutation severity group. Ten (13.5%) were in the "other" group and 17 (23%) participants did not have a *MECP2* mutation.

Table 5.1: Number of participants in the mild, mixed, severe, other and no mutation groups

Mutation Type	Number of subjects	%
Mild	22	29.7
C-Terminal	8	10.8
p. Arg133Cys	4	5.4
p. Arg294*	5	6.8
p. Arg306Cys	5	6.8
Mixed	14	18.9
p. Arg 106Trp	3	4.0
p. Arg 168*	6	8.1
p.Try158Met	5	6.8
Severe	11	14.7
Large Deletion	5	6.8
p. Arg255*	4	5.4
p. Arg 270*	2	2.7
Other	10	3.5
No mutation	17	23.0

*n=74

Between the baseline and follow-up scan, mean weight in relation to height declined. The mean BMI z-score at baseline was -0.90 (SD=2.53), which declined to -1.2 (SD=2.54) at the time of the follow-up scan. The annual mean BMI z-score decline was -0.07 (SD=0.55, range=-2.52-1.50). The menstrual stage and epilepsy diagnosis at follow-up and mobility level at baseline and follow-up for each mutation group are shown in Table 5.2. Eight participants who could walk with assistance at the time of the baseline scan were unable to walk by the follow-up scan, whereas 22

remained in the “walks with assistance” mobility level during the study. Two participants out of the original 14, who initially walked unaided, later required assistance to walk. The proportion unable to walk increased from 35.2% (n=26) at baseline to 46% (n=34) at follow-up. The severe mutation category had the highest percentage of wheelchair dependent participants at both baseline (n=7, 63.6%) and follow-up (n=8, 72.7%). One participant was newly diagnosed with epilepsy during the study, providing a total of 66 (89.2%) with epilepsy. Five participants sustained a fractured bone during the study period and 35 (47.3%) had experienced one or more fractures over their lifetime.

Table 5.2: Participants' mobility levels at baseline and follow-up and epilepsy diagnosis and menstrual staging at follow-up by mutation group

Mutation	Total		Mobility at baseline						Mobility at follow-up						Epilepsy diagnosis				Menstrual stage					
			walks unaided		assisted walking		doesn't walk		walks unaided		assisted walking		doesn't walk		no		yes		pre-menstrual		menstrual		post-menstrual	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	N	%	n	%	n	%	n	%
Mild	22	29.7	9	40.9	8	36.4	5	22.7	8	36.4	9	40.9	5	22.7	4	18.2	18	81.8	8	36.4	4	18.2	10	45.4
Mixed	14	18.9	2	14.3	7	50	5	35.7	2	14.3	5	35.7	7	50	1	7.1	13	92.9	7	50	1	7.1	6	42.9
Severe	11	14.9	1	9.1	3	27.3	7	63.6	0	0	3	27.3	8	72.7	2	18.2	9	81.8	7	63.6	1	9.1	3	27.3
Other	10	13.5	2	20	4	40	4	40	1	10	3	30	6	60	0	0	10	100	2	20	1	10	7	70
NM[a]	17	23	3	17.6	9	52.9	5	29.4	1	5.9	8	47.0	8	47.0	1	5.9	16	94.1	3	17.6	3	17.6	11	64.8
Total	74	100	17	22.9	31	41.9	26	35.2	12	16.2	28	37.8	34	46	8	10.8	66	89.2	27	36.5	10	13.5	37	50

[a]NM=no mutation

5.4.2 Densitometry findings

Table 5.3 shows the number of participants for whom each outcome measure was assessed. Most (96%) scans allowed HT adjusted LTM and BA to be measured, followed by both TB assessments (86.5-87.8%). Only 44.6% (n=33) were suitable for assessment of all bone outcome measures. Thirty-one (41.9%) LS scans could not be analysed due to movement artifact (n=9) or the presence of rods (n=22). In addition ten (13.5%) TB scans were excluded due to movement artifact reducing the number of TB scans to 64. Table 5.4 shows the age, mobility levels, menstrual stage and presence of *MECP2* mutation in those participants whose LS and TB scans were included and excluded. More of the 20-35year old group had their LS scans excluded (p=0.04). However, there was no difference in age group distribution between those whose TB scans were excluded or included in the study. There was also no difference in frequency of those in the mobility, mutation or menstrual stage groups for those whose TB or LS scans were included or excluded in this analysis. TB mean annual z-score changes did not differ for those who had lumbar scans included or excluded from the study (TB aBMD p=0.92, TB BMC p=0.48). This was also the case for the mean annual z-score changes for both LS scans for those who had TB scans included and excluded from the reported analysis (LSaBMD p=0.99, LS BMAD p=0.67).

Table 5.3: Number and proportion of participants for outcome measures

Bone and LTM Outcome/s measured	**Number of scans**	**%**
Ht adjusted LTM	71	96.0
Ht adjusted BA	71	96.0
TB BMC, TB BMC & Ht adjusted LTM	65	87.8
TB aBMD	64	86.5
LS aBMD	43	58.1
LS BMAD	35	47.3
All outcomes	33	44.6

Abbreviations: HT = height, LTM = lean tissue mass, BA = bone area, TB = total body, LS = lumbar spine, aBMD = areal bone mass density, BMC = bone mineral composition, BMAD = bone mineral apparent density

Table 5.4: Characteristics of participants whose lumbar spine and total body scans were included and excluded

	LS				TB			
	Included		Excluded		Included		Excluded	
	n	%	n	%	n	%	n	%
Total	43	58.1	31	41.9	64	86.5	10	13.5
Age group								
0-10yrs	11	68.7	5	31.3	15	93.8	1	6.2
>10-15yrs	11	68.7	5	31.3	15	93.8	1	6.2
>15-20yrs	12	70.6	5	29.4	15	88.2	2	11.8
>20yrs	9	36.0	16	64.0	19	76.0	6	24.0
Mobility level								
Walks unaided	16	84.2	3	15.8	21	84.0	4	16.0
Walks assisted	11	50.0	11	50.0	13	81.2	3	18.8
Unable to walk	16	48.5	17	51.5	30	90.9	3	9.1
Menstrual stage								
Premenarcheal	17	63.0	10	37.0	23	85.2	4	14.8
Menarcheal	7	70.0	3	30.0	10	100	0	0.0
Postmenarcheal	19	51.4	18	48.6	31	83.8	6	16.2
MECP2 mutation	30	52.6	27	47.4	47	82.5	10	17.5

Abbreviations: LS = lumbar spine, TB = total body

Table 5.5 shows the mean height standardised z-scores at baseline and follow-up and the change per annum for each outcome measure for all participants and also separately for those with a genetically confirmed mutation. The only bone outcome measure with a positive mean z-score change, representing an increase in bone density over time, was LS BMAD (mean annual z-score change=0.08, SD=0.3). There was a marginal decrease in aBMD from baseline to follow-up at the LS and TB, with rates of mean z-score changes of -0.04 and -0.03 per year, respectively. The TB BMC showed the greatest negative change (mean annual z-score change=-0.61, SD=0.6), representing a decrease in bone mineral content over time. Mean z-score changes were compared for all bone outcome measures between those that could walk unaided during the course of the study with those that initially could walk but later required assistance. Similarly mean z-score changes were compared for all bone outcome measures between those that could walk with assistance at the time of both scans, with those that initially could walk with assistance but by follow-up

could not walk. Although not statistically different, the mean z-score change was more negative in the TB BMC and LS aBMD in those who were able to walk assisted at baseline but could not walk at follow-up, compared to those who were still able to walk with assistance during the study period. However their TB BMD and LS BMAD z-score changes were more positive. The mean z-score at follow-up for the height adjusted LTM, was also much lower compared to the mean at baseline (mean annual z-score change=-0.21, SD=0.6, p=0.009) and for the height adjusted BA (mean annual z-score change=-0.14, SD=0.5, p=0.009), indicating a decrease in lean tissue mass and size of bones for height, respectively, over time. Overall, excluding LS BMAD, the bone mineral content and bone mineral density z-scores for all bone outcome measures declined, with the TB BMC showing significant decreases. Bone area and LTM z-scores also declined over the time between the baseline and follow-up scan. However, after adjusting TB BMC for height and lean tissue mass (TB BMC HT adjusted LTM), the z-score change of bone mineral content for the TB improved from -0.61 to 0.15 per annum. Thus TB BMC accrual over time appeared within normal limits once accommodations for reduced height and LTM accrual were made.

Table 5.5: Mean z-score at baseline and follow-up for each outcome

Outcome measure	**n**	**Baseline z-score**		**Follow-up z-score**		**Rate of change per annum**		**Rate of change excluding no mutation grp**	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
LS aBMD	35	-0.24	1.5	-0.22	1.5	-0.04	0.3	-0.05	0.3
LS BMAD	43	-0.61	2.2	-0.14	1.9	0.08	0.3	0.9	0.3
TB aBMD	64	-0.37	1.3	-0.53	1.4	-0.03	0.4	-0.09	0.4
TB BMC	65	-0.61	1.6	-2.63	2.0	-0.61	0.6[a]	-0.55	0.6
TB BMC HT LTM	65	-0.93	1.7	0.25	1.8	0.15	0.5	0.07	0.3
BA HT	71	-0.96	1.9	-1.40	2.2	-0.14	0.5[a]	-0.10	0.4
LTM HT	71	-1.00	1.5	-1.79	1.8	-0.21	0.6[a]	-0.17	0.5

Abbreviations: HT = height, LTM = lean tissue mass, BA = bone area, TB = total body, LS = lumbar spine, aBMD = areal bone mass density, BMC = bone mineral composition, BMAD = bone mineral apparent density

[a] p=<0.010

5.4.3 Multiple regression analysis

Table 5.6 shows the mean annual z-score change and confidence intervals obtained from univariate regression models for each bone outcome measure and the height adjusted LTM by age group, epilepsy, mobility level, menstrual stage and mutation group. For most bone measurements, there was no significant difference between those with and without epilepsy.

Table 5.6: Mean z-score change and 95% confidence interval for bone outcomes and lean tissue mass adjusted for height by age group, epilepsy diagnosis and mobility level at follow-up

	Total		LSBMAD			LSaBMD			TBBMD			TBBMC			LTM HT		
Age group[a]	n	%	Mean	LCI	UCI	Mean	LCI	UCI	Mean	LCI	UCI	Mean	LCI	UCI	Mean	LCI	UCI
4-<10	16	21.6	-0.09	-0.33	0.16	0.07	-0.13	0.28	-0.32	-0.49	-0.14	-0.40	-0.62	-0.17	-0.48	-0.77	-0.19
10-<15	16	21.6	-0.04	-0.29	0.22	0.21	-0.02	0.45	-0.17	-0.35	0.02	-0.71	-0.93	-0.50	-0.43	-0.62	-0.24
15-<20	17	23.0	-0.01	-0.11	0.10	0.03	-0.14	0.19	0.23	-0.02	0.48	-1.05	-1.32	-0.79	-0.23	-0.69	0.22
>20	25	33.8	-0.08	-3.17	3.00	0.01	-0.18	0.21	0.11	0.02	0.19	-0.27	-0.59	0.04	0.10	-0.08	0.28
Epilepsy																	
No	8	10.8	-0.04	-1.09	1.00	0.04	-0.25	0.33	-0.03	-0.27	0.21	-0.59	-0.94	-0.22	-0.09	-0.44	0.26
Yes	66	89.2	-0.04	-1.15	0.06	0.09	-0.01	0.19	-0.03	-0.13	0.08	-0.61	-0.77	-0.45	-0.20	-0.39	-0.07
Mobility level																	
Walks unaided	12	16.2	0.14	-0.10	0.38	0.13	-0.03	0.29	0.02	-0.16	0.20	-0.63	-0.97	-0.28	-0.07	-0.33	0.20
Walks with assistance	28	37.8	-0.02	-0.18	0.14	0.10	-0.08	0.28	-0.09	-0.23	0.05	-0.54	-0.82	-0.27	-0.21	-0.39	-0.03
Doesn't walk	34	50.0	-0.15	-0.32	0.02	0.04	-0.08	0.17	0.00	-0.17	0.17	-0.65	-0.86	-0.44	-0.26	-0.54	0.01
Menstrual stage																	
Pre	27	36.5	-0.11	-0.30	0.09	0.11	-0.08	0.30	-0.30	-0.44	-0.17	-0.62	-0.82	-0.43	-0.42	-0.62	-0.22
Menarcheal	10	13.5	0.03	-0.24	0.31	0.19	0.06	0.31	0.03	-0.17	0.23	-0.57	-0.88	-0.26	-0.29	-0.48	-0.10
Post	37	50.0	0.00	-0.10	0.10	0.02	-0.10	0.15	0.16	0.03	0.28	-0.60	-0.87	-0.34	-0.05	-0.29	0.19
Mutation group																	
No mutation	17	23.0	-0.03	-0.17	0.12	0.07	-0.06	0.21	0.14	-0.09	0.38	-0.78	-1.09	-0.46	-0.36	-0.82	0.09

Other	10	13.5	-0.02	-0.45	0.40	0.13	-0.21	0.46	-0.02	-0.32	0.28	-0.58	-1.21	0.04	0.04	-0.42	0.51
Mild	22	29.7	-0.06	-0.31	0.18	0.00	-0.18	0.19	-0.12	-0.30	0.05	-0.55	-0.86	-0.23	-0.23	-0.50	0.03
Mixed	14	18.9	0.01	-0.49	0.51	0.19	-0.20	0.59	-0.11	-0.32	0.09	-0.62	-0.95	-0.28	-0.20	-0.50	0.09
Severe	11	14.9	-0.12	-0.58	0.34	0.15	-0.21	0.50	-0.07	-0.33	0.19	-0.46	-0.64	-0.27	-0.22	-0.37	-0.07

Abbreviations: LCI = lower confidence interval, UCI = upper confidence interval, HT = height, LTM = lean tissue mass, TB = total body, LS = lumbar spine, aBMD = areal bone mass density, BMC = bone mineral composition, BMAD = bone mineral apparent density, Pre = premenarcheal, Post = postmenarcheal

[a]Age group as measured midway between baseline and follow-up scan

Figure 5.1 shows that the mean annual z-score changed for all bone outcome measures by mobility groups. The mean z-score change per annum for those 'who could walk' was more positive for LS BMAD (p =0.040), LS aBMD and marginally for the TB aBMD compared to the 'walks with assistance' and 'does not walk' groups. For all mobility levels the LS aBMD showed the most positive mean z-score change, whereas the TB BMC showed the most negative change.

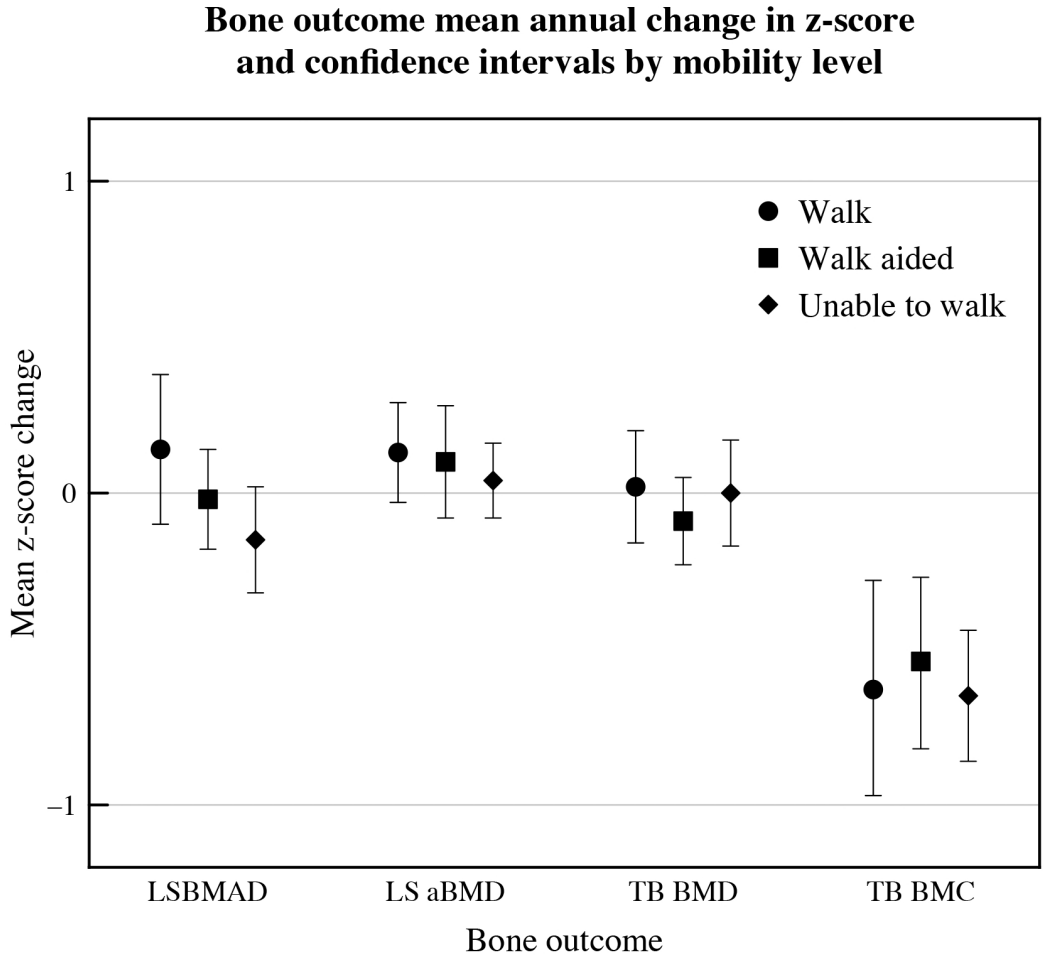

Figure 5.1: Bone outcome mean annual change in z-score and confidence intervals by mobility

Figure 5.2 illustrates the mean z-score change per annum in each bone outcome by menstrual groups. The pre-menarcheal group showed the greatest decline for all bone outcome measures, apart from the LS aBMD. The change was most favourable in the menarcheal group for both LS measurements and significantly more positive for TB aBMD (mean annual z-score change=0.03, p=0.040), compared with the premenarcheal group (mean annual z-score change=-0.30). As was seen for mobility, the TB BMC mean z-score change was the most negative

bone outcome for all menstrual groups.

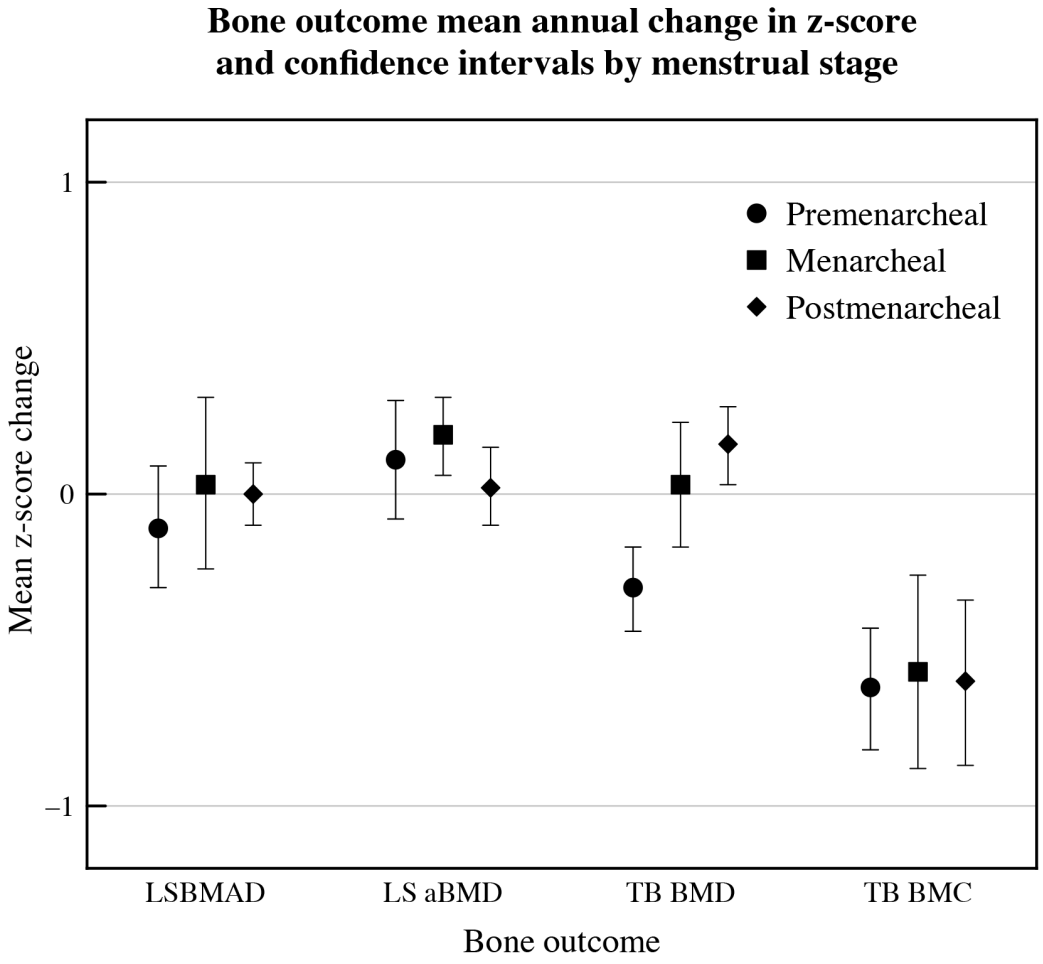

Figure 5.2: Bone outcome mean annual change in z-score and confidence intervals by menstrual stage

Multivariate linear regression models for the annual height standardised z-score change for each bone outcome included the height adjusted LTM z-score at baseline, age group and menstrual stage, as these were significant predictors in the univariate regression analysis. The 15-<20 years old (mean annual z-score change=-0.71, p=0.025) and the 10-<15 years old groups (mean annual z-score change=-0.71, p=0.009) had a significantly more negative z-score decline in TB BMC compared to the 4-<10 years old group (mean annual z-score change=-0.40). However, age group, menstrual stage and height adjusted LTM at baseline were no longer significant predictors of change in other bone outcomes such as height standardised BMC, volumetric or areal BMD. Regression models were repeated for each annual change in bone outcome z-score using data limited to those with a confirmed *MECP2* mutation. Age group, menstrual stage and height adjusted LTM

at baseline were not predictors of BMAD or aBMD annual z-score changes of the LS. Similar results to the full analysis were found for TB BMC z-score changes, where the 15-<20 years old (mean annual z-score change=-1.12, p=0.006) and the 10-<15 years old groups (mean annual z-score change=-0.75, p=0.033) had a significant decline compared to the 4-<10 years old group (mean annual z-score change=-0.40). However, compared to those who were premenstrual (mean annual z-score change=-0.34), those who had achieved menses between scans had improved TB aBMD z-score changes (mean annual z-score change=0.06, p=0.031). In addition, a more negative height adjusted LTM at baseline predicted a decrease in the annual TB aBMD change in z-score (p=0.014).

5.5 Discussion

To our knowledge this is the first longitudinal population-based study of changes in bone outcomes in Rett syndrome using densitometry. Forty-six percent (n=34) of study participants could not walk at the time of the follow-up scan. Levels of bone mineral density at the lumbar spine and total body and bone mineral content for the total body decreased over each year of study, with the exception of lumbar spine bone mineral apparent density. In addition, lean tissue mass volume, bone area for height and body mass index levels also decreased per annum. This means that with age, bone density measures in Rett syndrome were decreasing further from population norms adjusted for height (Lu et al., 1994). Total body bone mineral content declined significantly, especially in older age groups. However once adjusted for lean tissue mass and height, the total body bone mineral content approximated the population norm. Changes in bone density were more positive in those able to walk unaided. Furthermore, against expectations, those who had already achieved menarche had greater increases in total body bone density, compared to those who had not. The lean tissue mass or volume of muscle tissue also decreased, which correlated with the overall reduced mobility skills observed over time.

