THE ROLE OF SKELETAL MUSCLE ENERGETICS IN THE SYMPTOMS AND METABOLIC CHARACTERISTICS OF GROWTH HORMONE DEFICIENCY AND VITAMIN D DEFICIENCY:

A FUNCTIONAL MRS APPROACH

M.D. Thesis

Akash Sinha

Student number 089185643

Institute of Genetic Medicine

2013

''Your time is limited, so don't waste it living someone else's life. Don't be trapped by dogma — which is living with the results of other people's thinking. Don't let the noise of others' opinions drown out your own inner voice. And most importantly, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary''

''You can't connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future. You have to trust in something — your gut, destiny, life, karma, whatever. This approach has never let me down, and it has made all the difference in my life''

Steve Jobs, 2005

Abstract

Both growth hormone deficient (GHD) and vitamin D deficient adults complain of fatigue. Suboptimal skeletal muscle mitochondrial function has been implicated in several disorders where fatigue is a prominent feature. I examined *in vivo* dynamic skeletal muscle metabolism in GHD and vitamin D deficient adults using a non-invasive tool called phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS). ^{31}P MRS can quantify abnormalities in metabolic work-cost relationship during exercise.

In the first study, skeletal muscle metabolism was assessed in age, gender and physical activity matched untreated GHD adults, GH treated GHD adults and healthy volunteers. Fatigue perception was compared across the 3 groups using specific domains within a validated quality of life questionnaire (QoL-AGHDA). Whilst untreated GHD adults experienced more fatigue compared to treated GHD adults and normal volunteers, they did not demonstrate any perturbations in peripheral skeletal muscle metabolism as determined by ^{31}P MRS: maximal mitochondrial oxidative function, anaerobic glycolysis and proton clearance.

In the second study, the effect of cholecalciferol therapy on skeletal muscle mitochondrial function in vitamin D deficient adults who had presented to their local primary care team with fatigue was examined. ^{31}P MRS assessments were undertaken in 12 symptomatic, severely vitamin D deficient subjects before and after treatment with cholecalciferol. Cholecalciferol therapy was found to augment skeletal muscle mitochondrial oxidative phosphorylation in these individuals.

To summarise, I have examined dynamic skeletal muscle metabolism in two common endocrine disorders with contrasting results. There was no detectable perturbation in skeletal muscle metabolism in untreated GHD patients when compared to both matched GH replaced GHD patients and healthy controls. On the other hand, I have found that maximal mitochondrial function is improved and altered by vitamin D repletion in symptomatic vitamin D deficient adults, thereby suggesting a peripheral mechanism for fatigue in vitamin D deficiency. This is the first time that a link has been identified between vitamin D and the mitochondria in human skeletal muscle using ^{31}P MRS.

Dedication

I would like to dedicate my work to my Parents, my daughters and my wife Namrta. My parents because of all the love they gave me while growing up and providing me with the inspiration to work hard. My daughter(s) for all the happiness they have provided me in my life and finally, my wife Namrta, for always supporting me in my endeavours, through ‘thick and thin’. This work, although you will never read it, is for all of you!

Acknowledgements

Firstly, I would like to express my gratitude to my supervisors. I would like to thank Kieren Hollingsworth for not only teaching me the principles and techniques of ^{31}P MRS but also for his immense support and guidance whilst being a role model of a perfect scientist. I would like to thank Steve Ball for inspiring me to do better presentations and his clarity of thought. Finally, I would like to thank Tim Cheetham. Without his efforts and support, I would never have undertaken this degree and would never be a Paediatric Endocrinologist. I will always cherish my time with him. All I can say is if I become 10% like him, I will consider myself successful!

I would like to thank Pfizer and Serono Inc. for funding the 1st and 2nd year of this study including my salary. I would also like to thank the British Society of Paediatric Endocrinology for awarding me the money to undertake the Vitamin D study.

The following also deserve honourable mention:
Dr Mohammed Elemraid
Dr Prashant Mallya
All the Endocrine Specialist Nurses
Dr Cookey'surgery and their Nurse practitioner Jackie
MR centre radiographers and all the staff

Prizes, Publications and Presentations

Prize:

- 'Young Endocrinologist prize' for highly commended oral presentation at the British Endocrine Society [GBP 100] Mar 2013
- British Society of Paediatric Endocrinology and Diabetes National Research prize [GBP 15,000] Nov 2011

Peer reviewed Publications:

- Sinha A, Hollingsworth KG, Ball SG, Cheetham T. Improving the Vitamin D status of Vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle. Journal of Clinical Endocrinology and Metabolism. Feb 2013 (PMID: 23393184)
- Sinha A, Cheetham T, Pearce S. Prevention and Treatment of Vitamin D deficiency. Calcified Tissue International. Epub Nov 2012 (PMID: 23124916)
- Sinha A, Hollingsworth KG, Ball SG, Cheetham T. Impaired quality of life in growth hormone deficient adults is independent of the altered skeletal muscle oxidative metabolism found in conditions with peripheral fatigue. Clinical Endocrinology. Accepted May 2013

Abstracts and Presentations (Conferences):

- Sinha A, Hollingsworth K, Ball S, Cheetham T. Improving the Vitamin D status of Vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle. Oral presentation at the British Endocrine Society, Mar 2013 (Selected for Young Endocrinologists' Prize session)
- Sinha A, Hollingsworth K, Ball S, Cheetham T. *In vivo* characterisation of skeletal muscle metabolism in growth hormone deficient adults using phosphorus-31 Magnetic Resonance Spectroscopy. Judged poster presentation at the British Endocrine Society, Mar 2013
- Sinha A, Hollingsworth K, Ball S, Cheetham T. Examining the distribution of abdominal fat in Growth hormone deficiency using Magnetic Resonance Imaging. Poster presentation at the British Endocrine Society, Mar 2013

- Sinha A, Hollingsworth K, Ball S, Cheetham T. Does Vitamin D modulate mitochondrial oxidative phosphorylation? Oral presentation at the British Society of Paediatric Endocrinology & Diabetes, Nov 2012
- Sinha A, Cheetham T. Vitamin D trending: Trends in Vitamin D status, measurement and prescribing in Northern England, 2002-2011. Poster presentation at the British Society of Paediatric Endocrinology & Diabetes, Nov 2012
- Sinha A, Hollingsworth K, Ball S, Cheetham T. Mind over muscle: investigating the biology of fatigue in growth hormone deficiency using 31P-MR. Poster presentation at the British Society of Paediatric Endocrinology & Diabetes, Nov 2012
- Sinha A: Investigating fatigue in Growth hormone deficiency- A functional MRS approach. Oral presentation at the Scottish Paediatric Endocrinology Group, Dunkeld, Scotland (2012)

Abbreviations

Abbreviation	Meaning
MHC	Myosin heavy chains
MPHD	Multiple Pituitary Hormone Deficiency
GHD	Growth Hormone Deficiency
NICE	National Institute of Clinical Excellence
QoL	Quality of Life
HRQoL	Health related Quality of Life
AGHDA	Adult Growth Hormone Deficiency Assessment
MRS	Magnetic Resonance Spectroscopy
NMR	Nuclear Magnetic Resonance
IGF-1	Insulin-like Growth Factor-1
ITT	Insulin tolerance test
IGF-BP3	IGF-binding protein-3
ALS	Acid-labile subunit
GRS	Growth Hormone Research Society
hGH	human Growth Hormone
LBM	Lean Body Mass
KIMS	Pfizer International Metabolic Database Study
BMD	Bone mineral density
CO-GHD	Childhood onset-GHD
AO-GHD	Adult onset-GHD
MRI	Magnetic Resonance Imaging
GRS	Growth Hormone Research Society
TC	Total cholesterol
LDL-C	Low density lipoprotein-cholesterol
HDL-C	High density lipoprotein-cholesterol
TG	Triglyceride
HypoCCS	Hypopituitary Control and Complications Study
WHR	Waist Hip Ratio

Abbreviation	**Meaning**
ATP	Adenosine Triphosphate
PCr	Phosphocreatine/ Creatine Phosphate
Pi	Inorganic Phosphate
MVC	Maximum Voluntary Contraction
RCT	Randomised controlled trial
CFS	Chronic Fatigue Syndrome
AUC	Area under Curve
ITT	Insulin Tolerance Test
DXA	dual-energy x-ray absorptiometry
BMC	Bone Mineral Content
BSI	Bone strength Index
BBB	blood brain barrier
HPT	hypothalamo-pituitary-thyroid
11β-HSD	11β-hydroxysteroid dehydrogenase
UVB	Ultraviolet B
HPLC	High performance liquid chromatography
VDR	Vitamin D receptor
KO	knock out
mRNA	messenger ribonucleic acid

Table of Contents

Abstract ii

Dedication iii

Acknowledgements iv

Prizes, Publications and Presentations v

Abbreviations vii

Table of Contents ix

List of Figures xiii

List of Tables xv

Chapter 1 Background and Literature review *1*

1.1 Skeletal muscle Energetics 1

1.1.1 Physiology of ATP generation in the skeletal muscle (Energetics) 1

1.1.2 Skeletal fiber types 5

1.1.3 Muscle activity and energy expenditure 6

1.1.4 Muscle fatigue (Peripheral fatigue) 7

1.1.5 Summary 8

1.2 Fatigue 9

1.2.1 Overview 9

1.2.2 Why does fatigue occur? 9

1.2.3 Peripheral fatigue 10

1.2.4 Central Fatigue 10

1.2.5 Summary 12

1.3 Growth hormone Deficiency 14

1.3.1 GHD Adults 14

1.3.2 Diagnosis of Growth Hormone deficiency 16

1.3.3 Causes of growth hormone deficiency in adults 19

1.3.4 Treatment of GHD in Adults 23

1.3.5 The interplay between growth hormone and other pituitary hormones 25

1.3.6 Metabolic abnormalities in GHD adults 28

1.3.7 Growth hormone and skeletal muscle function 30

1.3.8 Quality of Life impairment in GHD 37

1.3.9 GHD and the brain 41

1.3.10 Growth Hormone and the mitochondria 44

1.3.11 Summary 46

1.4 Vitamin D deficiency 47

1.4.1 Vitamin D deficiency 47

1.4.2 Sources of vitamin D and metabolism 47

1.4.3 Vitamin D status assessment 48

1.4.4 Risk factors for vitamin D deficiency 50

1.4.5 Vitamin D and skeletal muscle function 51

1.4.6 Summary 55

1.5 Phosphorus-31 Magnetic Resonance Spectroscopy 57

1.5.1 Overview 57

1.5.2 Principles 58

1.5.3 Key bioenergetic pathways investigated 60

1.5.4 Mitochondrial oxidative phosphorylation during exercise and recovery 61

1.5.5 pH handling during exercise and recovery 64

1.5.6 Applications in Endocrine diseases 66

1.5.7 Advantages 68

1.5.8 Limitations 69

1.5.9 Summary 69

Chapter 2 Growth hormone and skeletal muscle energetics 71

2.1 Introduction 71

2.1.1 Background 71

2.1.2 Aims 72

2.1.3 Study subjects 74

2.1.4 Approval for study 76

2.1.5 Phosphorus-31 Magnetic Resonance Spectroscopy protocol 76

2.1.6 Assessing Quality of Life –AGHDA questionnaire 78

2.1.7 Biochemical evaluation and assays 79

2.1.8 Anthropometric evaluation 80

2.1.9 Physical activity evaluation 80

2.1.10 Outcome measures 81

2.1.11 Statistical analysis 81

2.2 Results 82

2.2.1 Subject clinical characteristics 82

2.2.2 Parameters of mitochondrial oxidative phosphorylation 86

2.2.3 Parameters of anaerobic glycolysis 87

2.2.4 Perception of fatigue and AGHDA scores 89

2.2.5 Pooled analyses and bivariate linear regression analysis to predict mitochondrial function ($\tau_{1/2}$PCr) 91
2.2.6 Multiple regression analysis to predict factors contributing to fatigue perception 92
2.2.7 Effect of radiotherapy and chemotherapy on fatigue perception 93
2.2.8 Assessment of adequacy of other pituitary hormone replacement 95

2.3 Conclusions 97

2.3.1 Study strengths 99
2.3.2 Study limitations 99
2.3.3 Summary 100

Chapter 3 Vitamin D and skeletal muscle energetics 101

3.1 Introduction 101

3.1.1 Background 101
3.1.2 Aims 101

3.2 Methods 102

3.2.1 Study subjects and general study protocol 102
3.2.2 Approval for the study 103
3.2.3 Phosphorus-31 Magnetic Resonance Spectroscopy protocol 103
3.2.4 Vitamin D regimen 105
3.2.5 Biochemistry 105
3.2.6 Outcome measures 105
3.2.7 Statistical analysis 106

3.3 Results 107

3.3.1 Subject clinical characteristics 107
3.3.2 Serum biochemistry 107
3.3.3 Parameters of oxidative phosphorylation 109

3.4 Conclusions 115

3.4.1 Study Strengths 120
3.4.2 Study Limitations 120
3.4.3 Summary 121

Chapter 4 Discussion 122

4.1 Summary 122

4.2 The future 124

4.2.1 GH study 124
4.2.2 Vitamin D study 125

Appendix..... 127
Appendix A AGHDA-QoL questionnaire..... 128
Appendix B IPAQ physical activity questionnaire..... 129
Appendix C Patient Information Sheet-GHD..... 130
Appendix D Healthy control-GHD..... 135
Appendix E Patient Information Sheet-Vitamin D..... 140
Appendix F Consent form..... 145
Appendix G Untreated GHD patient details..... 147
Appendix H Treated GHD patient details..... 150
Appendix I Publication-Improving the vitamin D status of vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle..... 153
Appendix J Publication-Prevention and treatment of vitamin D deficiency..... 159
References: 169

List of Figures

Figure 1 Schematic representation of the three mechanisms responsible for ATP resynthesis as a function of the time of exercise 2
Figure 2 Diagram showing the steps of oxidative phosphorylation within the mitochondria 4
Figure 3 Fiber-type composition of leg muscles in athletes. 6
Figure 4 Likelihood of reporting chronic fatigue (A) and chronic fatigue with impairment (B) among patients with cancer listed in a national registry, compared with individuals without cancer 12
Figure 5 Causes of GHD in adults 19
Figure 6 Growth hormone and its putative physical and functional effects on skeletal muscle tissue 36
Figure 7 Putative mechanisms of action of GH on brain 41
Figure 8 Growth hormone and phosphocreatine recovery in healthy adults 46
Figure 9 Summary of vitamin D metabolism 48
Figure 10 Genomic (Classic) vitamin D signaling pathway 53
Figure 11 Genomic and non-genomic effects of vitamin D on muscle 54
Figure 12 ^{31}P spectrum from gastrocnemius and soleus of a healthy volunteer at rest at 3T and the derived phosphorus spectrum showing relative concentrations of inorganic phosphate (Pi), phosphodiesters (PDE), phosphocreatine (PCr) and adenosine triphosphate (ATP). 58
Figure 13 Scheme of the variations of PCr, pH and ATP during a rest-exercise-recovery protocol quantified using ^{31}P MRS 61
Figure 14 ^{31}P MRS: After exercise and during recovery 63
Figure 15 Mitochondrial oxidative potential 64
Figure 16 pH recovery time 65
Figure 17 Mitochondrial oxidative capacity (k_{PCr}-PCr recovery rate constant) and insulin sensitivity 67
Figure 18 Growth Hormone Cross-sectional study design 74
Figure 19 Flowchart of recruitment 75
Figure 20 illustrates the apparatus used to permit exercise (plantar flexion) within the MR scanner (left) and the 0°-30° of plantar flexion involved (right) 78

Figure 21 shows lower IGF-1 levels in the untreated GHD group compared to the treated GHD group and the controls 83
Figure 22 Comparison of oxidative phosphorylation following exercise across the 3 groups 86
Figure 23 Comparison of pH recovery (proton excretion) following exercise across the 3 groups 87
Figure 24 shows a higher perception of fatigue in the untreated GHD group 90
Figure 25 shows impaired QoL in the untreated GHD group in comparison with the other groups 90
Figure 26 shows there is no correlation between serum IGF-1 and $\tau_{1/2}$PCr on bivariate analysis 91
Figure 27 Effect of radiotherapy on fatigue in GHD 93
Figure 28 Effect of chemotherapy on fatigue in GHD 94
Figure 29 Comparison of thyroxine levels in GHD patients 95
Figure 30 Comparison of testosterone levels in GHD patients 96
Figure 31 Vitamin D Longitudinal study 102
Figure 32 Serum 25OHD levels at presentation with vitamin D deficiency and then following calciferol therapy 108
Figure 33 Comparison of $\tau_{1/2}$ PCr kinetics before and after calciferol therapy 112
Figure 34 Comparison of $\tau_{1/2}$ ADP kinetics before and after calciferol therapy 112
Figure 35 Comparisons of mitochondrial function before and after calciferol therapy and with healthy controls. 113
Figure 36 A regression model built by pooling data from serum 25OHD and $\tau_{1/2}$PCr of all the participants. 114
Figure 37 Schematic diagram of the interaction of matrix Ca^{2+} with the processes involved in oxidative phosphorylation 119

List of Tables

Table 1 Congenital causes of GHD in adulthood 21
Table 2 Acquired causes of GHD in adulthood 22
Table 3 Summary of Studies reporting QoL changes with GH therapy 38
Table 4 Patient characteristics 84
Table 5 Comparison of bioenergetic parameters (^{31}P MRS) 88
Table 6 Comparison of AGHDA-QoL variables 89
Table 7 Effect of radiotherapy on fatigue perception 93
Table 8 Effect of chemotherapy on fatigue perception 94
Table 9 Biochemistry and ^{31}P MRS measurements on skeletal muscle in vitamin D deficient subjects before and after calciferol therapy 110

Chapter 1 Background and Literature review

1.1 Skeletal muscle Energetics

The primary evolutionary function of skeletal muscle is to promote locomotion which requires considerable energy expenditure. Skeletal muscles possess the unique ability to adapt to the increase in energy requirement by increasing ATP production. The increase in energy turnover can amount to 300-fold from the resting to the fully activated state and this can occur within a few milliseconds (Sahlin *et al.*, 1998). This process of metabolic regulation occurs primarily through 3 pathways, namely phosphocreatine hydrolysis, anaerobic glycolysis and oxidative phosphorylation. Muscle cells also vary according to their relative capacity for oxidative metabolism as opposed to anaerobic glycolysis which is determined by the relative proportions of their individual fiber types.

1.1.1 Physiology of ATP generation in the skeletal muscle (Energetics)

ATP homeostasis

A single muscle fiber may contain upto 15 billion thick filaments. When the muscle fiber is actively contracting, each thick filament breaks down approximately 2500 ATP molecules per second. Because even a small skeletal muscle contains thousands of muscle fibers, the ATP demands of a contracting skeletal muscle are enormous. In practical terms, the demand for ATP in a contracting muscle fiber is so high that it would be impossible to have all the necessary energy locally available as ATP before the contraction begins. Instead, a resting muscle fiber contains only enough ATP and other high-energy compounds to sustain a contraction until additional ATP can be generated. Throughout the rest of the exercise, the muscle fiber will generate ATP at roughly the same rate as it is used. This ability to sustain steady supplies of ATP in order to fuel the contracting muscles is called ATP homeostasis.

Sources of ATP generation

Since the contracting muscle needs a constant supply of energy in the form of ATP to provide its energy demands, the body relies on 3 sources of ATP generation. Energy from phosphocreatine hydrolysis is immediately available, and the maximal power of this mechanism is reached in a fraction of a second, but the mechanism rapidly fatigues and reaches a value close to zero in 10–20 s. At the other end of the spectrum, oxidative phosphorylation has a relatively lower maximal power but can be sustained for relatively longer periods of time. This mechanism is significantly slower in getting into action; about 2 min are needed before its asymptotic value is reached. The characteristics of the third mechanism, anaerobic glycolysis, are intermediate between those of the other two mechanisms (Figure 1).

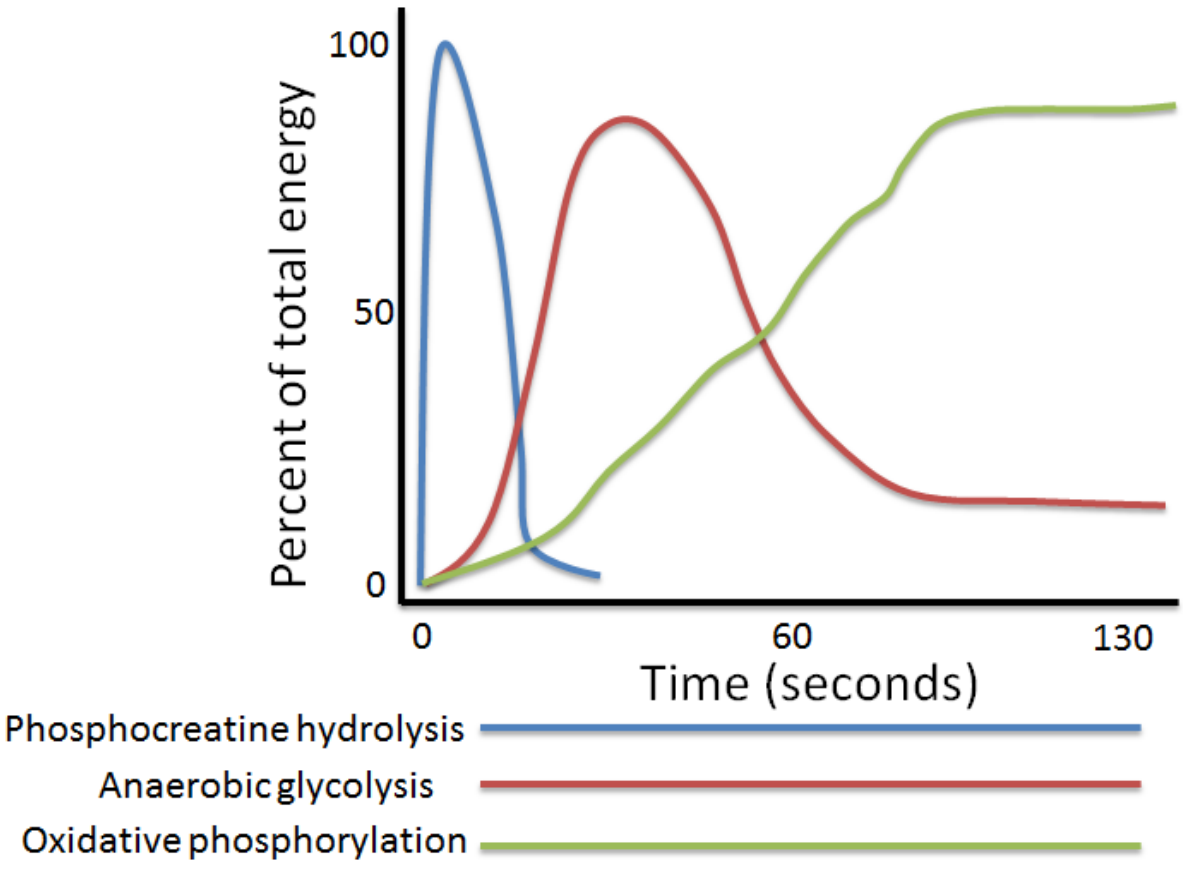

Figure 1 Schematic representation of the three mechanisms responsible for ATP resynthesis as a function of the time of exercise

I. Phosphocreatine hydrolysis

The primary function of ATP is the transfer of energy from one location to the other rather than the long-term storage of energy. During rest, a state of homeostasis exists where ATP production matches ATP consumption. During exercise, the demand for energy increases markedly and phosphocreatine (PCr), a high energy molecule, serves as a source of immediate energy. It releases energy-rich phosphate species which combine with ADP (released by the skeletal muscle) to form ATP and creatine.

$$ADP + PCr \rightarrow ATP + Cr$$

Therefore, in order to supply immediate energy for exercise, the skeletal muscle always has a richer content of PCr compared to ATP, reflected in a higher PCr peak compared to ATP when displayed in the ^{31}P MR spectra (Figure 14) (discussed in chapter 1.5). The high energy requirements of the contracting muscle during vigorous physical activity leads to the depletion of PCr stores in approximately 12 seconds.

Following exercise, the skeletal muscle replenishes the energy deficit incurred during exercise by transferring surplus energy produced to creatine leading to the replenishment of PCr stores in the skeletal muscle. The PCr consumed during the exercise is thereby replaced for future use.

$$ATP + Cr \rightarrow ADP + PCr$$

II. Glycolysis

This is an anaerobic process and occurs within the cytoplasm of the cell. It involves the breakdown of glucose to pyruvic acid and generates a net gain of 2 ATP whilst generating 2 pyruvic acid molecules from each glucose molecule. It is an inefficient process compared to aerobic metabolism. However, its advantage lies in that it does not require oxygen and therefore it can produce energy when the availability of oxygen limits ATP production aerobically.

III. Aerobic oxidative phosphorylation

This process occurs in the mitochondria of the cells in mammals. It normally provides 95% of the demands of the resting cells (Poehlman *et al.*, 1989). During this process, mitochondria absorb ADP, phosphate ions, oxygen and pyruvic acid from the surrounding cytoplasm. The substrates enter the *Krebs cycle/citric acid cycle,* a process that breaks down pyruvate and generates ATP in the process. Specifically, the oxidative phosphorylation and electron transport processes occur on the inner mitochondrial

membrane. The electron transport chain transports the electrons through its complexes, eventually reducing oxygen to water. The free energy generated pumps protons across the mitochondrial inner membrane. As the concentration of protons in the intra-membrane space increases, this gradient is relieved by the passage of protons through the complex V. This process is coupled to ATP synthase on the matrix side leading to ATP synthesis (Figure 2). Overall it is a very efficient process compared to glycolysis since it produces 30 ATP molecules per glucose molecule as opposed to 2 ATP molecules per glucose molecule (Frayn, 2009). This is due to the complete oxidation of substrates giving a much higher energy yield than the partial breakdown that occurs during glycolysis.

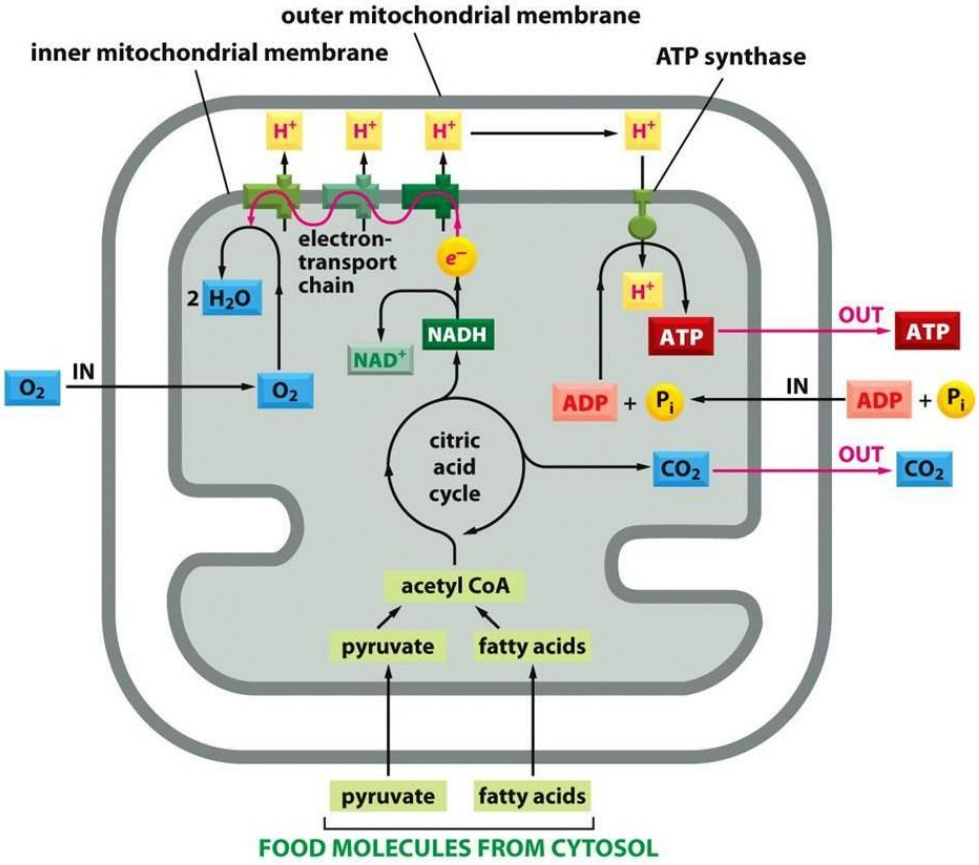

Figure 2 Diagram showing the steps of oxidative phosphorylation within the mitochondria

The key pathways are shown with food molecules entering the Krebs /citric acid cycle followed by the release of electrons which are transferred across the electron transport chain. The electron transport chain pumps protons across the inner mitochondrial membrane creating a gradient. Excess protons subsequently return across this gradient through complex V. This process is coupled to ATP synthase on the matrix side leading to ATP synthesis.

1.1.2 Skeletal fiber types

Since skeletal muscle have to cope with divergent activities ranging from long periods of standing to performing explosive movements, our muscles are composed of muscle cells with large differences in metabolic profile, contractile speed and cellular Ca^{2+} handling (Borina *et al.*, 2010). The mitochondrial densities of the skeletal muscle vary according to the energetic demands of the particular tissue. Muscle fibres can be classified, based on myosin heavy chain (MHC) isoforms, as type I (slow oxidative) or type II fibers. Type II fibers are further classified into subtypes IIa, IIx and IIb. Type IIb fibers (fast glycolytic) lie at the opposite end of the spectrum from type I fibers with the remaining type IIa (fast oxidative) and IIx fibers lying somewhere in between (Booth and Thomason, 1991). Type I and IIa muscle fibers tend to have a richer mitochondrial enzyme content and are, therefore, relatively fatigue resistant (Fitts, 1994). They are red in colour because of their rich myoglobin and mitochondrial content and rely primarily on mitochondrial oxidative phosphorylation as a means of energy production (Figure 3). Aerobic training can be expected to induce a greater increase in the oxidative capacity of the type I muscle fibers. On the other hand Type IIb fibers, also known as fast glycolytic fibers, are white which reflects a lower myoglobin content and mitochondrial density. They rely more substantially on anaerobic glycolysis as a means of fuelling energy demands. Intrinsic muscle strength is higher when there are a higher proportion of type II fibers. Thus resistance training and anabolic steroids would be expected to induce a greater increase in type II fibers than type I fibers. Hence, there are marked differences in contraction speed, metabolism and susceptibility to fatigue between the two fiber types.