Declining bone density and mineral content z-scores over time place any individual at a greater risk of fracture (Hernandez, Beaupre, & Carter, 2003). In the general pediatric population, previous fracture has been shown to increase the risk of future fracture 2-3 fold (Kontulainen et al., 2007). In this study, almost half of the participants had already experienced one or more fractures in their lifetime. This is in keeping with previous findings of an almost four fold greater incidence of fracture

in Rett syndrome compared to an equivalently aged female population (Downs, Bebbington, Woodhead, et al., 2008).

Childhood and adolescence represent a time of rapid growth of the skeletal system when bones increase in length and cross-sectional size. This growth is one of the most important determinants of bone strength (Rauch, 2007). Although up to 80% of peak bone mass is determined by genetic predisposition (Davies et al., 2005), gains in body weight as well as physical activity increase the mechanical loads placed on bone, influencing its growth (Cromer & Harel, 2000). This is particularly important during the pubertal growth spurt, where muscle development often precedes bone development (Cromer & Harel, 2000). Physical limitations and immobilisation in Rett syndrome may therefore have a negative impact on bone accrual due to reduced muscle loading. Over a third (35.2%) of the participants in our study were wheelchair dependent and a further 41.9% needed assistance to walk. These restrictions in mobility may explain the reduced height adjusted volume of lean tissue mass observed and consequently the smaller bone area (mean annual z-score change=-0.1) compared to age-matched controls. Our results support the view that reduced muscle mass can significantly impact bone, as when the total body bone mineral composition mean annual z-score change was adjusted for lean tissue mass and height, it improved considerably. The total body bone mineral composition measurements although low, may therefore be appropriate for the muscle tissue present (Binkley et al., 2008; Rauch, Bailey, Baxter-Jones, Mirwald, & Faulkner, 2004).

Due to the variability in motor function in those with Rett syndrome, intervention plans that improve both gross motor skills and physical activity levels need to be tailored to the individual and overseen by experts such as a physiotherapist (Lotan, Isakov, & Merrick, 2004). Increasing the amount of time walking and/or standing per day, with or without support depending on needs, is generally recommended. Although there are limited studies on physical training programs in Rett syndrome, one study involving four girls with Rett syndrome, who performed daily training on a treadmill for a two month period, found that their physical fitness and functional abilities improved significantly by the end of this period (Lotan et al., 2004).

The reduced body mass index may also partly explain the decreased bone mass and density in this cohort. Feeding problems, oromotor dysfunction and gastrointestinal dysmotility may often contribute to limited dietary intake (Motil,

Schultz, Browning, Trautwein, & Glaze, 1999; Oddy et al., 2007; Reilly & Cass, 2001; Thommessen, Kase, & Heiberg, 1992). In this study, the mean body mass index z-score at baseline was -0.90 and -1.2 at the time of the follow-up; an annual decrease in the mean z-score of -0.07. Failure to thrive and growth failure negatively impacts developing bone, reducing bone size and the mechanical strain placed on bones (Rauch, 2007). Therefore monitoring and maintenance of adequate weight and height in Rett syndrome should be a priority. Recently published guidelines for the management of nutrition and growth in Rett syndrome recommend biannual nutritional assessment until the age of 12 years, with annual assessments thereafter (Leonard et al., 2013). This assessment should include an analysis of energy intake, any mealtime issues and efficacy of swallowing (Leonard et al., 2013). In conjunction, the guidelines recommend that monitoring weight and height and vitamin D, calcium, alkaline phosphatase and other biochemical markers related to bone health should also be performed (Leonard et al., 2013). High-energy supplements may be incorporated in the diet and intense therapy can help improve feeding skills. When weight gain is poor despite intervention, gastrostomy may be required (Leonard et al., 2013), which has been shown to halt the decline in weight and height and increase fat free mass and body fat in Rett syndrome (Motil, Morrissey, Caeg, Barrish, & Glaze, 2009).

Poor weight gain and growth failure not only impact bone formation but also influence the interpretation of densitometry scans in children and adolescents because of the influence of size on this two dimensional measurement (Fewtrell, 2003). A strength of the current study was the calculation of lumbar spine volumetric bone density, referred to as bone mineral apparent density, as this reduces the influence small bones have on densitometry measurements. Bone mineral apparent density was the only bone outcome measure with a positive mean annual z-score change, representing a slight increase in bone accrual at the lumbar spine. An additional accommodation for the reduced height seen in Rett syndrome was our use of height-calculated z-scores rather than age z-scores and bone area and lean tissue mass calculated for height. Subsequently, total body bone mineral composition normalised once it was adjusted for both lean tissue mass and height.

In our study, those who achieved menarche during or prior to the study had greater increases in bone accrual in most bone outcomes. The onset of menarche appeared to have a protective effect on the development of bone density and mass in our Rett syndrome cohort. Growth hormones, insulin-like growth factor 1 and increasing sex

steroids, which surge during puberty, have been shown to have beneficial effects on the development of bone and muscle mass (Saggese, Baroncelli, & Bertelloni, 2002; Wang et al., 2004). Typically, at the onset of puberty, significant increases in oestrogen output, lead to rises in growth hormone and insulin-like growth factor 1 (Saggese et al., 2002). Oestrogen directly regulates the proliferation, differentiation, mineralisation and gene expression of osteoblastic cells, leading to increases in bone modeling (Saggese et al., 2002; Wang et al., 2004). Oestrogen also contributes to the regulation of bone calcium deposition and stimulates renal productions of 1,25(OH_2)D. These factors lead to increases in bone mass accumulation whereby, in the normal population, the skeletal mass approximately doubles between the onset of puberty and adulthood (Saggese et al., 2002).

Our recent investigation of the pubertal trajectory in an Australian cohort of 213 females with Rett syndrome, found that whilst premature adrenarche and menarche were observed in eight and six percent of girls respectively, the median age of onset of menarche at 14 years was slightly later than that reported or available for the normal population 12-13 years (Knight et al., 2013). Body mass index was shown to influence menarche, with those with normal or high body mass index experiencing earlier menarche than those who were underweight (Knight et al., 2013). In comparison to those without a *MECP2* mutation, those with C-terminal and early truncating mutations had an earlier age of onset of menarche, while a later onset was seen in those with a severe mutation such as p.Arg168* (Knight et al., 2013). Assessing pubertal development using Tanner staging in those with Rett syndrome could be important as delays in menarche place those at risk of low bone density (Marchand, 2009). Hormone replacement therapy, such as low dose oral or transdermal oestradiol, can be used to induce puberty, with dosage and duration following local protocols (Houlihan & Stevenson, 2009), but to date we are not aware of whether this practice is being followed in Rett syndrome.

Given the decreased bone accrual in Rett syndrome observed in our study and the paucity of intervention studies, intervention plans should focus on monitoring nutritional intake (Cass et al., 2003), including oro-motor functioning, and pubertal trajectory (Knight et al., 2013) and increasing the level of physical activity (Lotan et al., 2004). Regular densitometry assessments including body composition measurements from a young age are also likely to be important (Fewtrell, 2003; Houlihan & Stevenson, 2009). Although currently there is no published evidence on the exact age and frequency at which this should be assessed, a study of children

with cerebral palsy has suggested achieving a baseline scan from the age of six, with follow-up scans every one to two years depending on the individual's risk factors (Houlihan & Stevenson, 2009). As negative bone accrual values were identified at a young age in our study, this may well be an appropriate guideline for Rett syndrome.

The 97 participants who had densitometry scans in our baseline cross-sectional study (Jefferson et al., 2011), were representative of the 274 females with Rett syndrome in the Australian Rett Syndrome Database. Variables compared were age, mutation type, mobility level and epilepsy diagnosis, however weight and height were slightly lower in the study group (Jefferson et al., 2011). Although we have observed an association between low bone outcome values and genotype in our previous cross sectional study (Jefferson et al., 2011), this was not observed at follow-up. However our analysis was limited by sample size for the eight most common *MECP2* mutations, necessitating the merging of data into three mutation categories and thereby possibly obscuring any emerging relationships. An additional limitation was the smaller number of satisfactory lumbar spine measurements, reduced due to positioning difficulties and/or the presence of spinal rods. This may explain our lack of consistent findings for the lumbar spine bone outcomes. However our total body measurements, where the declines were greater, had a larger sample and may be viewed as a more representative measurement of bone accrual changes in Rett syndrome. Our results may also be influenced by survival bias, as eight participants who had baseline measurements died or were in poor health and were not able to attend the follow-up scan. Those individuals who were able to have both scans were likely more functional and in better health.

Almost all of the bone outcome measures showed annual decreases in bone density and mass. Due to the close relationship with height, weight and bone formation and the reduced body mass index values observed in this cohort, regular nutritional intake and gastrointestinal monitoring is recommended with use of gastrostomy where necessary (Leonard et al., 2013). This approach, in conjunction with pubertal monitoring, serial densitometry assessments and a physical activity plan tailored to the individual’s functional capabilities, will allow clinicians to monitor bone development with the aim of positively influencing bone mass and density and preventing its decline. The development of treatment protocols for assessing and improving bone density and mass in Rett syndrome are needed to improve bone health and reduce fracture risk.

Chapter Six

Clinical Guidelines for Management of Bone Health in Rett Syndrome Based on Expert Consensus and Available Evidence

Study publication:

Jefferson, A., Leonard, H., Siafarikas, A., Woodhead, H., Fyfe, S., Ward, L. M., Munns, C., Motil, K., Tarquinio, D., Shapiro, J. R., Brismar, T., Ben-Zeev, B., Bisgaard, A., Coppola, G., Ellaway, C., Freilinger, M., Geerts, S., Humphreys, P., Jones, M., Lane, J., Larsson, G., Lotan, M., Percy, A., Pineda, M., Skinner, S., Syhler, B., Thompson, S., Weiss, B., Witt Engerström, I., & Downs, J. (2016). Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence. *PLOS ONE*, 11(2), 1-18.

6.1 Abstract

We developed clinical guidelines for the management of bone health in Rett syndrome through evidence review and the consensus of an expert panel of clinicians. An initial guidelines draft was created which included statements based upon literature review and 11 open-ended questions where literature was lacking. The international expert panel reviewed the draft online using a 2-stage Delphi process to reach consensus agreement. Items describe the clinical assessment of bone health, bone mineral density assessment and technique, and pharmacological and non-pharmacological interventions. Agreement was reached on 39 statements which were formulated from 41 statements and 11 questions.

When assessing bone health in Rett syndrome a comprehensive assessment of fracture history, mutation type, prescribed medication, pubertal development, mobility level, dietary intake and biochemical bone markers is recommended. A baseline densitometry assessment should be performed with accommodations made for size, with the frequency of surveillance determined according to individual risk. Lateral spine X-rays are also suggested. Increasing physical activity and initiating calcium and vitamin D supplementation when low are the first approaches to optimizing bone health in Rett syndrome. If individuals with Rett syndrome meet the ISCD criterion for osteoporosis in children, the use of bisphosphonates is recommended. A clinically significant history of fracture in combination with low bone densitometry findings is necessary for a diagnosis of osteoporosis. These

evidence and consensus-based guidelines have the potential to improve bone health in those with Rett syndrome, reduce the frequency of fractures, and stimulate further research that aims to ameliorate the impacts of this serious comorbidity.

6.2 Introduction

Rett syndrome, although considered rare, is one of the most common causes of intellectual disability in females with an incidence of 1 in 8,500 by the age of 15 years (Fehr, Bebbington, Nassar, et al., 2011). Most individuals with Rett syndrome express a mutation in the *MECP2* gene, which either activates or represses neural transcription when it binds to methylated cytosines in DNA (Amir et al., 1999; Chahrour et al., 2008). However the severity of the disorder varies depending on the type of *MECP2* mutation (Bebbington et al., 2008; Cuddapah et al., 2014; Neul et al., 2008) and the pattern of X-chromosome inactivation (Archer et al., 2007). Clinical outcomes for this syndrome are complex, with varying degrees of autonomic dysfunction (Julu et al., 2001), motor impairments influencing mobility and oromotor control (Bebbington et al., 2008; Downs, Bebbington, Jacoby, et al., 2008; Foley et al., 2011; Reilly & Cass, 2001), epilepsy (Glaze et al., 2010; Jian et al., 2006) and poor growth (Motil et al., 2012; Oddy et al., 2007). A high proportion also develop skeletal abnormalities such as scoliosis (Downs et al., 2009; Percy et al., 2010), low bone density and mass and a high frequency of fractures (Jefferson et al., 2011). As almost 60% of those with Rett syndrome will live beyond 37 years, physicians must begin to address these chronic health issues early in life (Anderson et al., 2014).

Studies assessing bone mineral content (BMC, g) and areal bone mineral density (aBMD, g/cm^2) in Rett syndrome have shown that these parameters were lower compared to the gender matched control groups (Gonnelli et al., 2008; Jefferson et al., 2011; Motil, Ellis, Barrish, Caeg, & Glaze, 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011). Bone mineral content and aBMD z-scores for age and height tended to become more negative with age (Jefferson et al., 2011; Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011; Shapiro et al., 2010), however individuals as young as three and four years of age had low BMC and aBMD values in the lumbar spine (Jefferson et al., 2011; Shapiro et al., 2010), total body and femoral neck (Jefferson et al., 2011).

Fractures are also a common occurrence in Rett syndrome. The fracture incidence in an Australian Rett syndrome population was shown to be 43.3 per 1000 person years, which equates to a rate nearly four times that of the general population

(Downs, Bebbington, Woodhead, et al., 2008), within whom fractures are often associated with sport and playing activities (Cooper, Dennison, Leufkens, Bishop, & Van Staa, 2004). Fractures can occur spontaneously, with trivial trauma or a fall, and occur predominantly in the long bones of the upper and lower limbs (Downs, Bebbington, Woodhead, et al., 2008; Roende, Ravn, Fuglsang, Andersen, Vestergaard, et al., 2011). Fractures have also been closely linked with mobility levels and ability to bear weight, and in a Danish study there were significantly more non-ambulant patients who had fractured compared to the ambulant non-fractured healthy control group (Roende, Ravn, Fuglsang, Andersen, Vestergaard, et al., 2011). To date the prevalence and incidence of vertebral fractures in Rett syndrome remains unknown. As vertebral fractures, often considered a manifestation of osteoporosis, may be asymptomatic, they may escape medical attention (Halton et al., 2009).

Our Australian study, which assessed the BMC and aBMD in the lumbar spine, femoral neck and total body using dual energy X-ray absorptiometry, found that subjects who were unable to walk or wheelchair dependent had lower mean height calculated BMC and aBMD z-score values compared to those who were ambulant (Jefferson et al., 2011). The z-scores were predicted for height and sex, as these were more appropriate to use than comparisons with normal values by age, given the generally small stature of those with Rett syndrome. In a US study (n=49), one third of individuals who were non-ambulant had decreased bone mass in the lumbar spine (Shapiro et al., 2010). Small bone size and reduced lean tissue mass have also been observed and correlated with low bone density and mass values (Jefferson et al., 2011; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011). Lean tissue mass has been shown to be a strong predictor of bone density in young women in the general population, particularly during adolescence (Wang et al., 2005). Reduced lean tissue mass, often seen in those who are immobilised, decreases the physiological adaptations that occur within bone in response to muscle forces. This negatively affects bone development (modeling), leading to narrower bones with thin cortices and reduced trabecular bone mass (Davies et al., 2005; Janz et al., 2006).

Despite the high frequency of fractures and low bone mass and density identified in Rett syndrome, information about best clinical practice for the diagnosis, treatment and prevention of osteoporosis in this disorder is lacking. Due to its rarity, many

clinicians may have little or no experience with Rett syndrome. This study therefore developed clinical guidelines for the management of bone health in Rett syndrome.

6.3 Methods

This project was coordinated from the Telethon Kids Institute in Western Australia (WA). Ethics approval was obtained from Curtin University in Western Australia.
Guideline development followed the format described by the National Health and Medical Research Council, Australia (1999) and consensus was achieved using a modified Delphi technique as used in previous studies (Leonard et al., 2013). Potential expert panel members were invited to participate via email which provided information about the study and described the development process. As approved by the Curtin University ethics committee, the expert panel members provided their consent to participate in the study via email, which was then stored on a password-protected server at the Telethon Kids Institute.

6.3.1 Literature review and parent perspectives

The systematic literature search performed in 2013, reviewed the following databases: Medline, ProQuest Health and Medical Complete, PsychINFO, ScienceDirect, Web of Science, The Cochrane Library and EMBASE. Online libraries reviewed were those of the World Health Organisation, Canadian Medical Association Infobase Clinical Practice Guidelines, Geneva Foundation for Medical Education and Research, the National Guidelines Clearinghouse, National Institutes for Health and Care Excellence, Scottish Intercollegiate Guidelines and the Trip search engine. Keywords used in the search were a combination of Rett syndrome with bone, bone density, osteoporosis, osteopenia, fracture, vitamin D, calcium and exercise.

In addition, Rettnet, which is an online forum provided by Rettsyndrome.org allowing the exchange of information on aspects of Rett syndrome, was searched using the key words osteoporosis, bone, bone density, fracture, calcium, broke/broken, vitamin D, movement, exercise and brittle. Online communications were sourced from June 2009 to January 2011.

6.3.2 Expert panel

Potential panel members from around the world with clinical expertise in Rett syndrome or relevant medical specialties were identified from the literature, networks of investigators, recommendation of colleagues and RettSearch. Relevant

specialties included pediatrics, pediatric neurology, endocrinology, clinical genetics, radiology, gastroenterology, nursing, physiotherapy and dietetics.

6.3.3 Guidelines development using a modified Delphi technique

Based on findings from the literature, referenced statements and questions were developed and divided into two sections. Part A contained sections on clinical assessment, bone density assessment and related techniques. Part B focused on non-pharmacological and pharmacological intervention strategies. Members of the Consumer Reference Group (CRG) for the Australian Rett Syndrome Study, comprising parents of females with Rett syndrome, were given the opportunity to review the draft guidelines and comment on their comprehensiveness before they were made available to expert panel members. In addition, a clinical geneticist and a pediatric endocrinologist in Australia, both of whom have extensive knowledge and experience with Rett syndrome, tested the online version of the guidelines before it was made available to panel members.

Panel members had online access to the draft guidelines document, which was developed using HTML form and PHP script, and were able to log in using a personalised username and password. They were then able to respond to each statement by rating their level of agreement using a 5-point Likert scale (strongly agree, agree, neither disagree or agree, disagree, strongly disagree). There was also the option for panel members to provide a comment in relation to each statement. Additional questions not addressed in the literature were constructed based on the content of Rettnet postings. Panel members who felt they were not qualified to respond to a particular statement or question could choose the option “not my area of expertise”. All online responses could be edited, saved, and submitted in stages. Data were stored on a secure site in a MySQL database (Sun Microsystems, Cupertino, CA) at the Telethon Kids Institute in Perth, Western Australia.

Consensus for each statement was determined where at least 70% of responses were within one response category of the median response. New statements were formed from responses to each of the questions, and statements where consensus was not reached, were modified in accordance with panel members’ comments. Panel members were then asked to review a second guidelines draft that included the new statements formulated following first round responses. They were not given access to their previous responses or information regarding the median responses

to each statement as clear consensus had been reached in round one, negating the need for panel members to modify their responses. This is therefore a Modified Delphi process. Using the Scottish Intercollegiate Guidelines Network grading scheme (SIGN, 2008), statements where consensus was reached were rated according to their level of evidence. Systematic reviews and randomized controlled trials were rated as level 1, level 2 included case control or cohort studies, level 3 case report or case series and level 4 statements were supported by expert opinion.

6.4 Results

6.4.1 Literature review and parent perspectives

Search of seven databases and seven online libraries identified 214 citations as potentially relevant. Of these, 70 articles and three sets of guidelines were retrieved and reviewed in full text. Thirty-eight articles provided statements for inclusion in the draft guidelines. One hundred and ninety-five relevant Rettnet postings were identified. These postings were used to construct questions in the draft guidelines if statements had not already raised the issues. Seven members in the Australian CRG reviewed the first guidelines draft and provided feedback. Although the CRG supported the document, they requested additional statements be included on fracture vigilance and specific recommendations regarding vitamin D and calcium supplementation. The CRG also expressed concerns regarding the effects of contraceptive use on bone health and the efficacy of bisphosphonates on bone density.

6.4.2 Expert panel participation

Of the 62 clinicians who were contacted, 45 (72.6%) agreed to participate in the study, ten did not respond and seven declined to participate. Thirty-eight (84.4%) of the 45 who agreed to participate provided data. Eleven (29%) were pediatric neurologists, six (15.8%) adult endocrinologists, five (13.2%) clinical geneticists, four (10.5%) pediatricians, three (7.9%) physiotherapists, two (5.3%) pediatric endocrinologists, two (5.3%) dieticians, two (5.3%) gastroenterologists, one (2.6%) nurse, one (2.6%) was a pediatric orthopaedic surgeon, and one (2.6%) was a radiologist. Participant locations were as follows; 17 (44.7%) from the United States of America, four (10.5%) from Australia, three (7.9%) from Sweden and Israel, two (5.3%) from Denmark, Italy, Canada and one (2.6%) from France, Austria, Japan, Spain and the United Kingdom.

6.4.3 Initial guidelines draft and redrafting using a modified Delphi technique

The draft guidelines included 41 statements, 11 questions (representing all RettNet topics) and a reference list. The comments made by expert panel members were either supportive of the responses made to statements or used to construct statements for round two. Of the 38 participants who responded to the first round, 33 (86.7%) responded to the second round, which included nine new statements. Agreement was achieved for all statements in the second round, allowing these statements to form part of the final guidelines. The final document contained 39 separate statements, all of which reached consensus and are listed in Tables 7.1-7.5.

Table 6.1: Assessment of bone health

Statements	Level of Evidence	Median Response	n/N (%)[1]
All children with a clinical diagnosis of Rett syndrome should undergo genetic testing as genotype may influence the development and management of osteoporosis	2	Neither Agree or Disagree	29/31 (93.5)
Fractures in Rett syndrome can occur due to trivial trauma	2	Agree	35/35 (100)
Clinicians need to be vigilant for potential fractures	2	Strongly Agree	35/35 (100)
Measure weight and height to calculate Body Mass Index at each clinical visit	4	Strongly Agree	27/35 (77.1)
Identify all prescribed medications at each clinical visit, particularly those that can influence bone density: eg anti-epileptic medications, proton pump inhibitors, progesterone-only medications, vitamin supplements	2	Strongly Agree	36/36 (100)
Assess pubertal development using Tanner staging	2	Agree	32/32 (100)
Pubertal development may be delayed in girls or women with Rett syndrome which puts those affected at risk of low bone mineral density	2	Agree	23/29 (79.3)
Assess mobility level by asking about the following:			
The level of assistance needed for walking	2	Strongly Agree	34/35 (97.1)
The time spent walking each day	2	Agree	35/35 (100)
The distance walked each day	2	Agree	34/35 (97.1)
The amount of time standing in a standing frame if independent standing is not possible	2	Strongly Agree	33/35 (94.3)

Assess dietary intake including:			
24 hour diet recall	2	Agree	31/33 (93.9)
Recall of food high in vitamin D	2	Agree	28/33 (84.8)
Recall of food high in calcium	2	Agree	33/33 (100)
Assessment of sunlight exposure by asking about:			
Frequency of use of sunscreen and sun-protection factor/protective clothing	1,2	Agree	30/34 (88.2)
The time of the day when skin (equivalent to face and arms) is exposed to direct sunlight	1,2	Agree	31/34 (91.2)
Amount of time each day that skin (equivalent to face and arms) is exposed to direct sunlight	1,2	Agree	29/34 (85.3)
First line biochemical investigations include measurement of:			
Calcium (ideally also ionised calcium)	1,3	Agree	30/33 (90.9)
25 hydroxyvitamin D (25(OH)D)	1,3	Strongly Agree	32/33 (97.0)
Magnesium	1,3	Agree	30/33 (90.9)
Phosphorus	1,3	Agree	32/33 (97.0)
Alkaline Phosphatase (ALP)	1,3	Agree	28/32 (87.5)
Albumin	1,3	Agree	30/33 (90.7)
Second line biochemical investigations include measurement of:			
Electrolytes (ideally also ionised calcium)	4	Agree	25/27 (92.6)
Urine calcium/creatinine ratio (ideally also ionised calcium)	4	Agree	25/27 (92.6)

Bone turnover markers: N-telopeptide, collagen cross-links	4	Agree	24/27 (88.9)
Parathyroid hormone (PTH) if any pathological findings	4	Agree	29/33 (87.9)

*Scottish Intercollegiate Guidelines network, [1]Numerator is the number of responses with median response or 1 category either side and denominator is the number of clinicians in the panel whose expertise were relevant to this item

6.4.4 Clinical assessment of bone health, bone mineral density assessment and technique

Twelve items included in the clinical assessment of bone health section focused on factors that influence bone density in Rett syndrome such as genotype, fracture susceptibility, mobility level, height and weight, prescribed medications, pubertal development, calcium, dietary intake of calcium and vitamin D as well as vitamin D synthesis from sunlight exposure. This section also identified biochemical markers to be measured when assessing bone status in Rett syndrome. The statement "Pubertal development may be delayed in girls or women with Rett syndrome which puts those affected at risk of low bone mineral density", was added for round two in response to panel members' comments regarding the atypical pubertal development seen in Rett syndrome (Killian et al., 2014; Knight et al., 2013) (Table 6.1). Although all statements regarding assessment of mobility reached between 94-100% agreement, several comments were made regarding the difficulties in monitoring the duration and distance of walking. Panel members also commented on the need for a dietitian to assess dietary intake in Rett syndrome.

Table 6.2 includes five statements relating to bone mineral density assessment. Clinicians commented that risk factors for poor bone health need to be taken into consideration when deciding if and when bone mineral density assessments should be performed. All panel members agreed that baseline bone mineral density should be assessed by DXA. The initial guidelines draft posed a series of questions on timeframes for which scans should be performed based on specific z-score results at baseline and the presence of risk factors. In response to feedback, these questions were removed and replaced with a more generalized statement that monitoring of bone mineral density should occur every one-two years depending on clinical presentation, taking into account z-score results as well as previous occurrence of fractures.

Table 6.2: Bone mineral density assessment

Statements	Level of Evidence*	Median Response	n/N (%)[1]
Bone health needs to be considered early on in life and the following routine risk factors should be assessed:			
Ability to walk	2	Strongly Agree	32/33 (97.0)
Presence of either the p.R168X, p.R255X, p.R270X or p.T158M mutation	2	Strongly Agree	28/31 (90.3)
Prescribed anticonvulsant medication(s)	2	Strongly Agree	32/33 (97.0)
Oral and intramuscular progesterone medication(s)	2	Agree	31/32 (96.9)
In the presence of risk factors, a baseline bone mineral density measurement should be performed	4	Agree	32/33 (97.0)
Consider using the following techniques to assess bone health:			
Densitometry (DXA)	4	Strongly Agree	25/25 (100)
Lateral spine X-ray	4	Neither Agree or Disagree	20/25 (80.0)
Peripheral quantitative computed tomography (pQCT)	4	Neither Agree or Disagree	23/25 (92.0)
Monitor bone mineral density every 1-2 years depending on clinical presentation	4	Agree	29/34 (85.3)
If a long bone was fractured, the bone mineral density should also be measured in the alternate long bone	4	Agree	23/27 (85.2)

If a vertebra was fractured, the bone mineral density may be measured in adjacent vertebrae excluding measurement of the fractured vertebrae	4	Agree	25/27 (92.6)

*Scottish Intercollegiate Guidelines network

[1]Numerator is the number of responses with median response or 1 category either side and denominator is the number of clinicians in the panel whose expertise were relevant to this item

Table 6.3 includes seven statements related to techniques of assessing bone mineral density. Agreement by the panel for weight-calculated z-scores from DXA scans was not achieved (68.2% consensus) but was achieved for age and height-calculated z-scores. Techniques recommended to reduce unwanted movements during the DXA scan included listening to music, presence of parents or caregivers, swaddling and sedation when required.

Table 6.3: Bone mineral density assessment technique

Statement	Level of Evidence	Median Response	n/N (%)[1]
Where local normative data exists, measure the bone mineral content and areal bone mineral density in the total body minus the cranial bones (headless), and the postero-anterior lumbar spine	1	Agree	17/18 (94.4)
Total hip and proximal femur bone mineral content and areal bone mineral density measurements are not considered a reliable site for measurement due to difficulties with subject positioning	1	Agree	14/17 (82.4)
Z scores should be calculated from raw values for the following:			
Age	2	Agree	22/24 (91.7)
Height	2	Agree	23/23 (100)
Bone mineral apparent density (or volumetric bone mass density) adjustment is also recommended where possible	1	Agree	21/21 (100)
The same skeletal sites should be assessed when repeating densitometry measures longitudinally	4	Agree	27/27 (100)
In individuals with spinal rods, the bone mineral content and areal bone mineral density for the lateral distal femur and the total body minus the cranial bones (headless) should be measured	4	Agree	17/17 (100)

To reduce unnecessary movement during bone mineral density scan procedures, calming techniques such as music, the presence of carers/parents, swaddling or sedation may be used	4	Agree	31/31 (100)
Where possible densitometry measurements of lean tissue mass should be assessed	2	Agree	16/17 (94.1)

*Scottish Intercollegiate Guidelines network

[1]Numerator is the number of responses with median response or 1 category either side and denominator is the number of clinicians in the panel whose expertise were relevant to this item

6.4.5 Non-pharmacological and pharmacological interventions

The nine items describing the non-pharmacological intervention strategies, shown in Table 6.4, involved two major avenues for improving bone health; increasing physical activity and calcium and vitamin D supplementation. Although consensus was achieved for all statements, some panel members were nevertheless uncertain of the benefits of the use of a standing frame on bone density. This statement reached 84% consensus. The statement in round one that related to targeted exercise for those with limited mobility, was changed from recommending whole body vibration therapy to assisted walking due to lack of consensus. Subsequent to this change, 97% agreement was achieved.

Table 6.4: Non-pharmacological intervention

Statements	Level of Evidence*	Median Response	n/N (%)[1]
Increase physical activity in order to increase muscle strength and bone density	2	Strongly Agree	34/34 (100)
In order to increase physical activity, refer to a physiotherapist for development of an optimal physical activity plan	4	Strongly Agree	33/34 (97.0)
For those who are wheelchair bound, where possible:			
Encourage supported standing during transferring	1	Agree	33/34 (97.0)
Use a standing frame for at least 30 minutes a day	1	Strongly Agree	28/33 (84.8)
For those who are able to walk, aim to increase the distance and/or the length of time walked each day (aiming for 2 hours per day where possible)	1	Agree	29/32 (90.6)
Where mobility is limited, targeted exercise such as body weight supported treadmill or assisted walking is recommended	2	Strongly Agree	31/32 (96.9)
If calcium intake is low, increase dietary intake of calcium rich or calcium fortified foods	1,2,3	Strongly Agree	31/32 (96.9)

If dietary calcium intake is low and difficult to increase using dietary means, prescribe calcium supplements to meet the local recommended daily intake. The current recommended dietary intake levels within Australia are: 1-3yr 500mg/day, 4-8yr 700mg/day, 9-11yr 1000mg/day, 12-13yr 1300mg/day, 14-18yr 1300mg/day, >18yr 1000mg/day of elemental calcium. When prescribing medication please verify the content of elemental calcium in the preparation	1	Strongly Agree	31/32 (96.9)
If 25 hydroxyvitamin D levels are lower than 75nmol/L:			
Use local protocols for treatment and supplementation	1	Strongly Agree	27/32 (84.4)
Re-assess 25 hydroxyvitamin D levels after 4-8 weeks, then annually	4	Agree	32/32 (100)
Advise an appropriate amount of sunlight exposure based on latitude, time of day, season and skin type	1,2	Agree	27/31 (87.1)

*Scottish Intercollegiate Guidelines network

[1]Numerator is the number of responses with median response or 1 category either side and denominator is the number of clinicians in the panel whose expertise were relevant to this item

The six items in Table 6.5 address the pharmacological approaches to increasing bone density when there is a combination of low bone density and a fracture history. At the time of the study we followed the 2007 position statement by the International Society of Clinical Densitometry (ISCD) for classification of osteoporosis in children and adolescents (Baim et al., 2008). Following the development of these guidelines the ISCD updated this position statement (Bishop al., 2014). Whilst the group agreed that bisphosphonate therapy may be indicated, several panel members did make note of the lack of evidence for efficacy and safety of bisphosphonate use in Rett syndrome and that its mechanism of action may be different from disorders such as osteogenesis imperfecta in which its use is frequent (Glorieux, 2007). Three statements included in round one that related to combined, low dose or ultra low dose contraceptives and their effects on bone were removed due to lack of consensus. Panel members felt there was either a lack of evidence to support these statements or questioned their relevance in the Rett syndrome population.

Table 6.5: Pharmacological intervention

Statements	Level of Evidence*	Median Response	n/N (%)[1]
Bisphosphonates should be used if the International Society for Clinical Densitometry criteria for osteoporosis in children and adolescents are fulfilled	1,2	Agree	17/23 (73.9)
The intravenous dosage of Bisphosphonates should follow evidence-based protocols	4	Agree	16/22 (72.7)
Reassess bone mineral content and areal bone mineral density one year after Bisphosphonate therapy to decide on further therapy	4	Agree	24/26 (92.3)
If reassessment of bone mineral content and areal bone mineral density shows limited response, review the therapeutic approach	4	Agree	23/26 (88.5)
If hormonal intervention for regulation of the menstrual cycle is needed, use of Depot medroxyprogesterone acetate (DMPA) should be avoided	1,2	Agree	21/21 (100)
Although Levonorgestrel-releasing intrauterine system (LNG-IUS, Mirena) does not negatively affect bone density, communication difficulties during insertion need to be considered	4	Agree	15/15 (100)

*Scottish Intercollegiate Guidelines network

[1]Numerator is the number of responses with median response or 1 category either side and denominator is the number of clinicians in the panel whose expertise were relevant to this item

6.5 Discussion

Reduced bone mass and density have been identified in Rett syndrome throughout childhood and into adulthood (Cepollaro et al., 2001; Haas et al., 1997; Jefferson et al., 2011; Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011; Shapiro et al., 2010). In addition bone area is reduced (Jefferson et al., 2011; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011) and muscle mass is low (Jefferson et al., 2011; Leonard et al., 2013). One impetus for developing these guidelines was initially in response to concerns expressed by parents on Rettnet regarding the increased likelihood of bone fractures in Rett syndrome, as well as their questions regarding the assessment and improvement of bone density in their child. Furthermore, research indicating low bone mass and density (Jefferson et al., 2011) and increased fracture risk (Downs, Bebbington, Woodhead, et al., 2008) in Rett syndrome also highlighted the need for these guidelines.

This project identified relevant literature relating to Rett syndrome or developmental disabilities and aspects of bone growth during childhood, adolescence and adulthood. A series of statements and questions were developed from the literature and from the listserv Rettnet. An international expert panel and a parent reference group subsequently reviewed the statements. This comprehensive document, which aims to guide clinicians on best practices for maintaining bone health and reducing fractures in Rett syndrome, was compiled using a modified Delphi technique. The panel provided expert advice for the scope of the guidelines with limited “drop out” between the first and second round review. The option “not my area of expertise” made it possible for the clinicians to only express opinion in their respective field of experience, enabling inclusion of multiple health professions in the expert panel. Using similar methods as our previously developed guidelines for management of scoliosis (Downs et al., 2009) and assessment and management of nutrition and growth (Leonard et al., 2013) in Rett syndrome, the communication via email and the online review of the guidelines occurred in a timely and efficient manner.

Limitations of the study were the lack of supporting peer-reviewed literature specific to Rett syndrome for aspects of the guidelines, particularly statements relating to intervention strategies. Twenty-two of 63 statements (which includes the different parts of some statements), were formulated based on clinical experiences of the expert panel rather than peer-reviewed literature. These statements particularly related to the types of biochemical testing used to assess bone health, technique, frequency and skeletal site targeted for bone density measurements, vitamin D

measurements and use of bisphosphonates to treat low bone density. There is also limited evidence on the effects of physical activity on bone mass density in Rett syndrome. Where literature specific to Rett syndrome was absent, the guidelines not only relied on the opinion of the expert panel but literature on bone health in the general population and in those for other disabilities.

Biochemical assessments were described taking into consideration the likelihood of nutritional deficits observed in Rett syndrome (Reilly & Cass, 2001). Radiological investigations were also outlined with techniques and analyses tailored to accommodate the decreased stature (Oddy et al., 2007; Tarquinio et al., 2012) and movement problems (Bebbington et al., 2008) seen in Rett syndrome. For example, the guidelines include recommendations to reduce unnecessary movements during bone density scanning procedures as individuals with Rett syndrome often display stereotyped hand movements, dystonic sustained muscle contractions or abnormal postures (Segawa, 2005). Lastly both pharmacological and non-pharmacological approaches to improving bone density included focusing on realistic expectations of increasing weight bearing activities, calcium and vitamin D intake. These statements were made with the proviso that the tested efficacy of this approach on preventing fractures in at–risk children with Rett syndrome is unknown, although there is evidence in cerebral palsy that could be relevant. A randomised clinical trial in 26 non-ambulant children with cerebral palsy (CP) found that increasing normal standing time by 60%, with a total time between 180-675 minutes/week over the course of one year, improved vertebral volumetric trabecular bone mineral density compared to the control group with CP whose standing time was unchanged (Caulton et al., 2004). However, no change was observed in the volumetric trabecular bone mineral density of the proximal tibia (Caulton et al., 2004). A more recent study, which analysed factors associated with fracture in 536 children with CP, found that those who did not use a standing device were at a nearly four times greater risk of fracture (Uddenfeldt Wort, Nordmark, Wagner, Duppe, & Westbom, 2013). Panel members recommended the combination of increased time standing and vitamin D and calcium supplementation.

Poor growth, continuing into adulthood, has been observed in Rett syndrome from an early age (Oddy et al., 2007; Shultz et al., 1993; Tarquinio et al., 2012). Although first observed as a deceleration of head growth, height and weight are also reduced in Rett syndrome (Shultz et al., 1993), consistent with DXA findings of narrow bones for chronological age (Jefferson et al., 2011; Roende, Ravn, Fuglsang, Andersen,

Nielsen, et al., 2011). The cause of this growth failure is complex and often correlated with clinical severity and genotype (Bebbington al., 2010). However functional abnormalities of the digestive tract, oromotor problems and other factors that affect feeding may also lead to nutritional deficits and poor growth (Leonard et al., 2013; Reilly, Skuse, & Wolke, 2000). Assessing dietary intake, in particular consumption of foods high in calcium, and measuring serum levels of bone markers such as calcium and 25-hydroxyvitamin D (25(OH)D) (Motil et al., 2011), are essential steps when investigating bone health. A recent study found that dietary protein, calcium and phosphorus intakes expressed as a proportion of Dietary Reference Intakes for age and sex, showed significant positive associations with TB aBMD z-scores in Rett syndrome (Motil et al., 2014).

Typically 25(OH)D is used to assess vitamin D status, however there is some conjecture as to the optimal level to support skeletal health (Holick et al., 2012). The clinical practice guidelines for the evaluation, treatment and prevention of vitamin D deficiency developed by the Endocrine Society in the United States (US) and our guidelines, recommend that circulation 25(OH)D levels should reach a minimum threshold of 30ng/ml (75nmol/litre) (Holick et al., 2012). However, the committee at the Institute of Medicine in the US, suggest a lower threshold at 20ng/ml (50nmol/litre), as they felt there was little or no benefit to bone health above this value (Rosen et al., 2012). The current position statement for Australia and New Zealand and others, conclude that further research is needed to evaluate non-skeletal benefits of 25(OH)D levels >50 nmol/L /litre (30ngmL) (Paxton et al., 2013; Winzenberg, Powell, Shaw, & Jones, 2011). As the precise level recommended in Rett syndrome is unknown, given the altered bone in this condition, it may be beneficial to aim for a higher minimal threshold, although our panel recommended adherence to local protocols. The guidelines also include the recommended dietary reference intakes for calcium based on Australian standards (Sanders al., 2009). These serve as a guide only and may differ from a clinician's local protocol. We therefore suggest clinicians follow their local recommended calcium reference intakes. Where dietary intake is lacking or bone marker serum levels are low, supplementation is suggested.

Growth failure also needs to be considered when assessing bone mass and density using DXA analysis as the two-dimensional measurement is influenced by size and underestimates a person's BMC and aBMD when subjects are small for age (Fewtrell, 2003; Jefferson et al., 2011). The guidelines therefore recommends

calculation of height formulated z-scores and volumetric bone mineral density (bone mineral apparent density – BMAD) where possible (Carter, Bouxsein, & Marcus, 1992). Overall DXA assessment was agreed upon by the expert panel, however quantitative ultrasound (QUS) may also be considered given that it is less influenced by small stature as it measures the propagation of mechanical vibrations through tissue, rather than a two dimensional measurement. Quantitative ultrasound not only provides information on the density of peripheral bones such as the calcaneous, phalanx, tibia and fibula but their biomechanical properties such as elasticity and compressive strength. Furthermore, unlike DXA, QUS is non-invasive due to the absence of radiation exposure. Although QUS is not as widely accepted as DXA given that only peripheral bone density assessments can be made, its clinical use is increasing due in part to its cost effectiveness, portability and ease of use (Baroncelli, 2008). In addition, QUS has been shown to predict future fractures similarly to that of DXA (Moayyeri et al., 2009). With increasing normative data for peripheral bones (Baroncelli, 2008; Zadik, Price, & Diamond, 2003), QUS may be considered when assessing bone health in Rett syndrome.

A further risk factor for reduced bone density is poor mobility. In the Australian study of 97 females with Rett syndrome, we found that those with reduced mobility had lower bone mass and density measurements in the lumbar spine, femoral neck and total body (Jefferson et al., 2011). Decreased lean tissue mass, also identified in Rett syndrome, would reduce the physiological load placed on bone, thereby impairing bone development and decreasing bone strength (Janz et al., 2006; Jefferson et al., 2011). The recommendation to increase mobility, particularly through time spent walking or weight bearing, reached a high level of agreement by panel members. A daily training program in four independently mobile individuals with Rett syndrome aged between 8.5-11 years of age, found that both physical fitness and functional capabilities improved (Lotan et al., 2004). Due to the variability in physical capabilities in Rett syndrome, referral to a physiotherapist for development of a physical activity plan may be helpful. With reduced mobility comes the need for parents and caregivers to increasingly handle the children during transfers. It is therefore important to ensure that caregivers are trained in transfer methods and use of equipment and that the home and school environments are safe, so as to reduce the risk of accident and fracture.

Delayed puberty is another risk factor for low bone density that needs to be considered in Rett syndrome (Knight et al., 2013). Oestrogen plays an important

part in bone formation during puberty by stimulating endocortical bone formation, whilst inhibiting periosteal apposition (Schoenau, Neu, Rauch, & Manz, 2002). Delays in puberty may therefore negatively affect bone development. Our investigation of pubertal trajectory in 213 females with Rett syndrome in Australia, found that the median age of menarche (14 years) was slightly delayed compared to the normal population (12-13 years) (Knight et al., 2013) and a US study found that 19% of girls with Rett syndrome experience delayed menarche (Killian et al., 2014). Additionally, being underweight or having the presence of the C-terminal or p.Arg168* *MECP2* mutations correlated with delayed adrenarche, thelarche and menarche (Knight et al., 2013). The increased height velocity and weight gain, which typically occur during puberty, have also been shown to be absent in Rett syndrome and height has been shown to be below the normative mean as early as 17 months of age in Rett syndrome (Tarquinio et al., 2012). Therefore the clinical assessment of bone health should include measurement of height and weight and assessment of pubertal development using Tanner staging (Marshall & Tanner, 1969).

Furthermore, the prescription of medications to regulate the menstrual cycle should be reconsidered, in particular the use of depot medroxyprogesterone acetate (DMPA), as its use has been found to increase the risk of fracture in individuals with developmental disabilities (Watson, Lentz, & Cain, 2006). An additional class of medications that are highlighted as a risk factor for low bone density is the anticonvulsants (Watson et al., 2006), in particular valproate, which we have shown increases fracture risk three-fold in Rett syndrome, compared to the risk associated with no or any other prescribed antiepileptic drug (Leonard et al., 2010).

The ISCD 2013 position paper on “Fracture Prediction and the Definition of Osteoporosis in Children and Adolescents” has recently updated its definition of osteoporosis in the young. It is now recognized that vertebral fractures can occur in at-risk children with BMD z-scores better than the traditional -2.0 standard deviation threshold. As such, the new position paper states that BMD z-score criteria are no longer required to define osteoporosis in children with vertebral fractures. This underscores the importance of determining whether vertebral fractures are present in high-risk populations such as Rett syndrome. According to the current ISCD criteria in children without vertebral fractures, a clinically significant fracture history plus a low BMD ≤-2.0 standard deviations, are both required to diagnose osteoporosis. Clinically significant fractures include either two or more long bone

fractures by age 10 years or three or more long bone fractures at any age up to 19 years (Bishop et al., 2014; Schousboe et al., 2013). It should be noted that there is marked variability in the age and gender-matched BMD z-scores that are generated by different normative reference databases, which may place an individual above or below the absolute score of -2.0, depending on the normative data that is used (Ma et al., 2015). This further highlights the need to investigate for the presence of both vertebral and non-vertebral fractures and monitor the occurrence of future fractures when considering osteoporosis in children. Overall, the diagnosis of osteoporosis is now based on a more functional measure of bone health - fracture history (including vertebral fractures), rather than BMD alone (Hogler & Ward, 2015).

If vertebral fractures are present with or without low BMD z-scores or clinical significant fractures combined with low z-scores persist, the use of bisphosphonates should be considered (Bishop et al., 2014). Albeit there is only one single case study supporting its use in Rett syndrome (Lotan et al., 2013), bisphosphonates have become the standard of care in children with moderate to severe osteogenesis imperfecta where low BMC and aBMD are accompanied by recurrent long bone or vertebral compression fractures (Glorieux, 2007). However, it is still possible that the low bone mass observed in Rett syndrome may be due to fewer bone forming cells with decreased bone modeling as opposed to increased bone remodeling (Motil et al., 2014). Since bisphosphonates increase bone density by inhibiting osteoclasts, reducing resorption of existing bone (Glorieux, 2007), they may have a less positive effect on bone mass in Rett syndrome than in the general population. With growth, there is continued bone accretion, which together with reduced bone resorption, increases cortical bone thickness and trabecular bone number (Glorieux, 2007). During the first 12 months of bisphosphonate therapy in children with secondary osteoporosis, spine BMC and aBMD tend to increase significantly more than total body values (Simm et al., 2011). Due to the paucity of evidence in Rett syndrome, re-evaluation after one year of treatment is recommended.