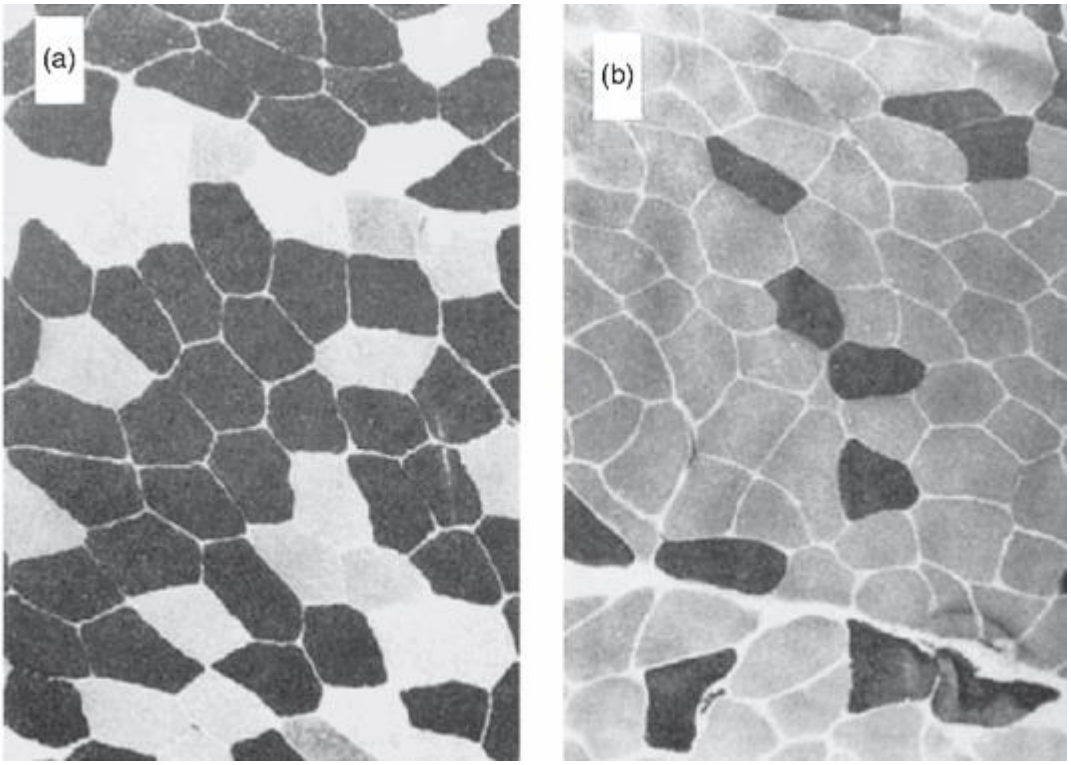

Figure 3 Fiber-type composition of leg muscles in athletes.

Different types of muscle fibers are shown in a cross-section of muscle, by staining for the enzyme myosin-ATPase (which reflects fast-twitch muscles): dark stained fibers are type II and lighter stained fibers are type I. Left (a) quadriceps muscle from a high jumper and on the right (b) from a marathon runner (Frayn, 2009).

1.1.3 Muscle activity and energy expenditure

The source of ATP generation to preserve ATP homeostasis in anaerobic exercise differs from those used in aerobic exercise. The fuel selection during these different forms of exercise illustrates many important facets of energy transduction and metabolic integration. Depending upon the level of muscle exertion, the body responds to ensure a constant supply of energy for the contracting muscle. The salient features of different levels of activities are described below:

Resting muscle: In the resting skeletal muscle, ATP production matches consumption and a steady state is maintained. The majority of energy is provided by aerobic metabolism.

Moderate levels of activity: As the demand for ATP increases, the mitochondrial ATP production rises. This leads to an increase in mitochondrial oxygen consumption. Oxygen availability is not a limiting factor, because oxygen can diffuse into the muscle fiber fast enough to meet mitochondrial needs but all the ATP produced is needed by the muscle

fiber, and no surplus is available. The skeletal muscle now relies primarily on the aerobic metabolism of pyruvic acid to generate ATP. The pyruvic acid is provided by glycolysis, which breaks down glucose molecules obtained from glycogen in the muscle fiber. If glycogen reserves are low, the muscle fiber can also break down other substrates, such as lipids or amino acids. As long as the demand for ATP can be met by mitochondrial activity, the ATP provided by glycolysis makes a relatively minor contribution to the total energy budget of the muscle fiber.

Peak levels of activity: The ATP demands are huge and mitochondrial ATP production rises to a maximum. This maximum rate is determined by the availability of oxygen, and oxygen cannot diffuse into the muscle fiber fast enough to enable the mitochondria to produce the required ATP. At peak levels of exertion, mitochondrial activity can provide only about one-third of the ATP needed (Conley *et al.*, 2001). The remainder is produced through glycolysis. When glycolysis produces pyruvic acid faster than it can be utilized by the mitochondria, pyruvic acid levels rise in the sarcoplasm. Under these conditions, pyruvic acid is converted to lactic acid thereby, lowering the intracellular pH. This change in pH can eventually alter the functional characteristics of key enzymes which can culminate in an inability to contract (Mainwood and Renaud, 1985).

Recovery period: In the recovery period, the conditions in muscle fibers are returned to their normal, pre-exertion levels. PCr reserves are replenished and pH is normalised. Immediately following exercise, ATP production exceeds its consumption. It is utilised to replenish energy stores by converting creatine to phosphocreatine (PCr) and glucose to glycogen. Since there is an abundant supply of oxygen to the mitochondria, efficient aerobic metabolism is the source of ATP generation. This is, therefore, an important stage for exercise physiologists since maximal mitochondrial oxidative phosphorylation is stimulated by by-products of ATP turnover such as ADP and inorganic phosphate (Pi). Based on this phenomenon, dynamic ^{31}P MRS studies serve to measure mitochondrial function during this critical window.

1.1.4 Muscle fatigue (Peripheral fatigue)

Skeletal muscle fatigue sets in when it can no longer contract despite continued neural stimulation. The cause of fatigue depends on the level of physical activity.

After short peak levels of activity such as a 100m sprint, fatigue develops because of the exhaustion of the PCr reserves and the accumulation of lactic acid or inorganic phosphate (Westerblad *et al.*, 2002). However, if the muscle fiber is contracting at moderate levels and ATP demands can be met through aerobic metabolism, fatigue will not occur until glycogen, lipid, and amino acid reserves are depleted. This type of fatigue affects the muscles of long-distance athletes, such as marathon runners, after hours of exertion.

1.1.5 Summary

The components of ATP homeostasis ensure that levels of ATP are maintained in the exercising muscle. The high ATP turnover rates yield by-products such as ADP and Pi which stimulate an increase in the oxidative phosphorylation rate which during recovery ensures that PCr concentrations return to quiescent levels. Muscle cells with high potential for ATP consumption, although powerful, are susceptible to rapid fatigue because of accumulation of by-products. On the other hand, cells with high capacity to restore ATP following exercise possess excellent endurance because ATP use and restoration are balanced.

Approximately 50% of the potential chemical energy released from foodstuffs is captured in the common chemical intermediate, ATP. ATP, together with its storage form, phosphocreatine (PCr), then serves as the immediate cellular energy source on which endergonic processes depend. ATP and PCr not only supply immediate cellular energy sources, but their relative levels also stimulate or inhibit processes of energy metabolism. At rest, normally high levels of ATP and PCr maintain the rate of oxidative phosphorylation and anaerobic metabolism at an appropriate level. When exercise starts, however, the utilization and decreased levels of ATP and PCr, and the increased levels of ADP and Pi stimulate processes of energy metabolism. Enzymes interact with products of energy metabolism to regulate the rate at which specific processes proceed. Muscles utilize three different systems of energy release during exercise, each of which differs in mechanism, capacity and endurance. Consequently, the rate and capacity for muscular power output is determined by the ability of these three systems to maintain cellular ATP homeostasis.

1.2 Fatigue

1.2.1 Overview

During voluntary contractions, muscles are activated by complex pathways originating in the α-motor neurons within the cortex leading to excitation of lower motor neurons in the spinal cord. The lower motor neuron axon carries the action potentials to the neuromuscular junction of the muscle. Interruptions in the pathway at the spinal cord and above are referred to as central fatigue whereas those occurring in the peripheral nerve, neuromuscular junction or muscle are referred to as peripheral fatigue. The brain accounts for 20% of the body's energy consumption at rest (Frayn, 2009). This is because the adult human brain is estimated to consist of around 100 billion nerve cells and 900 billion glial cells which have a high rate of metabolism. This high metabolic requirement requires a high rate of blood supply (50ml blood per minute per 100gm of brain tissue) (Frayn, 2009). However, during strenuous exercise, the high energy demands of the exercising skeletal muscle impose an approximate 18 fold increase in whole-body energy requirement over the resting level (Frayn, 2009). This involves major changes in substrate supply through the blood (50ml blood per minute per 100gm of muscle tissue during vigorous exercise). Hence reduced energy production or supply within the brain or muscle can lead to fatigue. Since mitochondrial oxidative phosphorylation is the principal source of energy supply in humans, it is not surprising that impairments in mitochondrial function often result in neuromuscular signs and symptoms, thereby reflecting the high energy 'footprint' of the brain and skeletal muscle.

Fatigue can encompass diverse phenomena that are the consequence of different physiological processes. Therefore, when discussing fatigue, all the dimensions of the problem must be examined. Firstly, subjective dimensions or the patients' perception of fatigue must be addressed. Generally, this dimension has three aspects: physical, emotional and cognitive. Secondly, objective dimensions namely the functional parameters of the skeletal muscle system must be examined.

1.2.2 Why does fatigue occur?

In simple terms fatigue occurs because of impairments in one or several of the processes that enable contractile proteins to generate a force. However, a more holistic view

acknowledges that the site of impairment depends on the task being performed. This has been described as the task dependency of fatigue (Bigland-Ritchie *et al.*, 1995). Since fatigue is task dependent, it is impossible to identify a single causal mechanism for fatigue. However, one approach to this problem is to consider central and peripheral fatigue separately.

1.2.3 Peripheral fatigue

Metabolic changes in the muscle may occur during fatigue, thereby affecting muscle contraction. Generally, peripheral fatigue is more objectively defined and evaluated since its pathophysiology is better understood (Cantor, 2010). The key target organ implicated in peripheral fatigue is the skeletal muscle which harbours the mitochondria. Perturbations in skeletal muscle metabolism such as delayed mitochondrial phosphocreatine resynthesis, delayed proton clearance or the accumulation of metabolic by-products are the cellular mechanisms responsible for the symptoms of peripheral fatigue (Allen *et al.*, 2008). One approach to studying peripheral fatigue is to examine muscle energetics, the metabolic pathways by which skeletal muscle generates ATP. Abnormal muscle energetics has been implicated in diverse conditions in which fatigue is a prominent feature (Wong *et al.*, 1992; Kuhl *et al.*, 1994; Pfeifer *et al.*, 1999; Hollingsworth *et al.*, 2008). Muscle energetics can be examined using phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) which is described in detail in chapter 1.5.

1.2.4 Central Fatigue

Central fatigue is a complex pathophysiological entity and is not well defined (Chaudhuri and Behan, 2004). Generally, the assessment of primary central fatigue is based upon self-reporting questionnaires (Lee *et al.*, 1991). Central fatigue may occur as a consequence of impaired α-motor neuron activation. The complex interplay between the nervous system and skeletal muscle during most strenuous exercise makes it difficult to design experiments to unequivocally assess the extent of central fatigue. Although various researchers have attempted to delineate the precise pathophysiological processes responsible, this has proved to be difficult. Since elements of central fatigue are often observed independent of muscle fatigue, both the cortex and the spinal cord at the level of motoneurons are implicated (Gandevia, 2001). Recently, Cotel *et al.*

provided a cellular mechanism for central fatigue. They demonstrated that during prolonged activity, serotonin spillover reaches the axon initial segment where it activates serotonin receptors, which inhibit the generation of action potentials in motoneurons and in turn, muscle contraction, thereby, providing a possible cellular mechanism for central fatigue (Florence Cotel, 2013). Further insight may also be gained by studying various biological models of central fatigue.

Chronic Fatigue Syndrome (CFS) Prototype

Chronic Fatigue Syndrome is a heterogeneous disorder characterised by debilitating fatigue which is severe enough to cause a significant loss of physical and social function for a minimum of 6 months. Four of the following symptoms must also be present if diagnostic criteria are to be met: sleep disturbance (usually unrefreshing sleep or hypersomnia), concentration impairment, muscle pain, multi-joint pains, headaches, post-exertional exacerbation of fatigue, sore throat, and tender lymph nodes (Fukuda *et al.*, 1994). Although CFS is often multifactorial in origin, neuroendocrine disturbances have been implicated in its aetiology. The hypo-thalamo-pituitary (HPA) axis has been a particular area of interest and it is quite likely that abnormalities of the HPA axis exist in at least some patients. Furthermore, given the many factors that may impinge on the HPA axis in CFS, such as inactivity, sleep disturbance, psychiatric comorbidity, medication, and on-going stress, it seems likely that HPA axis disturbance is heterogeneous and of multifactorial aetiology in CFS (Cleare, 2003). The growth hormone-insulin-like growth factor (GH-IGF-1) axis has also been investigated in CFS but there is no compelling evidence to suggest that GH dysfunction plays a significant role in CFS symptomatology (Moorkens *et al.*, 1998).

Cancer related fatigue prototype

Cancer related fatigue has been described as a “persistent, subjective sense of tiredness related to cancer and cancer treatment that interferes with usual functioning” (Hofman *et al.*, 2007). Fatigue occurs in a variety of cancers with patients reporting fatigue before diagnosis to beyond treatment completion (Jacobsen, 2004). In fact survivors of cancer may report persistent fatigue for several years (Figure 4). Several hypotheses have been proposed for the mechanisms underpinning cancer related fatigue including disruptions in the HPA axis, proinflammatory cytokines, circadian rhythm modulation, anaemia and

serotonin dysregulation (Wang, 2008). This again probably reflects the multidimensional nature of the problem. Treatment modality could also contribute to fatigue since >80% of outpatients receiving chemotherapy or radiotherapy reported some degree of fatigue as assessed using the Fatigue Symptom Inventory (Hickok *et al.*, 2005; Hofman *et al.*, 2007). A population-based, cross-sectional postal survey of hormone-naïve survivors with the diagnosis of prostate cancer who were treated with radical prostatectomy or radiotherapy revealed a higher prevalence of cancer related fatigue in the radiotherapy group (26.1%) as opposed to the surgically treated group (13.4%) (Kyrdalen *et al.*, 2010).

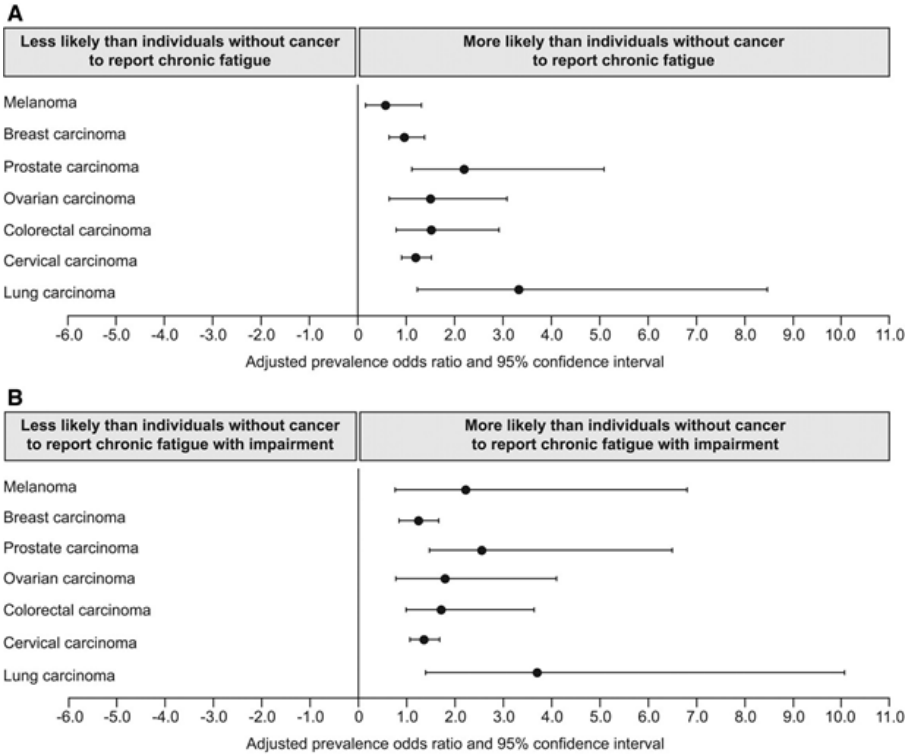

Figure 4 Likelihood of reporting chronic fatigue (A) and chronic fatigue with impairment (B) among patients with cancer listed in a national registry, compared with individuals without cancer

(Forlenza *et al.*, 2005)

1.2.5 Summary

Exercise begins in the brain and ends in the muscle. Hence any impairment in the entire pathway may contribute to fatigue. Whereas peripheral skeletal muscle fatigue is better understood, the mechanisms of chronic fatigue have not been fully resolved. The

prototypes discussed above provide independent and overlapping potential mechanisms for the pathophysiology of this complex phenomenon. Nevertheless, the general picture is that central fatigue could be of greater importance during prolonged low-intensity activities, where metabolic changes with muscle cells are likely to be limited, whereas intramuscular factors appear to dominate during activities of higher intensity (Place *et al.*, 2009). However, independent of the source, the impact of fatigue on the ability to undertake activities of daily living are both profound and pervasive. This has a knock-on effect on social and psychological domains leading to a lower perceived quality of life.

1.3 Growth hormone Deficiency

1.3.1 GHD Adults

Growth hormone (GH) promotes somatic growth and also regulates several other metabolic actions. The metabolic actions of GH were reported in the late fifties following the advent of pituitary derived GH preparations (Raben, 1962). Later on retrospective epidemiological studies revealed that patients with Multiple pituitary hormone deficiency (MPHD), when compared to age and sex matched controls, presented with fatigue, low mood, obesity and abnormal metabolic parameters despite being adequately supplemented with corticosteroid, thyroxine and sex hormones (Abs *et al.*, 1999). The development of recombinant growth hormone preparations in the 1980s led to investigations into the metabolic effects of GH replacement in GH deficient (GHD) adults.

The syndrome of GH deficiency is characterised by the variable presence of fatigue, decreased mood and well-being, reduced bone remodelling, altered body composition with centripetal obesity and hyperlipidaemia. However, it is important to appreciate that adults with GHD may also have potential confounding factors such as co-existing structural pituitary disease or cranial irradiation. The direct or indirect effects of suboptimal pituitary hormone replacement and other confounders may also contribute to some of the symptoms of GHD. Nonetheless, GH replacement leads to an improvement in certain signs and symptoms suggesting that GH deficiency may be responsible for some of these symptoms.

The National Institute of Clinical Excellence (NICE), UK has recommended that GH be used in severe GHD adults up to the age of 25 years in order to optimise bone mass accrual (NICE, 2003). GH is also recommended for use in older growth hormone deficient adults if there is a documented improvement in quality of life (QoL) on GH administration using a standardised and validated questionnaire developed specifically for GHD adults called the Adults with growth hormone deficiency assessment (AGHDA) questionnaire (McKenna et al., 1999).

One of the striking symptoms of GHD is fatigue and a resulting struggle to perform daily chores and tasks. Patients with GHD often have other co-morbidities which can also influence their outlook to life and mood but it is well documented that GH replacement

can lead to improvement in energy levels and QoL based on self-reporting assessments (Murray *et al.*, 1999; Mukherjee *et al.*, 2005; Koltowska-Haggstrom *et al.*, 2006; Jorgensen *et al.*, 2011b).

I shall be discussing the pertinent features related to GHD in adulthood in this chapter with a particular emphasis on the effects of GH on fatigue, skeletal muscle function, QoL and the brain.

1.3.2 Diagnosis of Growth Hormone deficiency

Since growth hormone secretion is pulsatile and circadian, random GH levels cannot be used to diagnose GHD. Moreover, isolated IGF-1 measurements, a downstream regulator of growth hormone action, do not serve as an accurate or reliable index of growth hormone status in adulthood (Hilding *et al.*, 1999; Biller *et al.*, 2002).

Definition

Severe GHD in adults is defined biochemically but within an appropriate clinical context. The syndrome of GHD in adults is characterized by alterations in body composition (such as increased abdominal obesity, decreased lean body mass, reduced muscle strength and exercise capacity), abnormal metabolic profiles (such as increased serum low density lipoprotein cholesterol and reduced insulin sensitivity) and impaired psychological well-being (Cuneo *et al.*, 1990; Rosen *et al.*, 1995; Carroll *et al.*, 1998; Sesmilo *et al.*, 2001; Koltowska-Haggstrom *et al.*, 2006).

Biochemical diagnosis

The diagnosis of GHD is established by provocative testing of GH secretion. The insulin tolerance test (ITT) is widely regarded as the gold standard (Molitch *et al.*, 2011). This test distinguishes GHD from the relatively low GH levels observed in the obese and the elderly, provided an adequate hypoglycaemic stimulus has been achieved (Kopelman *et al.*, 1979; Hoffman *et al.*, 1994). Because of the potential risks of hypoglycaemia, this test should be conducted in specialised endocrine units where it is undertaken frequently by staff that are adequately trained. Whereas most healthy subjects respond to insulin-induced hypoglycaemia with a peak GH production >5.1mcg/L, GHD patients produce a peak GH response of <5.1mcg/L (Biller *et al.*, 2002). However, GH response is a spectrum and it is important to be aware of the sensitivity and specificity issues surrounding a particular cut-off. Hypopituitary patients should also be on adequate hormone replacement during testing so as to ensure their other endocrine axes are intact and stable.

The combination of growth hormone releasing hormone (GHRH) with arginine also demonstrates considerable utility in separating hypopituitary patients from controls. In fact, diagnostic practices have shown a trend toward the GHRH-arginine testing regimen (Webb *et al*., 2009). This is probably because it is better tolerated and does not cause

hypoglycaemia. Caution must be applied in GHD patients of hypothalamic origin since a false positive result might be obtained because of the direct stimulation of the pituitary gland by GHRH.

Other tests available are the glucagon, arginine and clonidine provocation test but they are not well validated in the adult population (Darzy *et al.*, 2003).

Guidelines published by the GH research society (GRS) recommend that adults with known hypothalamic pituitary abnormalities and at least one known deficit of another pituitary hormone (excluding prolactin) should undergo a single diagnostic test to diagnose the presence of GH deficiency. However, those with childhood onset isolated GH deficiency (no evidence of hypothalamic pituitary abnormality or cranial irradiation), should be recommended two diagnostic tests once final adult height is achieved, except for those having low IGF-1 concentrations (SDS ≤2) where one test may be considered sufficient ('Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency,' 1998).

Biochemical surrogate markers

Of the several biochemical markers of GH action available, only serum IGF-1 concentrations are useful in titrating GH dosage therapy to age adjusted normal ranges. In adults, a normal serum IGF-1 does not exclude the diagnosis of GH deficiency because the sensitivity of serum IGF-1 as a marker of GHD declines with age, despite age-specific reference ranges. Whereas low IGF-1 concentrations correlate well with GHD in childhood and young men (mean age 26.5 years)(de Boer *et al.*, 1994), Toogood *et al*. found that only 21% of elderly GHD patients (aged 61-85 years) had a serum IGF1 lower than age matched controls (1996). Therefore, whereas serum IGF-1 concentrations may be a helpful adjunct in aiding diagnosis in younger patients, its diagnostic utility declines with increasing age.

To date, measurements of IGF-binding protein-3 (IGF-BP3) or acid-labile subunit (ALS) have not provided any advantage over the measurement of serum IGF-1.

Assay standardization

The above recommendations for cut-off values for the ITT are based on results obtained with polyclonal competitive radioimmunoassay calibrated against the pituitary-derived

preparation International Reference Preparation (IRP) 80/505. The GRS advocates future use of the recombinant hGH preparation IRP88/64. Further comparative studies are required to achieve standardization of GH assays ('Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency,' 1998).

1.3.3 Causes of growth hormone deficiency in adults

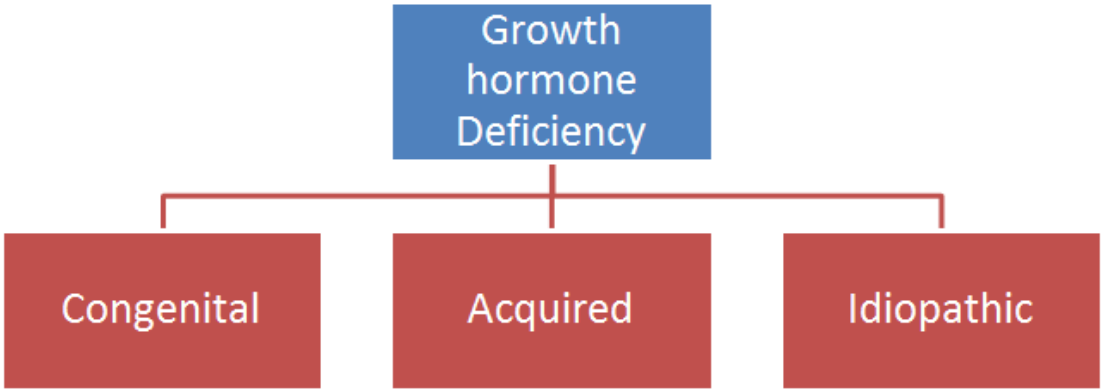

Figure 5 Causes of GHD in adults

Broadly speaking, GHD in adults is classified etiologically as congenital, acquired or idiopathic (Figure 5) (Molitch, 2011). Since the development of the pituitary gland depends on the sequential temporal and spatial expression of transcription factors and signaling molecules, mutations of these critical transcription factors can lead to congenital hypopituitarism. Whereas mutations in early transcription factors lead to multiple pituitary hormone deficiencies, others can cause isolated deficiencies (Alatzoglou and Dattani, 2009). GHD has also been reported due to mutations of other critical signaling molecules in the downstream pathway such as the genes encoding the GHRH receptor and the *GSα* gene leading to GHRH resistance (Mantovani *et al.*, 2003; Alatzoglou *et al.*, 2009). Congenital GHD is often associated with other congenital anatomical changes in the hypothalamic-stalk-pituitary region. The abnormalities range from pituitary hypoplasia and stalk agenesis to an ectopic posterior pituitary. These abnormalities are often associated with multiple pituitary hormone deficiencies (Maghnie *et al.*, 2001).

Pituitary and hypothalamic tumours may cause hypopituitarism primarily or following treatment with surgery or irradiation. Commonly occurring tumours are pituitary

adenomas and craniopharyngiomas. GHD risk is temporally linked and dose dependent on cranial irradiation. A younger age, a longer duration following radiotherapy and a higher dosage increase the probability of developing GHD (Mulder *et al.*, 2009). A dosage of >40Gy cranial radiotherapy is associated with > 50% likelihood of developing GHD (Gleeson *et al.*, 2004). Infiltrative diseases of the hypothalamus and stalk may also cause hypopituitarism and diabetes insipidus.

Finally, idiopathic GHD is very commonly observed in childhood GHD. These patients tend to present with biochemically defined GHD in childhood, often with normal neuroimaging. However, they are often found to have normal GH responses during reassessment at adulthood (Tauber *et al.*, 1997). This discrepancy can be ascribed to arbitrarily defined GHD diagnostic thresholds and poor reproducibility of the GH response to provocative testing within individuals. Moreover, children with constitutional delay in growth and puberty may also have a suboptimal GH response if they were not adequately primed with sex steroids at the time of testing. However, it is also possible that transient GHD is a true phenomenon in a fraction of these patients (Molitch, 2011). It is, therefore, an umbrella term used to describe a heterogeneous collection of patients with GHD of unknown cause, transient GHD and occasionally incorrectly diagnosed GHD. Idiopathic adult onset GH deficiency has also been described where its accurate diagnosis is challenged by the pulsatility of GH secretion, provocative test result variability, and suboptimal GH assay standardization. The spectrum between attenuated GH secretion associated with the normal aging process and with obesity, and truly well-defined Idiopathic adult onset GH deficiency is not distinct and may mislead the diagnosis. The reported prevalence of idiopathic adult onset GHD lies between 10-15% (Katznelson *et al.*, 2011).

Table 1 Congenital causes of GHD in adulthood

Congenital
Genetic
Transcription factor defects (PIT-1, PROP-1, LHX3/4, HESX-1, PITX-2)
GHRH receptor gene defects
GH secretagogue receptor gene defects
GH gene defects
GH receptor/post receptor defects
Associated with brain structural defects
Agenesis of corpus callosum
Septo-optic dysplasia
Empty sella syndrome
Holoprosencephaly
Encephalocele
Hydrocephalus
Arachnoid cyst
Associated with midline facial defects
Single central incisor
Cleft lip/palate
Idiopathic

(Molitch *et al.*, 2011)

Table 2 Acquired causes of GHD in adulthood

Acquired
Trauma
Perinatal
Postnatal
Central nervous system infection
Tumours of hypothalamus or pituitary
Pituitary adenoma
Craniopharyngioma
Rathke's cleft cyst
Glioma/astrocytoma
Germinoma
Metastatic
Other
Infiltrative/granulomatous disease
Langerhans cell histiocytosis
Sarcoidosis
Tuberculosis
Hypophysitis
Other
Cranial irradiation
Surgery of the pituitary or hypothalamus
Infarction
Spontaneous
Sheehan's syndrome

(Molitch *et al.*, 2011)

1.3.4 Treatment of GHD in Adults

Patient selection

In the United Kingdom, recombinant human growth hormone replacement is recommended by the National Institute for Clinical Excellence (NICE) for the treatment of adults with GHD if they fulfil all three of the following criteria (NICE, 2003):

I. Severe GH deficiency (defined as a peak GH response of <3 mcg/L [9mU/L] during an insulin tolerance test or a cross-validated GH threshold in an equivalent test).
II. A perceived impaired QoL, as demonstrated by a reported score of at least 11 in the disease-specific QoL-AGHDA questionnaire.
III. Already receiving appropriate pituitary hormone replacement.

It is important to note that the peak GH response of <3mcg/L stipulated by NICE above differs slightly from the Endocrine society which recommends a peak GH response of <5.1 mcg/L for the diagnosis of adult GHD to be made (Molitch *et al.*, 2011).

Dose selection

The objective of treatment is to maximise health benefits and minimise side-effects. However, there is considerable inter-individual sensitivity to treatment, particularly in the elderly. It is generally recommended that the initial dose should be started low (0.15-0.30 mg/day) and be gradually increased on the basis of clinical and biochemical responses. There is considerable variation in maintenance doses between individuals but the required dose rarely exceeds 1mg/day.

Clinical experience has demonstrated that the variability in subcutaneous absorption and individual responsiveness to GH makes dose determination based on body weight or body surface area less helpful than first anticipated. Therefore, GH replacement should be titrated to ensure optimum IGF-1 levels ('Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency,' 1998).

GH is generally continued until adult peak bone mass has been achieved (~25 years of age)(NICE, 2003). After adult peak bone mass has been attained, the decision to continue treatment primarily depends on the impact of GH withdrawal on the patient's Quality of Life.

Monitoring treatment efficacy

Treatment efficacy is evaluated using quality of life questionnaires and by titrating GH dosage according to IGF-1 levels. The best available bio-marker of GH action is serum IGF-1, which can be used to titrate the GH dosage and avoid over replacement. The objective is to maintain IGF-1 levels within an age-related normal range. During the initial stages of GH treatment, more frequent measurements are required but this eventually settles to once or twice yearly ('Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency,' 1998).

Safety issues and Contraindications

Fluid retention is sometimes observed with GH therapy. Hence, oedema and carpal tunnel syndrome can be experienced. However, these symptoms are often self-limiting and respond to a dose reduction. Markers of glycaemia should be monitored because GH can be associated with reduced insulin sensitivity.

Absolute contraindications include active malignancy, idiopathic intracranial hypertension and diabetic retinopathy. Although GH can safely be given in pregnancy, it should be stopped in the 2nd trimester since GH is endogenously produced by the placenta ('Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency,' 1998).

Long term care

GH replacement, like other pituitary hormones, can potentially be administered for life. The dose is usually reduced over time, in keeping with normal physiology.

1.3.5 The interplay between growth hormone and other pituitary hormones

The majority of adults with growth hormone deficiency have concurrent multiple pituitary hormone disease secondary to structural aplasia/damage of the pituitary gland. This may be congenitally acquired or secondary to the effects of surgery, radiotherapy or trauma. The effects of radiotherapy and surgery have potentially deleterious effects on long term health outcomes. Adults with GHD could also experience fatigue if there is suboptimal replacement of other hormones like thyroxine, cortisol or testosterone. Consequently, it is important to be aware of the implications and interactions of the thyroidal, adrenal and gonadal axes with the GH-IGF-1 axis since some of the features associated with GHD could also reflect the suboptimal replacement of other pituitary hormones.