Fracture represents a substantial burden on those with Rett syndrome and their caregivers (Laurvick et al., 2006) and can occur unrecognized, given the decreased pain sensitivity and communication issues observed in Rett syndrome (Downs et al., 2010). One goal of developing this document is to reduce the frequency of fractures and improve bone health of those with this disorder. The authors, along with an international clinical panel, have successfully developed guidelines using consensus methods, for the assessment, treatment and management of low bone density in

Rett syndrome, taking into consideration the nutritional deficits and limited mobility in this disorder as well as other risk factors. Given the rarity of this disorder, these guidelines may be used by clinicians with little or no experience managing patients with Rett syndrome. Wherever possible, statements were supported by peer-reviewed literature. However in the absence of evidence, the guidelines relied heavily on expert panel opinion. The lack of supporting literature opens the door for further research to enhance our understanding of how to improve bone density in Rett syndrome and the identification of successful treatment protocols when bone mass and density are reduced.

Chapter Seven

General Discussion and Recommendations

This final chapter presents the conclusions, significance and recommendations drawn from this research project, with reference to the three study objectives. A decrease in bone density and an almost four-fold increase in fracture risk in Rett syndrome have previously been reported (Downs, Bebbington, Woodhead, et al., 2008). Therefore, this thesis sought to identify the pattern of bone accrual in this disorder, as well as identify the factors that inhibit bone development, placing individuals at greater risk of fracture. Knowledge and understanding of the longitudinal changes in bone in Rett syndrome and the associated risk factors were then used to help define effective prevention and management strategies in the form of a clinical guideline.

7.1 Baseline and follow-up densitometry studies

7.1.1 Densitometry findings

Bone mineral density and content were measured using dual energy X-ray absorptiometry (DXA). Ninety-seven participants were sourced from the Australian Rett Syndrome Database, a population-based registry of individuals diagnosed with Rett syndrome. Baseline measurements were taken at the lumbar spine, femoral neck and total body, which were then repeated three to four years later in 74 of the initial 97 participants. All DXA scans were performed using the Lunar Prodigy machine using a standardised scanning protocol. In place of age z-scores, height and gender matched z-scores were calculated for bone outcome measures, and bone area and lean tissue mass z-scores were adjusted for height.

At baseline, between 41-78% of all bone outcome measures were greater than -1.0 standard deviation away from the mean, with femoral neck producing the lowest mean z-scores greater than two standard deviations below the population mean. There were 10/83 (12.05%) participants who had a total body aBMD height calculated z-score less than -2.0, 15/83 (18.07%) in the total body BMC, 15/64 (23.4%) in the lumbar spine aBMD, 6/62 (9.7%) in the lumbar spine volumetric BMD and 44/73 (60.3%) in the right femoral neck aBMD. Annual changes in mean z-scores for lumbar spine and total body areal bone mineral density (aBMD) were slightly negative at -0.04 and -0.03 respectively. However, total body bone mineral

content (BMC) annual mean z-score change was considerably lower at -0.61. Bone area at baseline was -1.02, which declined annually by a z-score of -0.14. Similar findings were observed for lean tissue mass which, when first measured, produced a mean z-score of -1.10, but declined annually by a z-score of -0.21. When adjusting the total body BMC measurement at follow-up for bone area and lean tissue mass, the mean z-score change, which was -0.61, was normalized to 0.15.

Although compromised bone density in Rett syndrome has been previously documented (Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011), the decline in bone accrual with age was not known. These findings provide insight into the declining nature of bone density in Rett syndrome, showing that as each year passes, bone outcome measures fall further from population norms. Similar patterns of negative change were seen for bone size and lean tissue mass, a surrogate of muscle mass, in this study population. The strong influence of these parameters was seen when, once adjusted for the narrowness of bones and reduced muscle, total body BMC changes reflected z-scores expected in individuals of the same height in the general population.

Low bone density has been identified in other conditions where motor impairment is prevalent such as cerebral palsy (CP). Impairment of bone accretion in CP, the most common form of chronic motor disability in children, has been seen to be low in the lumbar spine, tibia and femur, however more pronounced in those with prolonged immobilization (Al Wren, Lee, Kay, Dorey, & Gilsanz, 2011; Chen et al., 2011; Henderson, Kairalla, Barrington, Abbas, & Stevenson, 2005; Simm et al., 2011). Although mobility levels have been shown to strongly influence bone density in CP, other predictors such as under-nutrition, fracture history and anticonvulsant use, have also been shown to negatively affect bone accrual (Rimmer, Chen, McCubbin, Drum, & Peterson, 2010; Simm et al., 2011). These risk factors, which were investigated in the DXA studies in this thesis, are also present in Rett syndrome. However this disorder is unique due to the presence of a mutation in the *MECP2* gene, which may have direct effects on bone cellular processes or secondary effects due to neurological impairment (O'Connor et al., 2009). One study found that bone properties, such as stiffness, hardness and tensile strength were reduced in MeCP2 protein deficient mice (Kamal et al., 2015). Furthermore, a separate study which used MeCP2 deficient mice, observed a reduced cortical and trabecular bone volume in the femur and calvaria (O'Connor et al., 2009). A decrease in osteoid deposition may be the cause of this reduced bone volume, with osteoblast

dysfunction leading to reduced bone formation and thus density rather than an increase in osteoclast function, with numbers of these cells appearing normal compared to wild type mice (O'Connor et al., 2009). The theory of low bone density in Rett syndrome occurring due to reduced bone formation is also supported by a study which investigated biomarkers of bone formation in 50 females with Rett syndrome. This study showed that osteocalcin was reduced compared to gender and age matched reference ranges, even in the presence of normal C-telopeptide concentrations (Motil et al., 2014). Therefore, low bone density in Rett syndrome may be due to a decrease in bone formation, as opposed to an increase in bone resorption (Motil et al., 2014). Markers of bone formation and resorption have also been shown to be reduced in females with Rett syndrome, younger than 25 years, which suggests an altered bone metabolism and a decrease in bone turnover (Roende et al., 2014). Given the phenotype genotype relationship identified in Rett syndrome and the varied degree of severity (Bebbington et al., 2008), isolating the impact of genotype on bone metabolism and formation is challenging. Nonetheless, these studies have identified cellular and metabolic changes which may at least contribute to the onset of low bone density in Rett syndrome.

7.1.2 Predictors of low bone density

To address the second objective of this thesis, univariate regression analysis was performed for both baseline and follow-up bone outcome measures in order to investigate the predictive power of the variables on bone accrual.

At baseline, univariate models included age, presence of epilepsy, BMI, mobility level, fracture history, Tanner stage, anticonvulsant use and mutation type. Age was divided in to four groups with the aim of placing participants together with similar pubertal development. As described by Marshall and Tanner (1969), the development of secondary female sexual characteristics and appearance of breast buds occurs at approximately 11.15 years old, with the average age of menarche seen at 13.47 years. Furthermore, menstrual status collected from 2510 females in the US aged between eight to 20 years, showed that between ten to 90% of females had achieved menarche by the age of 11.11 years (Chumlea et al., 2003). The ages included in each group were supported by findings from an Australian study which investigated the pubertal trajectory in 233 females with Rett syndrome, where the average age of onset of menarche was between 11-12 years, with a median of 14 years (Knight et al., 2011). Therefore, placing individuals into the following age groups: 4-8, >8-14.5, >14.5-20, >20, increased the likelihood of participants being

placed with others who were also at a similar sexual maturation point. Age group cut off points were slightly changed for the longitudinal study, as participants were older, however the grouping still followed the typical ages of pubertal development and age of onset of menses according to Tanner and Marshall (1969).

Mutation types were grouped into the eight most common single point mutations, the C-terminal and large deletion mutations. The less common mutations were also grouped together, with final categories being those who did not have a *MECP2* mutation and those who had not been tested. Variables, which were shown to be significant predictors of bone density, were then included in multivariate regression models, and these included age, epilepsy, BMI, mobility and mutation type. Regression models were performed for TB BMC but not TB aBMD and LS aBMD but not LS BMAD as the results for these bone outcome measures were similar.

At follow-up, the variables chosen to incorporate in the univariate regression models were guided by the findings from the baseline DXA study and the literature and included: age, mutation group, previous fracture, epilepsy diagnosis, BMI, mobility and height adjusted lean tissue mass. In light of findings of delayed menarche in Rett syndrome (Killian et al., 2014; Knight et al., 2013), pubertal development was classed as premenarcheal, menarcheal and postmenarcheal instead of Tanner staging. These groups relate to stages during development when pubertal hormones which influence bone growth and bone mineral accrual differ (Wang et al., 2004). As there were only seven participants in the menarcheal group, compared to 17 and 19 in the premenarcheal and postmenarcheal groups respectively, the effects of menses on bone outcome measures may be underestimated. Furthermore, due to limited numbers of participants with some of the more common *MECP2* mutations, these were grouped as mild, mixed or severe mutations, according to phenotypic severity (Bebbington et al., 2008). The significant predictors identified in univariate regression models were age, menstrual group and height adjusted lean tissue mass, which were then included in multivariate regression analysis. This model was also repeated without the data from individuals who were *MECP2* mutation negative. The diagnosis of epilepsy, was not shown to correlate with low bone outcome measures in both DXA studies, which may be explained by the homogeneity of participants, with large proportions having epilepsy at baseline (75%) and follow-up (89%). Although the studies in this thesis did not request information regarding the way in which an epilepsy diagnosis was made, the use of

anticonvulsant medication was sourced for each participant and used as a surrogate for a diagnosis of epilepsy.

The major findings suggest that the capacity to be mobile was a strong predictor of low aBMD and BMC, with those who were least mobile having the lowest values. Furthermore, annual changes in lumbar spine bone outcome measures and total body aBMD were more positive in those who could walk unaided compared to those who could not walk. Mobility problems are common in Rett syndrome (Downs, Bebbington, Jacoby, et al., 2008), as was seen in our study group, with over half of the participants at baseline and 46% at follow-up wheelchair dependent. This suggests that maintaining mobility in those with Rett syndrome who have achieved ambulatory skills, could serve as a protective mechanism against fractures and reduced bone density. The negative effects of immobility on bone density in Rett syndrome correlate with the reduced lean tissue mass and bone area identified at baseline and follow-up.

Muscles place the largest mechanical loads on bone, whereby the forces drive bone to adapt by changing shape and increasing bone mass and therefore strength (Rauch et al., 2004). Maximal rates of muscle development have been shown to correlate with maximal rates of bone accumulation, which shows the functional relationship between these two tissue types. Therefore, it is likely that the immobility observed in Rett syndrome, affects muscle development which reduces the mechanical stress placed on bone leading to bones that are smaller and less dense (Rauch et al., 2004). These findings highlight the need for further investigation in to the types of physical activity feasible in Rett syndrome with reference to the varied degrees of motor deficits seen in this disorder and the impact these programs have on muscle mass and bone density. A standing program may be the best approach for those who are wheelchair dependent, with the use of orthoses or adaptive equipment (Stuberg, 1992). For those with Rett syndrome who are more mobile, exercise programs using a treadmill (Lotan et al., 2004) or walking programs aimed at increasing the time or distance walked, may positively influence bone density and muscle development in this population (Stuberg, 1992).

The mutation types p.Arg168* and p.Thr158Met, were also shown to be predictors of low bone density in the lumbar spine and total body baseline DXA scans, although this was not seen in the follow-up scans. This may be due to the smaller number of participants in the follow-up study, which led to fewer individuals having

certain mutation types. On a phenotypic severity scale, the p.Thr158Met is regarded as having a mixed or intermediate phenotype and the p.Arg168* a more severe phenotype (Bebbington et al., 2008; Cuddapah et al., 2014). Our findings provide new insight into the influence that mutation type may have on bone density in Rett syndrome and alert clinicians to the possibility that bone health may be compromised in individuals with particular mutations associated with a more severe or complex phenotype. This may lead to earlier monitoring and intervention programs in the presence of these genotypes.

Age was also a predictor of decreased TB BMC z-score changes in those who were 10 years or older, which normally represents a period when bones should be growing in length, width and increasing in density (Davies et al., 2005). Childhood and adolescence are critical periods of bone mineralisation, with between 40-60% of adult bone mass accrued during adolescence (Golden, Abrams, & Committee on Nutriton, 2014). Twenty-five percent of peak bone mass (PBM) accrual occurs during the two-year period of peak height velocity. However there is a six to 12 months delay in PBM accrual compared to increases in height, placing individuals at greater risk of bone fragility (Golden et al., 2014) and fracture during this period (Bonjour & Chevalley, 2014). Although the rate of bone mass accrual slows by the age of 16-18 in females and 17-20 years in males, bone mass does not reach its peak until between 25-35 years of age (Davies et al., 2005).

The finding that bone accrual appeared to decrease with age, suggests that attempts to improve bone density in Rett syndrome need to occur early in life. Intervention programs which implement daily exercise programs, to improve bone accrual in children in the general population, have shown the greatest impact occurs when implemented during the period of time prior to the onset of menses (Bonjour & Chevalley, 2014). Prior to this period, may be a beneficial time to investigate bone density in Rett syndrome and initiate preventative and treatment protocols for low bone density, such as improving mobility and ensuring an adequate intake of foods high in vitamin D and calcium to support bone metabolism.

Multivariate analysis restricted to mutation positive participants of the longitudinal change in z-scores, produced similar results to the cross sectional models, particularly for the effects of age. However observing over time, achieving menses appeared to have a protective effect on TB aBMD changes. During normal development, hormonal secretions such as growth hormone, insulin like growth

factor 1 and insulin, cause increases in cortical thickness, leading to larger bones in males and endosteal deposition in females (Bonjour & Chevalley, 2014). Under neuroendocrine control, the timing of increased height correlates with pubertal maturation (Bonjour & Chevalley, 2014; Golden et al., 2014). Pubertal timing, in particular menarcheal age, has been shown to have an inverse relationship with aBMD, such that those with delayed menarche have reduced aBMD in skeletal sites such as the radius, femoral neck and distal tibia (Bonjour & Chevalley, 2014).

There have been two studies investigating pubertal development in Rett syndrome, whereby both found delayed menarche compared to the general population (Killian et al., 2014; Knight et al., 2013). Thus the pubertal trajectory in Rett syndrome does not appear to be normal, which may negatively impact bone development due to the increased period of higher bone turnover (Eastell, 2005). It is therefore important for pubertal development to form part of the clinical assessment in this disorder, so that if delays are present investigations can occur to identify the cause, such as abnormal hormone levels or malnutrition (Bonjour & Chevalley, 2014). The influence of nutritional status should also be considered when monitoring pubertal trajectory, given its influence on pubertal maturation rates, whereby peri-pubertal sub-nutrition has been shown to delay the onset of puberty in females in the general population (Soliman, De Sanctis, & Elalaily, 2014). Given the small stature observed in this study with a mean BMI z-scores of -0.9 at baseline and -1.2 at follow up, translating to a negative annual change in BMI z-score of 0.7, malnutrition may be a contributing factor to the lack of growth. It would be interesting to investigate the change in BMI z-score by age groups in order to identify the age at which those with Rett syndrome are at risk of delayed growth. Nutritional status during infancy, childhood and adolescence has been shown to affect pubertal development, which suggests that assessment of dietary intake should occur early in life to assure proper timing and progress of pubertal development (Soliman et al., 2014). However, improving nutritional status in Rett syndrome is challenging given the feeding and gastrointestinal problems experienced in individuals with this condition with severe cases often requiring gastrostomy (Leonard et al., 2013; Motil et al., 2012).

In both DXA studies a history of fracture was not shown to be a predictor of decreased aBMD or BMC, although other studies have identified this correlation in Rett syndrome (Motil et al., 2008; Roende, Ravn, Fuglsang, Andersen, Nielsen, et al., 2011). Greater than one third of the study participants had a history of fracture at

baseline. Three quarters of these fractures involved a long bone of the upper or lower limb or a vertebral compression fracture, which are classed as “significant” fractures according to the International Society of Clinical Densitometry (ISCD) position statement (Gordon et al., 2014; Schousboe et al., 2013). At the time of follow-up, nearly half of the study sample had sustained a fracture. According to the guidelines, those with two or more long bone fractures by age 10 years, three or more long bone fractures at any age up to 16 years or a vertebral compression fracture combined with BMC or BMD z-scores less than or equal to -2.0, can be classified as osteoporotic (Schousboe et al., 2013). In light of the negative changes in total body and lumbar spine BMC and aBMD z-scores over a one-year period and the increasing proportion of the sample whom had sustained fractures, those who may not presently meet the ISCD criteria may be on a trajectory to osteoporosis.

In summary, the findings of reduced bone accrual and muscle mass observed in the study participants, provide insight into the risk factors for inadequate bone development in Rett syndrome. Given the broad range in severity and presence of risks factors for low bone density in this disorder, some individuals will be at greater risk than others. Nonetheless, this thesis highlights intervention and treatment strategies for all those with Rett syndrome such as maintenance of adequate nutrition, improved mobility and weight-bearing and normalising pubertal development, which may improve bone accrual in this condition.

7.2 The development of clinical guidelines to manage bone health document

Development of guidelines for the diagnosis, treatment and prevention of low bone density in Rett syndrome relates to the last objective in this thesis and provides a mechanism for addressing the reduced bone accrual and muscle mass identified in the DXA studies. The final guidelines document focused on two areas; the assessment of skeletal health and the prevention and treatment of low bone density. Where possible, each statement was supported by peer reviewed journal articles that rate highly on the ‘level of evidence’ scale, set by the Scottish Intercollegiate Guidelines Network (SIGN, 2011). Links to the publications supporting each statement were available for panel members to refer to when completing their review of the guidelines (Appendix B). However, there were many key areas where there was a paucity of evidence in the literature to inform the monitoring, prevention and treatment of poor bone health in Rett syndrome. After a two-round review

process using the Delphi Method, which is described in Section 3.18, the final guideline document consisted of 39 statements.

As a method of identifying stakeholder concerns and incorporating consumer input into the guidelines, an online forum Rettnet (Leonard et al., 2004) was reviewed. Rettnet, made available by Rettsyndrome.org, is a member only forum which fosters a sense of community and support for those wishing to discuss any topic related to Rett syndrome such as care, treatment and research. Information sourced from Rettnet was identified as a valuable resource during the development of previous guidelines in Rett syndrome, with one guideline focusing on management of scoliosis (Downs et al., 2009) and the other management of nutrition in Rett syndrome (Leonard et al., 2013).

The comments on Rettnet that related to the guidelines were grouped into five key topics. The first key topic was bone density, which posed questions as to how bone density is measured, the safety of this technique and interpretation of results. The second topic related to fractures, in particular how are they identified, how they were being managed and the decreased pain sensitivity observed in Rett syndrome, leading some parents to be unaware a fracture had occurred. The third topic related to treatment and prevention strategies for low bone density in Rett syndrome. The next topic which involved a significant amount of discussion on Rettnet, was in reference to feeding problems and nutritional deficits including vitamin D and calcium in Rett syndrome and how this might impact bone development. Lastly, parents felt that their child's bone density was being negatively impacted by the limited amount of weight-bearing activities performed each day.

A consumer reference group (CRG) for the Australian Rett Syndrome study, whose members included six mothers and one father of a daughter with Rett syndrome, was invited to review the draft guidelines document and provide feedback. The CRG identified four key areas not clear in the draft, including: the need for heightened fracture vigilance as one parent had a daughter who experienced a low impact fracture, specifying vitamin D and calcium supplementation rather than recommending non-specific nutrient supplements, questions relating to the use of progesterone based contraceptives or inclusion of all oral contraceptives and lastly bisphosphonate treatment, its negative side effects and efficacy. These key areas of interest by the CRG were further clarified in the draft.

The draft guideline was then presented to an expert panel comprising 38 members representing a broad range of clinical disciplines and research expertise. Figure 7.1 displays the number of health professionals invited to be a panel member and those who participated in round one and two of the Delphi process. Experts were identified via several approaches including review of the literature, discussions with an already established network of investigators at the Telethon Kids Institute and RettSearch, which is an online International consortium of Rett syndrome clinical researchers. Additional health professionals were invited to participate upon recommendation by their colleagues, who had already agreed to be a panel member. With a variety of clinical experts in the panel, members were not pressured to provide responses to areas with which they were not familiar, allowing them to choose the option “Not my area of Expertise”.

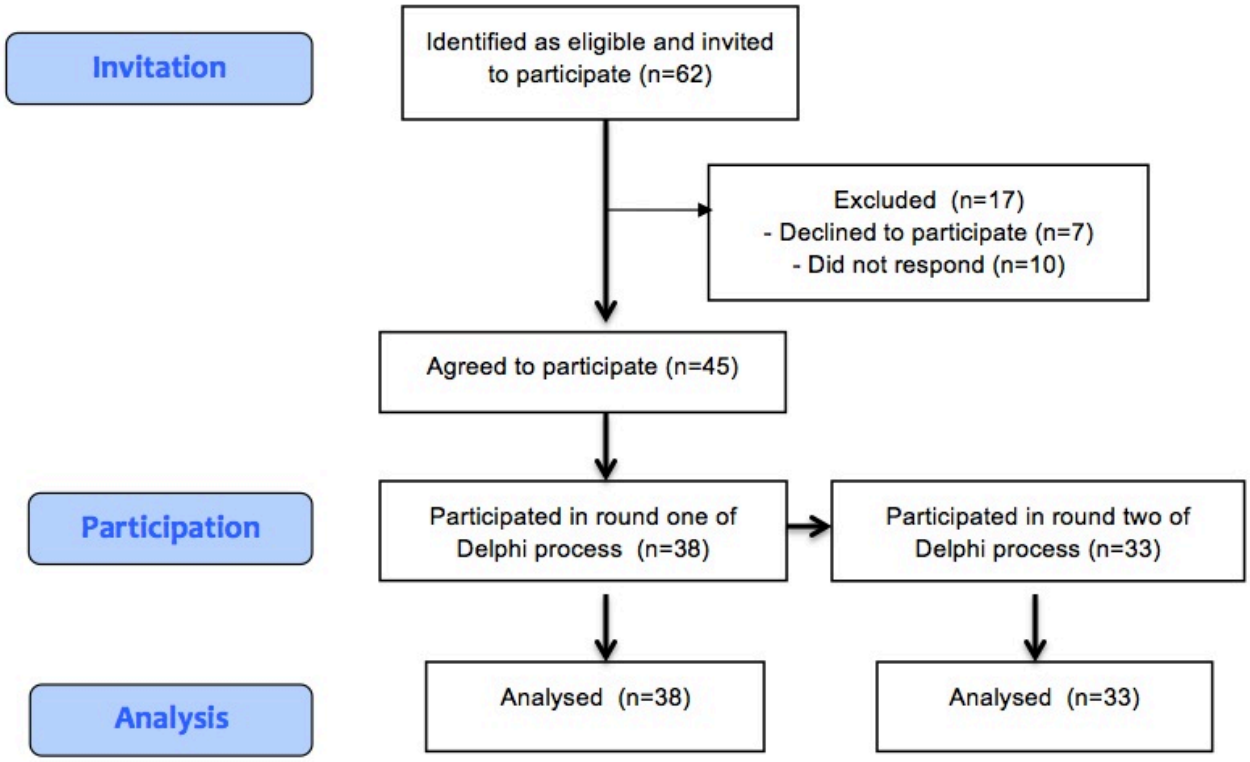

Figure 7.1: Expert panel recruitment and participation

The Delphi technique was used to gather data from respondents and develop consensus of opinion. A benefit of the Delphi technique is that it creates anonymity due to the controlled feedback mechanism, which limits group interaction and the pressure of conformity (Hsu & Sandford, 2007). Confidentiality was maintained by the geographical spread of panel members and their password protected access to the online version of the guidelines. Members only participated in a two round review process and were not given feedback on overall responses, so that they felt

no pressure to adopt a certain viewpoint. The Delphi technique also allowed the collection of data which could be statistically analysed, providing an unbiased objective analysis of whether consensus had been achieved. Consensus was accepted when 70% of responses were within one response category of the median response. New statements were formed based on comments related to questions and changes made to statements where consensus was not reached.