GH axis and the thyroid axis

The relationship between the GH-IGF-1 axis and the hypothalamo-pituitary-thyroid (HPT) axis is complex and interdependent. Untreated primary hypothyroidism is associated with reduced IGF-1 and IGF binding protein-3 that increases with thyroxine replacement (Akin *et al.*, 2009). Similarly several studies have demonstrated a variety of alterations in the HPT axis following GH administration in both normal and GHD adults and children. Whereas conflicting data have been reported in healthy individuals, studies by Porretti *et al.* and Losa *et al.* demonstrated that GH replacement at a dose that achieves a serum IGF-1 in the upper reference range unmasks hypothyroidism in patients with organic pituitary disease (Porretti *et al.*, 2002; Losa *et al.*, 2008). It has also been demonstrated that apparently euthyroid patients who developed *de novo* biochemical hypothyroidism following GH replacement had reduced QoL compared to those patients who remained euthyroid on GH, despite similar QoL scores at the beginning and the attainment of similar serum IGF-1 concentrations in both groups (Agha *et al.*, 2007).

Therefore, it is essential to monitor thyroid function diligently following GH commencement particularly in the first 6 months to identify those patients who have underlying secondary hypothyroidism.

GH axis and cortisol metabolism

Cortisol metabolism is dependent on the interconversion of cortisol and cortisone which is modulated by the isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD-1 functions as a net reductase converting cortisone to cortisol. GH inhibits 11β-HSD-1 resulting in a shift from active cortisol to inactive cortisone with increased cortisol clearance. This phenomenon may be responsible for precipitating secondary adrenal failure after GH commencement (Moore *et al.*, 1999). Tschop *et al.* studied the pharmacokinetics of hydrocortisone substitution in hypopituitary patients following GH replacement (Tschop *et al.*, 2000). Using serum and salivary cortisol profiles after hydrocortisone administration at baseline and 6 and 12 months following initiation of GH replacement, they found no clinically relevant alterations in the pharmacokinetics of cortisol following initiation of GH replacement in hypopituitary adults. However, because of the variation in cortisol-cortisone conversion within a particular organ or tissue, one cannot rule out tissue specific effects.

GH axis and oestrogen metabolism

The close interplay between GH and oestrogen leads to the attainment of gender specific body composition during puberty. Furthermore, oral oestrogen (due to its first pass metabolism in the liver) but not transdermal oestrogen impairs the metabolic action of GH on the liver, causing a fall in IGF-I production and fat oxidation. This may contribute to a loss of lean tissue and a gain in body fat in postmenopausal women and could contribute to an impairment of GH effect in hypopituitary women on GH replacement (Leung *et al.*, 2004).

GH axis and testosterone

There is evidence to suggest that a synergistic relationship exists between GH and testosterone. IGF-1 mRNA (messenger ribonucleic acid) increases when testosterone is administered to healthy elderly men and decreases in healthy young men when they are rendered hypogonadal (Ferrando *et al.*, 2002). Several studies have demonstrated a more pronounced improvement in body composition, muscle strength, endurance and QoL with the simultaneous administration of GH and testosterone than with either alone (Brill *et al.*, 2002; Gibney *et al.*, 2005; Giannoulis *et al.*, 2006). This suggests that GH and testosterone may exert independent and additive effects on protein metabolism, lipid

oxidation and resting energy expenditure. This again highlights the importance of ensuring adequate testosterone replacement in hypopituitary patients on GH replacement.

1.3.6 Metabolic abnormalities in GHD adults

Body composition

GH and IGF-1 manipulate, and are in turn influenced by, body composition with lean healthy individuals demonstrating higher GH but lower IGF-1 levels than their obese counterparts. GHD is associated with a typical phenotype characterised by centripetal obesity and a reduction in Lean Body Mass (LBM). Some of the deleterious metabolic effects of GHD may actually be caused by this centripetal fat distribution (Carroll *et al.*, 1998).

Irrespective of the underlying aetiology of GHD, the disorder is consistently associated with body composition changes. However, GHD secondary to craniopharyngioma is associated with a higher degree of obesity than non-functioning pituitary adenomas. This is due to the hyperphagia which develops secondary to the hypothalamic damage which may be sustained during tumour growth and subsequent management (Verhelst *et al*., 2005). A KIMS (Pfizer International Metabolic Database) study reported that patients undergoing pituitary radiotherapy had a higher mean body fat mass than patients with non-radiated tumours. It was suggested that this may be due to the direct detrimental effects of radiotherapy on the brain. However, the observed differences in these two groups could also be ascribed to the longer duration of untreated GHD in the irradiation group. Both of the groups benefitted from GH replacement (Maiter *et al*., 2006).

Although the consequences of GHD continue into later life, its effects in the elderly are a less important cause of centripetal weight gain than in younger patients.

Lipids

Patients with GHD often have adverse cardiovascular profiles and this may be partially responsible for the increased mortality observed in hypopituitary patients. An analysis of 2589 patients from the KIMS database revealed that 40% of GHD patients aged <20 years had total serum cholesterol (TC) above 5.3mmol/L. Mean TC increased linearly across age groups until it plateaued at 49 years so that in those aged 40 years, 75% of patients had a TC above 5.3mmol/L. Similar findings were seen in LDL-cholesterol (LDL-C) and to a lesser extent in triglycerides with reciprocal changes in HDL-cholesterol (HDL-C)

too. GH replacement was associated with significant improvements in TC, LDL-C and HDL-C but no changes in TG (Abs *et al.*, 2006). Older patients also demonstrated an improved lipid profile with GH replacement. In fact Attanasio *et al*. reviewed results from 242 HypoCCS (The Hypopituitary Control and Complications Study) patients and reported the greatest improvement in LDL/HDL ratio in patients aged 40-60 years when compared with both younger and older patients (2002a).

1.3.7 Growth hormone and skeletal muscle function

The anabolic effects of GH are apparent in patients with acromegaly and can be contrasted with the phenotype of GHD patients. Fatigue (26%) and weakness are commonly described by patients with acromegaly and may be prominent symptoms. These symptoms may reflect the associated medical consequences of acromegaly, including sleep apnea syndrome, cardiomyopathy with reduced function, hypopituitarism, hyperthyroidism, depressed mood, and diabetes mellitus (Katznelson *et al.*, 2011). *In vitro* and animal studies have demonstrated the stimulating effect of GH on skeletal muscle growth. Several methods have also been used to measure the *in vivo* effects of GH on muscle metabolism in humans including whole body nitrogen retention and total and regional muscle protein metabolism using labelled amino acids.

Adults with chronic, untreated GHD may have multiple somatic impairments including compromised muscle performance. They may experience functional limitations, diminished productivity, social isolation and excessive fatigue leading to an impaired QoL.

GH purportedly alleviates some of these impairments by increasing circulating levels of IGF-1, skin thickness, bone mineral content, lean body mass and reducing fat mass. However, whether alleviation of the impairments translates into meaningful improvements in physical function remains more controversial.

In vitro effects of GH/IGF-1 on differentiated skeletal muscle

Both GH and IGF-1 exert an anabolic effect on skeletal muscle tissue which includes myogenic differentiation (Florini *et al.*, 1991). The precise mechanism by which the GH/IGF-1 axis influences differentiated skeletal muscle tissue remains unclear. While it is clear that IGF-1 is an important myogenic signal that mediates the effects of GH on the skeletal muscle (Quinn *et al.*, 1994), the direct role of GH on skeletal muscle tissue using cell culture studies is less convincing (Florini *et al.*, 1977; Merrill *et al.*, 1977). A study where foetal sheep myoblasts were grown *in vitro* to examine protein turnover when exposed to growth hormone, showed no stimulation of protein synthesis. However, an inhibition of protein breakdown was found at high GH concentrations. In contrast,

exposure to IGF-1 resulted in a stimulation of protein synthesis at low levels.(Harper *et al.*, 1987).

In vivo effects of GH on skeletal muscle-Human studies

Woodhouse and co-workers demonstrated an increase in type I fiber size in adults with untreated GHD before and after 6 months of GH treatment (Woodhouse *et al*., 1999). These findings are in contrast to healthy control subjects who typically have larger type II muscle fibers that are recruited during brief high intensity exercise and smaller type I fibers that are recruited during more prolonged lower intensity activity. Because the proportion of fiber types are a major influence on contractile properties of skeletal muscle, altered fiber composition may be partly responsible for the impaired strength in adults with untreated GHD. However, the finding of larger type I fibers is counterintuitive and does not explain the increased perception of fatigue experienced by adults with GHD, since type I fibers are typically more fatigue resistant (Woodhouse *et al.*, 2006). However, this finding is not consistent. Previous studies which compared muscle biopsies from adults with untreated GHD revealed qualitatively normal muscle, with no obvious features of myopathy and no significant difference in mean fiber size or proportion (type I vs. type II fibers) compared with age- and gender-matched healthy adult controls (Whitehead *et al.*, 1989; Cuneo *et al.*, 1992).

There is also discrepancy when interpreting the true impact of GH replacement on skeletal muscle. Whereas early muscle biopsy findings demonstrate minimal changes in muscle fiber size (Cuneo *et al.*, 1992), subsequent imaging studies (both MRI and CT) reveal a significant increase in thigh muscle mass (Cuneo *et al.*, 1992; Janssen *et al.*, 1999). However, since GH replacement is associated with increased water retention, the measurement of cross-sectional areas of the muscle may simply reflect a rise in water content. Whether muscle fiber size is truly increased following GH replacement remains unclear.

Effects of GH/IGF-1 on protein metabolism

Adults with GHD have reduced lean body mass (LBM) and skeletal muscle mass when compared with healthy control subjects, suggesting possible impairment in protein anabolism. However, because the loss in LBM and muscle mass cannot persist indefinitely, metabolic adaptations must occur so that LBM and muscle mass stabilize,

albeit at reduced levels, in adults with GHD. Hoffman *et al*. demonstrated that a decline in the rate of protein breakdown occurs to offset the initial fall in protein synthesis so that net protein loss is minimized. They hypothesized that normalization of protein oxidation may be a homeostatic mechanism that helps to partially reduce protein loss in adults with GHD (1998).

The majority of studies to date suggest that IGF-1 mediates a reduction in protein degradation whilst administration of GH results in enhanced protein synthesis.

Similar to untreated GHD, there must be a homeostatic adaptation to protein anabolism in patients with GH replacement, since LBM cannot accrue indefinitely. The time-course of this metabolic adjustment remains controversial. However, the majority of placebo-controlled studies report that the positive effects of GH administration on protein synthesis and body composition usually occur within the first few months of treatment (Jorgensen et al., 1989; Salomon et al., 1989). In fact a 20 day in-patient study in 4 patients commencing GH therapy revealed that the initial response of acute nitrogen retention, and thus protein accretion, occurred within 2-5 days and subsided after 1-2 weeks of GH administration. Therefore, protein kinetics during the initial few months of GH administration in adults with GHD, as their LBM is increasing, appears to be different from that during chronic GH treatment when gains in LBM have stabilised and steady state has been achieved (Valk *et al.*, 1994). The duration of enhanced nitrogen retention after GH treatment remains controversial and warrants further investigation.

An interesting discovery has been that infusion of GH and IGF-I into the brachial artery increases forearm blood flow several fold (Fryburg *et al.*, 1991; Turkalj *et al.*, 1992). This effect appears to be mediated through stimulation of endothelial nitric oxide release leading to local vasodilatation. Moreover, co-infusion of a nitric oxide inhibitor with IGF-I appeared to blunt the stimulatory effect of IGF-I on forearm protein synthesis (Boger *et al*., 1996). Hence an IGF-I mediated increase in muscle nitric oxide release could account for some of the effects of GH on skeletal muscle protein synthesis.

It, therefore, appears that the acute effects of GH infusion result from increased protein anabolism. Conversely, the anabolic effects associated with the long term use of GH on skeletal muscle appear to be the result of inhibition of proteolysis, most likely mediated through IGF-1, resulting in reduced protein catabolism.

Effects of GH/IGF-1 on Lean body mass

Extensive cross-sectional studies suggest that adults with GHD have increased subcutaneous and visceral fat mass with reduced LBM when compared with matched healthy control subjects (De Boer *et al.*, 1992; Amato *et al.*, 1993; Beshyah *et al.*, 1995b; Hoffman *et al.*, 1995). However, a key shortcoming in all these cross-sectional comparisons is the failure to compare with GHD adults on GH replacement. It is possible that the increased fat mass observed is due to the morbidity of hypopituitarism rather than the effects of GH per se. The reduction in LBM is greater in untreated childhood onset GHD (CO-GHD) adults compared with adult onset GHD (AO-GHD) (Attanasio *et al.*, 2002b) suggesting that GH may play a role in somatic development.

Although there is general consensus that fat mass is higher and LBM slightly lower in adults with GHD (Carroll *et al.*, 1998), it has yet to be conclusively proven that the reduced LBM associated with GHD reflects a reduction in metabolically active muscle as opposed to reduced water content. However, abnormalities in body composition maybe normalised with GH therapy over time (Carroll *et al.*, 1998).

Hazem *et al.* undertook a meta-analysis to investigate the effects of GH therapy on body composition. Only randomised placebo-controlled trials of GH therapy in adults (>18 years) were included in the analysis with a minimum treatment period of 3 months. GH administration significantly and consistently reduced weight and body fat content while increasing lean body mass. However, BMI was not significantly affected. Since the majority of included studies had a follow up of 6 months (range 3-24 months), the clinical implications of the above findings remain uncertain since no study has shown that these changes are maintained on a long term basis (Hazem *et al.*, 2012).

Effects of GH/IGF-1 on skeletal muscle strength

Adults with GHD have reduced absolute maximal isometric (where the muscle does not shorten i.e. remains the same length) and possibly isokinetic (where the muscle shortens at a consistent rate i.e. maintains the same motion) muscle strength that is at least partly due to reduced muscle mass. Although findings from earlier studies suggested that intrinsic muscle strength might be reduced in these patients, more recent data refute this notion (Woodhouse *et al*., 2006). There are discordant findings between the substantial increase in muscle fiber size that occurs in adults treated with GH and the

lack of improvement in muscle strength. Interpretation of these findings is complicated by differences in severity of GHD, dose of administered GH and considerable individual variations in muscle strength. In addition the majority of placebo-controlled trials (4-6 months) used a shorter time period than the open-labelled studies (1-3 years).

Johannsson *et al.* observed an increase in isometric and isokinetic muscle strength in proximal muscle groups following 2 years of GH administration in GH deficient adults but this was accompanied by reduced local muscle endurance. They postulated that an inability of the skeletal muscle to metabolically adapt in terms of muscle glucose storage and utilization, oxidative enzyme activity and capillarization could explain this deficit (Johannsson *et al.*, 1997). A 10 year, single-centre open-label study investigated the long term effects of GH replacement on muscle strength. While there was a sustained increase in lean muscle mass and isometric knee flexor strength, other measures of upper leg and grip strength showed a transient increase in the 1st five years (0-5 years) of the study followed by a decrease in strength to baseline values or below during the 2nd five years of the study (5-10 years) (Gotherstrom *et al.*, 2009). When the investigators made corrections for age and gender using observed/predicted value ratios, GH partly protected against the normal age-related decrease in muscle strength. It is not clear, however, how the age and sex adjustments were made.

Widdowson *et al.* undertook a meta-analysis to determine the effect of GH replacement on muscle strength. Eight randomised, double-blinded, placebo-controlled studies were included involving 239 patients. The average duration of GH replacement was 6.7 months. Analysis revealed no significant improvement when all studies were combined nor when measured individually (isometric and isokinetic). Thus, in contrast to open-labelled longer term studies, short term controlled studies fail to demonstrate a benefit on muscle strength with GH therapy (Widdowson and Gibney, 2010).

Effects of GH/IGF-1 on aerobic performance

Studies evaluating maximal aerobic capacity (VO_2max) have consistently demonstrated markedly reduced VO_2max in GHD adults when compared with age, weight and height matched predictive values (Cuneo *et al.*, 1991; Whitehead *et al.*, 1992; Nass *et al.*, 1995). The postulated mechanisms for this are reduced skeletal muscle mass or altered metabolism (Cuneo *et al.*, 1991), diminished cardiac capacity (Colao *et al.*, 2001) and

reduced red blood cell volume due to impaired IGF-1 mediated erythropoiesis (Christ *et al.*, 1997). Another cardiopulmonary test called the ventilation threshold (VeT) or lactate threshold is used as an objective measure of the ability to perform submaximal, prolonged work (Woodhouse *et al.*, 2006). The effect of GH replacement on VeT remains controversial. Two studies (Cuneo *et al.*, 1991; Woodhouse *et al.*, 1999) reported a significant increase (16-18%) in VeT whilst another showed no change when compared to a placebo group (Nass *et al.*, 1995). The precise mechanism behind the possible increase in Vet remains unclear. Reduced glycolytic anaerobic metabolism (Jones and Ehrsam, 1982), improved proton clearance (Jones and Ehrsam, 1982) and delayed recruitment of type II fibers (Mateika and Duffin, 1994) are some of the proposed mechanisms.

Widdowson *et al.* undertook a meta-analysis to examine the effects of GH replacement on exercise performance. They included 11 randomised, double blinded placebo controlled studies involving 268 patients. GH replacement was associated with a significant improvement in maximal power output and maximal oxygen uptake (Widdowson and Gibney, 2008). The results of this study must be interpreted cautiously because the magnitude of benefit is difficult to ascertain.

Functional Limitations

Adults with GHD may suffer functional limitations because muscle power is known to be important for performance of daily activities such as rising from a chair, climbing stairs and for walking. However, the majority of activities of daily living are performed using aerobic metabolism. Thus, perhaps more importantly, it is the aerobic effects of GH that are likely to be more relevant in abating fatigue.

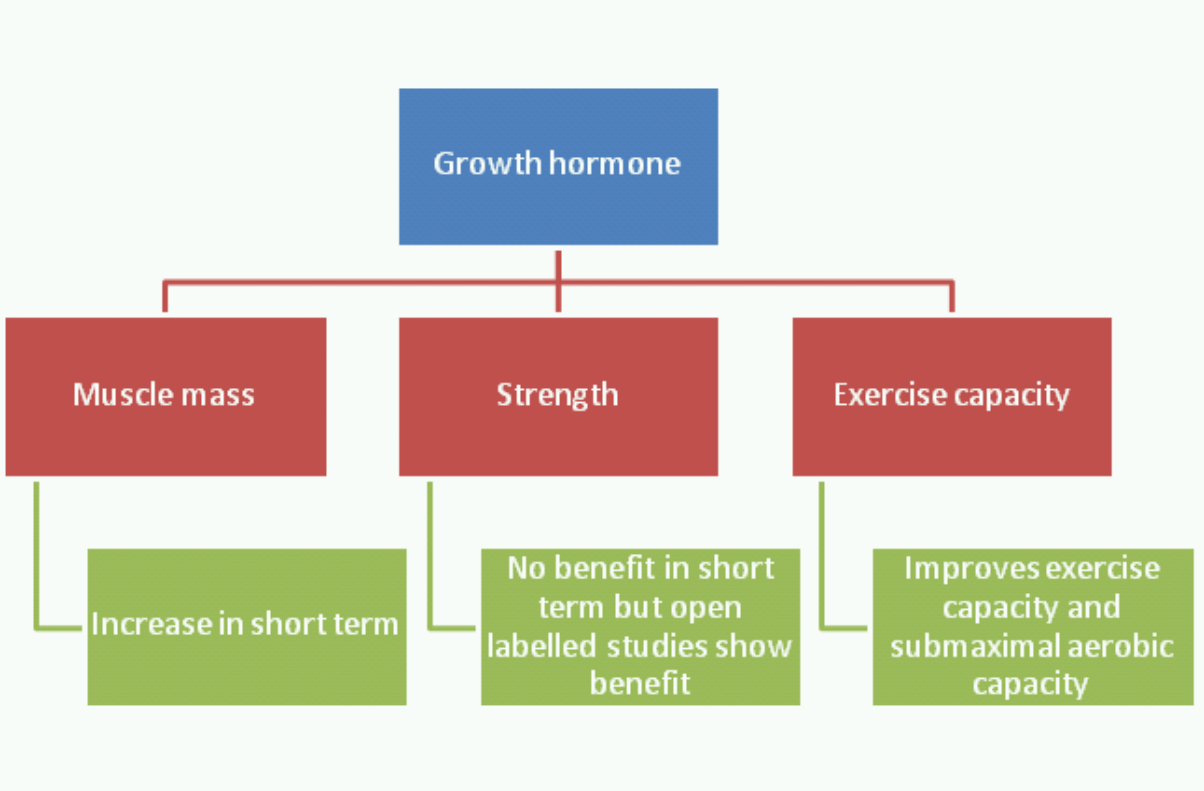

Figure 6 Growth hormone and its putative physical and functional effects on skeletal muscle tissue

1.3.8 Quality of Life impairment in GHD

Definition

Quality of life (QoL) is a ubiquitous concept that has different philosophical, political and health-related definitions. Health related QOL (HRQoL) includes the physical, function, emotional and social well-being of an individual. It is a patient-reported outcome usually measured with carefully designed and validated tools such as questionnaires. Often these instruments measure multidimensional domains of an individual's functional health and well-being.

QoL in GHD

Impairment of QoL in hypopituitary adults not receiving GH replacement therapy has been evident for a long time (Carroll *et al.*, 1998). Untreated adults patients with GHD most often complain of reduced energy levels, vitality, mental fatigue, emotional reactions, social isolation and anxiety (Bjork *et al.*, 1989; McGauley *et al.*, 1990). Additionally, some of these patients may also complain of a reduced sex drive, poor body image, reduced motor mobility, lack of self-confidence, a poor memory, reduced cognitive function, decreased mood and attention deficits (Deijen *et al.*, 1996; van Dam *et al.*, 2005).

The majority of these findings originate from small patient cohorts followed for a limited time period. The benefits of GH therapy in improving QoL as assessed by the 'gold standard' of clinical studies, randomised controlled trials (RCT), remains controversial. Table 3 illustrates the results of the RCT's that have examined whether GH therapy leads to an improved QoL. In the studies that demonstrated improvements in QoL there was inconsistency in terms of the instrument used and the effects measured. The variability in the assessment of QoL by different studies may reflect the different tests used in measuring QoL. There are both generic and disease specific tests available and therefore one would expect the generic tests to have a lower sensitivity to GH-dependent QoL indices. This also highlights the multifactorial nature of QoL. There may also be a temporal influence of GHD on well-being as Burman *et al*. demonstrated that there was an inverse correlation between duration of GHD and QoL (1995). On the contrary, the severity of GHD has not been shown to impact on QoL (Zenker *et al.*, 2002). A meta-

analysis by Ahmad *et al*. was unable to pool the findings of the trials assessed because of the heterogeneity in outcome measures and the lack of quantitative data to perform the meta-analysis. Nonetheless, 11/16 of the trials reported a statistically significant improvement in at least one construct of the QoL tool in patients who received active treatment (2001).

Table 3 Summary of Studies reporting QoL changes with GH therapy

Improvement in QoL indices reported	No benefit in QoL indices reported
(n=233) (Ma°rdh G and investigators, 1994)	(n=166) (Cuneo *et al*., 1998)
(n=173) (Attanasio *et al.*, 1997)	(n=40) (Beshyah *et al.*, 1995a)
(n=148) (Verhelst *et al.*, 1997)	(n=40) (Baum et al., 1998)
(n=73) (Chihara *et al.*, 2006)	(n=34) (Sathiavageeswaran et al., 2007)
(n=48) (Deijen *et al.*, 1998)	
(n=42) (Carroll *et al.*, 1997)	
(n=36) (Burman *et al.*, 1995)	
(n=32) (Wallymahmed *et al.*, 1997)	

Qol-AGHDA: A Disease specific questionnaire

There are several generic and disease specific test available to quantify QoL. Researchers have quantified QoL in hormone deficient patients in order to define the importance of the deficient hormone in determining QoL. However, patients with a chronic illness often adapt, matching their activities to their ability. Since adaption is not detected using conventional QoL instruments (Jorgensen, 1999), the relevance of the deficient hormone to QoL is often underestimated by the generic investigative approach. Older studies have usually relied on well-validated, generic tests of overall health and psychiatric well-being, such as the Nottingham Health Profile (NHP). These questionnaires have been criticized, however, for their lack of sensitivity to subtle changes in QoL (McKenna *et al*., 1999). In addition, generic tools do not address symptoms that are important to adult patients with GHD and concentrate on areas that are of little relevance (McKenna *et al*., 1999). Therefore, for the purpose of the studies described in this thesis, I shall be utilising a specific test developed particularly to assess GHD adults, namely QoL-AGHDA (Adults with growth hormone deficiency assessment).

QoL-AGHDA is a disease-specific, need-based measure which was developed based on in-depth qualitative interviews with adult patients with GHD (n=35; 14 men; age range 20–59 years) attending the Christie Hospital in Manchester, UK (Holmes SJ, 1995). Almost all patients were dissatisfied with their body image and complained of lack of energy (94% and 91% respectively), 83% had problems with memory and concentration, 71% described themselves as being short-tempered and easily irritated, 66% suffered from lack of strength and stamina, 63% experienced reduced physical and mental drive and 57% had difficulties coping with stressful situations and avoided external stimulation (Wiren *et al.*, 1998).

The measure is constructed of 25 items that evoke yes/no answers, acknowledging or denying certain problems. The total score is computed by quantifying a range of problems so that each 'yes' is given a score of 1 and each 'no' is attributed a score of 0. Therefore, a higher numerical score depicts a poor QoL. It has been shown to have good reliability, reproducibility, internal consistency and construct validity across a range of languages (McKenna *et al.*, 1999).

Possible mechanisms for GH-induced impaired QoL

A cross-sectional case-control study identified abnormalities in the sleep architecture of GHD adults. They postulated that increased hypothalamic GH releasing hormone (GHRH) activity produced through the loss of negative feedback in GHD could contribute to the daytime somnolence and fatigue in GHD (Copinschi *et al.*, 2010). Hence, the fatigue perceived by these patients could partly be central in origin.

Chrisoulidou *et al.* explored the possible mechanisms of action of GH in improving QoL. They discussed the possible mechanisms by which GH modulates QoL including its action at various neural sites, modifying mood, memory and cognition. It may also influence subjective well-being via somatic changes in body composition, cardiovascular health, reproductive function and skin (1998).

Limitations

One of the limitations of longitudinal self-reported outcome assessments is the response shift phenomenon. Response shift is a change in one's internal perception or understanding of a construct and is thought to be made up of three components:

reconceptualisation, reprioritization and recalibration (Schwartz and Sprangers, 1999; Sprangers and Schwartz, 1999; Rapkin and Schwartz, 2004).

- Reconceptualisation is the change in the understanding of which elements or components are included in a target construct.
- Reprioritisation is the change in the preference regarding the relative importance of certain components within the construct.
- Recalibration is the change in the value of scaling of certain health states in relation to others.

In summary, response shift may cause paradoxical or incorrect findings during longitudinal questionnaire trials. It is a naturally occurring process believed to be part of natural coping and adaptive mechanisms. However, it has the potential to invalidate study findings. Although no solution has been found to this problem, research is on-going to minimise this phenomenon.

1.3.9 GHD and the brain

The reported poor memory and reduced cognitive function observed in GHD patients has led to considerable interest in the effects of GH on the central nervous system (CNS). I shall briefly summarise an overview of the work undertaken in this particular field.

Evidence for the presence of GH in the brain

There are three possible mechanisms by which GH may exert its effect on brain function (Figure 7). Firstly, GH may release secondary mediators such as IGF-1 from peripheral tissues which may cross the blood brain barrier (BBB) (Coculescu, 1999). Secondly, GH may be degraded enzymatically into bioactive fragments which reach the brain and act on peptidergic receptors (Stanley *et al.*, 2011). Thirdly, GH may enter the brain itself and directly influence the nervous system. This notion is supported by the fact that GH has been detected in the brain and CSF (Nyberg and Burman, 1996).

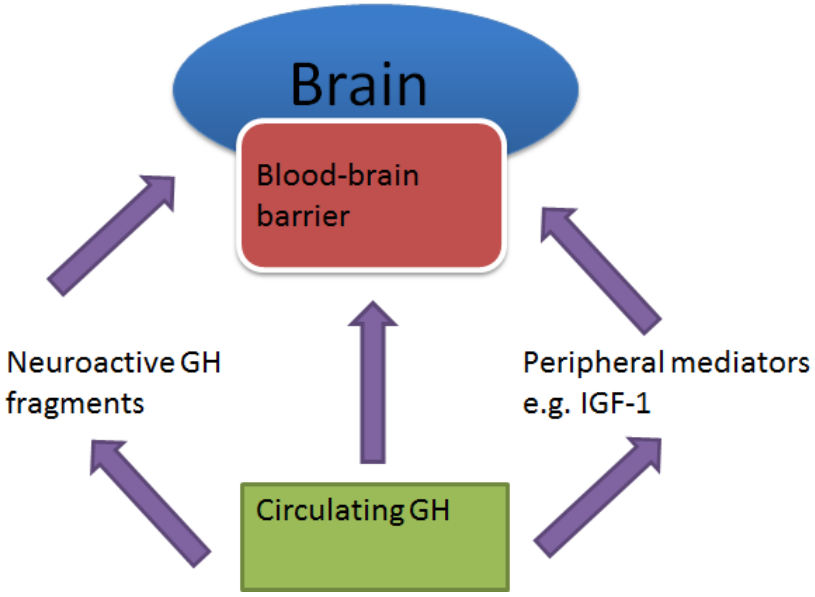

Figure 7 Putative mechanisms of action of GH on brain

Growth hormone receptors in the brain

Specific binding sites for GH in the human brain have been identified and characterised. The highest density of GH binding, using iodine-labelled GH was found in the choroid plexus followed by the hippocampus, the hypothalamus and the pituitary (Lai *et al.*, 1991).

Evidence of cognitive impairment in GH deficient adults and the effects of GH replacement

Several neuropsychological studies suggest that patients with GHD experience significant cognitive impairment (Deijen *et al.*, 1996; Lijffijt *et al.*, 2003; van Dam *et al.*, 2005) and that this can be ameliorated by GH replacement (Sartorio *et al.*, 1995; Deijen *et al.*, 1998; Arwert *et al.*, 2005a).

However, most of these conclusions have been drawn from cross-sectional comparisons between GHD patients and matched healthy controls. Hence the reliability of inferences regarding the presence of cognitive impairment in GHD is limited. GHD patients can have low mood and low motivation which may impair their performance on cognitive testing. The use of heterogeneous GHD patient groups may complicate interpretation due to the variability in degree and duration of GHD. Although the various cognitive tests measure similar cognitive domains, different tests have been used in different studies, thus, limiting their generalisability. Since multiple comparisons were undertaken, lack of the adjustment of error rates (such as Bonferroni correction) for multiple post hoc analyses could also lead to Type 1 errors.

Falleti *et al.* undertook a meta-analysis of all of the neuropsychological literature to date to examine the nature and magnitude of cognitive impairment in GHD patients and the extent to which GH replacement improves cognitive function (2006). They concluded that patients with GH deficiency have neuropsychological impairments in the domains of attention, memory and executive functions when compared to matched controls. When comparisons were made in cross-sectional studies between GH treated patients and matched healthy controls, the cognitive impairments persisted. However, this method did not include comparisons with GHD patients before GH replacement and so we cannot determine whether or not the impairments detected actually represented an improvement from prior cognitive dysfunction or whether there was no benefit at all. Prospective data showed that, compared to a non-treatment baseline, all aspects of cognitive function improved following 3-6 months of GH treatment. This improvement continued for up to 12 months across the domains of attention, spatial ability, memory and executive functions (Deijen *et al.*, 1998) and for as long as 10 years in the memory domain (Arwert *et al.*, 2005a). In contrast, Arwert *et al.* concluded from their meta-analysis that there was no evidence that GH substitution improved patient reported

outcomes in GHD adults. They observed a progressive decrease in effect sizes when treatment duration increased, thereby, pointing to the presence of a placebo effect, which was most prominent in the start of the treatment period. More importantly they found that GH treatment was no more effective than placebo in terms of improving patient-reported outcomes (Arwert *et al.*, 2005b).

Functional MR (fMRI) studies in GHD adults

Functional MRI is a neuroimaging technique for measuring brain activity. It works by detecting the changes in blood oxygenation and flow that occur in response to neural activity. When a brain area is more active it consumes more oxygen and to meet this increased demand, blood flow increases to the active area. fMRI can be used to produce activation maps showing which parts of the brain are involved in a particular mental task.

Arwert *et al.* compared cognitive function in GHD adults with matched controls using both neuropsychological tests and fMRI. They concluded that GH-deficient patients have a subnormal memory speed, but no impaired quality of memory performance, which may be due to compensatory recruitment of dorsal prefrontal brain regions (Arwert *et al.*, 2005c). They then investigated the effects of GH substitution on cognitive functioning in GHD adults using neuropsychological tests and fMRI. They concluded that 6 months of GH treatment improved the long-term as well as working memory in patients with GHD, and this was associated with decreased brain activation in the ventrolateral prefrontal cortex (Arwert *et al.*, 2006).

Effect of GH on developing brain structure and function in GHD children

Webb *et al*. investigated the effects of GH on developing brain structure and function. Reductions in white matter integrity in the corticospinal tract and corpus callosum and selective reductions in neural volume in isolated GHD children were identified. The abnormalities in white matter fiber density and neural volumes correlated significantly with cognitive function and motor skill abilities, which were also significantly impaired in isolated GHD children (Webb *et al.*, 2012).

1.3.10 Growth Hormone and the mitochondria

It is well recognized that the actions of GH include protein anabolism, promotion of lipolysis and reduced sensitivity to insulin-induced glucose metabolism in liver and peripheral tissues (Davidson, 1987; Moller and Norrelund, 2003; Jessen *et al.*, 2005). Since GH is an anabolic hormone, GH administration could increase the energetic demands on the mitochondria. Skeletal muscle mitochondrial activity maybe a particularly relevant and interesting candidate to interrogate since skeletal muscle tissue is not only the richest source of ATP generation in the body but is also one of the key target sites of growth hormone action along with its downstream regulator IGF-1. However, the effects of GH on mitochondrial function are not well established. In this section, I shall be discussing the work undertaken (*in vitro*, *in vivo* and ^{31}P MRS) so far to elucidate the role of GH on mitochondrial function.

In Vitro and in vivo Studies

Leung *et al.* developed an *in vitro* bio-assay to investigate the role of GH in the stimulation of mitochondrial fatty acid oxidation in human fibroblasts. They demonstrated that GH directly stimulated fatty acid oxidation *in vitro* (1997). However, IGF-1 had no detectable effect on fatty acid oxidation. In contrast, an *in vivo* study undertaken by Hussain *et al.* demonstrated that IGF-1 infusion enhances lipid oxidation in humans (1994). Therefore in order to explain this dichotomy, Leung *et al.* postulated that the *in vivo* stimulation of lipid oxidation by IGF-1 occurs indirectly through the suppression of insulin secretion and by reducing the inhibitory effect of insulin on whole-body lipid oxidation. However, the precise mechanism by which GH stimulates fatty oxidation still remains unclear. Unterluggauer *et al.* also demonstrated that IGF-1 dependent signaling stimulates mitochondrial respiration in prostate cancer cell lines (2008). Short *et al.* attempted to determine whether GH had an impact on mitochondrial regulation. They investigated the acute effects of GH by infusing GH or saline in 9 young healthy volunteers in a randomised crossover study design. Muscle biopsies were used to measure the mitochondrial ATP production rate, mitochondrial protein synthesis rate and the abundance of several gene transcripts that regulate muscle oxidative phenotype. They demonstrated that a 14 hour infusion of GH with an associated physiological elevation of GH in healthy people resulted in an increase in skeletal muscle

mitochondrial oxidative capacity, as shown by increased mitochondrial ATP production rates and increased citrate synthase activity. It also led to a higher muscle content of mRNA transcripts encoding oxidative proteins in mitochondria (COX3 and COX4), a nuclear transcription factor that regulates mitochondrial biogenesis (TFAM) and the glucose transport protein (GLUT4). However, they observed no effect on muscle protein synthesis, including mitochondrial proteins (2008).

^{31}P MRS and growth hormone production in healthy adults

Makimura *et al.* investigated the association between parameters of the GH axis and mitochondrial function non-invasively using ^{31}P MRS in healthy adults. At a single centre, they investigated 37 healthy adults between the ages of 18-55years. All subjects underwent GHRH-arginine testing after an overnight fast. Parameters of the GH axis measured included serum IGF-1, peak stimulated GH and GH area under the curve (AUC). Mitochondrial function was determined using ^{31}P MRS to assess PCr re-synthesis after sub maximal exercise (3 min X 40% maximal voluntary contraction of bilateral quadriceps contractions followed by a 5 min recovery). Mitochondrial function was measured using τPCr (mitochondrial phosphorylation potential) and Vi (initial rate of PCr recovery post-exercise). Both τPCr and Vi were studied as end points. They found a gender specific association between PCr recovery and parameters of the GH axis in otherwise healthy adult males but not in women (Makimura *et al.*, 2011). Moreover, obesity is associated with GH insufficiency (Murray *et al*., 2007) and they demonstrated that 4 of the 26 obese subjects had peak stimulated GH ≤ 4.2 mcg/L. Since obese subjects are more likely to have been physically inactive than their slimmer counterparts, it could be that an association exists between physical fitness and mitochondrial oxidative function (Johansen and Quistorff, 2003) rather than parameters of the GH axis. Besides, no explanations were provided for the gender dimorphic effect (Figure 8).

In contrast, Janssen *et al.* investigated the influence of GH on muscle phosphate metabolites at rest in GHD adults using ^{31}P MRS and found no evidence of an effect (Janssen *et al.*, 1999). Importantly, however, this method was not undertaken during exercise and hence dynamic markers of oxidative phosphorylation were not measured.

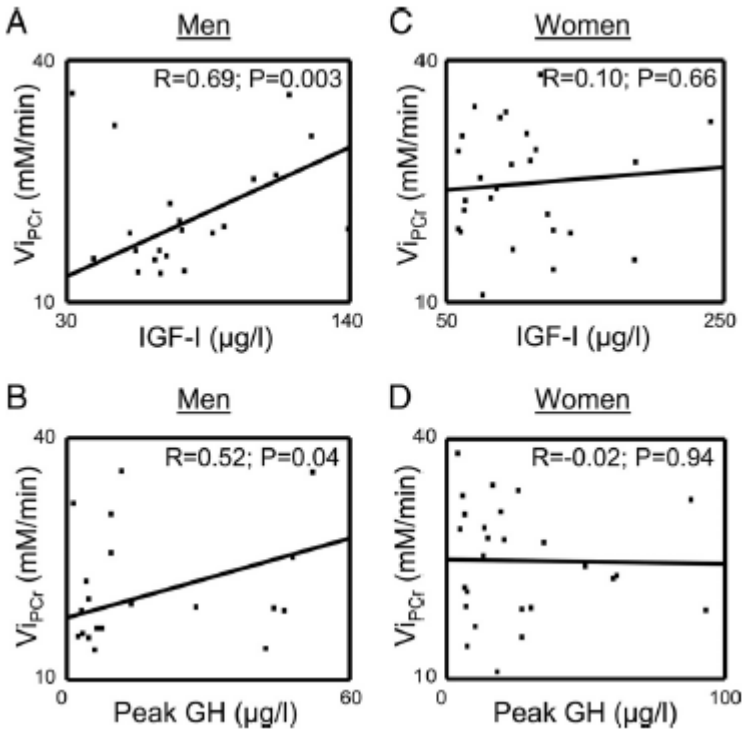

Figure 8 Growth hormone and phosphocreatine recovery in healthy adults

Bivariate analyses demonstrating association of (A) IGF-1 and (B) peak stimulated GH with Vi (phosphocreatine recovery) in men and (C) IGF-1 and (D) peak stimulated GH with Vi in women (Makimura *et al.*, 2011)

1.3.11 Summary

GHD adults present with a characteristic phenotype with fatigue being a predominant symptom. The fatigue also contributes to the impaired QoL often reported by these patients. There are multiple lines of evidence pointing towards the effects of GH on skeletal muscle metabolism although this does not necessarily translate into enhancements in functional outcomes following GH replacement in GH deficient subjects. Nonetheless, GH is a regulator of muscle function that potentially enhances muscle mass and improves exercise capacity. Skeletal muscle is thus a candidate for a common effector tissue responsible for mediating the symptoms and metabolic characteristics of GHD. Furthermore, because of the central role of mitochondria in energy generation and reports linking an association between GH and mitochondrial function, skeletal muscle mitochondrial function is a logical candidate to investigate when studying the pathophysiology of fatigue in GH deficiency.

1.4 Vitamin D deficiency

1.4.1 Vitamin D deficiency

Vitamin D is a prohomone essential for regulating calcium absorption in the gut. Vitamin D deficiency is associated with rickets in children and osteomalacia and myopathy in adults. Despite being freely available through UVB sunlight exposure of exposed skin, and to a lesser degree through eating oily fish, vitamin D deficiency is widespread in many developed and developing countries. This topic consequently figures prominently in a rapidly growing scientific literature, as well as in the popular press. This focus is appropriate and reflects the many uncertainties about vitamin D status, particularly its emerging role in extra-skeletal health, as well as its relationship to musculoskeletal disease. This section reviews the assessment of vitamin D status and the risk factors for vitamin D deficiency. It also examines the relationship between vitamin D and skeletal muscle.

1.4.2 Sources of vitamin D and metabolism

Most circulating vitamin D is synthesised from cutaneous exposure to solar rays with diet generally being a poorer source of vitamin D (Sinha *et al.*, 2012). Vitamin D exists as either ergocalciferol (Vitamin D2) or colecalciferol (Vitamin D3). Whereas ergocalciferol is derived from UV irradiated ergosterol of fungus or yeast origin, colecalciferol is animal derived. It is formed endogenously in the skin of mammals from its prohormone, 7-dehydrocholesterol, following solar ultraviolet B (UVB) radiation. It is then metabolised in the liver to 25-hydroxyvitamin D (25OHD) and then in the kidneys to either a metabolically active form (1, 25-dihydroxyvitamin D [1,25OHD]) or an inactive form (24, 25-dihydroxyvitamin D) (Figure 9). The activation of vitamin D in the liver and kidney occurs by hydroxylation which is catalysed by the mitochondrial cytochrome P450 system. 1, 25-dihydroxyvitamin D synthesis is stimulated by hypocalcemia, PTH and hypophosphataemia.

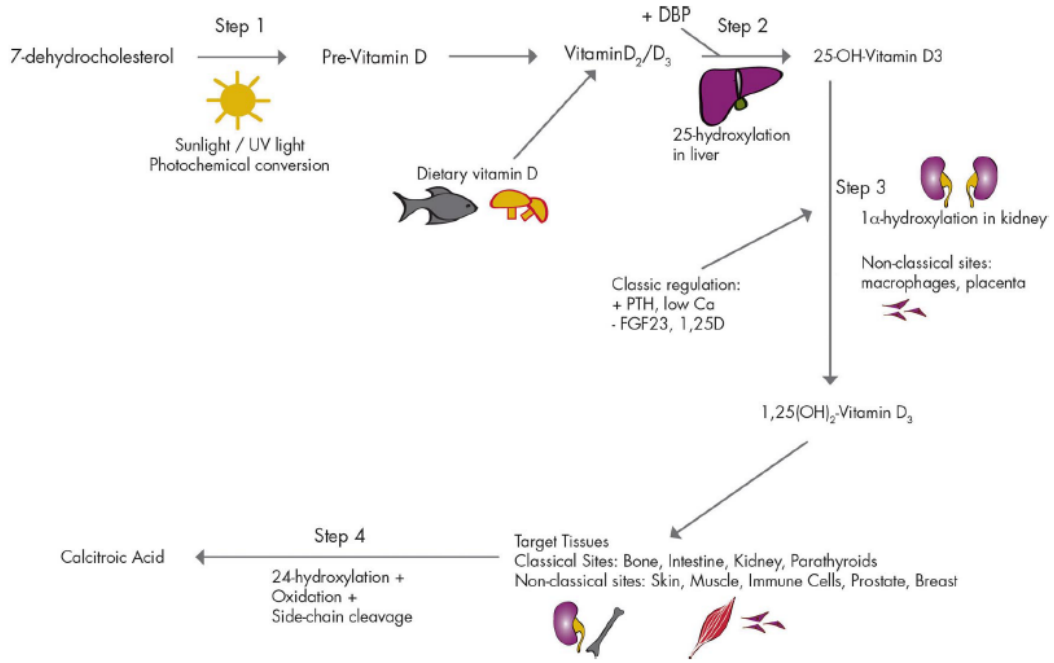

Figure 9 Summary of vitamin D metabolism

(Girgis *et al.*, 2012)

Biologically active 1,25OHD exerts its effect on target organs by binding to an intracellular vitamin D receptor (VDR). This binding leads to conformational changes with the receptor that allows it to interact with its heterodimer partner, retinoid X receptor (RXR). This liganded complex binds to vitamin D response elements in the DNA to regulate gene transcription (Figure 10). VDRs are steroid hormone receptors that are proteins with a binding site for a particular steroid molecule. Their discovery on several tissues in the body along with several epidemiological studies have raised interest in the potential role of vitamin D in several extra-skeletal diseases such as immune disorders, cancer and diabetes (Misra *et al.*, 2008). However, the possibility of confounding variables in those studies needs to be considered. Long term prospective studies examining the effects of vitamin D in preventing these conditions that may be related to vitamin D are awaited.

1.4.3 Vitamin D status assessment

Serum 25OHD concentration is the best marker of bodily vitamin D status, with a circulating half-life of around 3 weeks. Serum 1,25OHD, the active vitamin D hormone,

should not be measured as it has a short circulating half-life and does not reflect body vitamin D status (DeLuca, 2004; Holick, 2007; Holick, 2009; Bischoff-Ferrari *et al.*, 2010; Greene-Finestone *et al.*, 2011). Both adults and children with symptomatic vitamin D deficiency (rickets, myopathy and musculoskeletal pain) generally have serum 25OHD levels that are less than 30nmol/l.

What constitutes an optimum level of serum 25OHD remains uncertain, and is different depending on whether skeletal or non-skeletal outcomes are being considered, and may also be different at different stages of life. Many authorities are now recommending a serum 25OHD concentration of 75nmol/L or more (Vieth *et al.*, 2007; Holick *et al.*, 2011), largely based on evidence from physiological analysis of peak intestinal calcium absorption (Heaney *et al.*, 2003), and an association with better health outcomes in large epidemiological studies. Histomorphometric analysis of iliac crest biopsies from 675 individuals demonstrated that pathological mineralization defects only occurred in the context of serum 25OHD <75nmol/L (Priemel *et al.*, 2010). Furthermore, studies using serum PTH response as an index of serum 25OHD have recommended wide ranges varying from 30-100nmol/L (Sahota *et al.*, 2004; Dawson-Hughes *et al.*, 2005). Nevertheless, a serum 25OHD level of 50nmol/l or more can also be viewed as adequate for skeletal health (Mithal *et al.*, 2009; Aloia, 2011; Medicine, 2011; Ross *et al.*, 2011), as there is currently no level 1 (RCT) evidence that supports an improvement in health from elevating 25OHD levels from 50 to 75nmol/l.

There are several different assays available for measuring 25OHD concentrations but it is now accepted that liquid chromatography tandem mass spectroscopy is the 'gold standard'. The National Institute of Standards and Technology (NIST), in collaboration with the National Institutes of Health's Office of Dietary Supplements (NIH-ODS) has developed a Standard Reference Material for the determination of 25-hydroxyvitamin D [25(OH)D] in serum (Carter *et al.*, 2004a; Carter *et al.*, 2004b; Phinney, 2008; Phinney *et al.*, 2012). Rigorous use of this standard should improve the assessment, interpretation and generalisability of serum 25OHD levels.

1.4.4 Risk factors for vitamin D deficiency

Those individuals with pigmented skin living in northern latitudes are at major risk of developing vitamin D deficiency. This is also the key risk factor in sunnier climates such as Australia, India and the Middle East where vitamin D deficiency has also been widely reported (Sedrani *et al.*, 1983; Sachan *et al.*, 2005; Robinson *et al.*, 2006). With global migration and changes in diet towards processed food over the past century, millions of other people are now at risk of vitamin D deficiency as well (Lips, 2010). Elderly and institutionalised individuals are also at risk because of the relatively large amount of time spent indoors.

A prospective study conducted in Manchester, UK (53^{o}N) examined whether personal sunlight exposure levels could provide sufficient vitamin D (25OHD ≥50nmol/L) in white adults. This not only confirmed that vitamin D synthesis was negligible during the winter months in the UK, but also determined that attaining a 'peak' late summer 25OHD level of approximately 80nmol/L was required to ensure a 'trough' winter level of at least 50nmol/L [17]. 72% of the population did not attain the 'peak' level of 80nmol/L (Webb *et al.*, 2010). The same group also examined similar UVB -25OHD dose-responses in South Asians, and found that all participants had a vitamin D level <50nmol/L during the winter 'trough' (Farrar *et al.*, 2011). This indicates that South Asians cannot rely on solar vitamin D production to maintain an optimal Vitamin D status in northern latitudes. There were similar findings in African-American children living in Texas, North America (32^{o}N) (Shah *et al.*, 2000). It, therefore, seems unlikely that dark skinned people, or that the majority of fair skinned people, living in Northern latitudes, will be able to maintain adequate vitamin D levels throughout the year if current population habits with relation to sun exposure go unchanged.

Individuals with malabsorption, short bowel, renal and liver disease, survivors of cancer and those taking anticonvulsants, rifampicin or highly active antiretroviral drugs are also at higher risk. Vegetarians, people with photosensitive skin disorders and those with agoraphobia are at also at high risk of vitamin D deficiency. Obesity is a risk factor for low serum 25OHD, although total body fat vitamin D stores may be higher, so the clinical significance of the biochemical finding is currently uncertain (Drincic *et al.*, 2012).

1.4.5 Vitamin D and skeletal muscle function

Adults with vitamin D deficiency commonly present with myalgia and myopathy (Glerup *et al.*, 2000; Plotnikoff and Quigley, 2003; Mouyis *et al.*, 2008; Badsha *et al.*, 2009). The role of vitamin D on bone health and calcium metabolism is well established. Over the past decade, both clinical and laboratory studies have attempted to understand the precise link between vitamin D and muscle strength. Vitamin D may influence muscle function through the binding of 1,25 OHD to vitamin D receptors (VDR) in skeletal muscle (Meyer and Jones, 1957).

Lessons from VDR knockout (KO) mouse models

The VDR knockout mouse model has provided novel insights into the direct effect of vitamin D on skeletal muscle tissue. VDR mutant mice are characterised by alopecia, reduced body size and weight and impaired motor coordination (Park *et al.*, 1994). The VDR KO mouse model has also helped to clarify whether VDR or metabolic abnormalities are primarily responsible for the symptoms and clinical findings in this condition. VDR mutant mice were found to have muscle fiber diameters that were approximately 20% smaller than those of the wild type mice at 3 weeks of age. By 8 weeks of age, these muscle fiber changes were more prominent in the VDR KO mutant mice compared to the wild type suggesting that either these abnormalities progress over time or that as these mice age the metabolic alterations that occur contribute to the morphological changes. These morphological changes occur independent of any metabolic abnormalities such as hypocalcaemia and hypophosphatemia. The muscle fiber abnormalities were noted diffusely without any preference for type I or type II fibers (Endo *et al.*, 2003). This differs from the histological changes seen in vitamin D deficient myopathy in humans.

Muscle morphology

Muscle biopsies in adults with vitamin D deficiency show type IIa muscle fiber atrophy (Sorensen *et al.*, 1979). Type IIa or fast oxidative fibers are rich in myoglobin, mitochondria and vascular capillaries. These fibers have a high capacity for generating ATP by the mitochondrial oxidative process. Type IIb or fast glycolytic fibers contain less myoglobin, mitochondria and blood capillaries. These fibers are rich in glycogen and are susceptible to fatigue. A RCT found that treating elderly stroke survivors with

ergocalciferol resulted in an increase in muscle strength by reducing atrophy of type IIa muscle fibers, which may lead to decreased falls and hip fractures (Sato *et al.*, 2005).

Molecular mechanisms- Vitamin D receptors (VDR) in muscle tissue

1,25dihydroxyvitamin D (1,25OHD), the biologically active form of vitamin D, mediates its action by binding to the vitamin D receptor (VDR). VDRs are expressed in muscle tissue in rodents, chick and cloned human cell lines (Wong *et al.*, 1992; Thambyrajah *et al.*, 2000; Ryan and Jones, 2004; Ceglia *et al.*, 2010). However, this was not corroborated recently by Wang *et al.* when they used highly specific antibodies to identify VDRs in the skeletal muscle of humans (Wang and DeLuca, 2011). VDRs may act as either nuclear receptors (genomic action) or cell membrane receptors (non-genomic action).

- Genomic effects of 1,25OHD

The nuclear VDR is a ligand dependent nuclear transcription factor. Intranuclear staining detecting its existence was first demonstrated *in situ* in human skeletal muscle tissue by Costa *et al.* (1986). Following transportation to the nucleus by an intracellular binding protein, 1,25OHD binds to the nuclear receptor thereby resulting in mRNA gene transcription and *de novo* protein synthesis. The activation of the nuclear VDR results in heterodimerization between the VDR and a steroid receptor called retinoic receptor (RXR) (Figure 10). This genomic pathway is involved in muscle calcium uptake, transmembrane phosphate transport, phospholipid metabolism and muscle cell proliferation and differentiation. Both *in vitro* and *in vivo* experiments in chick skeletal muscle have demonstrated that 1,25OHD regulates muscle calcium uptake by modulating the activity of calcium pumps in the sarcoplasmic reticulum. The calcium influx is also regulated by voltage-sensitive calcium channels which thereby regulate intracellular calcium (Jones *et al.*, 1948). Modifications in intracellular calcium levels control contraction and relaxation of muscle, thus impacting muscle function (Boland *et al.*, 1995).

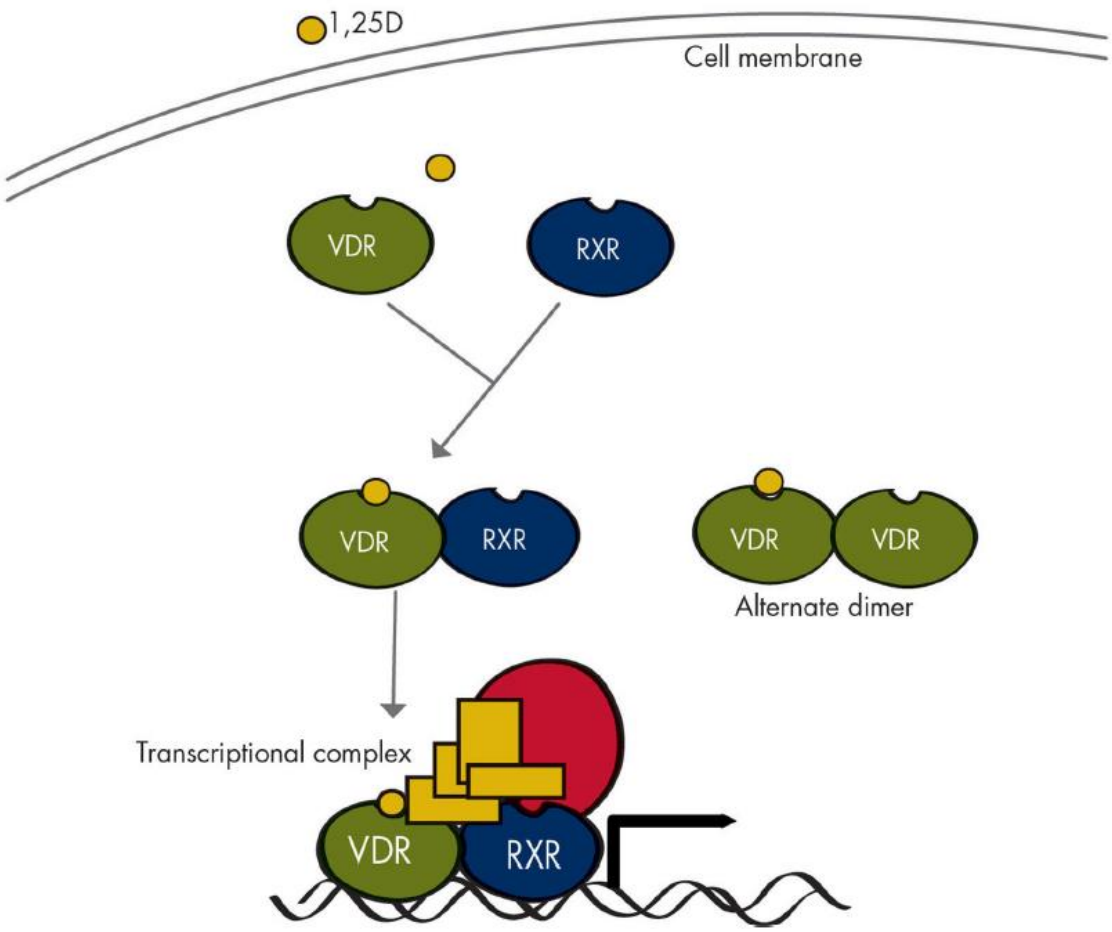

Figure 10 Genomic (Classic) vitamin D signaling pathway

(Girgis *et al.*, 2012)

- Non-genomic effects of 1,25OHD

Apart from its genomic effects, 1,25OHD induces a rapid, non-transcriptional response through a cell surface receptor. This mechanism may also be responsible for 1,25OHD mediated muscle contractility. *In vitro* studies on vitamin D deficient chicks showed that when 1,25OHD was added to skeletal muscle cells, it had a rapid effect (1-15min) on calcium uptake (Tarnopolsky and Parise, 1999). Inhibitors of RNA and protein synthesis did not inhibit these rapid effects suggesting no involvement of the nuclear VDR. Calcium channel blockers did suppress these effects indicating that 1,25OHD was acting at the membrane and influencing calcium influx into the cell (Tarnopolsky and Parise, 1999) (Figure 11).

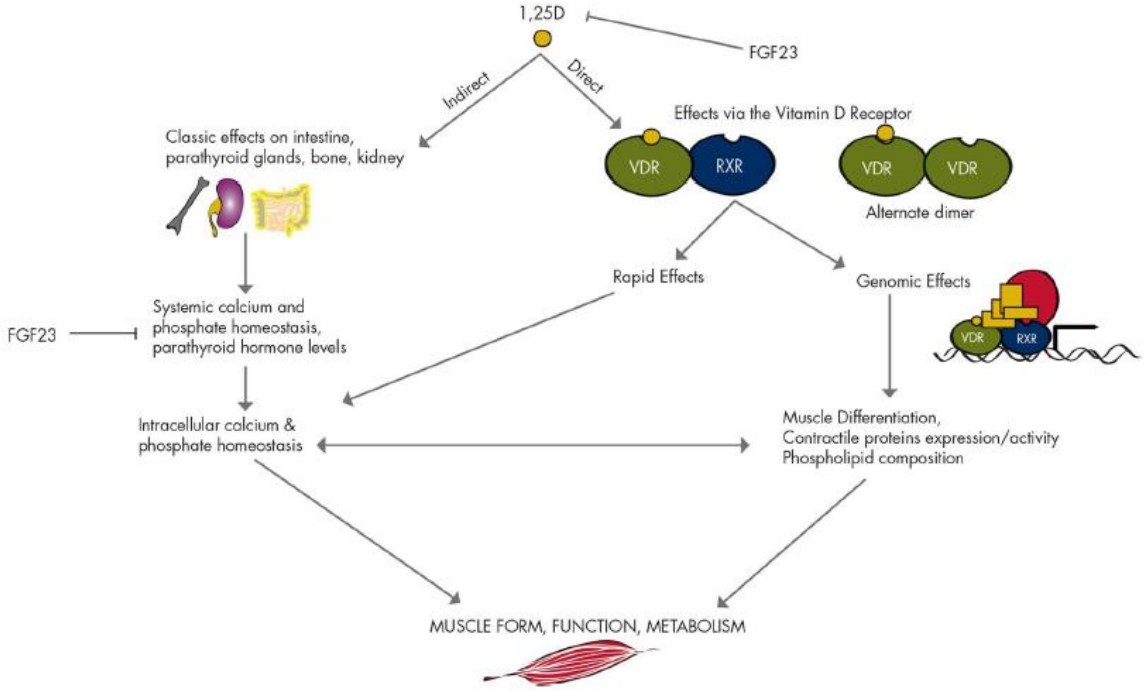

Figure 11 Genomic and non-genomic effects of vitamin D on muscle

Data on direct effects come predominantly from *in vitro* studies and are yet to be confirmed *in vivo*, where the presence of the VDR is currently under debate (Girgis *et al.*, 2012).

Clinical evidence

Several large cross-sectional studies have investigated the relationship between vitamin D status and muscle strength. Generally, muscle strength is evaluated by handgrip strength and/or thigh strength measured by a dynamometer.

Several cross-sectional studies have shown that lower serum 25OHD levels correlate with reduced muscle strength in the elderly (Gerdhem *et al.*, 2005; Houston *et al.*, 2007). However, other association studies in a more diverse age range, including healthy individuals, have not replicated these findings (Ceglia *et al.*, 2011). A systematic review conducted to examine the effects of low vitamin D concentrations and vitamin D supplementation on muscle, balance and gait performance in the elderly (≥65 years) was also inconclusive (Annweiler *et al.*, 2009). A cross sectional study conducted by Ward *et al.* in post-menarchal adolescent girls in Manchester, UK determined that vitamin D was positively associated with muscle power and force (2009). In summary, the cross-sectional designs of the studies described make it difficult to establish a causal

relationship. The elderly age range of the patients studied also makes it difficult to differentiate between weakness secondary to aging or vitamin D deficiency.

Randomised controlled trials to study the effects of vitamin D supplementation on muscle strength have been conflicting too. Improvements in quadriceps strength and body sway were noted in a RCT where community-dwelling ambulant older women were supplemented with ergocalciferol and calcium (Prince *et al.*, 2008). Six months of cholecalciferol and calcium supplementation improved muscle strength and physical performance in vitamin D deficient (mean serum 25OHD-25nmol/L) healthy adults in India (age 31.5 ± 5.0 years) (Gupta *et al.*, 2010). On the contrary, when Ward *et al.* conducted a RCT where post-menarchal adolescent females were supplemented with ergocalciferol over one year, there were no improvements in muscle force or power despite improvements in serum 25OHD levels (2010). A recent double blinded RCT from the same centre in India studied 173 young Indian women (mean age-22 years) with vitamin D deficiency who were randomised to one of four groups: calcium 500 mg twice daily, vitamin D, calcium + vitamin D, and placebo. Vitamin D was given at 60,000 IU/week for 8 weeks, and then every 2 weeks for 6 months. Mean serum 25OHD was 23nmol/L at baseline and increased to approximately 70nmol/L in the two vitamin D-treated groups and was unchanged in the calcium or placebo groups. At 6 months, there were no significant differences in handgrip strength between the four groups, no difference in the 6-min walking test, and no difference in the quality of life assessment (Goswami *et al.*, 2012).

The demonstration of unequivocal improvements in muscle strength in vitamin D deficiency remains elusive. Possible reasons for this include heterogeneity in patient demographics and outcome measured (grip strength, knee strength, hip strength, 6-min walk test, sway test) The fact that muscle strength is a function of several variables such as age, genetics, body size and physical fitness also needs to be considered.