One risk of the Delphi method is the potential for low response fractions (Hsu & Sandford, 2007). However, nearly 85% of those who agreed to participate in this study provided data and nearly 87% who provided responses to the first round, provided responses to the second round. Follow-up via email and maintenance of communication with panel members and the low number of review rounds, was likely to have positively influenced the response fraction. The ability for panel members to complete parts of the review, save and return later, may have also led to an increase in panel member participation.

The section of the guidelines which focused on preventative and treatment measures for low bone density in Rett syndrome, required the greatest reliance on the panel's views. With the absence of supporting literature and input largely weighted towards panel member opinion, the guidelines serve to identify areas of future research as currently no studies have investigated changes in bone density in Rett syndrome in response to increases in physical activity, dietary supplementation or bisphosphonate use. As physical activity has been shown to have a positive effect on BMC and lean tissue mass in children and adolescents without a disability (Hind & Burrows, 2007), improving weight-bearing activities may benefit these parameters in Rett syndrome. Furthermore, due to the decreased 25(OH)D levels (Motil et al., 2011), nutritional deficits and gastrointestinal disorders (Leonard et al., 2013) observed in Rett syndrome, maintenance of an adequate diet for growth and calcium and vitamin D supplementation are likely to improve bone health in this disorder.

Bisphosphonate use was the only medication recommended for treatment of low bone density in Rett syndrome in the guidelines, as long as a significant fracture history was present and aBMD z-score was lower than -2.0, in accordance with the ISCD criteria for osteoporosis diagnosis in children and adolescents (Bishop et al., 2014). A majority of studies investigating bisphosphonate use in children have centered on its use to treat osteogenesis imperfecta (Shapiro, Thompson, Wu,

Nunes, & Gillen, 2010; Ward et al., 2011; Wei et al., 2012) and cerebral palsy (Fehlings et al., 2012; Henderson et al., 2002). However, it is not known if this treatment will have the same positive effect in Rett syndrome as it requires a healthy population of osteoblasts for bone formation, which have been observed to be dysfunctional in the presence of a *MECP2* mutation in rodents (O'Connor et al, 2009). Furthermore, the identification of reduced biomarkers of bone formation such as osteocalcin in individuals with Rett syndrome provides further support to the possible dysfunction of osteoblasts (Motil et al, 2014). For this reason, the guidelines highlighted the need to measure biomarkers such as collagen cross links and N-telopeptide in Rett syndrome patients. As described in section 2.6.2, N-telopeptide is a breakdown product released during bone resorption and provides information on rate of bone turnover (Dalskov et al., 2014). Whereas collagen cross links increase the tensile strength of bone and have been thought to play a role in impaired mechanical properties of bone in aging and osteoporosis (Saito & Marumo, 2010). Therefore, investigations in to the efficacy of bisphosphonate use in Rett syndrome form an important recommendation for future work.

7.3 Limitations

The main limitation of the DXA studies related to the number of participants enrolled in the study. Firstly, the age of participants only included those four years and older as this was the youngest age in the normal reference population. This prevented investigation of longitudinal changes in bone density in the very young. Furthermore, due to the wide geographical spread of those with Rett syndrome throughout Australia, with only seven scan locations participating in the studies, some females in the ARSD could not participate as they were too far from the scan location or were too unwell to travel distances.

Participant numbers were also reduced between the baseline and follow-up scan, with 97 participants at baseline and 74 at follow-up. Three females were not included in the longitudinal study as they died between the baseline and follow-up scans. A consequence of the reduced number of participants at follow-up was the decreased number with certain common mutations. Instead of grouping mutation types based on the eight common point mutations, they were grouped based on severity (mild, mixed and severe). This may explain why two mutations, p.Arg168* and p.Thr158Met, were identified as strong predictors of low bone density at baseline but not longitudinally. Furthermore, the DXA studies did not investigate the possible influence of X chromosome inactivation patterns of the mutated *MECP2*

gene on bone outcome measures. As X chromosome inactivation can have an appreciable impact on phenotypic severity (Amir et al., 2000), investigating the impact of skewed X chromosome inactivation on aBMD and BMC measurements, may identify those with Rett syndrome at greater risk of low bone density.

An additional limitation of the DXA studies related to the decrease in total eligible lumbar spine scans due to the exclusion of those with lumbar rods. There were nine lumbar scans removed at baseline and 31 removed in the follow-up scan. Although there were considerably less lumbar scans compared to total body scans, similar patterns of decreased bone accrual in aBMD and BMC in these sites were seen, however to a lesser degree in the lumbar spine. The reduced number of lumbar spine scans may also explain why no predictors for low bone density were identified in the multiple regression models for this bone outcome, but were observed in the total body BMC.

Femoral neck scans, which produced the lowest mean z-scores at baseline, were not analysed longitudinally. Achieving the same position each time the femoral neck is scanned is problematic, as changes in the amount of internal rotation of the hip and placement of the trochanters and pelvis can influence bone density estimations (Bishop et al., 2014). Not achieving consistent positioning can therefore make it difficult to differentiate between actual changes in the amount of bone mineral present and changes due to different areas of bone being assessed (Bishop et al, 2014). Therefore the decision against undertaking longitudinal analysis of the femoral neck scans was made on the basis of ISCD recommendations for skeletal sites measured using DXA and in light of the physical restrictions in the study cohort.

Although this study was able to source information on the number of fractures participants had experienced and the date of occurrence from completed questionnaires, the means in which a fracture diagnosis was made were not collected, nor was the source of the diagnosis where some parents or caregivers may have made the fracture diagnosis themselves. In addition, a history of fracture was investigated via univariate regression analysis as a predictor of bone outcome measures. However, it would be meaningful to compare aBMD and BMC z-scores between those with and without a fracture history and include the skeletal site of fracture. For future studies investigating bone outcome measures in Rett syndrome, a greater understanding of how diagnosis was made would ensure greater data

accuracy and comparison between the fracture and non-fractured group, as well as more detail on the bone fractured, providing a greater understanding of how fracture influences bone density in this syndrome.

The normative data used to compare bone outcome and body composition measurements was sourced from Westmead Children's Hospital in Sydney. I did not have access to this data set as the raw data were sent to Westmead Children's Hospital for calculation of z-scores for age, height and weight, Consequently, I was not able to compare pubertal and menarcheal status between those in the data set and participants in the DXA studies. Given the influence pubertal maturation has on bone development, I recommend investigators assessing the skeletal health in Rett syndrome, collect information such as pubertal status from their chosen normative comparative group.

The main limitation in the guidelines development was the reliance on expert panel opinion, rather than peer reviewed literature. Furthermore, fewer numbers of expert panel members commented on statements in the 'Bone Mineral Density Assessment Technique" and 'Non-pharmacological Treatment' sections as they felt it was beyond their areas of expertise, which supports the need for further research in these areas. Although Rett syndrome is a rare disorder, there has been extensive research into the many facets of the disorder such as the understanding of the trajectory of scoliosis in Rett syndrome and outcomes following spinal fusion (Downs et al., 2009). The same model of comprehensive outcomes investigation that has occurred in comorbidities such as scoliosis (Downs et al., 2015; Marr, Leonard, Torode, & Downs, 2015) is still necessary for bone health in Rett syndrome.

7.4 Recommendations for future work

The findings of the research presented in this thesis have established a valuable knowledge base to inform future work. In light of the findings of decreased height which may reflect growth problems and possible nutritional deficits, bone area and lean tissue mass, keys areas of future research should focus on development and evaluation of intervention programs and investigations into the efficacy of treatment protocols for low bone density. The lack of supporting literature opens the door for further research to enhance our understanding of how to improve bone density in Rett syndrome and the identification of successful treatment protocols when bone mass and density are reduced. Identified through the findings and limitations of this

research, two major foci of research are recommended; Investigating physical activity in Rett syndrome and gaining an understanding of the effects of bisphosphonate use in Rett syndrome.

7.4.1 Investigating the effects of physical activity on bone mass and lean tissue mass in Rett syndrome

Weight-bearing exercises have been shown to positively influence bone mineral accrual in children and adolescents (Hind & Burrows, 2007) and BMC and volumetric density in children with cerebral palsy (Chad, Bailey, McKay, Zello, & Snyder, 1999). Weight-bearing exercises have also been shown to slow the progression of bone loss associated with immobility (American Association of Clinical Endocrinologists, 2003). Furthermore, high frequency, low intensity vibration therapy in motor impaired children, led to increases in bone mass and muscle strength (Reyes, Hernandez, Holmgren, Sanhueza, & Escobar, 2011).

There is currently only one study to have investigated the affects of increasing physical activity in four females with Rett syndrome (Lotan et al., 2004). However, in this study, the outcome measure was change in functional skills, and the authors did not investigate changes in bone parameters. How bone density and lean tissue mass in Rett syndrome respond to increases in weight-bearing exercises and increased muscle loads remains to be established.

A study investigating the impact of physical activity on lean tissue mass, bone area, fat mass and aBMD and BMC in Rett syndrome is recommended. Such a study may investigate factors that influence osteogenic activity in those with Rett syndrome such as the duration, frequency and mode of activity. This study may need to develop different exercise models according to mobility levels, such that supported standing using a standing-frame may be suitable for the less mobile, while the more mobile individuals may perform exercises with a higher weight-bearing force on bone, such as walking or running. Bone outcome and body composition changes could also be measured before and after participation in an exercise program, using DXA scans or using peripheral quantitative computed tomography (pQCT). Of particular importance to Rett syndrome given their reduced stature, volumetric density may be measured using pQCT, which is not achieved using DXA (Adams, Engelke, Zemel, & Ward, 2014). The pQCT bone imaging technique is also able to separate cortical from trabecular bone and can measure bone strength features including cortical porosity and the microstructure of bone (Adams et al., 2014).

Furthermore, several studies in the general population have shown that when exercise is combined with nutrient supplementation, greater improvements in bone density are observed, compared to exercise alone (Julian-Almarcegui et al., 2015). Although most normative studies have investigated the effects of calcium supplementation, other micronutrients such as magnesium and potassium or vitamin D may also impact bone status when combined with exercise (Julian-Almarcegui et al., 2015). Investigating the difference between the individual and combined effects of these parameters on bone density in Rett syndrome may provide evidence to support the recommendation of increasing exercise and vitamin D and calcium intake in Rett syndrome in order to optimise bone health.

7.4.2 The effect of bisphosphonate use in Rett syndrome on bone mineral density and content and fracture risk

One of the statements in the clinical guidelines suggested that use of bisphosphonates in Rett syndrome could be of value in the presence of a significant fracture history and an aBMD or BMC z-score of the lumbar spine or total body less than -2.0. This statement was formed based on the expert opinion of the panel, rather than findings from the literature.

Bisphosphonates act as an antiresorptive compound which disrupts osteoclast activity, leading to an increase in BMD (Boyce, Tosi, & Paul, 2014). This medication was first used to treat osteoporosis in adults, leading to improved bone density and a reduced fracture risk. However its efficacy in children, where bone metabolism differs from adults, is limited (Boyce et al., 2014). A large proportion of the studies on bisphosphonate use in children have focused on conditions such as osteogenesis imperfecta (Shapiro et al., 2010; Ward et al., 2011; Wei et al., 2012) and cerebral palsy (Fehlings et al., 2012; Henderson et al., 2002), showing improvements in bone density. However, there is debate as to whether they reduce fracture risk in these disorders, with some showing reductions and others finding no difference (Boyce et al., 2014).

Whether bisphosphonate use in Rett syndrome leads to an increase in BMD and a reduction in fracture occurrence, in addition to identification of the side effects of this medication remains to be established. Therefore a population-based assessment of aBMD and BMC in the lumbar spine and total body and fracture history should be performed on those with Rett syndrome. In individuals who meet the diagnosis criteria of low bone density according to the ISCD pediatric position statement

(Schousboe et al., 2013), where bisphosphonate use is warranted, assessment of bone density and content should be repeated after one year of treatment, in the hopes of improving the current knowledge gap in treating low bone density in Rett syndrome.

7.5 Conclusion

Rett syndrome is a complex disability, associated with vulnerability to poor bone health, which is likely linked to genotype and influenced by lifestyle factors. This thesis provides a greater understanding of bone accrual in Rett syndrome and the risk factors for low bone density including age, poor mobility, pubertal delay and genotype. The clinical guidelines provide therapeutic options to improving bone density in Rett syndrome including assessment and monitoring of nutritional status and implementing physical activity programs tailored to the individual. Adequate dietary intake, including vitamin D and calcium, benefits growth and bone development leading to larger bones. Adequate nutrition may also help support pubertal development in this disorder and increases in weight-bearing activity promote muscle development, increasing muscle size and strength, which have an anabolic effect on bone. However, the precise effects of nutrition and physical activity programs on bone health in Rett syndrome are not known. Whether the use of bisphosphonates to treat low bone density in Rett syndrome leads to positive changes needs further investigation. Nonetheless, these non-pharmacological and pharmacological prevention and management strategies have the potential to improve bone health and reduce the fracture incidence in Rett syndrome, and this thesis sets the scene for the development of studies to test their effectiveness.

Appendix A

Densitometry Instructions Letter

Ethical approval for sedation has not been requested. We appreciate that in a few subjects there may be difficulty with the procedure. Below are some suggestions for ease of management.

Regions

It is recommended that scans be in the following order:

1st scan: Lumbar spine as it gets individuals used to the noise of the machine first.

2nd scan: The left femoral neck tried initially and if possible also the right.

3rd scan: Total body.

Positioning

– Use the standard manufacturer positioning.

– For pediatric patients (up to and including **20** years) have hands placed in a loose fist (if possible), as this was how normative data was obtained.

Rett syndrome patients

– Previously it has been easier to have a sheet wrapping to hold the patient still, especially for the 3.5 mins needed to do the TB. Extra time for the scans is required because of this.

– You may need to play a video (Wiggles and High Five popular!), use a dummy, ask mum for ideas on how to keep them calm. When parents are first contacted to make an appointment they will be asked to bring something along that may make things a little easier on their daughter.

– Individuals with button or rods will have this mentioned on the consent form. Scan as normal and this subgroup may be dealt with as a separate analysis.

– We certainly appreciate that the results may not be perfect but a note of difficulties or presence of rods etc may be recorded on the report.

– To diminish time wasting and distress, parents will be advised before their children come not to be wearing zips, press-studs, earrings, hook and eyes etc in their clothing.

– For LS and FN having Mum/Dad at the head end made the process easier and kept each child calmer.

Prodigy depth

– Depth is used based on Lunar normals.

– Less than 13cm depth = thin mode

– 13-25cm depth = standard mode

– Greater than 25cm = thick mode

– Note: if on the cusp of the depth cut-off on the first scan, it is probably better to assume that the child will grow and choose the larger scan mode to allow for the growth, such that the same scan mode can be used over the 2 years. If the patient grows a lot over the 2 years and needs to up a scan mode, I suggest doing the 2nd scan on both modes.

Quality Assurance

– Please use your standard manufacturer recommended quality assurance procedures.

– Please use Document machine changes on the Schewart charts, thin + standard

Transmission of data to central site

– The process of transmission of data is negotiable in each study location.

Raw Data

– Each individual already has an identification number which will be on the consent form that will be bought with them to the appointment. We would also appreciate it if you could send a copy of the raw data for our own analysis to aussierett@ichr.uwa.edu.au or download onto a CD and send to the address below.

Standard report

– AussieRett has sent out the consent form to each family which informed them that they will have to bring this with them. A copy of this consent form can be made for your own records and a copy forwarded to Dr Helen Leonard. Please also include a copy of your standard report, which should also be sent to the clinician noted on the consent form.

Australian Rett Syndrome Study

Telethon Kids Institute

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Appendix B

Chapter 6 Clinical Guidelines Tables with the addition of the references provided to expert panel members

Table 6.1: Assessment of bone health

Statements	Level of evidence	References
All children with a clinical diagnosis of Rett syndrome should undergo genetic testing as genotype may influence the development and management of osteoporosis	2	(Jefferson et al., 2011), (Motil et al., 2008), (Zysman et al., 2006), (Roende et al., 2011)
Fractures in Rett syndrome can occur due to trivial trauma	2	(Downs et al., 2008), (Roende et al., 2011)
Clinicians need to be vigilant for potential fractures	2	(Downs et al., 2008), (Roende et al., 2011)
Measure weight and height to calculate Body Mass Index at each clinical visit	4	Expert opinion
Identify all prescribed medications at each clinical visit, particularly those that can influence bone density: eg anti-epileptic medications, proton pump inhibitors, progesterone-only medications, vitamin supplements	2	(Leonard et al., 2010), (Coppola at al., 2009), (Lopez, Grimes, Schultz & Kurtis, 2009), (Watson, Lentz & Cain, 2006), Cromer & Harel, 2000)
Assess pubertal development using Tanner staging	2	(Marchand, 2009)

Pubertal development may be delayed in girls or women with Rett syndrome which puts those affected at risk of low bone mineral density	2	(Knight et al., 2013), (Killian et al., 2014)
Assess mobility level by asking about the following:		
The level of assistance needed for walking	2	(Downs et al., 2008)
The time spent walking each day	2	(Downs et al., 2008)
The distance walked each day	2	(Downs et al., 2008)
The amount of time standing in a standing frame if independent standing is not	2	(Downs et al., 2008)
Assess dietary intake including:		
24 hour diet recall	2	(Marchand, 2009), (Issacs, Murdock, Iane & Percy, 2003), (Schwartzman, Vitolo, Schwartzman & Morais, 2008), (Oddy et al., 2007)
Recall of food high in vitamin D	2	(Marchand, 2009), (Issacs et al., 2003), (Schwartzman et al., 2008), (Oddy et al., 2007)
Recall of food high in calcium	2	(Marchand, 2009), (Issacs et al., 2003), (Schwartzman et al., 2008), (Oddy et al., 2007)
Assessment of sunlight exposure by asking about:		

Frequency of use of sunscreen and sun-protection factor/protective clothing	1,2	(Working Group of the Australian New Zealand Bone Mineral Society 2005), (Vanlint, Nugent & Durvasula, 2008)
The time of the day when skin (equivalent to face and arms) is exposed to direct sunlight	1,2	(Working Group of the Australian New Zealand Bone Mineral Society 2005), (Vanlint et al., 2008)
Amount of time each day that skin (equivalent to face and arms) is exposed to direct sunlight	1,2	(Working Group of the Australian New Zealand Bone Mineral Society 2005), (Vanlint et al., 2008)
First line biochemical investigations include measurement of:		
Calcium (ideally also ionised calcium)	1,3	(Cass et al., 2003), (Bergman et al., 2009)
25 hydroxyvitamin D (25(OH)D)	1,3	(Cass et al., 2003), (Bergman et al., 2009)
Magnesium	1,3	(Cass et al., 2003), (Bergman et al., 2009)

Phosphorus	1,3	(Cass et al., 2003), (Bergman et al., 2009)
Alkaline Phosphatase (ALP)	1,3	(Cass et al., 2003), (Bergman et al., 2009)
Albumin	1,3	(Cass et al., 2003), (Bergman et al., 2009)
Second line biochemical investigations include measurement of:		
Electrolytes (ideally also ionised calcium)	4	Expert opinion
Urine calcium/creatinine ratio (ideally also ionised calcium)	4	Expert opinion
Bone turnover markers: N-telopeptide, collagen cross-links	4	Expert opinion
Parathyroid hormone (PTH) if any pathological findings	4	Expert opinion

Table 6.2: Bone mineral density assessment

Statements	**Level of evidence**	**References**
Bone health needs to be considered early on in life and the following routine risk factors should be assessed:		

Ability to walk	2	(Jefferson et al., 2011), (Leonard et al., 2010), (Motil et al., 2008), (Zysman et al., 2006), (Roende et al., 2011)
Presence of either the p.R168X, p.R255X, p.R270X or p.T158M mutation	2	(Jefferson et al., 2011), (Leonard et al., 2010), (Motil et al., 2008), (Zysman et al., 2006), (Roende et al., 2011)
Prescribed anticonvulsant medication(s)	2	(Jefferson et al., 2011), (Leonard et al., 2010), (Motil et al., 2008), (Zysman et al., 2006), (Roende et al., 2011)
Oral and intramuscular progesterone medication(s)	2	(Jefferson et al., 2011), (Leonard et al., 2010), (Motil et al., 2008), (Zysman et al., 2006), (Roende et al., 2011)
In the presence of risk factors, a baseline bone mineral density measurement should be performed	4	Expert opinion
Consider using the following techniques to assess bone health:		

Densitometry (DXA)	4	Expert opinion
Lateral spine X-ray	4	Expert opinion
Peripheral quantitative computed tomography (pQCT)	4	Expert opinion
Monitor bone mineral density every 1-2 years depending on clinical presentation	4	Expert opinion
If a long bone was fractured, the bone mineral density should also be measured in the alternate long bone	4	Expert opinion
If a vertebra was fractured, the bone mineral density may be measured in adjacent vertebrae excluding measurement of the fractured vertebrae	4	Expert opinion

Table 6.3: Bone mineral density assessment technique

Statements	**Level of evidence**	**References**
Where local normative data exists, measure the bone mineral content and areal bone mineral density in the total body minus the cranial bones (headless), and the postero-anterior lumbar spine	1	(Rauch et al., 2008)

Total hip and proximal femur bone mineral content and areal bone mineral density measurements are not considered a reliable site for measurement due to difficulties with subject positioning	1	(Rauch et al., 2008)
Z scores should be calculated from raw values for the following:		
Age	2	(Jefferson et al., 2011), (Hogler et al., 2003), (Zacharin, 2009)
Height	2	(Jefferson et al., 2011), (Hogler et al., 2003), (Zacharin, 2009)
Bone mineral apparent density (or volumetric bone mass density) adjustment is also recommended where possible	1	(Rauch et al., 2008)
The same skeletal sites should be assessed when repeating densitometry measures longitudinally	4	Expert opinion
In individuals with spinal rods, the bone mineral content and areal bone mineral density for the lateral distal femur and the total body minus the cranial bones (headless) should be measured	4	Expert opinion
To reduce unnecessary movement during bone mineral density scan procedures, calming techniques such as music, the presence of carers/parents, swaddling or sedation may be used	4	Expert opinion
Where possible densitometry measurements of lean tissue mass should be assessed	2	(Jefferson et al., 2011), (Hogler et al., 2003), (Zacharin, 2009),

Table 6.4: Non-pharmacological intervention

Statements	Level of evidence	References
Increase physical activity in order to increase muscle strength and bone density	2	(Baxter-Jones et al., 2008), (Fulkerson et al., 2004), (Fragala-Pinkham, Haley, Rabin & Kharasch, 2005), (Lotan et al., 2004)
In order to increase physical activity, refer to a physiotherapist for development of an optimal physical activity plan	4	Expert opinion
For those who are wheelchair bound, where possible:		
Encourage supported standing during transferring	1	(Downs et al., 2008)
Use a standing frame for at least 30 minutes a day	1	(Downs et al., 2008)
For those who are able to walk, aim to increase the distance and/or the length of time walked each day (aiming for 2 hours per day where possible)	1	(Downs et al., 2008)
Where mobility is limited, targeted exercise such as body weight supported treadmill or assisted walking is recommended	2	(Lotan et al., 2004), (Fragala-Pinkham et al., 2005), (Ruck, Chabot & Rauch, 2010)

If calcium intake is low, increase dietary intake of calcium rich or calcium fortified foods	1,2,3	(Sanders et al., 2009), (Salamoun et al., 2005), (Heaney, 2003), (Branca 2001), (Bailey et al., 2000), (Baer et al., 1997)
If dietary calcium intake is low and difficult to increase using dietary means, prescribe calcium supplements to meet the local recommended daily intake. The current recommended dietary intake levels within Australia are: 1-3yr 500mg/day, 4-8yr 700mg/day, 9-11yr 1000mg/day, 12-13yr 1300mg/day, 14-18yr 1300mg/day, >18yr 1000mg/day of elemental calcium. When prescribing medication please verify the content of elemental calcium in the preparation		
If 25 hydroxyvitamin D levels are lower than 75nmol/L:	1	(Working Group of the Australian New Zealand Bone Mineral Society, 2005)
Use local protocols for treatment and supplementation	1	(Working Group of the Australian New Zealand Bone Mineral Society, 2005)
Re-assess 25 hydroxyvitamin D levels after 4-8 weeks, then annually	4	Expert opinion

Advise an appropriate amount of sunlight exposure based on latitude, time of day, season and skin type	1,2	(Vanlint et al., 2008), (Working Group of the Australian New Zealand Bone Mineral Society, 2005)

Table 6.5: Pharmacological intervention

Statements	**Level of evidence**	**References**
Bisphosphonates should be used if the International Society for Clinical Densitometry criteria for osteoporosis in children and adolescents are fulfilled	1,2	(Rauch, 2008), (Ward et al., 2009), (Drake, Clarke & khosia, 2008)
The intravenous dosage of Bisphosphonates should follow evidence-based protocols	4	Expert opinion
Reassess bone mineral content and areal bone mineral density one year after Bisphosphonate therapy to decide on further therapy	4	Expert opinion
If reassessment of bone mineral content and areal bone mineral density shows limited response, review the therapeutic approach	4	Expert opinion

If hormonal intervention for regulation of the menstrual cycle is needed, use of Depot medroxyprogesterone acetate (DMPA) should be avoided	1,2	(Kuohung, Borgatta & Stubblefield, 2000), (Lopez 2009), (Cromer & Harel, 2003)
Although Levonorgestrel-releasing intrauterine system (LNG-IUS, Mirena) does not negatively affect bone density, communication difficulties during insertion need to be considered	4	Expert opinion

Appendix C

Coauthor Publication permission and contribution statements

TO WHOM ITY MAY CONCERN: 02/18/2016

I, Jay R. Shapiro, MD as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND

Drafting the work or revising it critically for important intellectual content; AND

Final approval of the version to be published; AND

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:

Drafting the work AND revising it critically for important intellectual content

Jay R. Shapiro, MD

From: Anne-Marie Bisgaard Pedersen <Anne-Marie.Bisgaard.Pedersen@regionh.dk>
Date: Thursday, 11 February 2016 4:18 PM
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Subject: SV: PLOS ONE guidelines publication and permission statement

I Anne-Marie Bisgaard as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Mary Jones, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Alan Percy, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
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Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content.