1.4.6 Summary

Vitamin D insufficiency and deficiency remains a major health issue affecting, to differing degrees, a large proportion of the population of many countries, both affluent and developing. This indicates a widespread failure of public health policy in many societies. One can conclude that sunlight in isolation is insufficient to generate sufficient vitamin D

production in most people living in northern latitudes, including the UK. Diet is a poor source of vitamin D for most of the population. There has been considerable interest in the relationship between vitamin D and muscle strength. The present body of evidence suggests that treatment with vitamin D does not equivocally lead to improvements in muscle strength in otherwise healthy vitamin D deficient adults. However, the study of muscle function entails not only the assessment of strength but also of endurance. However, rather than focussing on muscle strength which has already been examined by several investigators, a novel approach would be to examine skeletal muscle oxidative metabolism in symptomatic, vitamin D deficient adults and observe the impact of vitamin D therapy on skeletal muscle metabolism post therapy. This technique measures the energy cost of an activity and therefore provides insights into the energy efficiency of the vitamin D deficient skeletal muscle.

1.5 Phosphorus-31 Magnetic Resonance Spectroscopy

1.5.1 Overview

Almost three decades ago, Chance *et al.* reported for the first time that phosphorus metabolites could be observed *in vivo* using Phosphorus-31 Magnetic Resonance Spectroscopy (^{31}P MRS) (Chance *et al.*, 1980), thus, offering the prospect of examining muscle energetics *in vivo* under strictly non-invasive conditions. From that time onwards, MRS technology has rapidly evolved with the availability of high field and wide bore superconducting magnets and methodological developments (such as dedicated pulse sequences, and the spatial localization of the NMR signal).

Before ^{31}P MRS, the measurement of phosphorylated compound concentrations in living cells was non-trivial. Traditional methods such as percutaneous needle biopsy and freeze clamping are limited by the possible partial degradation of phosphorylated metabolites during extraction. Furthermore, consecutive measurements cannot be made during exercise, thereby, making it impossible to obtain high time-resolution kinetics. Compared to analytical methods, ^{31}P MRS offers the opportunity of measuring non-invasively and continuously with a high time-resolution, the concentration of phosphorylated compounds involved in muscle energetics using radiofrequency pulses and strong magnetic fields. It generates a spectrum which is a plot of signal intensity versus frequency that shows the chemical shift. The chemical shift is the resonant frequency of a nucleus relative to a standard with the position and number of the chemical shift being diagnostic of the molecule. Interestingly, direct biopsy derived biochemical and ^{31}P MRS measurements give comparable results when measured during exercise (Bangsbo *et al.*, 1993; Tarnopolsky and Parise, 1999).

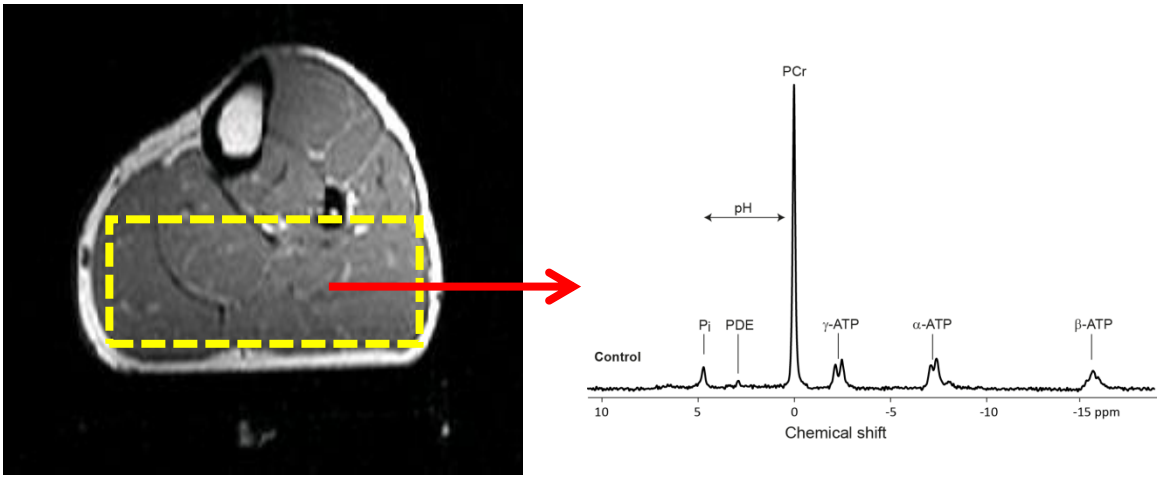

Figure 12 ^{31}P spectrum from gastrocnemius and soleus of a healthy volunteer at rest at 3T and the derived phosphorus spectrum showing relative concentrations of inorganic phosphate (Pi), phosphodiesters (PDE), phosphocreatine (PCr) and adenosine triphosphate (ATP).

Please note that due to the bandwidth performance of the adiabatic excitation pulse centred on PCr, the beta-ATP moiety is excited to a lesser degree than the alpha and gamma moieties. Although ADP is at too low a concentration to be visible in the spectrum, the β-ADP resonance would have a chemical shift of -3.09ppm, the α-ADP resonance at -7.05ppm (Graaf, 2007). ATP concentrations are buffered by transfer of a phosphate group from PCr to ADP as indicated.

1.5.2 Principles

Magnetic Resonance Spectroscopy (MRS) gathers information using technology similar to Magnetic Resonance Imaging (MRI). However, rather than providing a spatial image, MRS offers a frequency spectrum containing discrete signals from compounds of a selected resonating nucleus (Figure 12). Commonly studied nuclei using *in vivo* MRS include biologically active metabolites such as ^{31}P, ^{1}H and ^{13}C. The magnitude of a metabolite peak in the frequency spectrum is proportional to its concentration in the volume of tissue being examined, provided the spectrum is corrected for T1 weighting (Figure 12). Measuring changes in these peak areas, which result from either normal function, disease processes or from the effects of drugs, enables monitoring of intracellular changes which are not necessarily accompanied by structural changes. ^{31}P Magnetic Resonance Spectroscopy (^{31}P MRS) offers an opportunity to probe oxidative mitochondrial function non-invasively by measuring the kinetics of high energy phosphate species involved in muscle energy metabolism during and after perturbation by exercise, thereby providing a dynamic window into the 'mitochondrial fitness' of

contracting skeletal muscle. Moreover, it can also be used to measure intracellular pH in 'real time' and quantify the rate of pH recovery (proton efflux) which is a function of the vascular supply to the muscle.

Skeletal muscle is an ideal tissue to interrogate using ^{31}P MRS. It is not only abundant in energy rich phosphate metabolite stores but it can be examined within an exercise protocol, thus, offering the opportunity to measure fluctuations of phosphate rich metabolites continuously in a high time resolution. Moreover, ^{31}P MRS can be utilised to study different muscle groups whose phosphorus spectrum may differ because of the inherent variations in their fiber compositions. Commonly investigated muscle groups are the gastro-soleus complex, vastus lateralis, forearm flexor and thenar muscles (Nishida *et al.*, 1992; Pfeifer *et al.*, 1999; De Feyter *et al.*, 2008; Jones *et al.*, 2010). The gastro-soleus complex serves as a particularly useful muscle group to investigate mitochondrial oxidative potential. Not only is it rich in mitochondrial content but it can also be effectively isolated during plantar flexion exercise, thereby, preventing the recruitment of other muscles during the examination.

During the procedure, the candidate muscle group is placed within a magnetic field inside the bore of the MR scanner. This creates two distinct energy levels for the angular momentum of the phosphorus nuclei. The strength of the magnetic field, measured in Tesla, generally vary between 1.5-3.0 Tesla. A higher magnetic field strength gives enhanced spectral definition, and a higher signal to noise ratio. Low-energy radiofrequency pulses are then applied which cause the phosphorus nuclei to move between energy states and allow us to detect their presence.

^{31}P-MR spectra from skeletal muscle display 7 peaks: 3 from the three phosphate moieties of ATP (α,β and γ), 1 from inorganic phosphate(Pi), 1 from phosphocreatine (PCr) and 2 from the two smaller resonances of phosphomonoesters (PME) and phosphodiesters (PDE), which both contain several distinct chemical species (Figure 12). The PME and PDE peaks are only visible when present in sufficiently high concentrations or with sufficiently long acquisition times. The area under the curve of each resonance is proportional to the concentration of the corresponding metabolite *in vivo*. Intracellular pH can be calculated by measuring the distance between the Pi and PCr peaks, since the frequency of the Pi resonance is pH dependent (Moon and Richards, 1973). In order to be detectable on the spectra, the corresponding metabolite should be present in

concentrations of 1mM or greater to generate a sufficient signal to noise ratio in a reasonable acquisition time. The α, β and γ moieties of ATP have a multiplet structure owing to J-couplings between the phosphorus nuclei of medium strength (Schroder *et al.*, 2005). However, one of the key active metabolites involved in the regulation of ATP synthesis, adenosine diphosphate (ADP), does not produce a measurable signal since its resting concentration is approximately 10 µM. Nevertheless, this can be calculated indirectly using the creatine kinase equilibrium reaction:

[ADP] = [ATP][Cr]/([PCr][H+] Keq)

where Keq is the creatine kinase equilibrium constant (1.66 x 109 M-1) (Veech *et al.*, 1979), [ATP] is assumed to be 8.2 mM throughout. [Cr] is estimated from [PCr], assuming that the total creatine pool ([PCr]+[Cr]) is constant and that at rest [Cr]/[PCr] = 0.15/0.85. [H+] can be obtained from the measured pH, where pH = -log10 [H+]. The calculations are derived from the γ-ATP moiety of the ATP (Jeneson *et al.*, 1997; Cea *et al.*, 2002). Skeletal muscle bioenergetics in several conditions such as McArdle's disease (Ross *et al.*, 1981), glycogen storage disease type XIII (Vorgerd and Zange, 2002), dermatomyositis (Cea *et al.*, 2002), chronic fatigue syndrome (Wong *et al.*, 1992; Jones *et al.*, 2010), muscular dystrophy (Lodi *et al.*, 1999b), Friedrich's ataxia (Lodi *et al.*, 1999a), mitochondrial diseases (Kuhl *et al.*, 1994; Taivassalo *et al.*, 1998; Lodi *et al.*, 2004), type 2 diabetes (Scheuermann-Freestone *et al.*, 2003; De Feyter *et al.*, 2008) and aging (McCully *et al.*, 1991; Taylor *et al.*, 1997) have been investigated using this technique.

1.5.3 Key bioenergetic pathways investigated

^{31}P MRS can be used to investigate different energetic pathways in skeletal muscle. Skeletal muscle tissues have the unique ability to sustain very large-scale changes in ATP turnover to meet the increased tissue demands when work is initiated. Importantly, the ATP concentration is maintained in near steady state despite a several fold increase in ATP turnover, often referred to as ATP homeostasis (Hochachka and McClelland, 1997) (Figure 13). This ensures that energy is always available to the contracting muscle cells to fuel its activity through either oxidative phosphorylation or anaerobic glycolysis.

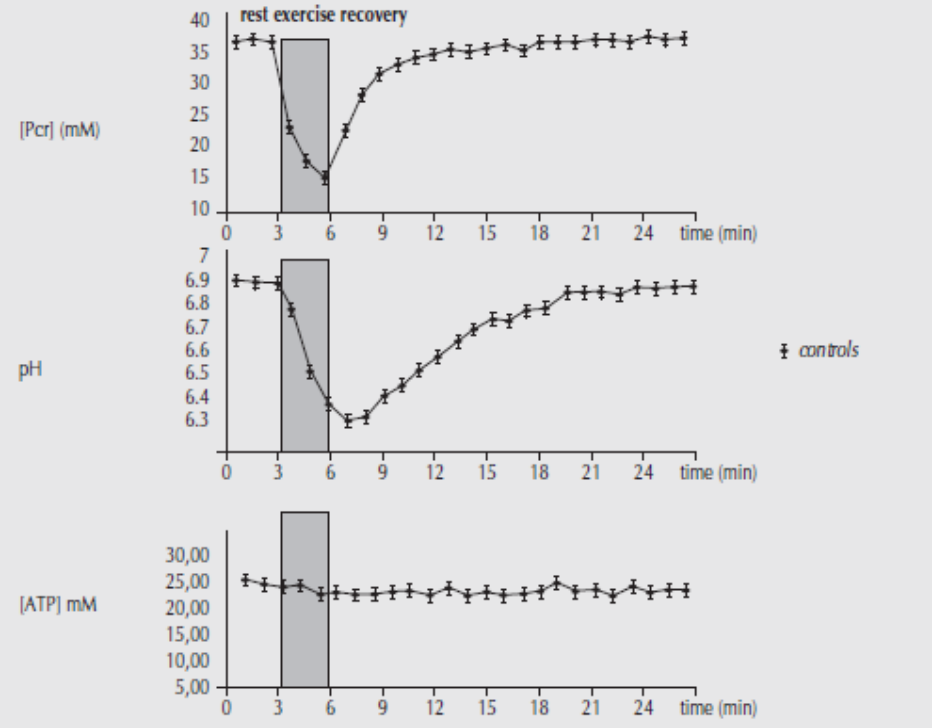

Figure 13 Scheme of the variations of PCr, pH and ATP during a rest-exercise-recovery protocol quantified using ^{31}P MRS.

Note that ATP remains constant (ATP homeostasis) during exercise in a normal subject due to its continuous resynthesis by three different metabolic pathways (Park *et al.*, 2000)

1.5.4 Mitochondrial oxidative phosphorylation during exercise and recovery

Skeletal muscle is rich in phosphocreatine reserves which serve as an immediate source of energy for muscular activity. The consumption of energy-rich phosphocreatine reserves to fuel muscle contraction is referred to as phosphocreatine hydrolysis. Using predetermined parameters, for example 25% maximal voluntary contraction (MVC), the skeletal muscle can be exercised so as to deplete the PCr reserves, whilst ensuring intracellular pH is stable. Avoiding lowering pH too much during this exercise avoids lengthening the half-time of PCr recovery and avoids stimulation of the alternative anaerobic glycolysis (S Iotti, 1993).

This can be observed in the MR spectra with the progressive depletion of the PCr peak and the corresponding increase in the Pi peak (Figure 14). After the exercise ceases, oxidative phosphorylation is maximally stimulated by high concentrations of ADP and the mitochondria synthesise ATP which is then converted through the reverse creatine kinase reaction to replenish the phosphocreatine (PCr) stores. Since exercise has

stopped at this juncture, the rate of increase in PCr is proportional to the rate of generation of ATP in the mitochondria which cannot be directly measured. During recovery, ATP synthesis relies exclusively on oxidative metabolism because glycolysis is inactive. Hence surrogates of maximum mitochondrial oxidative phosphorylation can subsequently be computed by measuring the recovery constant for PCr replenishment such as the half-time of PCr recovery ($\tau_{1/2}$ PCr) (Taylor *et al.*, 1983). This statement holds true only if the muscles are adequately perfused since optimal substrate delivery to the mitochondria is essential in order to measure maximal mitochondrial oxidative phosphorylation. Furthermore, the assumption that PCr resynthesis is a function of mitochondrial ATP synthesis is supported by observations that PCr resynthesis is absent when the muscle is kept ischemic following muscle contraction, and PCr recovery occurs only upon reperfusion of the limb(Quistorff *et al.*, 1993). Therefore, the recovery period is an important phase of the experiment during which mitochondrial oxidative phosphorylation 'capacity' is measured (Figure 15).

Endurance training has been shown to increase mitochondrial content and its associated oxidative enzymes and capillary density of the skeletal muscle which link to an improvement in metabolic capacity and exercise performance. However, determination of local metabolic capacity and profile changes that occur as a response to training usually requires an invasive method such as muscle biopsy. Aerobically trained individuals such as rowers and long distance runners demonstrate faster PCr recovery after exercise ($\tau_{1/2}$ PCr) suggesting improved muscle oxidative capacity when compared to untrained adults or sprint trained runners (McCully *et al.*, 1989; Johansen and Quistorff, 2003).

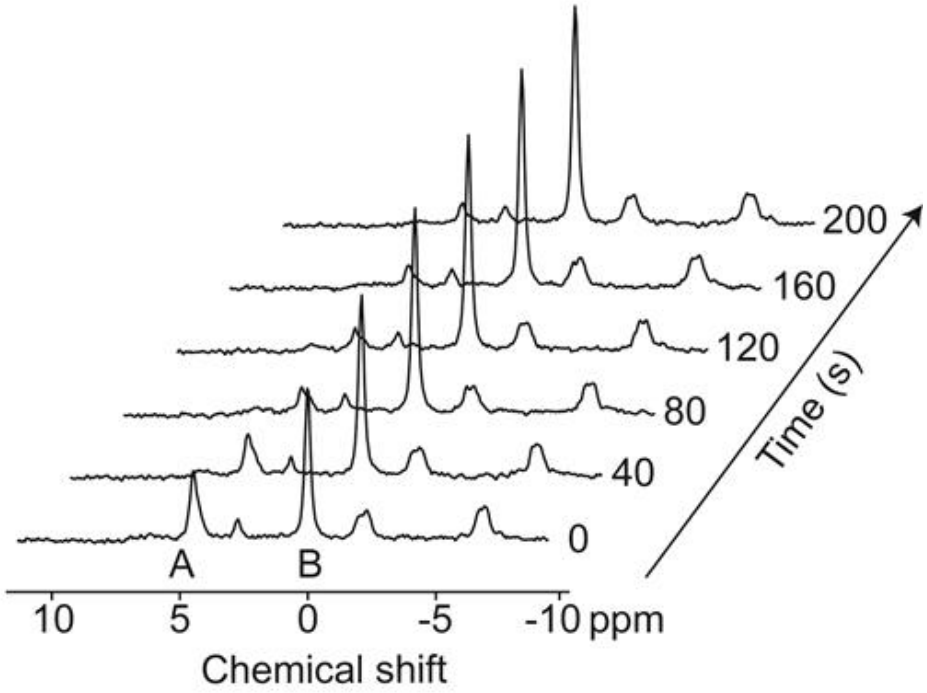

Figure 14 ^{31}P MRS: After exercise and during recovery

A selection of phosphorus spectra acquired from the gastrocnemius and soleus of a volunteer at 3.0T during recovery from a plantar flexion exercise. A decreasing concentration of inorganic phosphate (A) and the replenishment of phosphocreatine (B) can be measured. The rate of recovery of phosphocreatine acts as a surrogate measure for maximal mitochondrial function.

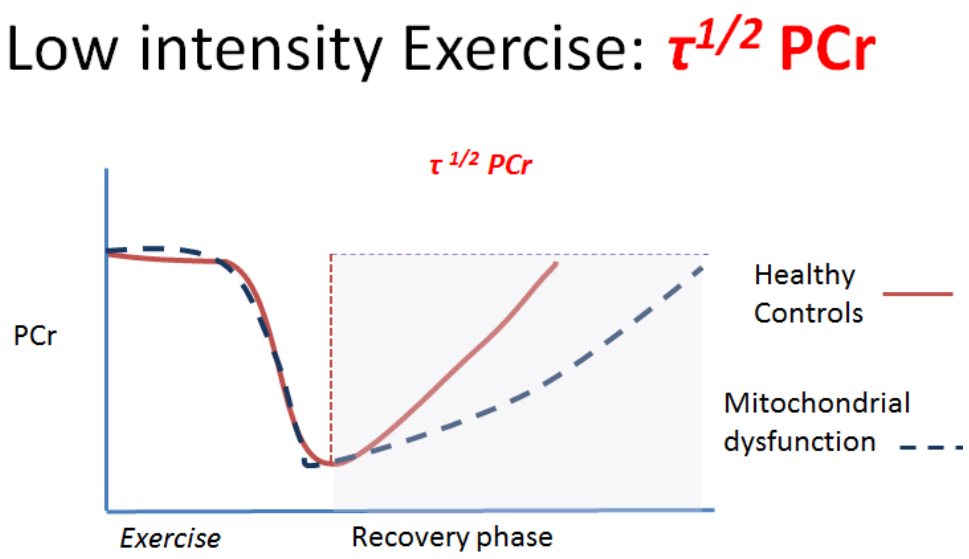

Figure 15 Mitochondrial oxidative potential

This approach measures the kinetics of PCr recovery using progressive low-level exercise that produces relatively small changes in metabolites and therefore, a rapid re-attainment of the steady-state condition. The rate of increase in PCr is proportional to the maximal mitochondrial oxidative phosphorylation rate. Patients with mitochondrial dysfunction typically have delayed rates of phosphocreatine resynthesis.

1.5.5 pH handling during exercise and recovery

Anaerobic glycolysis is activated during moderate to high intensity activities when the cellular energy demand outstrips the supply of oxygen to the mitochondria. Although not as efficient as oxidative phosphorylation (in terms of ATP production/mole of glucose), it serves as a critical source of energy during more intense activities. It produces lactic acid as a by-product resulting in intracellular acidification. Again using predetermined parameters (such as 35% MVC), the skeletal muscle can be stressed to stimulate the glycolytic pathway. Cellular pH increases initially during exercise secondary to H^+/proton consumption during PCr hydrolysis. Subsequently, pH decreases following anaerobic metabolism due to lactate production and then PCr resynthesis. The cell consequently excretes the excess proton load through the Na^+/H^+ antiporter, lactate-H^+ cotransporter (membrane transporter systems) and others; this is critical for acid resolution (Kemp *et al.*, 1994). Intracellular acidosis may be deleterious to the contractile functioning of the myocytes, hence contributing to fatigue (Bangsbo *et al.*, 1996). Active proton excretion following fatigue-inducing exercise is

associated with the resolution of that fatigue and perception of the ability to return to exercise (Mainwood and Alward, 1982). Since the pH can be measured and the rate of recovery of phosphocreatine is known at each time point post exercise, and no further protons are produced during the recovery phase, the rate of proton efflux can be calculated. The pH recovery time which is the time taken to excrete the excess lactic acid from the cell can also be measured (Figure 16). Other indices such as nadir pH and maximum rate of proton efflux can be measured or derived. If acid transporter function is normal then proton efflux rate becomes a surrogate for peripheral vascular supply. The pH recovery time is delayed in conditions such as dermatomyositis and polymyositis, thereby implicating impaired peripheral vascular supply as a contributor to fatigue (Cea *et al.*, 2002). Apart from intracellular acidosis as a cause of fatigue, there is evidence to suggest that fatigue from high intensity exercise may actually arise secondary to accumulation of inorganic phosphate (Pi) (Fryer *et al.*, 1995; Dahlstedt *et al.*, 2001; Hoybye *et al.*, 2007). Pi is a by-product of anaerobic metabolism in skeletal muscle involving hydrolysis of phosphocreatine to creatine and Pi.

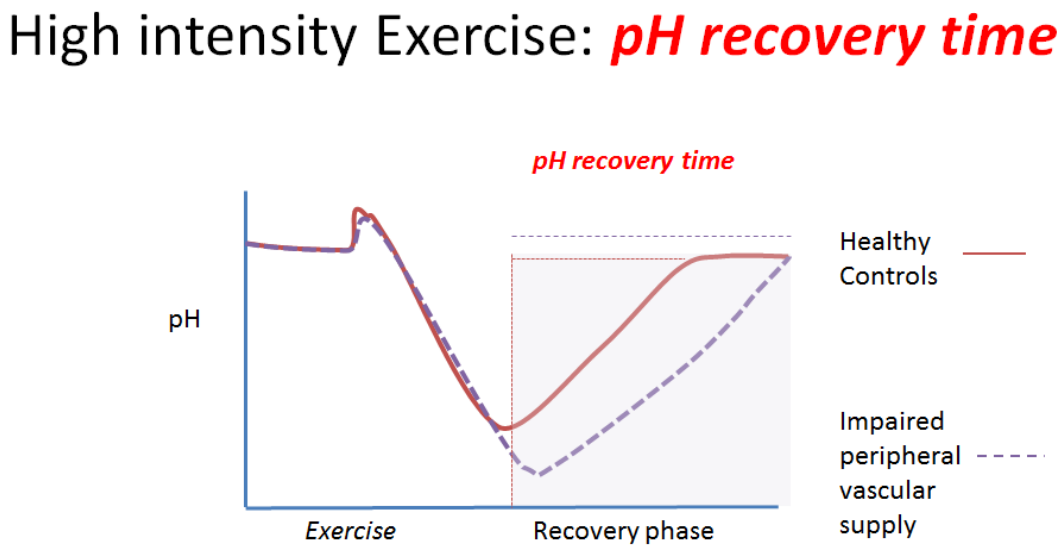

Figure 16 pH recovery time

In contrast to low intensity exercise, this involves the study of muscle energetics during high-intensity exercise. Anaerobic metabolism contributes significantly to energy production resulting in decreased intracellular pH. Active proton excretion following exercise is associated with the perception of the ability to return to exercise. Diseases with impaired peripheral vascular supply may present with fatigue because of delayed proton excretion.

1.5.6 Applications in Endocrine diseases

Hormones initiate biological responses by binding to target cell receptors with high affinity and specificity. Target cell receptors, both cell surface and nuclear, initiate a signalling cascade which result in physiological changes within the cellular milieu. Hormone levels are tightly regulated through a feedback mechanism to ensure homeostasis. This physiological equilibrium is disrupted in endocrine disorders resulting in metabolic alterations within the cellular milieu which in turn manifest as clinical signs and symptoms. ^{31}P MRS has been used to examine mitochondrial function in several endocrine diseases. It can provide information regarding either the cause or consequence of disease by examining specific components of cellular metabolic function.

Type 2 Diabetes:

Insulin has a critical role in the energy transduction pathway. Insulin enhances mitochondrial function by stimulating mitochondrial enzymes, increasing protein synthesis and suppressing protein degradation (Gelfand and Barrett, 1987; Stump *et al.*, 2003). Mitochondrial dysfunction has been implicated in the pathogenesis of insulin resistance and type 2 diabetes (Patti and Corvera, 2010). Mitochondrial oxidative potential in type 2 diabetes has been investigated *in vivo* using ^{31}P MRS. Delayed post exercise recovery kinetics suggested that mitochondrial dysregulation may be implicated in the pathogenesis of insulin resistance and type 2 diabetes (Sirikul *et al.*, 2006; Schrauwen-Hinderling *et al.*, 2007). However, when healthy controls were carefully matched mitochondrial dysfunction was not associated with either insulin resistance or type 2 diabetes (De Feyter *et al.*, 2008; Trenell *et al.*, 2008). This has been supported by recent work confirming that mitochondrial function and insulin resistance did not depend on each other. Prolonged exercise training could, at least partly, reverse the mitochondrial impairments associated with the longstanding diabetic state (Figure 17) (van Tienen *et al.*, 2012).

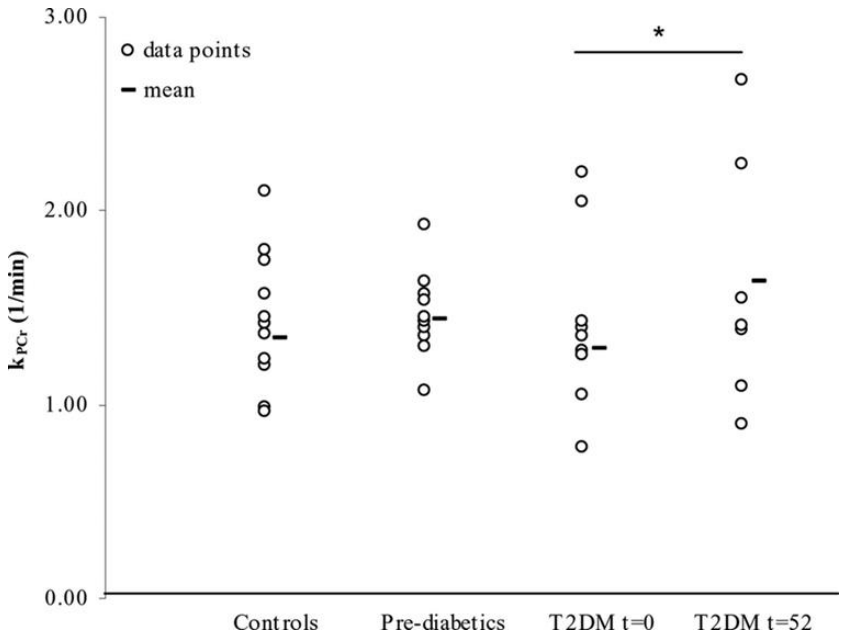

Figure 17 Mitochondrial oxidative capacity (k_{PCr}-PCr recovery rate constant) and insulin sensitivity

The k_{PCr} activity in controls, prediabetics, and T2DM before (t=0) and after 52 wk exercise training (t=52). k_{PCr}* significantly changed in T2DM group at 52 wk compared with T2DM at time zero, analysed with paired *t* test, $P < 0.05$ (van Tienen *et al.*, 2012).

Thyroid disease:

Thyroid hormone is the major endocrine regulator of energy expenditure. Thyroid status in mammals is intricately linked with their basal metabolic rate (BMR). Mitochondrial function is, therefore, a logical candidate to investigate when determining the metabolic effects of thyroxine. *In vitro* experiments, primarily on rat liver preparations, have revealed that both genomic and non-genomic mechanisms exist by which the mitochondrial function may be modulated (Harper and Seifert, 2008). The genomic pathway involves the induction of mitochondriogenesis (i.e. synthesis of new mitochondria) in order to meet the increased demand for energy by the thyroid hormone. This process is mediated by complex interactions between nuclear and mitochondrial genomes leading to increased expression of mitochondrial transcription factors, nuclear respiratory factor-1(NRF-1), and peroxisome-proliferator-activated receptor gamma coactivator-1α (PGC-1α) (Wu *et al.*, 1999; Weitzel *et al.*, 2003; Lee and Wei, 2005). Some of these genes are also responsible for the increased expression of mitochondrial proteins (Short *et al.*, 2007). Thyroid hormone effects on the expression of oxidative phosphorylation genes can occur through the interaction of the thyroid

hormone receptor complex with hormone response elements (HRE) in nuclear and mitochondrial genes (Scheller and Sekeris, 2003). Thyroid hormones also induce substantial modifications in mitochondrial inner membrane protein and lipids. Although the precise mechanisms still remain unclear, modifications in the inner mitochondrial membrane are responsible for the uncoupling of oxidative phosphorylation which subsequently leads to the hypermetabolic effects of thyroxine (Brasitus and Dudeja, 1988). Both Kaminsky and Argov *et al.,* demonstrated bioenergetic impairments in PCr resynthesis in the hypothyroid skeletal muscle using ^{31}P MRS, thereby, implicating reduced mitochondrial oxidative capacity (Argov *et al.*, 1988; Kaminsky *et al.*, 1992) . Moreover, this was reversible with thyroxine therapy (Argov *et al.*, 1988). Taylor *et al.,* however, concluded that proton handling was abnormal too as evident by a slow pH recovery (Taylor *et al.*, 1992). Therefore, it seems that a combination of both of these factors could be responsible for the debilitating fatigue in hypothyroidism.

1.5.7 Advantages

The most obvious advantage of ^{31}P MRS lies in its ability to non-invasively determine muscle biochemistry, thereby obviating the need for invasive muscle biopsies. Moreover, it is undertaken in 'real time' thus, reflecting serial changes in the muscle biochemical milieu. Therefore, it is ideal for monitoring dynamic metabolic processes and providing measurements of important metabolite concentrations. Reliable and reproducible parameters can be calculated and compared. Larson-Meyer *et al.* confirmed that ^{31}P MRS measurements of mitochondrial function such as $\tau_{1/2}$ PCr, yielded reproducible results that correlated well with whole-body maximal oxygen uptake (VO_{2max}) (CV of 4.6-7.1%) (Larson-Meyer *et al.*, 2000). The results are sensitive and correlate well with *in vitro* measurements of oxidative capacity in mitochondria isolated from muscle biopsies too (Lanza *et al.*, 2011). Moreover, it is safe and does not involve exposure to ionising radiation.

A cross-sectional design can be employed to investigate abnormal bioenergetic footprints in chronic conditions such as Type 2 diabetes and chronic fatigue syndrome. It, however, precludes us from concluding whether the underlying defect is secondary to the cause of the disease or consequent to the inactivity and reduced well-being as a result of it. This can be overcome by designing a longitudinal study. In fact longitudinal

studies have been implemented to investigate the effects of drugs/hormones such as statins and thyroxine respectively (Sesmilo *et al.*, 2000; Wu *et al.*, 2011).

1.5.8 Limitations

^{31}P MRS does have limitations. Considerable inter-individual variations in metabolic transactions may occur. For example, the rates of PCr consumption and re-synthesis may differ by a factor of 2 among healthy individuals (Kushmerick *et al.*, 1992). While the recovery of PCr after exercise is independent of the exercise undertaken, it is not independent of the reperfusion of the limb leading to variations between oxidative capacity and PCr recovery (Kemp *et al.*, 2002). In other words, abnormal mitochondrial respiration could be a primary event due to inherent mitochondrial abnormalities or secondary to abnormal substrate delivery. Primary mitochondrial disorders arising from mitochondrial DNA defects have a reduced rate of mitochondrial ATP production which is typically associated with reduced intracellular acidosis during exercise and an increased or normal rate of H^+ extrusion from the cell during the initial recovery. On the other hand, conditions with abnormal substrate supply will have a reduced rate of mitochondrial ATP production but with an increased rate of acidification during exercise and a reduced rate of H^+ efflux during recovery (Cea *et al.*, 2002).*In vivo* MRS is a relatively insensitive technique compared to *in vitro* mass spectrometry since concentrations in the millimolar range are required for detection with MRS. It is also expensive and requires dedicated hardware and software packages. Moreover, compared to muscle biopsy, analytical information regarding the oxidative phosphorylation pathway is not available.

1.5.9 Summary

Since the phosphorus species present in sufficient concentration for *in vivo* MRS are mostly high-energy compounds, ^{31}P MRS can be used to investigate bioenergetics in the human skeletal muscle. It not only provides a window into the *in vivo* energy transactions occurring within the contracting skeletal tissue but also permits quantitative measurements of energy rich phosphorus species within the muscle, therefore, providing a robust and reliable method to interrogate and measure skeletal tissue behaviour.

Over the past decade, there has been emerging evidence to suggest that crosstalk between endocrine hormones and the skeletal muscle tissues exist which is essential to maintain optimum muscle function (Pedersen and Febbraio, 2008). Moreover, the mitochondrion is a critical regulator of muscle energy and the discovery of hormone responsive elements (HRE) on the mitochondria confirms the role of hormones in ensuring optimal function. Thus, ^{31}P MRS forms a useful adjunct to investigate novel parameters in endocrine disease and may provide insight into the various pathophysiologies involved in endocrine myopathies.

Chapter 2 Growth hormone and skeletal muscle energetics

2.1 Introduction

2.1.1 Background

Adults with growth hormone deficiency (GHD) experience fatigue, reduced vitality and reduced energy levels (Bjork *et al.*, 1989; McGauley *et al.*, 1990). These symptoms improve with growth hormone (GH) replacement (Gilchrist *et al.*, 2002; Jorgensen *et al.*, 2011a), although the precise basis for both the symptoms and their response to treatment remain unclear.

Fatigue may be classified as either central or peripheral. Generally if fatigue occurs at rest it suggests a central/psychological origin. Central fatigue is a well-recognised symptom and a feature of many conditions. It is a complex pathophysiological entity and may well reflect contributions from multiple factors (Chaudhuri and Behan, 2004). The objective assessment of central fatigue is primarily based upon validated self-reporting questionnaires (Lee *et al.*, 1991). The diagnosis of central fatigue is usually made when there is a significant perception of fatigue in the absence of specific skeletal tissue (peripheral) abnormalities.

Commonly patients, who relate their fatigue to physical exercise, tend to experience peripheral fatigue. Abnormal skeletal muscle metabolism has been implicated in the pathophysiology of peripheral fatigue. Delayed mitochondrial phosphocreatine re-synthesis, delayed proton clearance and aberrant accumulation of metabolic by-products have all been described across a range of conditions in which fatigue is a prominent feature(Wong *et al.*, 1992; Kuhl *et al.*, 1994; Pfeifer *et al.*, 1999; Allen *et al.*, 2008; Hollingsworth *et al.*, 2008). The role of mitochondria in cellular energy metabolism raises the possibility that impairments in skeletal muscle energy metabolism underlie the fatigue in GHD patients. GH has been reported to promote mitochondrial oxidative capacity (Short *et al.*, 2008). Recently Makimura *et al.* demonstrated an association between phosphocreatine recovery (a measure of mitochondrial function) and parameters of the GH axis in healthy males (Makimura *et al.*, 2011). In contrast, Janssen *et al.* investigated the influence of GH on muscle phosphate metabolites at rest in GHD

adults and found no evidence of an effect(Janssen *et al.*, 1999). The latter study was not undertaken during exercise, thereby not measuring sensitive markers of dynamic oxidative phosphorylation. The skeletal muscle is a logical tissue to study peripheral fatigue in GHD adults since both GH receptors and IGF-1 receptors are purportedly expressed in skeletal muscle (Beguinot *et al.*, 1985; Frick *et al.*, 1998).

Phosphorus-31 Magnetic Resonance Spectroscopy (^{31}P MRS) is a non-invasive tool that has been used by muscle physiologists to examine for abnormalities in metabolic work-cost relationships during exercise. Specifically it assesses the oxidative capacity of skeletal muscle *in vivo* by measuring the kinetics of energy rich phosphate species involved in muscle energy metabolism (such as those involved in the creatine kinase reaction) during and after exercise. It provides temporal information regarding the metabolic changes occurring within the skeletal muscle, thereby providing a dynamic window into the 'mitochondrial fitness' of skeletal muscle. It can also be used to measure intracellular pH in 'real time' and quantify the rate of pH recovery (proton efflux) which is a measure of the cell's ability to eliminate acid i.e. proton load. The technique can also measure concentrations of key cellular metabolites implicated in fatigue, such as inorganic phosphate (Hoybye *et al.*, 2007).

In this study, I used quantitative ^{31}P MRS to establish whether *in vivo* deficits of skeletal muscle mitochondrial function or other bioenergetic abnormalities occur in untreated GHD adults and their role in the pathogenesis and clinical expression of fatigue in GHD.

2.1.2 Aims

The aim of the present study was to investigate the role of skeletal muscle mitochondrial function in the pathophysiology of fatigue in GHD by:

a. Characterising and comparing *in vivo* oxidative ($\tau_{1/2}$PCr) and anaerobic (pH recovery time) skeletal muscle metabolism in adults with untreated GHD, treated GHD and healthy volunteers.
b. Correlating these data with parallel comparisons of the perception of fatigue across the same 3 groups using specific domains within a validated quality of life (AGHDA-QoL) questionnaire.

My hypothesis was that the fatigue experienced by untreated GHD adults is due to perturbations in their skeletal muscle metabolism.

2.1.3 Study subjects

This cross-sectional study investigated skeletal muscle metabolism in 3 groups: untreated GHD adults, treated GHD adults and healthy controls using ^{31}P MRS. Twenty two untreated GHD adults and twenty three treated GHD adults were identified and recruited from the KIGS and KIMS database (a pharmaco-epidemiological database of all GHD patients maintained by Pfizer Inc.) across Northern England. Twenty healthy controls were also recruited through local advertisements. All subjects underwent ^{31}P MRS, anthropometry and body composition assessment. They also completed AGHDA and physical activity assessment (IPAQ) questionnaires. The study design is summarised in Figure 18.

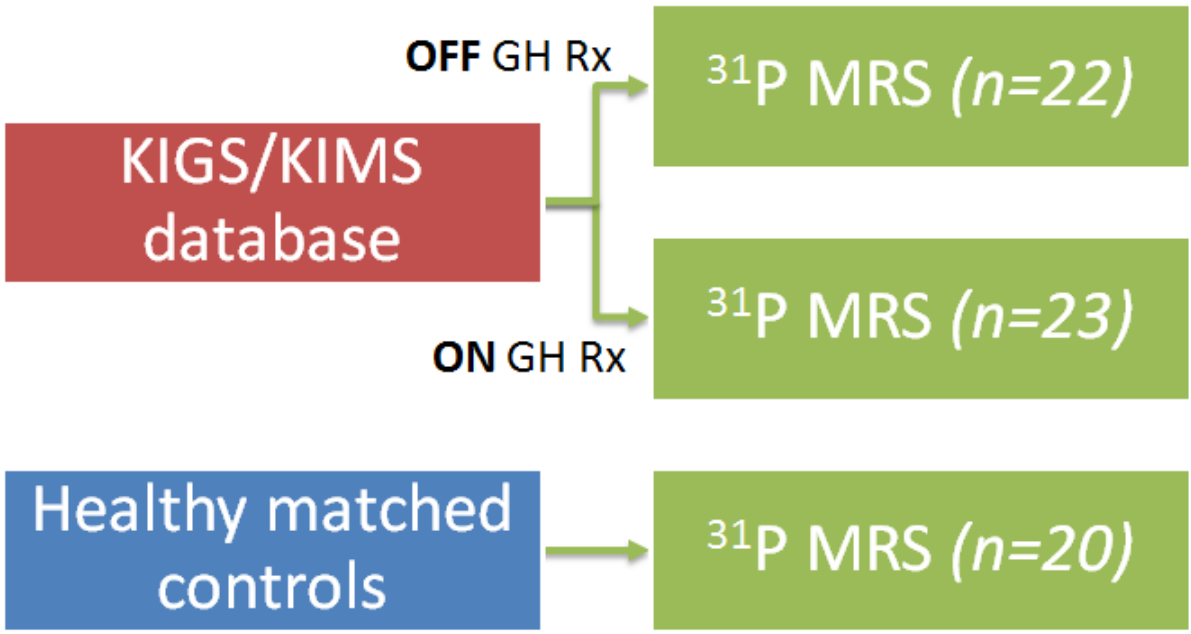

Figure 18 Growth Hormone Cross-sectional study design

Inclusion criteria:

Patients between 16-50 years of age were eligible for the trial. Although still a matter of debate, this age group was chosen in order to eliminate the age associated changes in skeletal muscle metabolism (Kent-Braun and Ng, 2000; Kutsuzawa *et al.*, 2001). Following the identification of 119 GHD patients using the KIGS/KIMS database, potential eligible candidates were screened with their clinicians namely Dr Steve Ball, Dr Tim

Cheetham, Prof Simon Bailey and Sr Margaret Miller to ensure appropriate selection of volunteers i.e. those who were mentally and physically able to understand the consenting process and undergo the exercise MR scan. Prospective subjects were subsequently sent Patient Information Sheets (Appendix) and recruited at the Endocrine clinics at Newcastle University Hospitals NHS trust (Figure 19). All three groups were matched based on age, gender and physical activity levels (Table 4). Subjects were diagnosed with Adult GHD if their peak GH on GHRH-arginine testing was less than 4.1µg/liter (Molitch *et al.*, 2011). Subjects were deemed to be untreated if they had not received GH in the past one year. The other abnormal endocrine axes were being replaced in hypopituitary subjects. Fasting bloods were obtained in all subjects to ensure subjects were receiving adequate therapy.

Exclusion criteria:

Exclusion criteria included those with:

I. MR incompatible implants since they would be unsuitable for MR imaging,
II. Learning difficulty due to concerns regarding obtaining consent and comprehending instructions
III. Profound physical impairments which would preclude them from undertaking the exercise under the MR scanner

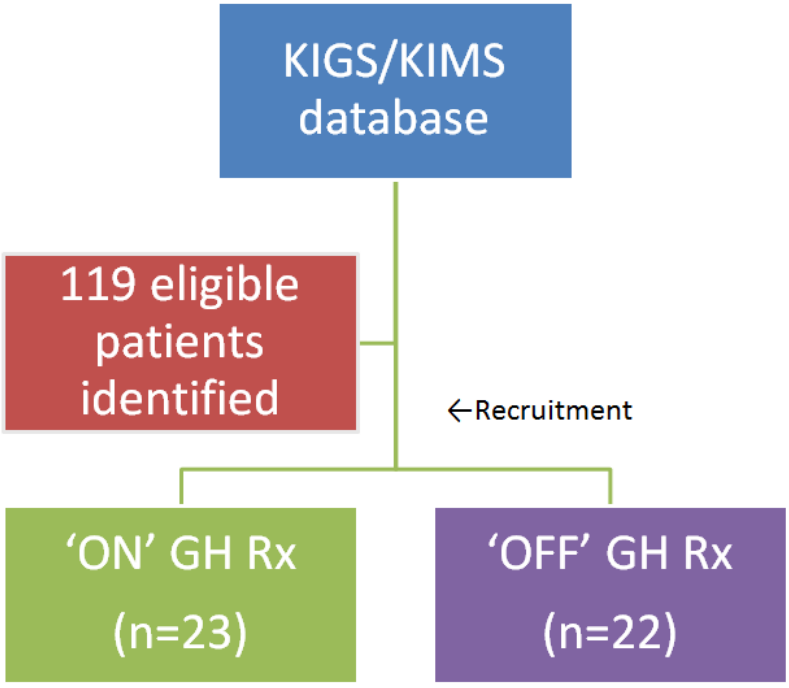

Figure 19 Flowchart of recruitment

2.1.4 Approval for study

The study titled 'The role of skeletal muscle function and energetics in the symptoms and metabolic characteristics of Growth Hormone deficiency: a functional NMR approach; REC Reference no: 10/H0907/71' was granted a favourable opinion by the Newcastle and North Tyneside 2 Research Ethics Committee on 21st December 2010. The Newcastle upon Tyne Hospitals NHS foundation Trust granted NHS R&D approval for the conduct of this project (Trust R&D project-5519) on 26th January 2011.
Written informed consent was obtained from all participants (see Appendix for details regarding PIS and consent form). The investigation was conducted according to the principles of the Declaration of Helsinki.

2.1.5 Phosphorus-31 Magnetic Resonance Spectroscopy protocol

Magnetic resonance spectroscopy data were acquired using a 3T Achieva scanner (Philips, Best, NL) with a 14cm diameter phosphorus surface coil for transmission/reception of signal and the in-built body coil for anatomical imaging (Boska, 1994). Imaging to localise to gastrocnemius and soleus muscles is performed by axial T1 turbo spin-echo imaging around the fattest part of the calf (TR = 671ms, TE = 10ms, 256x153 matrix, resolution 1.37x1.71, 16 slices with 10mm gap). A purpose-built exercise apparatus was developed for operation with the magnetic resonance imaging (MRI) scanner (Figure 20). This apparatus permitted a controlled plantar flexion (between 0° to 30°) to isolate and exercise the soleus and gastrocnemius muscles with the patient lying supine, whilst using restraining straps to prevent the recruitment of the quadriceps. Subjects performed a validated exercise protocol which contained two periods of exercise, consisting of three minutes of rest, three minutes of plantar flexion at 0.5 Hz (sufficient time to deplete the PCr stores which would in turn stimulate the mitochondria for recovery analysis) and six and a half minutes of rest post exercise to measure recovery to equilibrium (Hollingsworth *et al.*, 2008). The first period used a fixed load of 25% of the Maximum Voluntary Contraction (MVC, determined prior to spectroscopy) to accurately measure oxidative metabolism in recovery whilst attempting to maintain a stable pH as possible (Kemp and Radda, 1994). The second period, used a higher fixed load (35% of MVC) to produce greater anaerobic stimulus and allow evaluation of pH handling. TR = 5s was used with paired subtracted spectra to perform

1D ISIS selection in the anterior-posterior direction, so effectively there are 2 averages per final spectrum at time resolution 10s. The dimension of the 1D anterior-posterior ISIS selection was 48mm. The gradient strength used in the ISIS localisation was 3.09 mT/m. The extent of spatial shift between Pi and γ-ATP (since α and β-ATP are not used in the calculations) is 7mm (The frequency difference induced across the selected 1D slab is 2556Hz and Pi and γ-ATP are 389Hz apart on the spectrum). Deviation from the prescribed slab is minimised by putting PCr on resonance.

Quantification of the spectral peaks and calculation of pH was performed using the AMARES time domain fit routine in the jMRUI processing software (v 3.0) (Kemp *et al.*, 1997; Vanhamme *et al.*, 1999). The prior knowledge model used assumed single Lorentzian resonances for Pi, PDE and PCr, with the α and γ moieties of ATP modelled as doublets of equal area with 17Hz separation, and the β-ATP moiety modelled as a triplet with 17Hz separation and area ratio 1:2:1 (Hamilton *et al.*, 2003). Parameters of oxidative metabolism and pH handling were calculated. Exponential fits to the recovery data were made to estimate the half-times for recovery to equilibrium of PCr ($\tau_{1/2}$ PCr) and ADP ($\tau_{1/2}$ ADP) (Taylor *et al.*, 1983). The relationship between ADP concentration and oxidation rate was used to estimate maximal oxidative ATP production (Q_{max}) as described in the equation: $Q_{max} = V(1+K_m / [ADP]$ end exercise) where Km is taken as 30 μM (Kemp *et al.*, 1993). Muscle pH was assessed using the chemical shift between phosphocreatine peak and the inorganic phosphate peak,

$pH = pK_a + 10 \log_{10} ((\delta_1-\delta)/(\delta-\delta_2))$

where $pK_a = 6.75$, δ is the chemical shift from PCr to Pi in ppm, $\delta_1 = 3.27$ppm, $\delta_2 = 5.63$ppm (Moon and Richards, 1973).

Proton efflux, E, was calculated for every time point after cessation of exercise,

$E = (\upsilon + m)d[PCr]/dt + \beta dpH/dt$

where ν,m and β are cytosolic buffering capacity constants, which can be estimated based on previous work and which themselves have a dependency on pH (Kemp *et al.*, 1997).

This allows the evaluation of the time point at which efflux reached its maximum. The pH at three key time points was examined: (i) baseline pH which can demonstrate resting metabolic imbalances, (ii) pH immediately post exercise which reflects the extent to which the anaerobic glycolytic pathway has been activated and (iii) the nadir pH after

exercise which indicates the maximum burden of acid clearance imposed by anaerobic metabolism and the resynthesis of phosphocreatine (Hoult *et al.*, 1974). Time to pH recovery was calculated by measuring the time from the cessation of exercise until pH returned to within 0.01 units of its pre exercise value. If pH was higher or equal to its initial value at cessation of exercise, due to the pH raising effect of PCr hydrolysis, pH recovery time was taken to be zero.

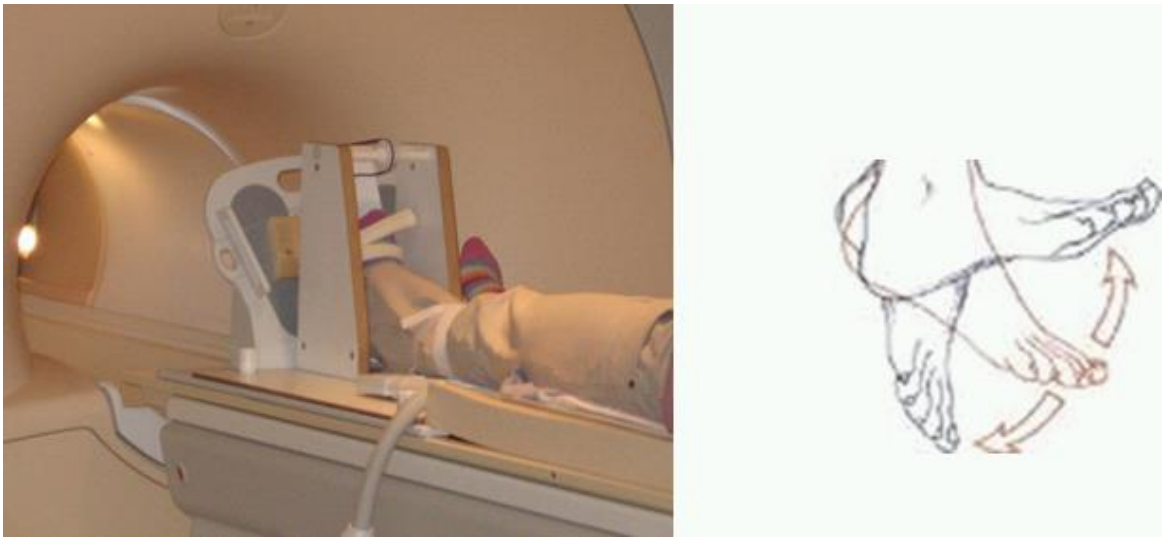

Figure 20 illustrates the apparatus used to permit exercise (plantar flexion) within the MR scanner (left) and the 0°-30° of plantar flexion involved (right)

2.1.6 Assessing Quality of Life –AGHDA questionnaire

The 'Assessment of growth Hormone Deficiency in Adults' (AGHDA) questionnaire is a self-administered instrument developed specifically to objectively evaluate the impaired quality of life (QoL) and fatigue experienced by adults with GHD (Holmes SJ, 1995). It has been validated in adults with GHD, besides forming an important criterion for qualification for GH therapy in adults in the United Kingdom (NICE, 2003). The measure is constructed of 25 items that evoke yes/no answers, acknowledging or denying certain problems. The total score is computed by quantifying a range of problems so that each 'yes' is given a score of 1 and each 'no' is attributed a score of 0. Therefore, a higher numerical score depicts a poor QoL. It has been shown to have good reliability, reproducibility, internal consistency and construct validity (McKenna *et al.*, 1999). The 25 items may be clustered into five domains: tiredness (seven questions), memory and concentration (six questions), tenseness (three questions), social isolation (five questions), and self-confidence (four questions) (Koltowska-Haggstrom *et al.*, 2006). For

each domain, the mean score per question was calculated (see Appendix A AGHDA-QoL questionnaire).

2.1.7 Biochemical evaluation and assays

Fasting bloods were obtained from all subjects. All biochemistry and quantitative hormone measurements were measured using standard methodologies in the medical biochemistry laboratory of Newcastle University Hospitals NHS Trust. The principles and performance assays are briefly described below:

Serum IGF-1:

Quantitative measurements of serum IGF-1 were undertaken using a solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulite 2000) with an intra-assay coefficient of variation (CV) of 2.3-3.9% and inter-assay CV of 3.7-8.1%.

Serum free thyroxine (fT4):

The Roche Free T4 assay, a competitive immunoassay using electro-chemi-luminesce technology was used to measure *f*T4. It has an intra-assay coefficient of variation (CV) of 1.6-2.5% and inter-assay CV of 3.0-3.8%.

Serum insulin:

The Mercodia Insulin kit, an enzyme linked immunosorbent assay (ELISA), was used for the quantitative measurement of serum Insulin. It has an intra-assay coefficient of variation (CV) of 2.8-3.2% and inter-assay CV of 3.0-3.9%.

Serum glucose:

Colorimetric detection was used based on the principle of glucose oxidase, in which H_2O_2 reacts with various hydrogen donors to produce a colour change that is proportional to the glucose concentration. It has an intra-assay coefficient of variation (CV) of 0.9-1.8% and inter-assay CV of 1.0-1.8%.

Glycated haemoglobin (HbA1c):

The Tosoh Haemoglobin A1c method which utilises the principles of ion exchange high performance liquid chromatography (HPLC) was used.

Total cholesterol:

The assay had an intra-assay coefficient of variation (CV) of 0.8-2.0% and inter-assay CV of 1.7-3.3%.

High density lipoproteins (HDL):

The assay had an intra-assay coefficient of variation (CV) of 0.1-2.5% and inter-assay CV of 3.9-5.7%.

HOMA assessment:

All subjects had their homeostasis model of assessment (HOMA) index calculated (Matthews *et al.*, 1985). The HOMA estimates steady state beta cell function and insulin sensitivity as percentages of a normal reference population. These measures correspond well, but are not necessarily equivalent, to non-steady state estimates of beta cell function and insulin sensitivity derived from stimulatory models such as the hyperinsulinaemic clamp and the oral glucose tolerance test. The HOMA index was calculated using the formula-(fasting glucose(mmol/L) x fasting insulin(mU/L) / 22.5)

2.1.8 Anthropometric evaluation

All subjects underwent anthropometric assessment with measurements of their standing height and weight using a calibrated vertical stadiometer and digital weight scale respectively. Waist circumference was measured using a non-elastic measuring tape. Body fat percentage was evaluated using a bio-impedance analysis scale (Bodystat 1500) which has a reported CV of 3% (Lukaski *et al.*, 1985).

2.1.9 Physical activity evaluation

Self-reported levels of physical activity and inactivity were assessed with IPAQ (International Physical Activity Questionnaire), a validated questionnaire (Booth, 2002). The long format questionnaire, developed specifically for research purposes, assesses the physical activities undertaken by the respondent over the past 7 days (Craig *et al.*, 2003). Continuous scoring (MET/metabolic equivalent of task-minutes per week) was calculated to ensure matching of physical activity levels between subjects. MET is a scale of the energy cost of various physical activities in multiples of the resting metabolic rate (see Appendix B IPAQ physical activity questionnaire).

2.1.10 Outcome measures

The primary outcome of this study was to compare recovery kinetics of phosphocreatine ($\tau_{1/2}$PCr), a direct marker of maximal mitochondrial oxidative function which is independent of work or power output, across the 3 groups (Binzoni *et al.*, 1992). $\tau_{1/2}$PCr was compared with serum IGF-1 levels to evaluate the relationship between growth hormone status and the mitochondria. Comparisons between fatigue perception (assessed with Qol-AGHDA) and peripheral fatigue (assessed with ^{31}P MRS) across the 3 groups were undertaken to determine the aetiology of fatigue in GHD.

2.1.11 Statistical analysis

The study was designed to recruit a minimum group size of 19 subjects, with a 90% power to detect 10% impairment in $\tau_{1/2}$PCr. Sample size was estimated from pilot studies of healthy individuals at our centre who underwent repeat ^{31}P MRS to determine repeatability. Using the Bland-Altman method, we expected to find a maximum of 9% variability in $\tau_{1/2}$PCr from repeated measurements (Bland and Altman, 1986). Statistical analyses were undertaken using Minitab v16 software package. All data are expressed as mean ±SD unless described otherwise. The Kolmogorov-Smirnov test of normality was used to determine the distribution of data. Overall differences between the untreated GHD, treated GHD and control groups were tested with an ANOVA test (parametric) and Tukey's family error rate was applied for *post hoc* pair-wise comparisons between the 3 groups. Where data was non-parametric, the Kruskal-Wallis test was used. Differences between unpaired groups were tested with an unpaired t-test (parametric) or a Mann Whitney test (non-parametric). To test the association and relationship between variables, the chi square test and Pearson's correlation coefficients were calculated respectively. Several bivariate regression models were independently created to identify predictors of mitochondrial function. A multiple linear regression was undertaken with continuous and categorical predictors to determine predictors of fatigue. All tests were two-sided and the level of significance was set at $P<0.05$.

2.2 Results

2.2.1 Subject clinical characteristics

Sixty five subjects were recruited between the ages of 16 and 50 years (See Table 4). The gender distribution was similar across the three groups with a male preponderance ranging from 59.2% to 65.2%. There was no difference in body mass indices (BMI) ($p=0.06$) or waist circumference ($p=0.09$) across the three groups. Controls had lower body fat percentage compared to both treated and untreated GHD adults ($p=0.03$). The proportion of subjects with multiple pituitary hormone deficiency and isolated growth hormone deficiency were similar in both the untreated and treated GHD groups ($p=0.40$). There was a preponderance of childhood onset GHD in both the untreated ($n=18/22$) and treated ($n=15/23$) GHD groups. The self-reported activity levels (MET min/week) amongst the 3 groups was similar ($p=0.25$).

Untreated subjects with GHD had lower serum IGF-1 levels compared to both treated GHD and healthy controls ($p<0.001$) (Figure 21). In order to minimise the effects of other hormones on skeletal muscle function, stable hormone replacement in all patients was ensured (Table 4). There was also no difference between parameters of insulin sensitivity in the 3 groups. Treated GHD patients had elevated high density lipoprotein (HDL) compared to controls and untreated GHD adults ($p=0.007$).

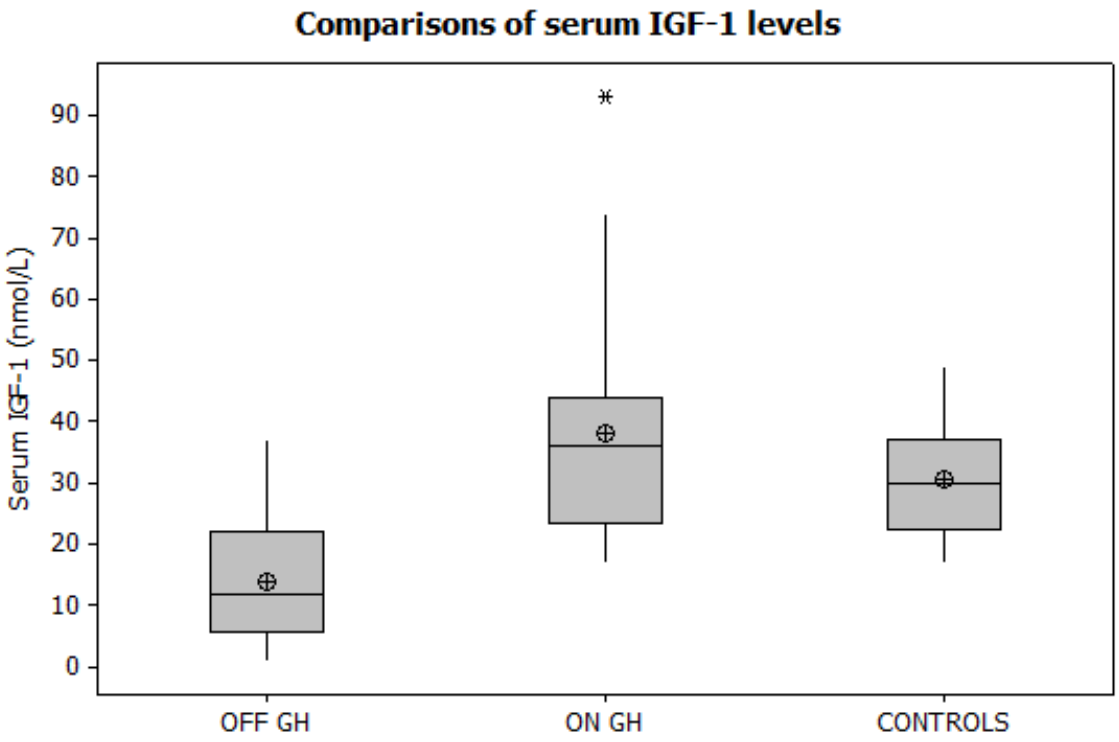

Figure 21 shows lower IGF-1 levels in the untreated GHD group compared to the treated GHD group and the controls

The boxplot depicts the sample minimum, lower quartile, median, upper quartile and sample maximum data.

*** refer to outliers.**

ʘ depicts the sample mean.