Alan Percy

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I, Leanne Ward MD, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Sue Thompson as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:

Revising it critically for important intellectual content

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I, Jane Lane, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
My contribution to the paper was as follows:
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To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
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Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Kathleen J. Motil, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

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I Steve Skinner, MD as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Dr. Peter Humphreys, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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I, Aris Siafarikas, as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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I Meir Lotan as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
or the acquisition, analysis, or interpretation of data for the work

Skellefteå,
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Statement

I, Gunilla Larsson, as co-author, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Participating in the expert panel
Revising the paper critically for important intellectual content

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I Prof Bruria Ben-Zeev as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows: Inserting the Israeli data and approach to bone health in Rett syndrome patients
Drafting the work or revising It critically for important intellectual content

Sincerely
Prof Bruria Ben-Zeev
Head of Pediatric Neurology Unit andIsraeli Rett Ctr
Safra Pediatric Hospital
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I Craig Munns, as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
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Subject: RE: Coauthor permission statement

I, Ami Bebbington, as coauthor, grant Amanda Jefferson permission to use the article titled "Bone mineral content and density in Rett syndrome and their contributing factors" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; and the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work and revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Statistical analysis of data collected & interpreted by Amanda with methodology directed by Amanda and other co-authors
Drafting the tables and figures of the work under direction from co-authors
Revising the work for statistical accuracy in the methods and results section

Kind Regards

Ami Bebbington

TELETHON KIDS INSTITUTE
Discover. Prevent. Cure.

From: "Julie Briody (SCHN)" <julie.briody@health.nsw.gov.au>
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Subject: RE: publication and permission statement

I, Julie Briody, as coauthor, grant Amanda Jefferson permission to use the article titled "Bone mineral content and density in Rett syndrome and their contributing factors" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Critical revision of the work
Calculation of BMD z-scores

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Date: Monday, 22 February 2016 12:18 PM
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I, Peter Jacoby, as coauthor, grant Amanda Jefferson permission to use the article titled "Bone mineral content and density in Rett syndrome and their contributing factors" and "Longitudinal bone mineral content and density in Rett syndrome and their contributing factors" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Analysis and interpretation of data for the work

Regards
Peter

From: Jenny Downs <Jenny.Downs@telethonkids.org.au>
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Subject: Re: Coauthor permission and contribution

I, Jenny Downs as coauthor, grant Amanda Jefferson permission to use the articles titled "Longitudinal bone mineral content and density in Rett syndrome and their contributing factors" and "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the papers as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the papers as co-supervisor was as follows:
Substantial contributions to the conception, design and the interpretation of data for the work; AND
Revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Best wishes,
Jenny

From: "Carolyn Ellaway (SCHN)" <carolyn.ellaway@health.nsw.gov.au>
Date: Friday, 12 February 2016 9:55 AM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Cc: Helen Leonard <Helen.Leonard@telethonkids.org.au>
Subject: RE: PLOS ONE guidelines publication and permission statement

I, Carolyn Ellaway as co-author, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work

Kind regards
Carolyn Ellaway MBBS PhD FRACP CGHGSA
Senior Staff Specialist | Genetic Metabolic Disorders Service I Senior Clinical Lecturer Sydney University
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The Sydney children's Hospitals Network

From: BirgitSyhler<bisy02@hotmail.com>
Date:Tue,1 Mar 2016 17:45:04+0100
To: HealthSciences<Amanda.Jefferson@curtin.edu.au>
Subject: Permission to use article

I, Birgit Syhler, as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

Best regards
Birgit Syhler

I, Ingegered Witt Engerström, as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND
Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

Ingegerd Witt Engerström
MD, Ph.D, Neuropediatrician
Swedish National Rett Center
Frösön
Sweden

From: "Dr. Vais Batya" <Batya.Vais@sheba.health.gov.il>
Date: Monday, 22 February 2016 2:40 PM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: RE: PLOS ONE guidelines publication and permission statement

I Batia Weiss as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content
Sincerely
Dr. Batia Weiss
Director, Pediatric Gastroenterology& Nutrition Unit Edmond & Lily Safra Children's Hospital, Tel- Hashomer weissb@sheba.health.gov.il
Tel:03-53028468, 03-5305006

From: Boyd Strauss <boyd.strauss@monash.edu>
Date: Tuesday, 23 February 2016 4:26 AM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: Re: Rett syndrome article: Coauthor permission and contribution

I, BOYD JG STRAUSS, as coauthor, grant Amanda Jefferson permission to use the article titled "Bone mineral content and density in Rett syndrome and their contributing factors" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
The acquisition, analysis, or interpretation of data for the work; AND Drafting the work or revising it critically for important intellectual content Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Boyd JG Strauss MBBS (Monash) PhD (Deakin) FRACP FRCPath FRCP (Edin)
Professor of Medicine (adjunct)
Dept. of Medicine, School of Clinical Sciences, Monash University
Clayton, Victoria, 3168
Australia

From: Daniel Tarquinio <danieltarq@gmail.com>
Date: Tuesday, 23 February 2016 11:58 PM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: Re: PLOS ONE guidelines publication and permission statement

I Daniel C. Tarquinio as coauthor, grant Amanda Jefferson permission to use the article titled "*Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence*" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND

Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

On 22/02/16 7:16 PM, "Mercedes Pineda Marfa" <pineda@hsjdbcn.org> wrote:

I M.Pineda as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

Dra Mercé Pineda
Barcelona
España

From: Suzanne Geerts <sgeerts@uab.edu>
Date: Monday, 22 February 2016 10:25 PM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: RE: PLOS ONE guidelines publication and permission statement

I Suzanne Geerts as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

From: Torkel Brismar <torkel.brismar@gmail.com>
Date: Monday, 22 February 2016 3:04 PM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: Re: FW: PLOS ONE guidelines publication and permission statement

I Torkel Brismar as coauthor, grant Amanda Jefferson permission to use the article titled "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the paper as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the paper was as follows:
Drafting the work or revising it critically for important intellectual content

From: Sue Fyfe <S.Fyfe@curtin.edu.au>
Date: Thursday, 25 February 2016 12:39 PM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: Re: coauthor contributions

I Sue Fyfe as coauthor, grant Amanda Jefferson permission to use the articles titled "Bone mineral content and density in Rett syndrome and their contributing factors", "Longitudinal bone mineral content and density in Rett syndrome and their contributing factors" and "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the papers as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the papers was as follows:
Study design and review
Contribution to analysis design
Draft review and writing

Kind regards
Sue
Adj Professor Sue Fyfe
Department of Medical Education | Faculty of Health Sciences
Email | mailto: s.fyfe@curtin.edu.au
Web | http://curtin.edu.au

From: Helen Leonard <Helen.Leonard@telethonkids.org.au>
Date: Monday, 29 February 2016 12:15 AM
To: Amanda Jefferson <amanda.jefferson@curtin.edu.au>
Subject: Coauthor statement

I, Helen Leonard as coauthor and Amanda's PhD supervisor, grant Amanda Jefferson permission to use the articles titled "Bone mineral content and density in Rett syndrome and their contributing factors", "Longitudinal bone mineral content and density in Rett syndrome and their contributing factors" and "Clinical guidelines for management of bone health in Rett syndrome based on expert consensus and available evidence" as part of her thesis.

To the best of my knowledge, Amanda's contribution to the papers as first author was as follows:
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

My contribution to the papers was as follows:
Contributions to the conception or design of the work; and the acquisition, analysis, and interpretation of data for the work; AND
Revising the work critically for important intellectual content; AND Final approval of the version to be published; AND
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Appendix D

Ethics approval: Development of best practice guidelines for prevention and management of low bone density and fracture in Rett syndrome

memorandum

To	Dr Jennepher Downs, Physiotherapy
From	A/Professor Stephan Millett, Chair, Human Research Ethics Committee
Subject	Protocol Approval **HR 44/2010**
Date	01 July 2010
Copy	Amanda Jefferson, School of Public Health Helen Leonard, Telethon Institute for Child Health Research Susan Fyfe, Physiotherapy Graduate Studies Officer, Faculty of Health Sciences

Curtin University of Technology
Office of Research and Development
Human Research Ethics Committee
TELEPHONE 9266 2784
FACSIMILE 9266 3793
EMAIL hrec@curtin.edu.au

Thank you for your application submitted to the Human Research Ethics Committee (HREC) for the project titled "*Development of the Best Practice Guidelines for Prevention and Management of Low Bone Density and Fracture in Rett Syndrome*". Your application has been reviewed by the HREC and is **approved**.

- You have ethics clearance to undertake the research as stated in your proposal.
- The approval number for your project is **HR 44/2010.** *Please quote this number in any future correspondence.*
- Approval of this project is for a period of twelve months **01-07-2010** to **01-07-2011.** To renew this approval a completed Form B (attached) must be submitted before the expiry date **01-07-2011.**
- If you are a Higher Degree by Research student, data collection must not begin before your Application for Candidacy is approved by your Faculty Graduate Studies Committee.
- The following standard statement **must be** included in the information sheet to participants:
  This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 44/2010). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participants. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Applicants should note the following:

It is the policy of the HREC to conduct random audits on a percentage of approved projects. These audits may be conducted at any time after the project starts. In cases where the HREC considers that there may be a risk of adverse events, or where participants may be especially vulnerable, the HREC may request the chief investigator to provide an outcomes report, including information on follow-up of participants.

The attached **FORM B** should be completed and returned to the Secretary, HREC, C/- Office of Research & Development when the project has finished, or:

- If at any time during the twelve months changes/amendments occur, or
- If a serious or unexpected adverse event occurs, or
- 14 days prior to the expiry date if renewal is required.
- An application for renewal may be made with a Form B three years running, after which a new application form (Form A), providing comprehensive details, must be submitted.

Regards,

A/Professor Stephan Millett
Chair Human Research Ethics Committee

Ethic approval: Factors affecting the skeletal integrity in an Australian Rett syndrome cohort

memorandum

To	Professor Sue Fyfe, Public Health
From	A/Professor Stephan Millett, Chair, Human Research Ethics Committee
Subject	Protocol Approval **HR 51/2010**
Date	7 May 2010
Copy	Amanda Jefferson, Public Health Dr Jennepher Downs, Physiotherapy Professor Helen Leonard, ICHR Graduate Studies Officer, Faculty of Health Sciences

Curtin University of Technology

Office of Research and Development

Human Research Ethics Committee

TELEPHONE 9266 2784
FACSIMILE 9266 3793
EMAIL hrec@curtin.edu.au

Thank you for your application submitted to the Human Research Ethics Committee (HREC) for the project titled "*Factors affecting the skeletal integrity in an Australian Rett Syndrome Cohort*". The Committee notes the prior approval by Princess Margaret Hospital (447/EP) and has reviewed your application consistent with Chapter 5.3 of the *National Statement on Ethical Conduct in Human Research*.

- You have ethics clearance to undertake the research as stated in your proposal.
- The approval number for your project is **HR 51/2010.** *Please quote this number in any future correspondence.*
- Approval of this project is for a period of twelve months **07-05-2010** to **07-05-2011**. To renew this approval a completed Form B (attached) must be submitted before the expiry date **07-05-2011**.
- If you are a Higher Degree by Research student, data collection must not begin before your Application for Candidacy is approved by your Faculty Graduate Studies Committee.
- The following standard statement **must be** included in the information sheet to participants:

  This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 51/2010). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participants. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Applicants should note the following:

It is the policy of the HREC to conduct random audits on a percentage of approved projects. These audits may be conducted at any time after the project starts. In cases where the HREC considers that there may be a risk of adverse events, or where participants may be especially vulnerable, the HREC may request the chief investigator to provide an outcomes report, including information on follow-up of participants.

The attached **FORM B** should be completed and returned to the Secretary, HREC, C/- Office of Research & Development:
When the project has finished, or

- If at any time during the twelve months changes/amendments occur, or
- If a serious or unexpected adverse event occurs, or
- 14 days prior to the expiry date if renewal is required.
- An application for renewal may be made with a Form B three years running, after which a new application form (Form A), providing comprehensive details, must be submitted.

Regards,

A/Professor Stephan Millett
Chair Human Research Ethics Committee

Bibliography

American Association of Clinical Endocrinologists (AACE). (2003). American Association of Clinical Endorcrinologists medical guidelines for clinical practice for the prevention and treatment of postmenopausal osteoporosis. *Endrocrine Practice*, 9(6), 544-564.

Adams, J. E., Engelke, K., Zemel, B. S., & Ward, K. A. (2014). Quantitative computer tomography in children and adolescents: The 2013 ISCD Pediatric Official Positions. *Journal of Clinical Densitometry*, 17(2), 258-274.

Ager, S., Fyfe, S., Christodoulou, J., Jacoby, P., Schmitt, L., & Leonard, H. (2006). Predictors of scoliosis in Rett syndrome. *Journal of Child Neurology*, 21(9), 809-813.

Al Wren, T., Lee, D. C., Kay, R. M., Dorey, F. J., & Gilsanz, V. (2011). Bone density and size in ambulatory children with cerebral palsy. *Developmental Medicine and Child Neurology*, 53(2), 137-141.

Amir, R. E., Van den Veyver, I. B., Schultz, R., Malicki, D. M., Tran, C. Q., Dahle, E. J., . . . Zoghbi, H. Y. (2000). Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes. *Annals of Neurology*, 47(5), 670-679.

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., & Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl- CpG-binding protein 2. *Nature Genetics*, 23(2), 185-188.

Anderson, A., Wong, K., Jacoby, P., Downs, J., & Leonard, H. (2014). Twenty years of surveillance in Rett syndrome: What does this tell us? *Orphanet Journal of Rare Diseases*, 9(87), 1-9.

Antoniazzi, F., Mottes, M., Fraschini, P., Brunelli, P. C., & Tato, L. (2000). Osteogenesis imperfecta: Practical treatment guidelines. *Paediatric drugs*, 2(6), 465-488.

Archer, H., Evans, J., Leonard, H., Colvin, L., Ravine, D., Christodoulou, J., . . . Clarke, A. (2007). Correlation between clinical severity in patients with Rett syndrome with a p.R168X or p.T158M MECP2 mutation, and the direction and degree of skewing of X-chromosome inactivation. *Journal of Medical Genetics*, 44(2), 148-152.

Bachrach, L. K. (2005). Osteoporosis and measurement of bone mass in children and adolescents. *Endocrinology and Metabolism Clinics of North America*, 34(3), 521-535.

Bachrach, L. K., & Sills, I. N. (2011). Clinical report-bone densitometry in children and adolescents. *Pediatrics*, 127(1), 189-194.

Baer, M. T., Kozlowski, B. W., Blyler, E. M., Trahms, C. M., Taylor, M. L., & Hogan, M. P. (1997). Vitamin D, calcium and bone status in children with developmental delay in relation to anticonvulsant use and ambulatory status. *American Journal of Clinical Nutrition, 65(4)*, 1042-1051.

Bailey, D. A., Martin, A. D., McKay, H. A., Whiting, S., & Mirwald, R. (2000). Calcium accretion in girls and boys during puberty: A longitudinal analysis. *Journal of Bone & Mineral Research*, 15(11), 2245-2250.

Baim, S., Binkley, N., Bilezikian, J. P., Kendler, D. L., Hans, D. B., Lewiecki, E. M., & Silverman, S. (2008). Official Positions of the International Society for Clinical Densitometry and executive summary of the 2007 ISCD Position Development Conference. *Journal of Clinical Densitometry*, 11(1), 75-91.

Baim, S., Wilson, C. R., Lewiecki, E. M., Luckey, M. M., Downs, R. W., Jr., & Lentle, B. C. (2005). Precision assessment and radiation safety for dual-energy X-ray absorptiometry: Position paper of the International Society for Clinical Densitometry. *Journal of Clinical Densitometry*, 8(4), 371-378.

Baroncelli, G. I. (2008). Quantitative ultrasound methods to assess bone mineral status in children: Technical characteristics, performance, and clinical application. *Pediatric Research*, 63(3), 220-228.

Baxter-Jones, A. D. G., Eisenmann, J. C., Mirwald, R. L., Faulkner, R. A., & Bailey, D. A. (2008). The influence of physical activity on lean mass accrual during adolescence: A longitudinal analysis. *Journal of Applied Physiology*, 105(2), 734-741.

Baxter-Jones, A. D. G., Kontulainen, S. A., Faulkner, R. A., & Bailey, D. A. (2008). A longitudinal study of the relationship of physical activity to bone mineral accrual from adolescence to young adulthood. *Bone*, 43(6), 1101-1107.

Bebbington, A., Anderson, A., Ravine, D., Fyfe, S., Pineda.M., de Klerk, N., . . . Leonard, H. (2008). Investigating genotype-phenotype relationships in Rett syndrome using an international dataset. *Neurology*, 70(11), 868-875.

Bebbington, A., Percy, A., Christodoulou, J., Ravine, D., Ho, G., Jacoby, P., . . . Leonard, H. (2010). Updating the profile of C-terminal MECP2 deletions in Rett syndrome. *Journal of Medical Genetics*, 47(4), 242-248.

Bergman, C., Gray-Scott, D., Chen, J.-J., & Meacham, S. (2009). What is next for the dietary reference intakes for bone metabolism related nutrients beyond calcium, phosphorus, magnesium, vitamin D, and fluoride. *Critical Reviews in Food Science and Nutrition*, 49(2), 136-144.

Berven, S., & Bradford, D. S. (2002). Neuromuscular scoliosis: Causes of deformity and principles for evaluation and management. *Seminars in Neurology*, 22(2), 167-178.

Bianchi, M. L., Leonard, M. B., Bechtold, S., Hogler, W., Mughal, M. Z., Schonau, E., . . . Ward, L. (2014). Bone health in children and adolescents with chronic diseases that may affect the skeleton: The 2013 ISCD Pediatric Official Positions. *Journal of Clinical Densitometry*, 17(2), 281-294.

Binkley, T. L., Berry, R., & Specker, B. L. (2008). Methods for measurement of pediatric bone. *Reviews in Endocrine & Metabolic Disorders*, 9(2), 95-106.

Binkovitz, L. A., & Henwood, M. J. (2007). Pediatric DXA: Technique and interpretation. *Pediatric Radiology*, 37(1), 21-31.

Bishop, N., Arundel, P., Clark, E., Dimitri, P., Farr, J., Jones, G., . . . Shaw, N. (2014). Fracture prediction and the definition of osteoporosis in children and adolescents: The ISCD 2013 pediatric official positions. *Journal of Clinical Densitometry: Assessment & Management of Musculoskeletal Health*, 17(2), 275-280.

Blake, G. M., & Fogelman, I. (1997). Technical principles of dual energy x-ray absorptiometry. *Seminars in Nuclear Medicine*, 27(3), 210-228.

Blake, G. M., & Fogelman, I. (2001). Bone densitometry and the diagnosis of osteoporosis. *Seminars in Nuclear Medicine*, 31(1), 69-81.

Blake, G. M., Naeem, M., & Boutros, M. (2006). Comparison of effective dose to children and adults from dual X-ray absorptiometry examinations. *Bone*, 38(6), 935-942.

Bolton, J., Patel, S., Lacey, H., & White, S. (2005). A prospective study of changes in bone turnover and bone density associated with regaining weight in women with anorexia nervosa. *Osteoporosis International,* 16(12), 1955–1962.

Bonjour, J., & Chevalley, T. (2014). Pubertal timing, bone acquisition, and risk of fracture throughout life. *Endocrine Reviews*, 35(5), 820-847.

Boskey, A. L. (2013). Bone composition: Relationship to bone fragility and antiosteoporotic drug effects. *BoneKEy Reports*, 2(447), 1-11.

Boyce, A. M., Tosi, L. L., & Paul, S. M. (2014). Bisphosphonate treatment for children with disabling conditions. *The Journal of Injury, Function and Rehabilitation*, 6(5), 427-436.

Branca, F., & Valtuena, S. (2001). Calcium, physical activity and bone health--building bones for a stronger future. *Public Health Nutrition*, 4(1A), 117-123.

Budden, S. S. (1997). Understanding, recognizing and treating Rett syndrome. *Medscape Womens Health*, 2(3), 1-11.

Budden, S. S., & Gunness, M. E. (2001). Bone histomorphometry in three females with Rett syndrome. *Brain and Development*, 23(1), S133-137.

Budden, S. S., & Gunness, M. E. (2003). Possible mechanisms of osteopenia in Rett syndrome: Bone histomorphometric studies. *Journal of Child Neurology*, 18(10), 698-702.

Caffarelli, C., Gonnelli, S., Tanzilli, L., Hayek, J., Vichi, V., Franci, M. B., . . . Nuti, R. (2012). The relationship between serum ghrelin and body composition with bone mineral density and QUS parameters in subjects with Rett syndrome. *Bone*, 50(4), 830-835.

Carter, D. R., Bouxsein, M. L., & Marcus, R. (1992). New approaches for interpreting projected bone densitometry data. *Journal of Bone Mineral Research*, 7(2), 137-145.

Cass, H., Reilly, S., Owen, L., Wisbeach, A., Weekes, L., Slonims, V., . . . Charman, T. (2003). Findings from a multidisciplinary clinical case series of females with Rett syndrome. *Developmental Medicine and Child Neurology*, 45(5), 325-337.

Caulton, J. M., Ward, K. A., Alsop, C. W., Dunn, G., Adams, J. E., & Mughal, M. Z. (2004). A randomised controlled trial of standing programme on bone mineral density in non-ambulant children with cerebral palsy. *Archives of Disease in Childhood*, 89(2), 131-135.

Centers for Disease Control and Prevention (CDC). (2005). Clinical Growth Charts. Retrieved www.cdc.gov/growthcharts/clinical_charts.htm

Cepollaro, C., Gonnelli, S., Bruni, D., Pacini, S., Martini, S., Franci, M. B., . . . Gennari, C. (2001). Dual X-ray absorptiometry and bone ultrasonography in patients with Rett syndrome. *Calcified Tissue International*, 69(5), 259-262.

Chad, K. E., Bailey, D. A., McKay, H. A., Zello, G. A., & Snyder, R. E. (1999). The effect of a weight-bearing physical activity program on bone mineral content and estimated volumetric density in children with spastic cerebral palsy. *The Journal of Pediatrics*, 135(1), 115-117.

Chahrour, M., Jung, S. Y., Shaw, C., Zhou, X., Wong, S. T. C., Qin, J., & Zoghbi, H., Y. (2008). MECP2, a key contributor to neurological disease, activates and represses transcription. *Science*, 320(1), 1224-1229.

Chen, C., Ke, J., Wang, C., Wu, K. P., Wu, C., & Wong, A. M. (2011). Factors associated with bone density in different skeletal regions in children with cerebral palsy of various motor severities. *Developmental Medicine and Child Neurology*, 53(2), 131-141.

Chen, P. Y., Toroian, D., Price, P. A., & McKittrick, J. (2011). Minerals form a continuum phase in mature cancellous bone. *Calcified Tissue International*, 88(5), 351-361.

Christodoulou, J., & Weaving, L. S. (2003). MECP2 and beyond: Phenotype-genotype correlations in Rett syndrome. *Journal of Child Neurology*, 18(10), 669-674.

Chumlea, W. C., Schubert, C. M., Roche, A. F., Kulin, H. E., Lee, P. A., Himes, J. H., & Sun, S. S. (2003). Age at menarche and racial comparisons in US girls. *Pediatrics*, 111(1), 110-113.

Chun, K. J. (2011). Bone densitometry. *Seminars in Nuclear Medicine*, 41(3), 220-228.

Clark, E. M., Tobias, J. H., & Ness, A. R. (2006). Association between bone density and fractures in children: A systematic review and meta-analysis. *Pediatrics*, 117(2), e291-297.

Colvin, L., Leonard, H., de Klerk, N., Davis, M., Weaving, L., Williamson, S., & Christodoulou, J. (2004). Refining the phenotype of common mutations in Rett syndrome. *Journal of Medical Genetics*, 41(1), 25-30.

Coppola, G., Fortunato, D., Auricchio, G., Mainolfi, C., Operto, F., Signoriello, G., . . . Salvatore, M. (2009). Bone mineral density in children, adolescents, and young adults with epilepsy. *Epilepsia, 50*(9), 2140-2146.

Cooper, C., Dennison, E. M., Leufkens, H. G., Bishop, N., & Van Staa, T. P. (2004). Epidemiology of childhood fractures in Britain: A study using the general practice research database. *Journal of Bone and Mineral Research*, 19(12), 1976-1981.

Crabtree, N., & Ward, K. (2009). Bone densitometry: Current status and future perspectives. *Endocrine Development*, 16(1), 58-72.

Cranney, A., Horsley, T., O'Donnell, S., Weiler, H., Puil, L., Ooi, D., . . . Mamaladze, V. (2007). Effectiveness and safety of vitamin D in relation to bone health. *Evidence Report Technology Assessment*, 158(1), 1-235.

Cromer, B., & Harel, Z. (2000). Adolescents: At increased risk for osteoporosis? *Clinical Pediatrics*, 39(10), 565-574.

Cuddapah, V. A., Pillai, R. B., Shekar, K. V., Lane, J. B., Motil, K. J., Skinner, S. A., . . . Olsen, M. L. (2014). Methyl-CpG-binding protein 2 (MECP2) mutation type is associated with disease severity in Rett syndrome. *Journal of Medical Genetics*, 51(3), 152-158.

Dallas, S. L., Prideaux, M., & Bonewald, L. F. (2013). The osteocyte: An endocrine cell ... and more. *Endocrine Reviews*, 34(5), 658-690.

Dalskov, S., Muller, M., Ritz, C., Damsgaard, C. T., Papadaki, A., Saris, W. H., . . . Molgaard, C. (2014). Effects of dietary protein and glycaemic index on biomarkers of bone turnover in children. *British Journal of Nutrition*, 111(1), 1253-1262.

Damilakis, J., Solomou, G., Manios, G. E., & Karantanas, A. (2013). Pediatric radiation dose and risk from bone density measurements using a GE Lunar Prodigy scanner. *Osteoporosis International*, 24(7), 2025-2031.

Davies, J. H., Evans, B. A. J., & Gregory, J. W. (2005). Bone mass acquisition in healthy children. *Archives of Disease in Childhood*, 90(4), 373-378.

Downs, J., Bebbington, A., Woodhead, H., Jacoby, P., Jian, L., Jefferson, A., & Leonard, H. (2008). Early determinants of fractures in Rett syndrome. *Pediatrics*, 121(3), 540-546.

Downs, J., Bergman, A., Carter, P., Anderson, A., Palmer, G. M., Roye, D., . . . Leonard, H. (2009). Guidelines for management of scoliosis in Rett syndrome patients based on expert consensus and clinical evidence. *Spine,* 34(17), E607-617.

Downs, J., G!eranton, S. M., Bebbington, A., Jacoby, P., Bahi-Buisson, N., Ravine, D., & Leonard, H. (2010). Linking MECP2 and pain sensitivity: The example of Rett syndrome. *American Journal of Medical Genetics*, 152A(5), 1197–1205.

Downs, J., Leonard, H., & Hill, K. (2012). Initial assessment of the StepWatch Activity MonitorTM to measure walking activity in Rett syndrome. *Disability and Rehabilitation*, 34(12), 1010-1015.

Downs, J., Stahlhut, M., Kingsley, W., Syhler, B., Bisgaard, M., Jacoby, P., & Leonard, H. (2016). Validating the Rett syndrome gross motor scale. *PLoS ONE*, 11(1), 1-11.

Downs, J., Torode, I., Wong, K., Ellaway, C., Elliott, E. J., Christodoulou, J., . . . Leonard, H. (2015). The natural history of scoliosis in females with Rett syndrome. *Spine*, 41(10), 856-863.

Downs, J. A., Bebbington, A., Jacoby, P., Msall, M. E., McIlroy, O., Fyfe, S., . . . Leonard, H. (2008). Gross motor profile in Rett syndrome as determined by video analysis. *Neuropediatrics*, 39(4), 205-210.

Dragich, J., Houwink-Manville, I., & Schanen, C. (2000). Rett syndrome: A surprising result of mutation in MECP2. *Human Molecular Genetics*, 9(16), 2365-2375.

Drake, M. T., Clarke, B. L., & Khosla, S. (2008). Bisphosphonates: mechanism of action and role in clinical practice. *Mayo Clinic Proceedings, 83*(9), 1032-1045.

Eastell, R. (2005). Role of oestrogen in the regulation of bone turnover at the menarche. *Journal of Endocrinology*, 185(2), 223-234.

Emaus, N., Berntsen, G. K. R., Joakimsen, R., & Fonnebo, V. (2005). Bone mineral density measures in longitudinal studies: The choice of phantom is crucial for quality assessment. The Tromso study, a population-based study. *Osteoporosis International*, 16(12), 1597-1603.

Engelke, K., & Gluer, C. C. (2006). Quality and performance measures in bone densitometry: Part 1: Errors and diagnosis. *Osteoporosis international*, 17(9), 1283-1292.

Fehlings, D., Switzer, L., Agarwal, P., Wong, C., Sochett, E., Stevenson, R., . . . Gaebler, D. (2012). Informing evidence-based clinical practice guidelines for children with cerebral palsy at risk of osteoporosis: A systematic review. *Developmental Medicine and Child Neurology*, 54(2), 106-116.

Fehr, S., Bebbington, A., Ellaway, C., Rowe, P., Leonard, H., & Downs, J. (2011). Altered attainment of developmental milestones influences the age of diagnosis of Rett syndrome. *Journal of Child Neurology*, 26(8), 980-987.

Fehr, S., Bebbington, A., Nassar, N., Downs, J., Ronen, G. M., De Klerk, N., & Leonard, H. (2011). Trends in the diagnosis of Rett syndrome in Australia. *Pediatric Research*, 70(3), 313-319.

Fewtrell, M. (2003). Bone densitometry in children assessed by dual x-ray absorptiometry: Uses and pitfalls. *Archives of Disease in Childhood*, 88(9), 795-798.

Flynn, J., Foley, S., & Jones, G. (2007). Can BMD assessed by DXA at age 8 predict fracture risk in boys and girls during puberty? An eight-year prospective study. *Journal of Bone and Mineral Research*, 22(9), 1463-1467.

Foley, K.-R., Downs, J., Bebbington, A., Jacoby, P., Girdler, S., Kaufmann, W. E., & Leonard, H. (2011). Change in gross motor abilities of girls and women with Rett syndrome over a 3- to 4-year period. *Journal of Child Neurology*, 26(10), 1237-1245.

Fragala-Pinkham, M. A., Haley, S. M., Rabin, J., & Kharasch, V. S. (2005). A fitness program for children with disabilities. *Physical Therapy, 85*(11), 1182-1200.

Frost, H. M., & Schnau, E. (2000). The "muscle-bone unit" in children and adolescents: A 2000 overview. *Journal of Pediatric Endocrinology and Metabolism*, 13(6), 571-590.

Fulkerson, J. A., Himes, J. H., French, S. A., Jensen, S., Petit, M. A., Stewart, C., . . . Jacobsen, K. (2004). Bone outcomes and technical measurement issues of bone health among children and adolescents: considerations for nutrition and physical activity intervention trials. *Osteoporosis International, 15*(12), 929-941.

Gazarian, M., Wiliams, K., Elliot, E., Chant, K., Longbottom, H., Mellis, C., . . . Ruben, A. (1999). Evaluation of a national surveillance unit. *Archives of Diseases in Children*, 80(1), 21-27.

Gilsanz, V., & Wren, T. (2007). Assessment of bone acquisition in childhood and adolescence. *Pediatrics*, 119(2), S145-149.

Glaze, D. G., Percy, A. K., Skinner, S., Motil, K. J., Neul, J. L., Barrish, J. O., . . . Lee, H. S. (2010). Epilepsy and the natural history of Rett syndrome. *Neurology*, 74(11), 909-912.

Glorieux, F. (2007). Experience with bisphosphonates in osteogenesis imperfecta. *Pediatrics*, 119(2), S163-165.

Golden, N. H., Abrams, S. A., & Committee on Nutrition. (2014). Optimizing bone health in children and adolescents. *Pediatrics*, 134(4), e1229-1243.

Gonnelli, S., Caffarelli, C., Hayek, J., Montagnani, A., Cadirni, A., Franci, B., . . . Nuti, R. (2008). Bone ultrasonography at phalanxes in patients with Rett syndrome: A 3-year longitudinal study. *Bone*, 42(4), 737-742.

Gordon, C. M., Leonard, M. B., Zemel, B. S., & International Society for Clinical Densitometry (ISCD). (2014). 2013 Pediatric Position Development Conference: executive summary and reflections. *Journal of Clinical Densitometry*, 17(2), 219-224.

Goulding, A., Taylor, R., Jones, I., McAule, K., Manning, P., & Williams, S. (2000). Overweight and obese children have low bone mass and area for their weight. *International Journal of Obesity*, 24(5), 627-632.

Haas, R. H., Dixon, S. D., Sartoris, D. J., & Hennessy, M. J. (1997). Osteopenia in Rett syndrome. *Journal of Pediatrics*, 131(5), 771-774.

Hagberg, B. (2002). Clinical manifestations and stages of Rett syndrome. *Mental Retardation and Developmental Disability Research Reviews,* 8(2), 61-65.

Hagberg, B. (2005). Rett syndrome: Long-term clinical follow-up experiences over four decades. *Journal of Child Neurology*, 20(9), 722-727.

Hagberg, B., Goutieres, F., Hanefield, F., Rett, A., & Wilson, J. (1985). Rett syndrome: Criteria for inclusion and exclusion. *Brain and Development*, 7(3), 372-373.

Hagberg, B. A., & Skjeldal, O. H. (1994). Rett variants: a suggested model for inclusion criteria. *Paediatric Neurology*, 11(1), 5-11.

Halton, J., Gaboury, I., Grant, R., Alos, N., Cummings, E. A., Matzinger, M., . . . Canadian, S. C. (2009). Advanced vertebral fracture among newly diagnosed children with acute lymphoblastic leukemia: Results of the Canadian Steroid-Associated Osteoporosis in the Pediatric Population (STOPP) research program. *Journal of Bone & Mineral Research*, 24(7), 1326-1334.

Harrison, D. J., & Webb, P. J. (1990). Scoliosis in the Rett syndrome: Natural history and treatment. *Brain and Development*, 12(1), 154-156.

Haas, R. H., Dixon, S. D., Sartoris, D. J., & Hennessy, M. J. (1997). Osteopenia in Rett syndrome. *Journal of Pediatrics*, 131(5), 771-774.

Hawkinson, J., Timins, J., Angelo, D., Shaw, M., Takata, R., & Harshaw, F. (2007). Technical white paper: bone densitometry. *Journal of American College of Radiology*, 4(5), 320-327.

He, S., Zurynski, Y., & Elliot, E. (2010). What do paediatricians think of the Australian Paediatric Surveillance Unit? *Journal of Paediatrics and Child Health*, 46(7-8), 412-418.

Heaney, R. P. (2003). How does bone support calcium homeostasis? *Bone, 33*(3), 264-268.

Hedstrom, E. M., Svensson, O., Bergstrom, U., & Michno, P. (2010). Epidemiology of fractures in children and adolescents. *Acta Orthopaedica*, 81(1), 148-153.

Henderson, R. C., Kairalla, J. A., Barrington, J. W., Abbas, A., & Stevenson, R. D. (2005). Longitudinal changes in bone density in children and adolescents with moderate to severe cerebral palsy. *The Journal of Pediatrics*, 146(6), 769-775.

Henderson, R. C., Lark, R. K., Kecskemethy, H. H., Miller, F., Harcke, T. H., & Bachrach, S. J. (2002). Bisphosphonates to treat osteopenia in children with quadriplegic cerebral palsy: a randomized, placebo-controlled clinical trial. The journal of pediatrics, 141(5), 644-651.

Hernandez, C. J., Beaupre, G. S., & Carter, D. R. (2003). A theoretical analysis of the relative influences of peak BMD, age-related bone loss and menopause on the development of osteoporosis. Osteoporosis International, 14(10), 843-847.

Heymsfield, S. B., Wang, Z., Baumgartner, R. N., & Ross, R. (1997). Human body composition: advances in models and methods. *Annual Review of Nutrition*, 17(1), 527-558.

Hind, K., & Burrows, M. (2007). Weight-bearing exercise and bone mineral accrual in children and adolescents: a review of controlled trials. *Bone*, 40(1), 14-27.

Hobbie, R. K., & Roth, B. J. (2007). *Intermediate physics for medicine and biology* (4th ed.). New York, USA: Springer Science + Business Media.

Hogler, W., Briody, J., Woodhead, H. J., Chan, A., & Cowell, C. T. (2003). Importance of lean mass in the interpretation of total body densitometry in children and adolescents. Journal of Pediatrics, 143(1), 81-88.

Hogler, W., & Ward, L. (2015). Calcium and bone disorders in children and adolescents. In J. Allgrove & N. J. Shaw (Eds.), *Osteoporosis in children* (2nd ed., Vol. 28, pp. 176-195). Basel, Switzerland: Karger.

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., . . . Weaver, C. M. (2011). Evaluation, treatment, and prevention of vitamin D deficiency: An Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology and Metabolism*, 96(7), 1911-1930.

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., . . . Weaver, C. M. (2012). Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. *Journal of Clinical Endocrinology and Metabolism,* 97(4), 1153-1158.

Houlihan, C. M., & Stevenson, R. D. (2009). Bone density in cerebral palsy. *Physical Medicine and Rehabilitation Clinics of North America,* 20(3), 493-508.

Hsu, C., & Sandford, B. A. (2007). The delphi technique: Making sense of consensus. *Practical Assessment Research and Evaluation*, 12(10), 1-8.

Hui, S. L., Slemenda, C. W., & Johnston, C. C., Jr. (1988). Age and bone mass as predictors of fracture in a prospective study. *Journal of Clinical Investigation*, 81(6), 1804-1809.

Huppke, P., Roth, C., Christen, H. J., Brockmann, K., & Hanefeld, F. (2001). Endocrinological study on growth retardation in Rett syndrome. *Acta Paediatrica*, 90(11), 1257-1261.

International Atomic Energy Agency (IAEA). (2013) *Dual energy x-ray absorptiometry for bone and composition assessment. Human health series* (Vol. 15). Austria. IAEA.

International Commission on Radiological Protection (ICRP). (2007). The 2007 recommendations of the International Commission on Radiological Protection. In J. Valentin (Ed.), *ICPR publication 103. Annals of the ICRP* (pp. 1-34): USA: Elsevier Ltd.

International Society of Clinical Densitometry (ISCD). (2015). *ISCD 2015 Official Positions – Adults.* USA: ISCD. Retrieved from http:// http://www.iscd.org/official-positions/2015-iscd-official-positions-adult/

Isaacs, J. S., Murdock, A., Lane, J., & Percy, A. K. (2003). Eating difficulties in girls with *Rett* syndrome compared with other developmental disabilities. *Journal of the American Dietetic Association, 103*(2), 224-230.

Janz, K. F., Gilmore, J. M., Burns, T. L., Levy, S. M., Torner, J. C., Willing, M. C., & Marshall, T. A. (2006). Physical activity augments bone mineral accrual in young children: The Iowa Bone Development study. *Journal of Pediatrics*, 148(6), 793-799.

Jefferson, A. L., Woodhead, H. J., Fyfe, S., Briody, J., Bebbington, A., Strauss, B. J., . . . Leonard, H. (2011). Bone mineral content and density in Rett syndrome and their contributing factors. *Pediatric Research*, 69(4), 293-298.

Jian, L., Archer, H., Ravine, D., Kerr, A., de Klerk, N., Christodoulou, J., . . . Leonard, H. (2005). p.R270X MECP2 mutation and mortality in Rett syndrome. *European Journal of Human Genetics*, 13(11), 1235-1238.

Jian, L., Nagarajan, L., de Klerk, N., Ravine, D., Bower, C., Anderson, A., . . . Leonard, H. (2006). Predictors of seizure onset in Rett syndrome. *The Journal of Pediatrics*, 149(4), 542-547.

Julian-Almarcegui, C., Gomez-Cabello, A., Huybrechts, I., Gonzalez-Aguero, A., Kaufman, J. M., Casajus, J. A., & Vicente-Rodriguez, G. (2015). Combined effects of interaction between physical activity and nutrition on bone health in children and adolescents: A systematic review. *Nutrition Reviews*, 73(3), 127-139.

Julu, P. O., Kerr, A. M., Apartopoulos, F., Al-Rawas, S., Witt Engerstrom, I., Engerstrom, L., . . . Hansen, S. (2001). Characterisation of breathing and associated central autonomic dysfunction in the Rett disorder. *Archives of Disease in Childhood*, 85(1), 29-37.

Kamal, B., Russell, D., Payne, A., Constante, D., Tanner, K. E., Isaksson, H., . . . Cobb, S. R. (2015). Biomechanical properties of bone in a mouse model of Rett syndrome. *Bone*, 71(1), 106-114.

Keret, D., Bassett, G. S., Bunnell, W. P., & Marks, H. G. (1988). Scoliosis in Rett syndrome. *Journal of Pediatric Orthopedics*, 8(2), 138-142.

Killian, J. T., Lane, J. B., Cutter, G. R., Skinner, S. A., Kaufmann, W. E., & Tarquinio, D. C. (2014). Pubertal development in Rett syndrome deviates from typical females. *Pediatric Neurology*, 51(6), 769-775.

Kling, J. M., Clarke, B. L., & Sandhu, N. P. (2014). Osteoporosis prevention, screening, and treatment: A review. *Journal of Women's Health*, 23(7), 563-572.

Knight, O., Bebbington, A., Siafarikas, A., Woodhead, H., Girdler, S., & Leonard, H. (2013). Pubertal trajectory in females with Rett syndrome: A population-based study. *Brain and Development*, 35(10), 912-920.

Kontulainen, S., Hughes, J., Macdonald, H., Johnston, J., Daly, R., & Petit, M. (2007). Optimizing bone mass and strength. The role of physical activity and nutrition during growth. *Medicine and Sport Science*, 51(1), 13-32.

Kuohung, W., Borgatta, L., & Stubblefield, P. (2000). Low-dose oral contraceptives and bone mineral density: an evidence-based analysis. *Contraception, 61*(2), 77-82.

Lang, F. T. (2010). Bone mineral assessment of the axial skeleton: Technical aspects. In R. A. Adler (Ed.), *Contemporary endocrinology: Osteoporosis: pathophysiology and clinical management* (2nd ed., pp. 23-50). LLC: Humana Press.

Larkin, A., Sheahan, N., O'Connor, U., Gray, L., Dowling, A., Vano, E., . . . Malone, J. F. (2008). QA/acceptance testing of DEXA x-ray systems used in bone mineral densitometry. *Radiation Protection Dosimetry*, 129(1-3), 279-283.

Laurvick, C. L., de Klerk, N., Bower, C., Christodoulou, J., Ravine, D., Ellaway, C., . . . Leonard, H. (2006). Rett syndrome in Australia: A review of the epidemiology. *Journal of Pediatrics*, 148(3), 347-352.

Laurvick, C. L., Msall, M. E., Silburn, S., Bower, C., de Klerk, N., & Leonard, H. (2006). Physical and mental health of mothers caring for a child with Rett syndrome. *Pediatrics*, 118(4), e1152-1164.

Lentle, B., Cheung, A. M., Hanley, D. A., Leslie, W. D., Lyons, D., Papaioannou, A., . . . Siminoski, K. (2011). Osteoporosis Canada 2010 guidelines for the assessment of fracture risk. *Canadian Association of Radiologists Journal*, 62(4), 243-250.

Leonard, H., Bower, C., & English, D. (1997). The prevalence and incidence of Rett syndrome in Australia. *European Child and Adolescent Psychiatry*, 6(1), 8-10.

Leonard, H., Downs, J., Jian, L., Bebbington, A., Jacoby, P., Nagarajan, L., . . . Woodhead, H. (2010). Valproate and risk of fracture in Rett syndrome. *Archives of Disease in Childhood*, 95(6), 444-448.

Leonard, H., Ravikumara, M., Baikie, G., Naseem, N., Ellaway, C., Percy, A., . . . Downs, J. (2013). Assessment and management of nutrition and growth in Rett syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 57(4), 451-460.

Leonard, H., Thomson, M. R., Glasson, E. J., Fyfe, S., Leonard, S., Bower, C., . . . Ellaway, C. (1999). A population based approach to the investigation of osteopenia in Rett syndrome. *Developmental Medicine and Child Neurology*, 41(5), 323-328.