Table 4 Patient characteristics

Mean ±SD	OFF GH	ON GH	Controls	P value
Total No	22	23	20	
Age	27.85±9.34	29.76±10.07	31.05±7.93	0.537
Sex ratio	59.2% males	65.2% males	60% males	
Aetiology of GHD:				
MPHD/ Isolated GHD	17/5	20/3	NA	0.396
Childhood onset/ Adult onset	18/4	15/8	NA	0.208
Surgery	14	9	NA	0.475
Radiotherapy	16	8	NA	0.475
Chemotherapy	6	1	NA	0.475
Anthropometry:				
BMI (kg/m^2)	29.9±8.7	29.6±6.7	25.1±4.2	0.058
Waist (cms)	94.8±21.9	99.5±16.9	86.84±10.8	0.085
Body fat %	31.2±13.4 (A)	30.1±9.9 (A,B)	22.5±9.1 (B)	**0.031**
Activity level (MET min/week)	1622†	2043†	2735†	0.246*
Endocrine parameters:				
IGF-1 (nmol/L)	14.0±11.1 (B)	38.1±19.9 (A)	30.7±9.5 (A)	**<0.001**
Thyroxine fT4 (pmol/L)	18.2±3.7	19.0±3.9	16.3±2.1	0.085
Testosterone (nmol/L)	20.6±9.5	15.9±7.9	20.7±5.5	0.250
25OHD (nmol/L)	57.9±34.4	53.9±20.2	44.2±29.4	0.418
Metabolic parameters:				
Fasting glucose (mmol/L)	4.9±0.6	4.9±0.6	5.5±1.6	0.172

Fasting insulin (µU/ml)	11.1±7.9	12.3±12.1	6.2±4.9	0.120
HOMA index	2.5±1.9	2.7±2.8	1.4±1.3	0.198
HbA1c (%)	5.6±0.8	5.5±0.4	5.2±0.4	0.254
Total cholesterol (mmol/L)	5.3±1.1	5.1±1.1	4.8±1.1	0.450
HDL (mmol/L)	1.2±0.3 (A)	1.5±0.4 (A,B)	1.4±0.3 (B)	**0.007**
LDL (mmol/L)	3.3±1.1	2.9±1.2	2.7±0.8	0.292

All data are expressed as mean ±SD unless expressed otherwise. † suggests median. The Kolmogorov-Smirnov method was used to test for normality ($p>0.05$ signifying parametric distribution). Overall differences (parametric data) between the untreated GHD, treated GHD and control groups was tested with an ANOVA test and Tukey's family error rate was applied for *post hoc* pair-wise comparisons between the three groups (means that do not share a letter were significantly different). Where the data was non-parametrically distributed the Kruskal-Wallis test was calculated (*)

MPHD multiple pituitary hormone deficiency, MET metabolic equivalent-minutes, 25OHD 25 Hydroxy vitamin D, HOMA homeostatic model assessment, HDL high density lipoprotein, LDL low density lipoprotein, testosterone levels were from males only

2.2.2 Parameters of mitochondrial oxidative phosphorylation

There was no difference in $\tau_{1/2}$PCr (p=0.54) nor $\tau_{1/2}$ADP (p=0.42) between the 3 groups (Table 5) (Figure 22). Maximal oxidative mitochondrial function, Q_{max}, was computed which showed no difference between the 3 groups (p=0.73). Neither males (r=-0.08, p=0.64) nor females (r= -0.37, p=0.08) demonstrated any association between $\tau_{1/2}$PCr and IGF-1 on bivariate analysis.

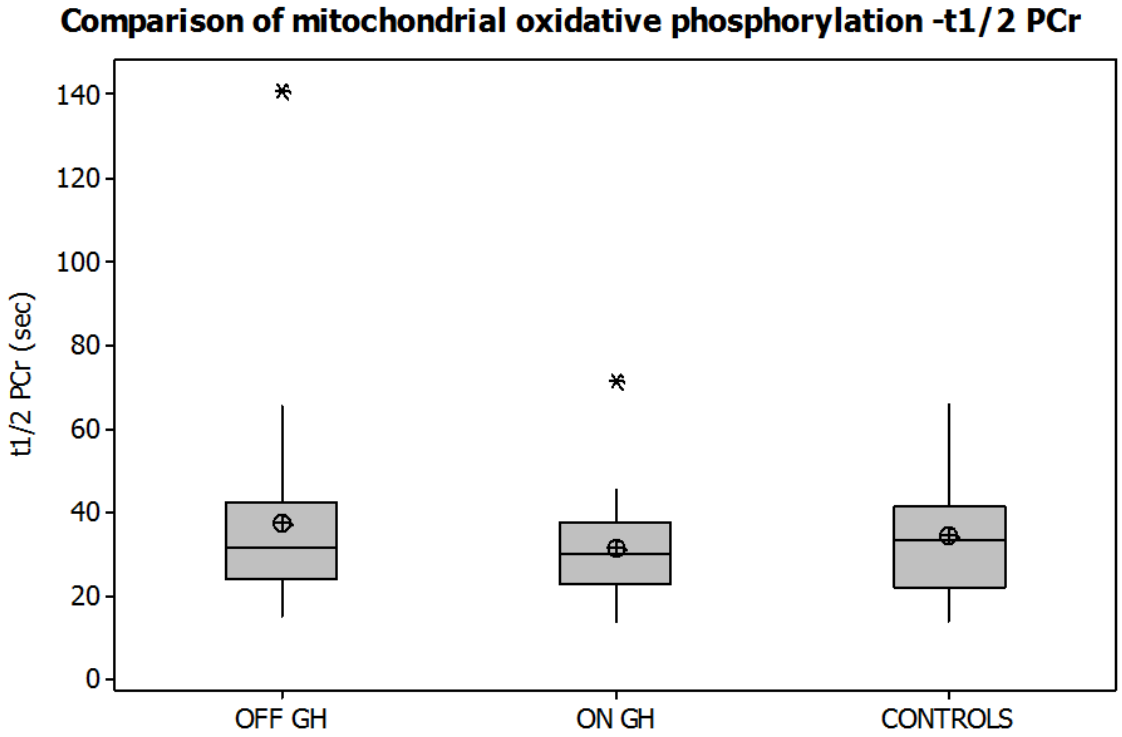

Figure 22 Comparison of oxidative phosphorylation following exercise across the 3 groups

There was no difference in oxidative function between the 3 groups (ANOVA, p=0.54). The asterisks (*) refer to outliers.

2.2.3 Parameters of anaerobic glycolysis

There was no difference in pH recovery times following cessation of exercise (p=0.38) amongst the 3 groups (Figure 23). There was no difference in the nadir pH of the skeletal muscle cells following exercise (p=0.68). There was also no difference in the maximum proton efflux rates following exercise (p=0.37). No difference between resting metabolites such as Pi (p=0.11), PCr (p=0.67) or pH (p=0.62) was detected across the 3 groups (Table 5). There was no gender difference when resting Pi (P=0.14), PCr (p=0.96), end exercise pH (p=0.31), nadir pH (p=0.07) and pH recovery times (p=0.33) were examined.

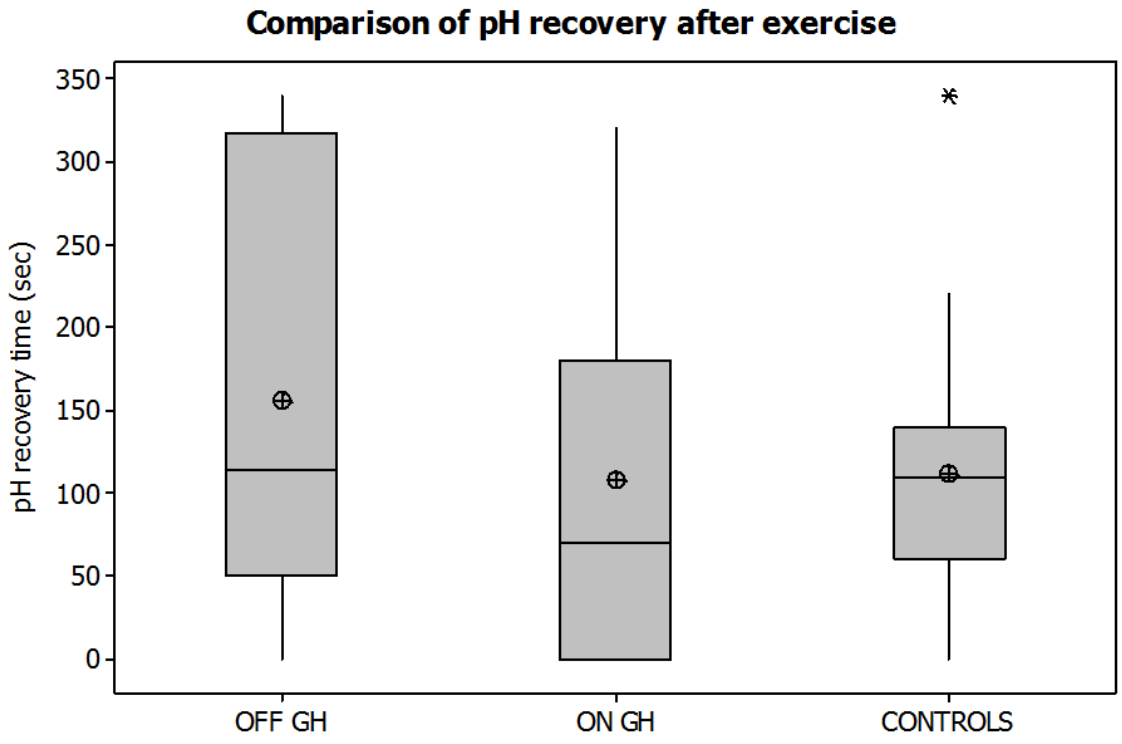

Figure 23 Comparison of pH recovery (proton excretion) following exercise across the 3 groups

There was no difference detected between the 3 groups (Kruskal-Wallis, p=0.38). The asterisks (*) refer to outliers.

Table 5 Comparison of bioenergetic parameters (^{31}P MRS)

Mean ±SD	OFF GH Rx	ON GH Rx	Controls	P value
REST				
Pi (mM)	2.9±0.4	3.2±0.5	2.8±0.6	0.109
PCr (mM)	31.8±2.9	32.4±2.8	32.4±1.5	0.668
pH (U)	7.03±0.02	7.04±0.02	7.05±0.02	0.615
END OF EXERCISE				
% PCr drop	23±0.09	22±0.08	21±0.07	0.823
pH recovery time (sec)	156.5±127.8 115†	108.3±113.3 70†	112.1±77.8 110†	0.297 0.383*
Nadir pH (U)	6.96±0.10	6.98±0.08	6.97±0.06	0.683
Maximum efflux (mM/min)	2.46±1.30	2.34±1.20	2.98±2.01	0.365
RECOVERY				
$\tau_{1/2}$PCr (s)	37.51±26.40	31.19±12.60	34.47±13.75	0.535
$\tau_{1/2}$ADP (s)	22.81±9.13	26.30±12.53	26.69±8.70	0.421
Q_{max} (mM/min)	18.2±10.9	20.4±8.6	18.5±11.6	0.730

All data are expressed as mean ±SD unless expressed otherwise. † suggests median. The Kolmogorov-Smirnov method was used to test for normality (p>0.05 signifying parametric distribution). Overall differences (parametric data) between the untreated GHD, treated GHD and control groups were tested with an ANOVA test. Where the data was non-parametrically distributed the Kruskal-Wallis test was used (*).

The 25% MVC protocol was used to determine indices of recovery whereas the 35% MVC protocol was used to measure end of exercise parameters.

2.2.4 Perception of fatigue and AGHDA scores

Untreated GHD adults complained of significantly increased fatigue when compared to treated GHD adults and healthy controls (p= 0.031) (Figure 24). Untreated GHD adults also complained of significantly decreased quality of life when compared to treated GHD adults and healthy controls (p=0.001) (Figure 25). There was no significant difference in the remaining QoL domains amongst the 3 groups (Table 6). A higher numerical score depicts a poor QoL (discussed in Qol-AGHDA: A Disease specific questionnaire).

Table 6 Comparison of AGHDA-QoL variables

Mean ±SD	OFF GH	ON GH	Controls	P value
Total AGHDA	7.5† (2.71)	5† (0.66)	1† (-3.51)	**0.001***
Fatigue	3.09±3.1	2.04±2.3	0.73±1.1	**0.009**
Memory & Concentration	2.62±1.5	2.46±1.4	2.71±1.3	0.92
Tenseness	2.18±1.2	1.50±0.9	1.33±0.6	0.24
Social Isolation	2.28±1.3	2.16±0.9	2.00±1.4	0.95
Self confidence	2.23±1.0	1.70±0.9	1.66±1.2	0.39

All data are expressed as mean ±SD unless expressed otherwise. † suggests median. The Kolmogorov-Smirnov method was used to test for normality (p>0.05 signifying parametric distribution). Overall differences (parametric data) between the untreated GHD, treated GHD and control groups were tested with an ANOVA test. Where the data was non-parametrically distributed the Kruskal-Wallis test is also included (*). The Z values for each group are expressed within brackets.

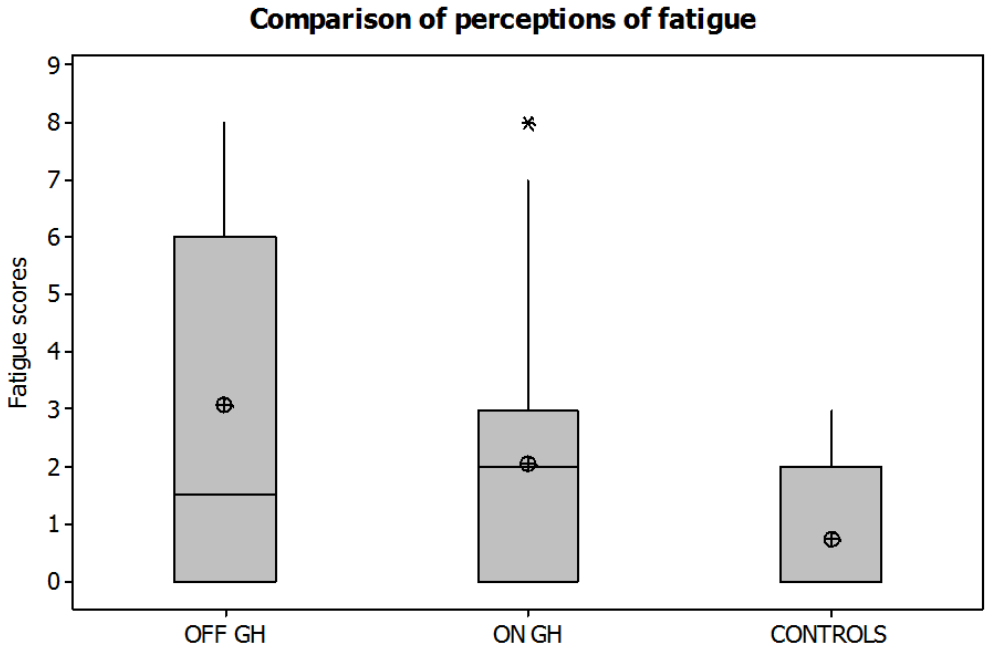

Figure 24 shows a higher perception of fatigue in the untreated GHD group

(ANOVA, p=0.009)

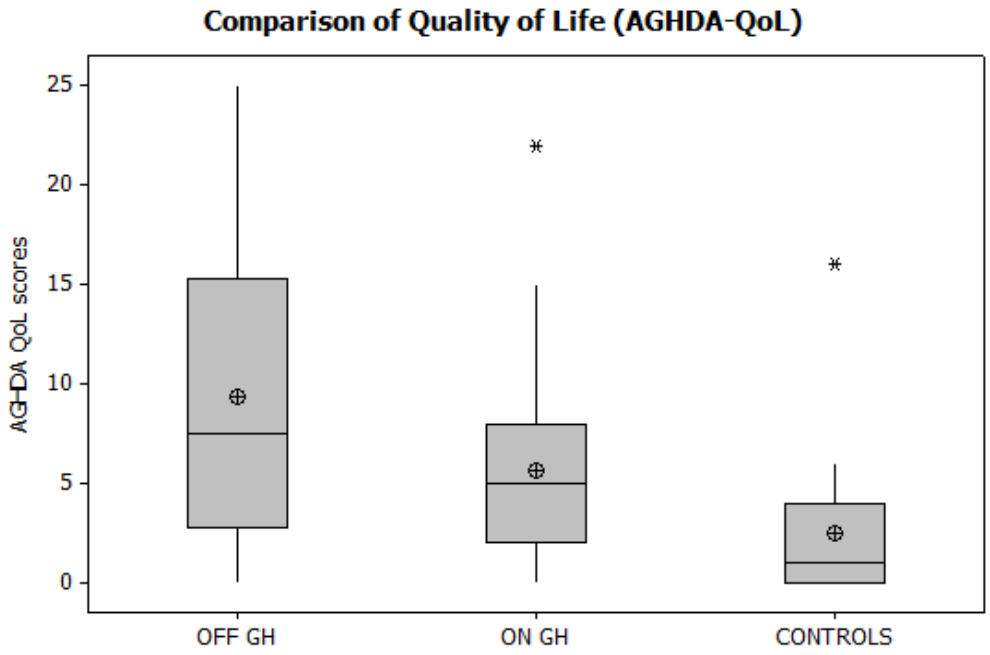

Figure 25 shows impaired QoL in the untreated GHD group in comparison with the other groups

(Kruskal-Wallis, p=0.001)

2.2.5 Pooled analyses and bivariate linear regression analysis to predict mitochondrial function ($\tau_{1/2}$PCr)

Neither $\tau_{1/2}$PCr (r=-0.13, p=0.32) (Figure 26) nor $\tau_{1/2}$ ADP (r=-0.13, p=0.05) was associated with serum IGF-1 in our patients when pooled analyses were undertaken between indices of *in vivo* mitochondrial function and IGF-1. Several other bivariate linear regression models were developed in order to explore the possible relationship between mitochondrial function and factors influencing it. Using $\tau_{1/2}$PCr as the response, the predictors tested in individual bivariate models included age, 25-hydroxy vitamin D (25OHD), body fat %, testosterone, thyroxine, BMI, waist circumference, AGHDA-QoL scores, fatigue-AGHDA scores and IPAQ scores. Only serum 25OHD was a significant predictor of $\tau_{1/2}$ PCr (r=-0.42, p=0.005).

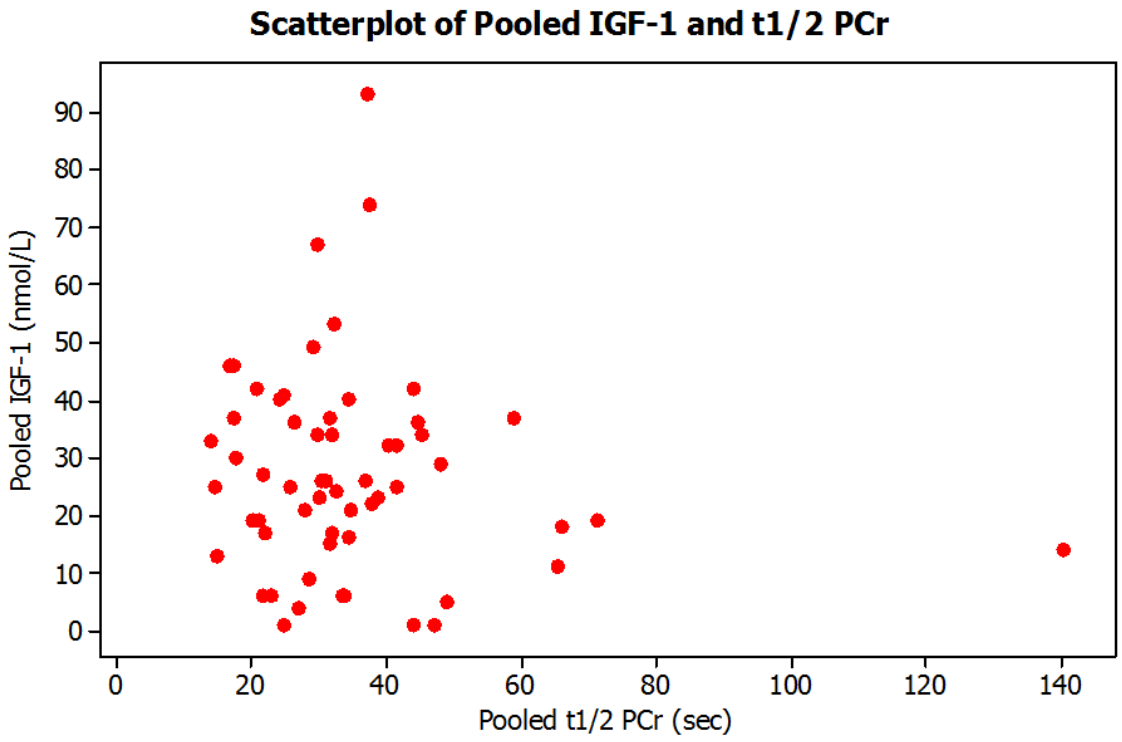

Figure 26 shows there is no correlation between serum IGF-1 and $\tau_{1/2}$PCr on bivariate analysis

2.2.6 Multiple regression analysis to predict factors contributing to fatigue perception

A multiple regression was undertaken in order to examine the relationship between fatigue and the potential factors influencing it. Using fatigue scores from QoL-AGHDA as the response, the predictors entered in the model were age, $\tau_{1/2}$PCr, pH recovery time, maximum proton efflux rate, IGF-1, testosterone (in males), vitamin D, IPAQ scores, BMI and body fat %. The R^2 for the overall model was 46.32% and there were no significant predictors of fatigue perception.

2.2.7 Effect of radiotherapy and chemotherapy on fatigue perception

There was no significant difference in fatigue perception between those treated with radiotherapy (Figure 27; Table 7) or chemotherapy (Figure 28; Table 8).

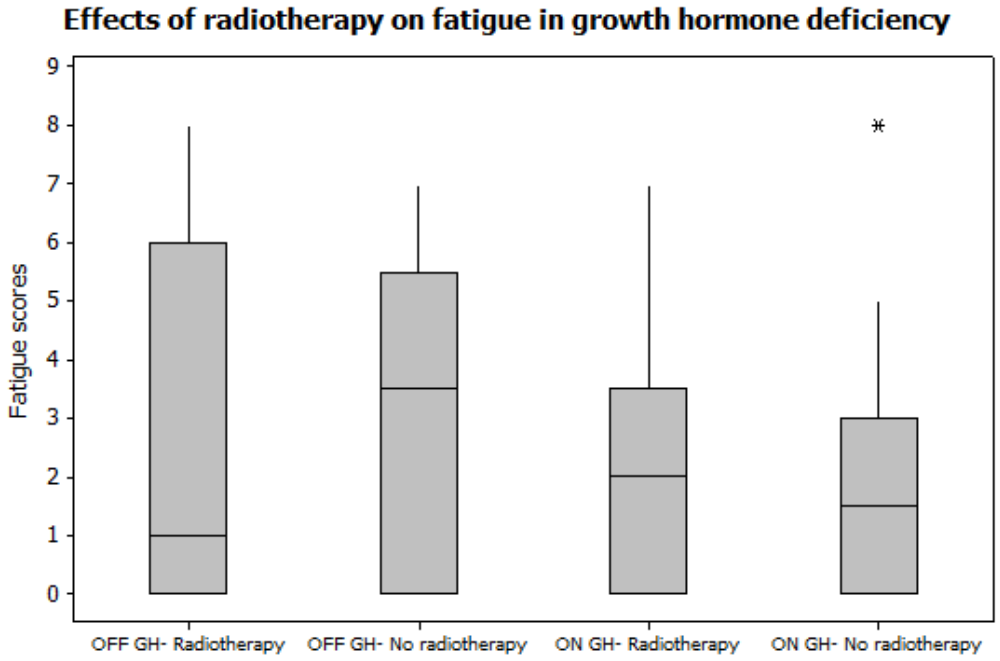

Figure 27 Effect of radiotherapy on fatigue in GHD

Table 7 Effect of radiotherapy on fatigue perception

	Patient numbers	Median fatigue score	P value
OFF GH Radiotherapy	16	1	1.00
OFF GH No radiotherapy	6	3.5	
ON GH Radiotherapy	9	2	0.74
ON Gh No radiotherapy	14	1.5	

Comparisons were undertaken with the Mann-Whitney test

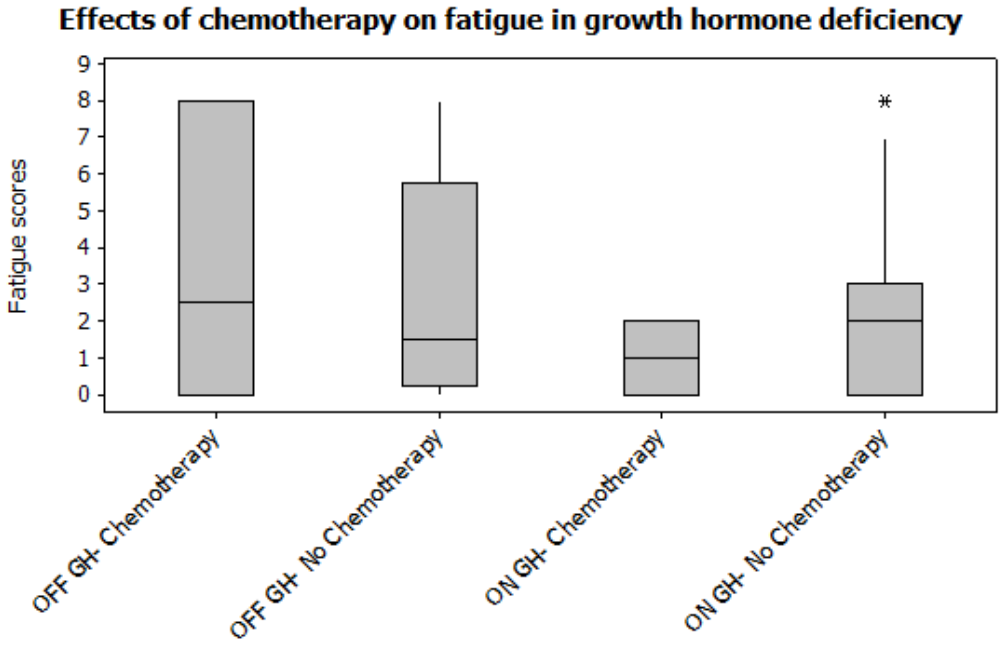

Figure 28 Effect of chemotherapy on fatigue in GHD

Table 8 Effect of chemotherapy on fatigue perception

	Patient numbers	Median fatigue score	P value
OFF GH Chemotherapy	6	2.5	1.00
OFF GH No chemotherapy	16	1.5	
ON GH Chemotherapy	2	1	0.62
ON Gh No Chemotherapy	21	2	

Comparisons were undertaken with the Mann-Whitney test

2.2.8 Assessment of adequacy of other pituitary hormone replacement

Since a significant proportion of the GHD patients had MPHD, it was important to ensure that the remaining pituitary hormones were being adequately replaced. There was no significant difference in free thyroxine levels between untreated (mean ±SD- 18.2±3.7pmol/L) and treated GHD adults (mean ±SD: 19.0±3.9pmol/L) (p=0.48). There was also no significant difference in testosterone levels between untreated (mean ±SD: 20.6±9.5nmol/L) and treated GHD adult males (mean ±SD- 15.9±7.9nmol/L) (p=0.23). There was also no difference in the hydrocortisone replacement regimen (mgs/m^2/day) between the untreated GHD (mean ±SD: 8.27±1.67, range: 6.12-13.15) and treated GHD groups (mean ±SD: 8.21±1.39, range: 6.79-10.77) (p=0.93).

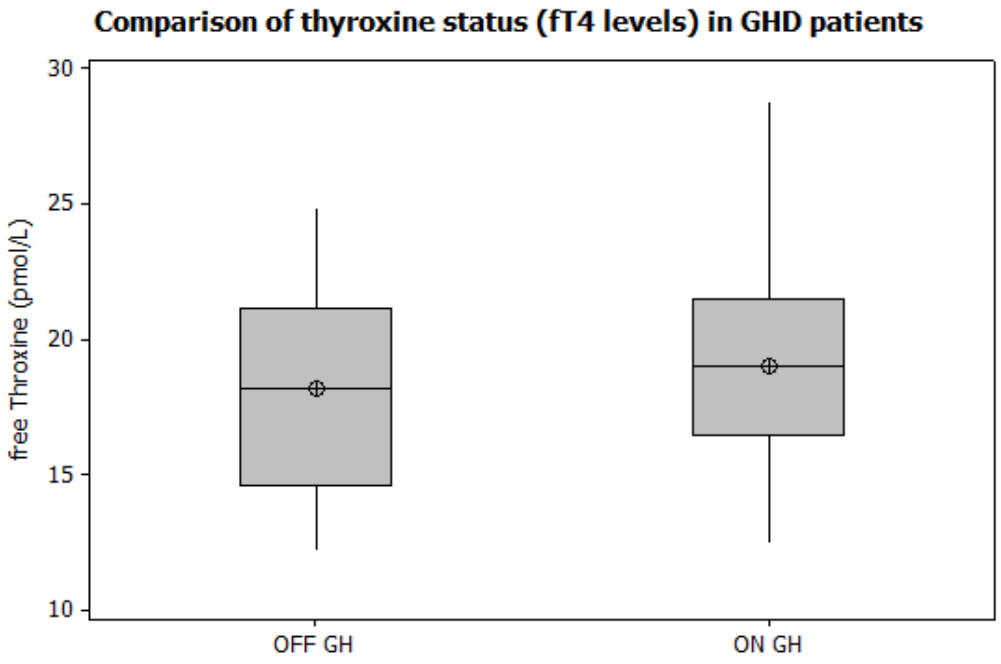

Figure 29 Comparison of thyroxine levels in GHD patients

There was no difference in *f*T4 levels between the untreated GHD and treated GHD adults (Mann Whitney, p=0.48)

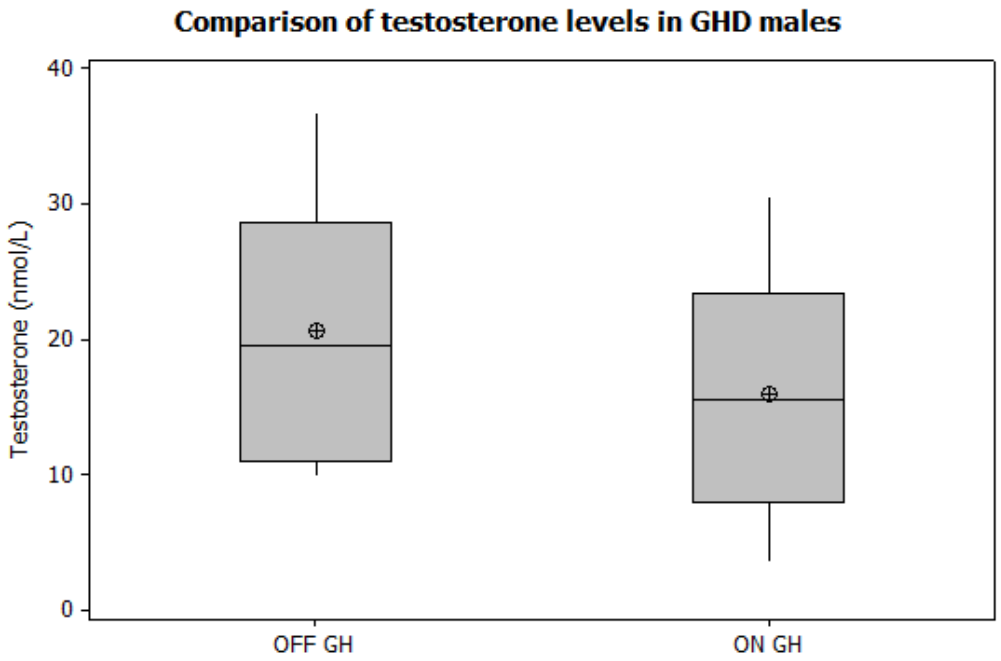

Figure 30 Comparison of testosterone levels in GHD patients

There was no difference in testosterone levels between the untreated GHD and treated GHD adult males (Mann Whitney, p=0.23)

2.3 Conclusions

The data confirm that untreated GHD adults report enhanced fatigue when compared to treated GHD adults and healthy controls. These differences are not associated with differences in skeletal muscle oxidative capacity, nor anaerobic glycolysis as assessed by ^{31}P MRS: both features are seen in a range of conditions in which muscle fatigue is a clinical feature. This suggests a predominant central component in the pathophysiology of fatigue in GH deficiency.