Leonard, H., Thomson, R., Bower, C., Fyfe, S., & Constantinou, J. (1995). Skeletal abnormalities in Rett syndrome: Increasing evidence for dysmorphogenetic defects. *American Journal of Medical Genetics*, 58(3), 282-285.

Leonard. H, Slack-Smith. L, Phillips. T, Richardson, S., D'Orsogna, L., & Mulroy, S. (2004). How can the internet help parents of children with rare neurological disorders? *Journal of Child Neurology*, 19(11), 902-907.

Lidstrom, J., Stokland, E., & Hagberg, B. (1994). Scoliosis in Rett syndrome. Clinical and biological aspects. *Spine*, 19(14), 1632-1635.

Lopez, L. M., Grimes, D. A., Schulz, K. F., & Curtis, K. M. (2009). Steroidal contraceptives: effect on bone fractures in women (review). *Cochrane Database of Systematic Review, (2), 1-62.*

Lotan, M., Isakov, E., & Merrick, J. (2004). Improving functional skills and physical fitness in children with Rett syndrome. *Journal of Intellectual Disability Research*, 48(8), 730-735.

Lu, P. W., Briody, J. N., Ogle, G. D., Morley, K., Humphries, I. R., Allen, J., . . . Cowell, C. T. (1994). Bone mineral density of total body, spine, and femoral neck in children and young adults: A cross-sectional and longitudinal study. *Journal of Bone and Mineral Research*, 9(9), 1451-1458.

Ma, J., Siminoski, K., Alos, N., Halton, J., Ho, J., Lentle, B., . . . Canadian STOPP Consortium. (2015). The choice of normative pediatric reference database changes spine bone mineral density Z-scores but not the relationship between bone mineral density and prevalent vertebral fractures. *Journal of Clinical Endocrinology and Metabolism*, 100(3), 1018-1027.

Macdonald, H. M., Kontulainen, S. A., Petit, M. A., Beck, T. J., Khan, K. M., & McKay, H. A. (2008). Does a novel school-based physical activity model benefit femoral neck bone strength in pre- and early pubertal children? *Osteoporosis International*, 19(10), 1445-1456.

Marchand, V. (2009). Nutrition in neurologically impaired children. *Paediatric Child Health*, 14(6), 395-40.

Marr, C., Leonard, H., Torode, I., & Downs, J. (2015). Spinal fusion in girls with Rett syndrome: Family experiences and postoperative recovery. *Child Care, Health & Developmen*t, 41(6), 1000-1009.

Marshall, W. A., & Tanner, J. M. (1969). Variations in pattern of pubertal changes in girls. *Archives of Disease in Childhood*, 44(235), 291-303.

Martin, K., Rosenberg, M., Miller, M., French, S., McCormack, G., Bull, F., . . . Pratt, S. (2008). *Move and Munch final report. Trends in physical activity, nutrition and body size in Western Australian children and adolescents: The Child and Adolescent Physical Activity and Nutrition Survey* (CAPANS). Perth, Australia: Government of Western Australia.

Mergler, S., Evenhuis, H. M., Boot, A. M., De Man, S. A., Bindels-De Heus, K. G., Huijbers, W. A., & Penning, C. (2009). Epidemiology of low bone mineral density and fractures in children with severe cerebral palsy: A systematic review. *Developmental Medicine and Child Neurology*, 51(10), 773-778.

Moayyeri, A., Kaptoge, S., Dalzell, N., Bingham, S., Luben, R. N., Wareham, N. J., . .. Khaw, K. T. (2009). Is QUS or DXA better for predicting the 10-Year absolute risk of fracture? *Journal of Bone and Mineral Research*, 24(7), 1319-1325.

Molgaard, C., Thomsen, B. L., Prentice, A., Cole, T. J., & Michaelsen, K. F. (1997). Whole body bone mineral content in healthy children and adolescents. *Archives of Disease in Childhood*, 76(1), 9-15.

Morton, R. E., Bonas, R., Minford, J., Tarrant, S. C., & Ellis, R. E. (1997). Respiration patterns during feeding in Rett syndrome. *Developmental Medicine and Child Neurology*, 39(9), 607-613.

Motil, K. J., Barrish, J. O., Lane, J. B., Geerts, S. P., Annese, F., McNair, L., . . . Glaze, D. G. (2011). Vitamin D Deficiency is Prevalent in Females with Rett Syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 53(5), 569-574.

Motil, K. J., Barrish, J. O., Neul, J. L., & Glaze, D. G. (2014). Low bone mineral mass is associated with decreased bone formation and diet in girls with Rett syndrome. Journal of Pediatric Gastroenterology and Nutrition, 59(3), 386-392.

Motil, K. J., Caeg, E., Barrish, J. O., Geerts, S. P., Lane, J. B., Percy, A., . . . Glaze, D. G. (2012). Gastrointestinal and nutritional problems occur frequently throughout life in girls and women with Rett syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 55(3), 292-298.

Motil, K. J., Ellis, K. J., Barrish, J. O., Caeg, E., & Glaze, D. G. (2008). Bone mineral content and bone mineral density are lower in older than younger females with Rett syndrome. *Pediatric Research*, 64(4), 435-439.

Motil, K. J., Morrissey, M., Caeg, E., Barrish, J. O., & Glaze, D. G. (2009). Gastrostomy placement improves height and weight gain in girls with Rett syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 49(2), 237-242.

Motil, K. J., Schultz, R. J., Abrams, S., Ellis, K. J., & Glaze, D. G. (2006). Fractional calcium absorption is increased in girls with Rett syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 42(4), 419-426.

Motil, K. J., Schultz, R. J., Browning, K., Trautwein, L., & Glaze, D. G. (1999). Oropharyngeal dysfunction and gastroesophageal dysmotility are present in girls and women with Rett syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, 29(1), 31-37.

Munns, C. F., & Cowell, C. T. (2005). Prevention and treatment of osteoporosis in chronically ill children. *Journal of Musculoskeletal & Neuronal Interactions*, 5(3), 262-272.

Murphy, M. K., Black, N. A., Lamping, D. L., McKee, C. M., Sanderson, C. F., Askham, J., . . . Marteau, T. (1998). Consensus development methods, and their use in clinical guideline development. *Health Technology Assessment*, 2(3), i-88.

Nan, X., Meehan, R. R., & Bird, A. (1993). Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Research*, 21(21), 4886-4892.

Nan, X., Tate, P., Li, E., & Bird, A. (1996). DNA methylation specifies chromosomal localization of MeCP2. *Molecular and Cellular Biology*, 16(1), 414-421.

Naranje, S. M., Erali, R. A., Warner, W. C. J., Sawyer, J. R., & Kelly, D. M. (2016). Epidemiology of Pediatric Fractures Presenting to Emergency Departments in the United States. *Journal of Pediatric Orthopaedics*, 36(4), e45-e48.

Neul, J. L., Fang, P., Barrish, J. O., Lane, J., Caeg, E. B., Smith, E. O., . . . Percy, A. (2008). Specific mutations in Methyl-CpG-Binding Protein 2 confer different severity in Rett syndrome. *Neurology*, 70(16), 1313-1321.

Neul, J. L., Kaufmann, W. E., Glaze, D. G., Christodoulou, J., Clarke, A. J., Bahi-Buisson, N., . . . RettSearch Consortium. (2010). Rett syndrome: revised diagnostic criteria and nomenclature. *Annals of Neurology*, 68(6), 944-950.

Njeh, C. F., Fuerst, T., Hans, D., Blake, G. M., & Genant, H. K. (1999). Radiation exposure in bone mineral density assessment. *Applied Radiation Isotope*, 50(1), 215-236.

Njeh, C. F., Samat, S. B., Nightingale, A., McNeil, E. A., & Boivin, C. M. (1997). Radiation dose and in vitro precision in paediatric bone mineral density measurement using dual X-ray absorptiometry. *The British Journal of Radiology*, 70(835), 719-727.

O'Connor, R., Zayzafoon, M., Farach-Carson, M., & Schanen, N. (2009). Mecp2 deficiency decreases bone formation and reduces bone volume in a rodent model of Rett syndrome. *Bone*, 45(2), 346-356.

Oddy, W. H., Webb, K. G., Baikie, G., Thompson, S. M., Reilly, S., Fyfe, S. D., . . . Leonard, H. (2007). Feeding experiences and growth status in a Rett syndrome population. *Journal of Pediatric Gastroenterology and Nutrition*, 45(5), 582-590.

Osteoporosis Society of Canada. (1996). Clinical practice guidelines for the diagnosis and management of osteoporosis. *Medical Association Journal*, 155(8), 1113-1133.

Paxton, G. A., Teale, G. R., Nowson, C. A., Mason, R. S., McGrath, J. J., Thompson, M. J., . . . Munns, C. F. (2013). Vitamin D and health in pregnancy, infants, children and adolescents in Australia and New Zealand: A position statement. *Medical Journal of Australia*, 198(3), 142-143.

Percy, A. K., Lane, J. B., Childers, J., Skinner, S., Annese, F., Barrish, J., . . . MacLeod, P. (2007). Rett syndrome: North American database. *Journal of Child Neurology*, 22(12), 1338-1341.

Percy, A. K., Lee, H. S., Neul, J. L., Lane, J. B., Skinner, S. A., Geerts, S. P., . . . Glaze, D. G. (2010). Profiling scoliosis in Rett syndrome. *Pediatric Research*, 67(4), 435–439.

Pereira, R. M. R., Carvalho, J. F. d., Paula, A. P., Zerbini, C., Domiciano, D. S., Goncalves, H., . . . Andrada, N. C. (2012). Guidelines for the prevention and treatment of glucocorticoid-induced osteoporosis. *Revista Brasileira De Reumatologia,* 52(4), 580-593.

Pietrobelli, A., Faith, M. S., Wang, J., Brambilla, P., Chiumello, G., & Heymsfield, S. B. (2002). Association of lean tissue and fat mass with bone mineral content in children and adolescents. *Obesity Research*, 10(1), 56-60.

Rauch, F. (2007). Bone accrual in children: Adding substance to surfaces. *Pediatrics*, 119(2), S137-140.

Rauch, F., Plotkin, H., DiMeglio, L., Engelbert, R. H., Henderson, R. C., Munns, C., . . . Zeitler, P. (2008). Fracture prediction and the definition of osteoporosis in children and adolescents: the ISCD 2007 Pediatric Official Positions. *Journal of Clinical Densitometry, 11*(1), 22-28.

Rauch, F. (2012). The dynamics of bone structure development during pubertal growth. *Journal of Musculoskeletal Neuronal Interactions*, 12(1), 1-6.

Rauch, F., Bailey, D. A., Baxter-Jones, A., Mirwald, R., & Faulkner, R. (2004). The 'muscle-bone unit' during the pubertal growth spurt. *Bone*, 34(5), 771-775.

Reilly, S., & Cass, H. (2001). Growth and nutrition in Rett syndrome. *Disability and Rehabilitation*, 23(3-4), 118-128.

Reilly, S., Skuse, D., & Wolke, D. (2000). *Schedule for oral motor assessment*. London & Philedelphia: Whurr Publishers Ltd.

Remer, T., Boye, K. R., Hartmann, M., Neu, C. M., Schoenau, E., Manz, F., & Wudy, S. A. (2003). Adrenarche and bone modeling and remodeling at the proximal radius: Weak androgens make stronger cortical bone in healthy children. *Journal of Bone and Mineral Research*, 18(8), 1539-1546.

Renieri, A., Meloni, I., Longo, I., Ariani, F., Mari, F., Pescucci, C., & Cambi, F. (2003). Rett syndrome: the complex nature of a monogenic disease. *Journal of Molecular Medicine*, 81(6), 346-354.

Rett, A. (1966). On a unusual brain atrophy syndrome in hyperammonemia in childhood. *Wiener Medizinische Wochenschrift*, 116(37), 723-738.

Reyes, M. L., Hernandez, M., Holmgren, L. J., Sanhueza, E., & Escobar, R. G. (2011). High-frequency, low-intensity vibrations increase bone mass and muscle strength in upper limbs, improving autonomy in disabled children. *Journal of Bone and Mineral Researc*h, 26(8), 1759-1766.

Rimmer, J. H., Chen, M. D., McCubbin, J. A., Drum, C., & Peterson, J. (2010). Exercise intervention research on persons with disabilities: What we know and where we need to go. *American Journal of Physical Medicine & Rehabilitation*, 89(3), 249-263.

Roende, G., Petersen, J., Ravn, K., Fuglsang, K., Andersen, H., Nielsen, J. B., . . . Jensen, J. E. (2014). Low bone turnover phenotype in Rett syndrome: Results of biochemical bone marker analysis. *Pediatric Research*, 75(4), 551-558.

Roende, G., Ravn, K., Fuglsang, K., Andersen, H., Nielsen, J. B., Brondum-Nielsen, K., & Jensen, J.-E. B. (2011). DXA measurements in Rett syndrome reveal small bones with low bone mass. *Journal of Bone & Mineral Research*, 26(9), 2280-2286.

Roende, G., Ravn, K., Fuglsang, K., Andersen, H., Vestergaard, A., Brondum-Nielsen, K., . . . Nielsen, J. B. (2011). Patients with Rett syndrome sustain low-energy fractures. *Pediatric Research*, 69(4), 359-364.

Rosen, C. J., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., Durazo-Arvizu, R. A., . . . Taylor, C. L. (2012). IOM committee members respond to Endocrine Society vitamin D guideline. *Journal of Clinical Endocrinology and Metabolism*, 97(4), 1146-1152.

Royal College of Paediatrics and Child Health. (2006). *Setting standards for development of clinical guidelines in paediatrics and child health* (4th ed). UK: RCPCH.

Ruck, J., Chabot, G., & Rauch, F. (2010). Vibration treatment in cerebral palsy: A randomized controlled pilot study. *Journal of Musculoskeletal Neuronal Interactions, 10*(1), 77-83.

Saggese, G., Baroncelli, G. I., & Bertelloni, S. (2002). Puberty and bone development. Best practice & research. *Clinical Endocrinology & Metabolism*, 16(1), 53-64.

Saito, M., & Marumo, K. (2010) Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. *Osteoporosis International*, 21(1), 195-214.

Salamoun, M. M., Kizirian, A. S., Tannous, R. I., Nabulsi, M. M., Choucair, M. K., Deeb, M. E., & El-Hajj Fuleihan, G. A. (2005). Low calcium and vitamin D intake in healthy children and adolescents and their correlates. *European Journal of Clinical Nutrition, 59*(2), 177-184.

Sambrook, P. N., Seeman, E., Phillips, S. R., & Ebeling, P. R. (2002). Preventing osteoporosis: Outcomes of the Australian Fracture Prevention Summit. Osteoporosis Australia National Prescribing Service. *The Medical Journal of Australia*, 176(8), 1-16.

Sanders, K. M., Nowson, C. A., Kotowicz, M. A., Briffa, K., Devine, A., Reid, I. R., . . . Osteoporosis Australia and the Endocrine Society of Australia. (2009). Calcium and bone health: Position statement for the Australian and New Zealand Bone and Mineral Society, Osteoporosis Australia and the Endocrine Society of Australia. *Medical Journal of Australia*, 190(6), 316-320.

Schoenau, E., Neu, C. M., Rauch, F., & Manz, F. (2002). Gender-specific pubertal changes in volumetric cortical bone mineral density at the proximal radius. *Bone*, 31(1), 110-113.

Schousboe, J. T., Shepherd, J. A., Bilezikian, J. P., & Baim, S. (2013). Executive summary of the 2013 International Society for Clinical Densitometry Position Development Conference on bone densitometry. *Journal of Clinical Densitometry*, 16(4), 455-466.

Schwartzman, F., Vitolo, M. R., Schwartzman, J. S., & Morais, M. B. D. (2008). Eating practices, nutritional status and constipation in patients with Rett syndrome. *Arquivos de Gastroenterologia, 45*(4), 284-289.

Segawa, M. (2005). Early motor disturbances in Rett syndrome and its pathophysiological importance. *Brain and Development*, 27(1), S54-58.

Seibel, M. J. (2005). Biochemical markers of bone turnover. Part I: Biochemistry and variablility. *Clinical Biochemist Reviews*, 26(1), 97-122.

Seibel, M. J. (2006). Biochemical markers of bone turnover. Part II: Clinical applications in the management of osteoporosis. *Clinical Biochemist Reviews*, 27(3), 123-138.

Shapiro, J. R., Bibat, G., Hiremath, G., Blue, M. E., Yablonski, T., Kantipuly, A., . . . Naidu, S. (2010). Bone mass in Rett syndrome: Association with clinical parameters and MECP2 mutations. *Pediatric Research*, 68(5), 446-451.

Shapiro, J. R., Thompson, C. B., Wu, Y., Nunes, M., & Gillen, C. (2010). Bone mineral density and fracture rate in response to intravenous and oral bisphosphonates in adult osteogenesis imperfecta. *Calcified Tissue International*, 87(2), 120-129.

Sherwood, L. (2009). *Human physiology: From cells to systems*. New York: Wadsworth Publishing Company.

Shultz, R. J., Glaze, D. G., Motil, K. J., Armstrong, D. D., del Junco, D. J., Hubbard, C. R., & Percy, A. K. (1993). The pattern of growth failure in Rett syndrome. *American Journal of Diseases of Children*, 147(6), 633-637.

Scottish Intercollegiate Guideline Network (SIGN). (2011). *SIGN 50: A guideline developer's handbook*. Edinburgh: Scottish Intercollegiate Guideline Network.

Simm, P. J., Johannesen, J., Briody, J., McQuade, M., Hsu, B., Bridge, C., . . . Munns, C. (2011). Zoledronic acid improves bone mineral density, reduces bone turnover and improves skeletal architecture over 2 years of treatment in children with secondary osteoporosis. *Bone*, 49(5), 939-943.

Soliman, A., De Sanctis, V., & Elalaily, R. (2014). Nutrition and pubertal development. *Indian Journal of Endocrinology and Metabolism*, 18(1), S39-47.

Soyka, L. A., Grinspoon, S., Levitsky, L. L., Herzog, D. B., & Klibanski, A. (1999). The effects of anorexia nervosa on bone metabolism in female adolescents. *Journal of Clinical Endocrinology & Metabolism*, 84(12), 4489-4496.

Squillaro, T., Hayek, G., Farina, E., Cipollaro, M., Renieri, A., & Galderisi, U. (2008). A case report: Bone marrow mesenchymal stem cells from a Rett syndrome patient are prone to senescence and show a lower degree of apoptosis. *Journal of Cellular Biochemistry*, 103(6), 1877-1885.

Stuberg, W. A. (1992). Considerations related to weight-bearing programs in children with developmental disabilities. *Physical Therapy*, 72(1), 35-40.

Tarquinio, D. C., Motil, K. J., Hou, W., Lee, H.-S., Glaze, D. G., Skinner, S. A., . . . Percy, A. K. (2012). Growth failure and outcome in Rett syndrome: Specific growth references. *Neurology*, 79(16), 1653-1661.

Thommessen, M., Kase, B. F., & Heiberg, A. (1992). Growth and nutrition in 10 girls with Rett syndrome. *Acta Paediatrica*, 81(9), 686-690.

Uddenfeldt Wort, U., Nordmark, E., Wagner, P., Duppe, H., & Westbom, L. (2013). Fractures in children with cerebral palsy: A total population study. *Developmental Medicine and Child Neurology*, 55(9), 821-826.

Vanlint, S., Nugent, M., & Durvasula, S. (2008). Vitamin D and people with intellectual disability. *Australian Family Physician, 37*(5), 348-351.

Van Rijn, R. R., & Van Kuijk, C. (2009). Of small bones and big mistakes: Bone densitometry in children revisited. *European Journal of Radiology*, 71(3), 432-439.

Wagner, C. L., & Greer, F. R. (2008). Prevention of rickets and vitamin D deficiency in infants, children, and adolescents. *Pediatrics*, 122(5), 1142-1152.

Wang, M. C., Bachrach, L. K., Van Loan, M., Hudes, M., Flegal, K. M., & Crawford, P. B. (2005). The relative contributions of lean tissue mass and fat mass to bone density in young women. *Bone*, 37(4), 474-481.

Wang, Q., Nicholson, P. H., Suuriniemi, M., Lyytikainen, A., Helkala, E., Alen, M., . . . Cheng, S. (2004). Relationship of sex hormones to bone geometric properties and mineral density in early pubertal girls. *Journal of Clinical Endocrinology and Metabolism*, 89(4), 1698-1703.

Ward, L., Tricco, A., Phuong, P. N., Cranney, A., Barrowman, N., Gaboury, I., . . . Moher, D. (2009). Bisphosphonate therapy for children and adolescents with secondary osteoporosis (Review). *Cochrane Database of Systematic Reviews 2007*, (2), 1-42.

Ward, L. M., Rauch, F., Whyte, M. P., D'Astous, J., Gates, P. E., Grogan, D., . . . Glorieux, F. H. (2011). Alendronate for the treatment of pediatric osteogenesis imperfecta: A randomized placebo-controlled study. *The Journal of Clinical Endocrinology and Metabolism*, 96(2), 355-364.

Watson, K. C., Lentz, M. J., & Cain, K. C. (2006). Associations between fracture incidence and use of depot medroxyprogesterone acetate and anti-epileptic drugs in women with developmental disabilities. *Womens Health Issues*, 16(6), 346-352.

Weatherholt, A. M., Fuchs, R. K., & Warden, S. J. (2012). Specialized connective tissue: Bone, the structural framework of the upper extremity. *Journal of Hand Therapy*, 25(2), 123-132.

Webber, C. E. (2006). Photon absorptiometry, bone densitometry and the challenge of osteoporosis. *Physics in Medicine and Biology*, 51(13), R169-185.

Wei, S., Cai, Q., Qiu, W., Zhao, L., Ye, J., Wang, X., . . . Gu, X. (2012). Clinical study of oral alendronate in the treatment of pediatric osteogenesis imperfecta. *Chinese Medical Journal*, 92(4), 246-249.

Whiting, S. J., Vatanparast, H., Baxter-Jones, A., Faulkner, R. A., Mirwald, R., & Bailey, D. A. (2004). Factors that affect bone mineral accrual in the adolescent growth spurt. *Journal of Nutrition*, 134(3), 696S-700S.

Winzenberg, T., Powell, S., Shaw, K. A., & Jones, G. (2011). Effects of vitamin D supplementation on bone density in healthy children: Systematic review and meta-analysis. *British Medical Journal*, 342, c7254.

Wong, C. C., & McGirt, M. J. (2013). Vertebral compression fractures: A review of current management and multimodal therapy. *Journal of Multidisciplinary Healthcare*, 6(1), 205-214.

Working Group of the Australian New Zealand Bone Mineral Society. (2005). Vitamin D and adult bone health in Australia and New Zealand: a position statement. *Medical Journal of Australia, 182*(6), 281-285.

Young, B., & Heath, J. W. (2000). *Wheater's functional histology* (3rd ed.). London: Harcourt Publishers Limited, Churchill Livingstone.

Zacharin, M. (2004). Current advances in bone health of disabled children. *Opinion in Pediatrics*, 16(5), 545-551.

Zacharin, M. (2009). Assessing the skeleton in children and adolescents with disabilities: Avoiding pitfalls, maximising outcomes. A guide for the general paediatrician. *Journal of Paediatrics and Child Health*, 45(6), 326-331.

Zadik, Z., Price, D., & Diamond, G. (2003). Pediatric reference curves for multi-site quantitative ultrasound and its modulators. *Osteoporosis International*, 14(10), 857-862.

Zysman, L., Lotan, M., & Ben-Zeev, B. (2006). Osteoporosis in Rett syndrome: A study on normal values. *Scientific World Journal*, 6(1), 1619-1630.