Phosphocreatine resynthesis rate after exercise ($\tau_{1/2}$PCr) is a sensitive, reliable and non-invasive index of mitochondrial oxidative capacity in exercising skeletal muscle (Lanza *et al.*, 2011). $\tau_{1/2}$PCr is delayed in both mitochondrial diseases and hypothyroidism confirming a significant peripheral basis of fatigue in these conditions (Kuhl *et al.*, 1994; Pfeifer *et al.*, 1999). It is important, however, to emphasise that ^{31}P MRS does not measure muscle strength which has been extensively investigated in GHD subjects (Widdowson and Gibney, 2010). Rather ^{31}P MRS provides insights into the metabolic processes occurring within the exercising skeletal muscle, specifically the aerobic oxidative ($\tau_{1/2}$PCr) and anaerobic glycolytic pathways.

My data does not support an association between impaired IGF-1 and $\tau_{1/2}$PCr in patients with GHD. While previous data have demonstrated an association between IGF-1 and $\tau_{1/2}$PCr in healthy males (Makimura *et al.*, 2011), the present study included both GHD adults and healthy volunteers, thereby examining mitochondrial function across a wider GH continuum. Furthermore although healthy controls had reduced body fat % compared to the GHD groups, they did not demonstrate any differences in oxidative function within the muscle compared to GHD adults.

Peripheral muscle fatigue may also be caused by impairments in the anaerobic glycolytic pathway. This may occur either secondary to excessive lactic acid (Bangsbo *et al.*, 1996) or inorganic phosphate (Dahlstedt *et al.*, 2001) accumulation or due to delayed clearance of acid which is measured as pH recovery time. Delayed pH recovery can reflect impaired peripheral vascular supply (Cea *et al.*, 2002). There was no difference in parameters of anaerobic metabolism between the 3 groups studied.

Given the major significance of fatigue in the clinical setting, it is important to assess and measure this symptom in a relevant and reproducible manner. In the current study,

perception of fatigue (measured with the fatigue domain of the validated AGHDA-QoL score) was significantly higher in untreated GHD adults, confirming the findings of others (Table 3). However, there was a wide variation in perception of fatigue, particularly in the untreated GHD group. This could be due to the heterogeneity within the patient groups being studied. A significant proportion of patients in the GHD groups were survivors of cancer where some had also undergone therapy with radiotherapy and chemotherapy. Both cancer and treatment with radiotherapy and chemotherapy may independently cause fatigue (Forlenza *et al.*, 2005). Besides there are challenges in quantifying fatigue in a clinical setting, especially where therapy remains unblinded. GHD adults also complain of reduced well-being, particularly due to social isolation, depressed mood and anxiety: features that are often observed in central fatigue (Lloyd, 1998; Gilchrist *et al.*, 2002).

There are several possible mechanisms for central fatigue in growth hormone deficiency. Copinschi *et al*. identified abnormal sleep architecture in GHD adults (Copinschi *et al.*, 2010). They postulated that increased hypothalamic GH releasing hormone (GHRH) activity produced through the loss of negative feedback, could contribute to the daytime somnolence and fatigue. Many adults with GHD have associated corticotroph failure secondary to pituitary dysfunction. The principal central component of stress response is corticotrophin-releasing hormone (CRH) which not only regulates the hypothalamic-pituitary-adrenal (HPA) axis but also modulates behavioural changes that occur during stress (Koob *et al.*, 1993). Loss of circadian rhythm is well documented in subjects with corticotroph failure and exogenous replacement (Debono *et al.*, 2009). Aberrations in the HPA axis may induce important biological and behavioural consequences leading to central fatigue in patients with GHD. Some biological models from chronic fatigue syndrome support this theory (Lloyd, 1998). In addition, CNS structures critical for cognitive functioning contain a high density of GH and IGF-1 receptors (Bondy and Lee, 1993; Lai *et al.*, 1993). Neuropsychological studies have demonstrated improvements in cognitive function in GHD adults with GH replacement (Deijen *et al.*, 1998). Hence compromised central GH/IGF-1 signaling may contribute to fatigue in patients with GHD.

2.3.1 Study strengths

The strengths of this study lie in the fact that unlike several other GHD studies, comparisons have been made with age and sex matched 'treated' GHD patients as well as healthy volunteers. Besides ^{31}P MRS is a validated technique which measures skeletal muscle bioenergetics. This is also the first study to assess and compare dynamic skeletal muscle energetics in GHD adults.

2.3.2 Study limitations

This study has a number of limitations in terms of both the patient groups being studied and the tools used to study them. Firstly, untreated GHD patients, despite having impaired QoL, were not on GH replacement because of personal choice. Although this could introduce bias, it is interesting to note that there was still no difference in the metabolic functioning of the skeletal muscle between the 3 groups. Both untreated and treated GHD cohorts were unblinded and hence, I cannot eliminate a 'nocebo' effect in the untreated GHD group. However, if such were the case, it reinforces the powerful interaction between growth hormone and the patient's mind-brain interaction (Brody and Miller, 2011).

Secondly, adults with GHD often had concomitant deficits of other pituitary hormones as isolated GHD in adulthood is relatively rare. Hence although attempts to minimise the impact of hormones other than GH through appropriate matching were undertaken, this process may have been imperfect. Exogenous hormone replacement rarely mimics the natural bioavailability and circadian rhythm of endogenous hormone secretion, especially glucocorticoids. However, the glucocorticoid replacement dosages were similar between the untreated and treated GHD group, thus minimising the likelihood of inappropriate glucocorticoid dosage causing the fatigue. The GH/IGF-1 axis also plays an important role in modulating the peripheral metabolism of glucocorticoids by inhibiting 11β-hydroxy steroid dehydrogenase 1 (11β-HSD1) which functions *in vivo* as a reductase, catalysing the conversion of cortisone to cortisol. Enhanced activity of 11β-HSD1 in GHD may result in increased local and/or systemic cortisol production in patients on hydrocortisone replacement (Agha and Monson, 2007). To date, however, 11β-HSD1 and glucocorticoid action have not been shown to play a significant role in skeletal muscle function in GHD (Maiter *et al.*, 2006). Hypogonadism has been reported to contribute to

cancer related fatigue but none of our patients were biochemically hypogonadal (Strasser *et al.*, 2006). We also ensured all our patients were biochemically euthyroid (normal *f*T4 levels) since hypothyroidism is a well-known cause of fatigue (Louwerens *et al.*, 2012). Some of my subjects were survivors of cancer and had undergone treatment with radiotherapy and chemotherapy which are independent causes of cancer related fatigue. However, neither radiotherapy nor chemotherapy was significantly associated with enhanced fatigue perception in my subject groups.

Thirdly, while this cross-sectional study was designed to detect overt differences between the three groups, subtle short-term effects of GH action on mitochondrial function may not have been detected. Therefore there is a small possibility of a type II error (false negative).

Fourthly, the measurement and comparison of fatigue perception amongst patient groups using self-reported outcomes has certain limitations. Differences in conceptualisation (understanding of which elements are included in a target construct), prioritisation (preference regarding the relative importance of certain components within the construct) and calibration (value of scaling of certain health states in relation to others) may often translate to wide inter-individual variations in outcome measurements.

Finally, a sampling error could result in an inability to detect a difference between the groups because of the heterogeneous aetiology of the underlying GHD.

2.3.3 Summary

In summary, this data demonstrates that although untreated GHD adults experience fatigue when compared to both treated GHD adults and healthy volunteers, they do not demonstrate any corresponding perturbations in skeletal muscle metabolism. This data indicates that abnormal muscle energetics is not responsible for the fatigue of GHD adults. Taken together, this data suggests that fatigue in GHD adults may have a central component.

Chapter 3 Vitamin D and skeletal muscle energetics

3.1 Introduction

3.1.1 Background

Recent randomised controlled trials by Ward *et al.* and Goswami *et al.* have refuted the notion that vitamin D might augment muscle strength (Ward *et al.*, 2010; Goswami *et al.*, 2012). Furthermore, the inability to accurately identify vitamin D receptors (VDR) in skeletal muscle recently has raised questions about its role within skeletal muscle (Wang and DeLuca, 2011).On the other hand, fatigue and myopathy are well recognised in the context of vitamin D deficiency and muscle symptoms may arise independent of derangements of bone biochemistry (Glerup *et al.*, 2000). Physicians frequently encounter vitamin D deficient children, adolescents and adults presenting with musculoskeletal symptoms which resolve with calciferol therapy (Ziambaras and Dagogo-Jack, 1997; Pearce and Cheetham, 2010). However, the underlying mechanism for the reversible symptoms of fatigue and myopathy in vitamin D deficiency remains unclear.

The assessment of muscle function involves measuring several parameters including endurance as well as strength. There are established approaches to studying these components but there are also novel tools that can focus on the functional relationship between vitamin D deficiency and skeletal muscle metabolism. One such approach is Phosphorus-31 MRS (^{31}P MRS) which can be used to examine skeletal muscle energetics *in vivo.* ^{31}P MRS can measure the metabolic work-cost relationship of the contracting skeletal tissue and thereby determine its oxidative capacity.

3.1.2 Aims

The aim of the study described in this chapter was to examine the effects of cholecalciferol therapy on skeletal mitochondrial oxidative function in vitamin D deficient subjects using ^{31}P MRS. The hypothesis was that cholecalciferol therapy would be associated with improved skeletal muscle mitochondrial oxidative function in symptomatic vitamin D deficient individuals (<25nmol/L).

3.2 Methods

3.2.1 Study subjects and general study protocol

Twelve individuals with severe vitamin D deficiency (<15nmol/L) were studied. All 12 subjects had their biochemistry assessed and underwent a standardised exercise protocol with an assessment of ^{31}P MRS prior to being treated with cholecalciferol therapy as detailed below. Biochemistry and the exercise protocol with ^{31}P MRS were then repeated following 10-12 weeks of calciferol therapy.

15 age-matched healthy volunteers were recruited as controls through local advertisements. The 15 healthy controls for the Vitamin D study were sampled from the 20 healthy controls used for the GH study. 15 patients were selected since only 15/20 controls had their 25OHD measured. The study design is summarised in Figure 31.

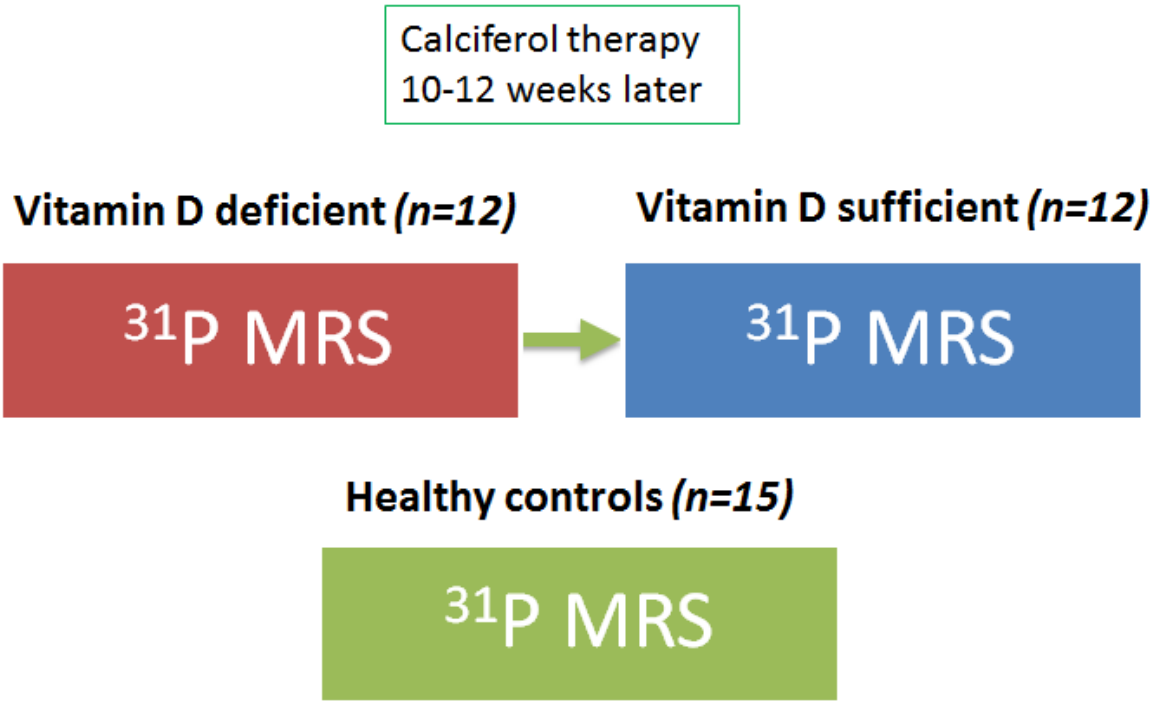

Figure 31 Vitamin D Longitudinal study

Inclusion criteria:

For patients to enter the study, they were deemed to be both severely vitamin D deficient (<25nmol/L) and symptomatic. They all presented to primary care with fatigue

and/or muscle cramps between March and May 2012. The primary care team approached the patients who met these criteria and gave them patient information sheets. The patients subsequently contacted the researcher, Dr Akash Sinha and expressed an interest in participating in the study.

Exclusion criteria:

Exclusion criteria included a history of thyroid, pituitary or inherited mitochondrial disease. Furthermore, those who were MR incompatible because of metallic implants were to be excluded as well.

3.2.2 Approval for the study

The study was approved by the Newcastle and North Tyneside 2 Research Ethics Committee (10/H0907/71-amendment 1) and written informed consent was obtained from all participants. The study was conducted according to the principles of the Declaration of Helsinki.

3.2.3 Phosphorus-31 Magnetic Resonance Spectroscopy protocol

MRS data were acquired using a 3T Achieva scanner (Philips, Best, NL) with a 14cm diameter Phosphorus surface coil for transmission/reception of signal and the in-built body coil for anatomical imaging (Boska, 1994). Imaging to localise to gastrocnemius and soleus muscles is performed by axial T1 turbo spin-echo imaging around the fattest part of the calf (TR = 671ms, TE = 10ms, 256x153 matrix, resolution 1.37x1.71, 16 slices with 10mm gap). A purpose-built exercise apparatus was developed for operation with the imaging scanner. This apparatus permitted a controlled plantar flexion (between 0° to 30°) to isolate and exercise the soleus and gastrocnemius muscles with the patient lying supine, whilst using restraining straps to prevent recruitment of the quadriceps. Subjects performed an exercise protocol consisting of three minutes of rest, three minutes of plantar flexion at 0.5 Hz and six and a half minutes of rest post exercise to measure recovery to equilibrium. A fixed load of 35% of Maximum Voluntary Contraction (determined prior to spectroscopy) was employed to accurately measure oxidative metabolism in recovery whilst maintaining a stable pH (Kemp and Radda, 1994). It is important to highlight that this differs from the 25% MVC employed in the GHD study including the healthy controls. 35% MVC in the vitamin D deficient patients was

deliberately selected since 25% MVC failed to adequately deplete their PCr reserves and hence skeletal muscle mitochondrial function ($\tau_{1/2}$PCr, $\tau_{1/2}$ADP) would not be accurately measured. Importantly, 35% MVC exercise in the vitamin D deficient group led to a similar % PCr depletion and nadir pH to the 25% MVC exercise in the healthy controls. Phosphorus spectra were collected at 10s intervals throughout the exercise using a fully adiabatic 1D-ISIS sequence to localise signal to gastrocnemius and soleus muscles. TR = 5s was used with paired subtracted spectra to perform 1D ISIS selection in the anterior-posterior direction, so effectively there are 2 averages per final spectrum at time resolution 10s. The dimension of the 1D anterior-posterior ISIS selection was 48mm. The gradient strength used in the ISIS localisation was 3.09 mT/m. The extent of spatial shift between Pi and γ-ATP (since α and β-ATP are not used in the calculations) is 7mm (The frequency difference induced across the selected 1D slab is 2556Hz and Pi and γ-ATP are 389Hz apart on the spectrum). Deviation from the prescribed slab is minimised by putting PCr on resonance.

Quantification of the spectral peaks and calculation of pH was performed using the AMARES time domain fit routine in the jMRUI processing software (v 3.0) (Kemp *et al.*, 1997; Vanhamme *et al.*, 1999). The prior knowledge model used assumed single Lorentzian resonances for Pi, PDE and PCr, with the α and γ moieties of ATP modelled as doublets of equal area with 17Hz separation, and the β-ATP moiety modelled as a triplet with 17Hz separation and area ratio 1:2:1 (Hamilton *et al.*, 2003). Parameters of oxidative metabolism and pH handling were calculated. Exponential fits to the recovery data were made to estimate the half-times for recovery to equilibrium of PCr ($\tau_{1/2}$ PCr) and ADP ($\tau_{1/2}$ ADP) (Taylor *et al.*, 1983). The relationship between ADP concentration and oxidation rate was used to estimate maximal oxidative ATP production (Q_{max}) as described in the equation: $Q_{max} = V(1+K_m$ / [ADP] end exercise) where Km is taken as 30 μM (Kemp *et al.*, 1993). Muscle pH was assessed using the chemical shift between phosphocreatine peak and the inorganic phosphate peak,

$pH = pK_a + 10 \log_{10} ((\delta_1-\delta)/(\delta-\delta_2))$

where $pK_a = 6.75$, δ is the chemical shift from PCr to Pi in ppm, $\delta_1 = 3.27$ppm, $\delta_2 = 5.63$ppm (Moon and Richards, 1973).

Proton efflux, E, was calculated for every time point after cessation of exercise,

$E = (\upsilon + m)d[PCr]/dt + \beta dpH/dt$

where ν,m and β are cytosolic buffering capacity constants, which can be estimated based on previous work and which themselves have a dependency on pH (Kemp *et al.*, 1997).

This allows the evaluation of the time point at which efflux reached its maximum.

3.2.4 Vitamin D regimen

All subjects were studied before and after treatment with Cholecalciferol 20,000IU on alternate days for 10-12 weeks for correction of their Vitamin D deficiency (Sinha *et al.*, 2012).

3.2.5 Biochemistry

Serum samples were obtained from participants at both visits. Serum concentrations of 25OHD were measured using the DiaSorin 25Hydroxyvitamin D radio-immunoassay (RIA) (Cat # 68100E Stillwater, Minnesota USA). The DiaSorin RIA assay involves a two-step procedure. The first is a rapid extraction step using acetonitrile to isolate 25 Hydroxy vitamin D (25OHD) and other hydroxylated metabolites of vitamin D. Equilibrium RIA is then performed with 25ul (in duplicate) extracted sample, antibody to 25OHD and iodinated tracer to 25OHD. Phase separation is achieved by the addition of a second antibody and polyethylene glycol. Radioactive counts in the centrifuged pellet are then inversely proportional to the 25OHD concentration in the original sample. Serum samples used in this study were stored at -20ºC prior to analysis. Two quality controls are routinely used in the assay with mean values of 39 and 134 nmol/L and interassay coefficient of variations (CV's) of 8.4% and 12.6% respectively. The intraassay CV for this method is < 8% with a functional sensitivity of 6nmol/L.

3.2.6 Outcome measures

The primary outcome of this study was to compare skeletal muscle mitochondrial function ($\tau_{1/2}$PCr, $\tau_{1/2}$ADP) in 12 symptomatic vitamin D deficient subjects before and after cholecalciferol therapy using ^{31}P MRS.

3.2.7 Statistical analysis

Sample size was estimated from pilot studies of healthy individuals at our centre who underwent repeat ^{31}P MRS to determine repeatability. Using the Bland-Altman method, we expected to find a maximum of 9% variability in $\tau_{1/2}$PCr from repeated measurements (Bland and Altman, 1986). In order to detect a 10% change in $\tau_{1/2}$PCr at a significance p=0.05 with 90% power, an estimation of a minimum group of 10 subjects was made. Statistical analyses were undertaken using Minitab v16 software package. All data are expressed as mean ±SD. After ensuring parametric distribution with the Kolmogorov-Smirnov method (p>0.15), paired t-tests were undertaken to make comparisons pre and post cholecalciferol. A linear regression model was built to examine the relationship between 25OHD and $\tau_{1/2}$PCr. Significance was set at P<0.05.

3.3 Results

3.3.1 Subject clinical characteristics

Participants presented to primary care with fatigue and myopathy. The mean age (±SD) of the subjects was 33.7±9.8years (range 18.1-50.4 years). One patient had a complex medical history including Hepatitis C, depression, epilepsy and anaemia and was on a thiamine, folic acid, vitamin B, levetiracetam and amitriptyline. The remaining participants had no other relevant past illness. 2 participants were Caucasian, 2 were Afro-Caribbean and 8 were of South-East Asian ethnicity. 5 females and 7 males participated. There was no change in diet, medication or exercise pattern between the two study visits. Symptoms of fatigue and myopathy were confirmed on the first visit with all patients reporting an improvement in symptoms at their second visit. The 15 healthy controls were 31.3±7.0 years of age (range 22.3-45.9 years).

3.3.2 Serum biochemistry

All patients were severely vitamin D deficient at their first assessment (mean serum 25OHD 8.83±4.28nmol/L). All were noted to have a significant improvement in 25OHD status (mean serum 25OHD 113.8±51.5nmol/L) following cholecalciferol therapy prior to the repeat MRS muscle study ($p<0.001$) (Figure 32). There was no significant difference in serum calcium, phosphate or alkaline phosphatase levels before and after cholecalciferol (Table 9). The mean serum 25OHD of the healthy controls was 44.2±29.4nmol/L.

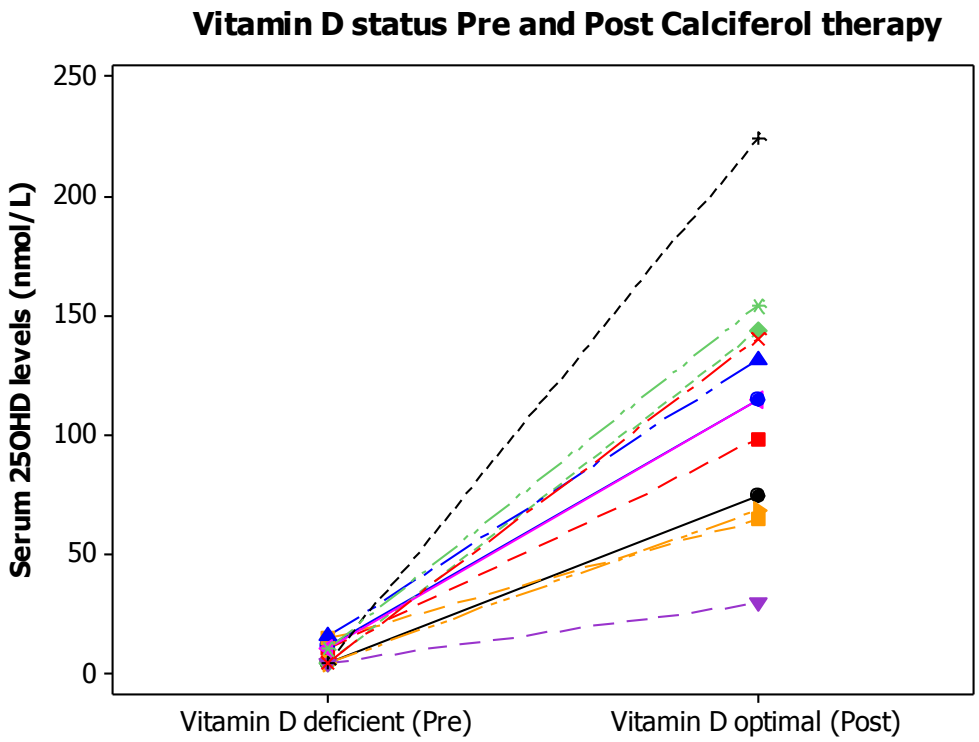

Figure 32 Serum 25OHD levels at presentation with vitamin D deficiency and then following calciferol therapy

3.3.3 Parameters of oxidative phosphorylation

Post-exercise PCr and ADP recovery ($\tau_{1/2}$PCr, $\tau_{1/2}$ADP), indices of mitochondrial oxidative function improved significantly following cholecalciferol therapy ($p < 0.001$, $p=0.003$, Figure 33 and Figure 34 respectively) (Table 9) $\tau_{1/2}$PCr improved from 34.44±8.18s (vitamin D deficient) to 27.84±9.54s (vitamin D optimal). The $\tau_{1/2}$PCr of the healthy controls was 35.66±14.62s. The resting metabolites including Pi, PCr and pH did not differ significantly. There was also no difference in PCr depletion (%PCr drop) or differences in nadir pH and maximum proton efflux at the end of exercise (Table 9). Figure 35 shows a box plot of $\tau_{1/2}$PCr comparisons between the vitamin D deficient, vitamin D optimal and the healthy controls. A pooled regression analysis which included vitamin D deficient, optimal and healthy controls showed lower serum 25OHD levels correlated with longer $\tau_{1/2}$PCr ($r=-0.41$, $p=0.009$) (Figure 36).

Table 9 Biochemistry and ^{31}P MRS measurements on skeletal muscle in vitamin D deficient subjects before and after calciferol therapy

Mean ±SD	Baseline (Vitamin D deficient)	Follow-up (Vitamin D optimal)	P value	Healthy controls
BIOCHEMISTRY				
Serum 25OHD (nmol/L)	8.83±4.28	113.8±51.5	**<0.001**	44.2±29.4
Serum calcium (mmol/L)	2.29±0.1	2.35± 0.1	0.24	NA
Serum phosphate (mmol/L)	0.99±0.1	1.12±0.2	0.19	NA
Alkaline phosphatase (U/L)	86.7± 15.5	78.5± 9.5	0.16	NA
REST				
Pi (mM)	2.63±0.48	2.78±0.47	0.38	2.94±0.59
PCr (mM)	31.77±3.20	32.09±4.19	0.77	32.39±1.28
Pi/PCr	0.08±0.02	0.09±0.02	0.38	0.09±0.02
pH (U)	7.04±0.03	7.05±0.03	0.28	7.04±0.03
END OF EXERCISE				
% PCr drop	0.25±0.14	0.24±0.10	0.81	0.21±0.08
Nadir pH (U)	6.99±0.08	7.00±0.05	0.34	6.91±0.28
Maximum proton efflux (mM/min)	3.63±4.37	3.70±1.83	0.95	2.59±1.69
RECOVERY				
$\tau_{1/2}$PCr (s)	34.44±8.18	27.84±9.54	**<0.001**	35.66±14.62
$\tau_{1/2}$ADP (s)	26.84±6.58	21.93±6.81	**0.003**	27.49±9.12

Data are expressed as mean± SD. Paired t-tests were undertaken for comparisons pre and post calciferol therapy. The healthy control data (n=15) was obtained from the GH study controls (n=20). Since only 15/20 of the healthy controls had their vitamin D analysed, only they could be selected. It is also important to highlight that the recovery

indices in the controls were calculated from 35% MVC exercise. Importantly, however, the % PCr depletion and nadir pH was similar across the 3 groups.

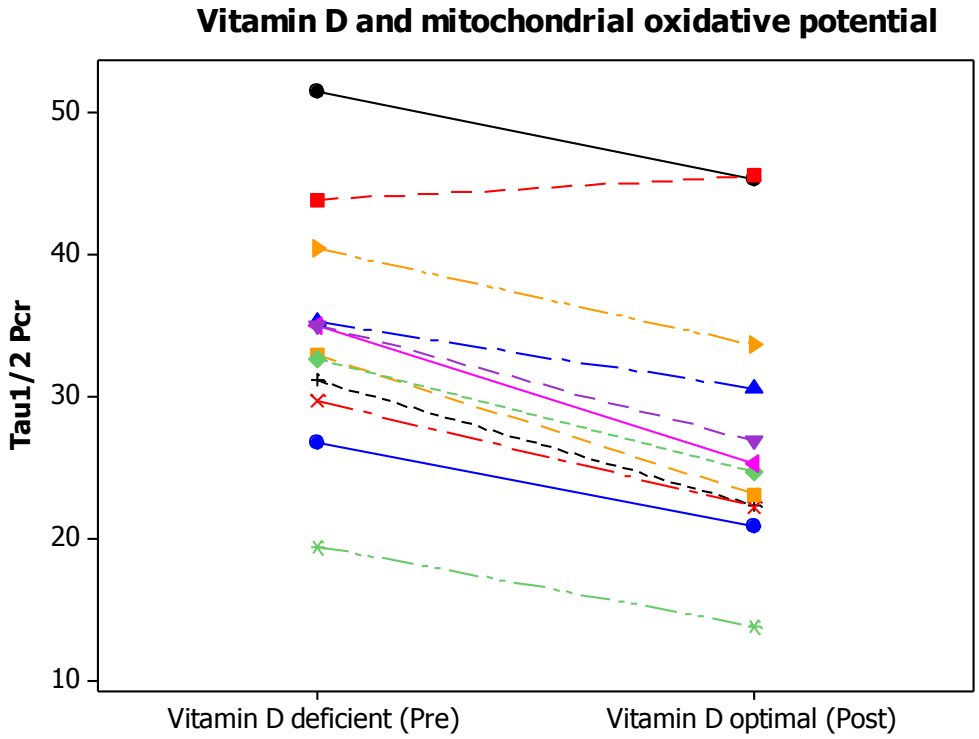

Figure 33 Comparison of $\tau_{1/2}$ PCr kinetics before and after calciferol therapy

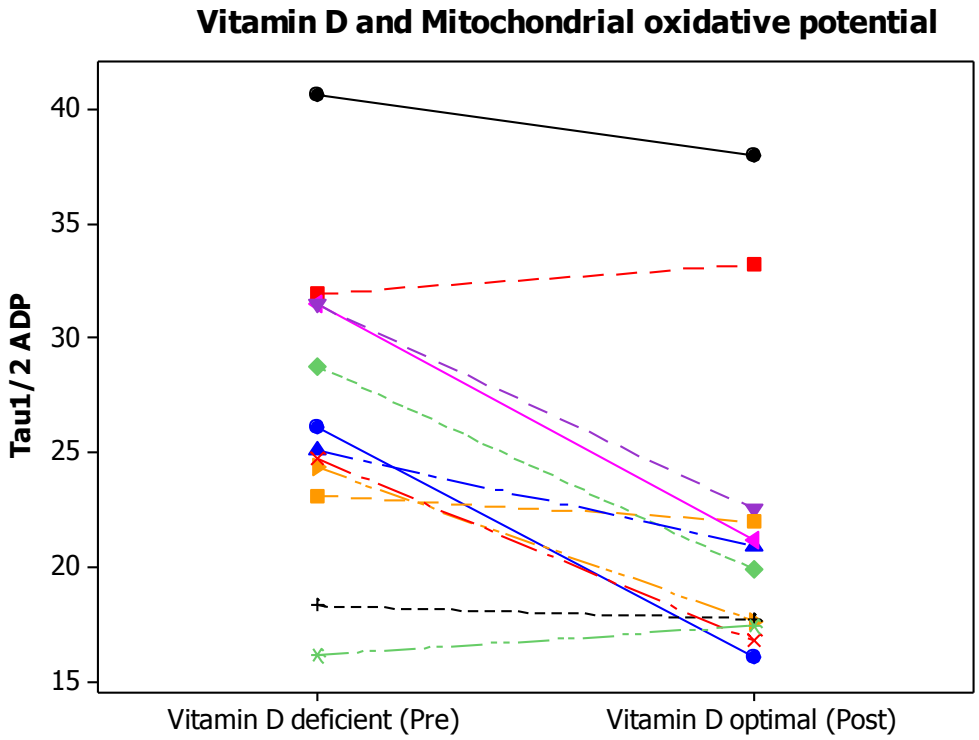

Figure 34 Comparison of $\tau_{1/2}$ ADP kinetics before and after calciferol therapy

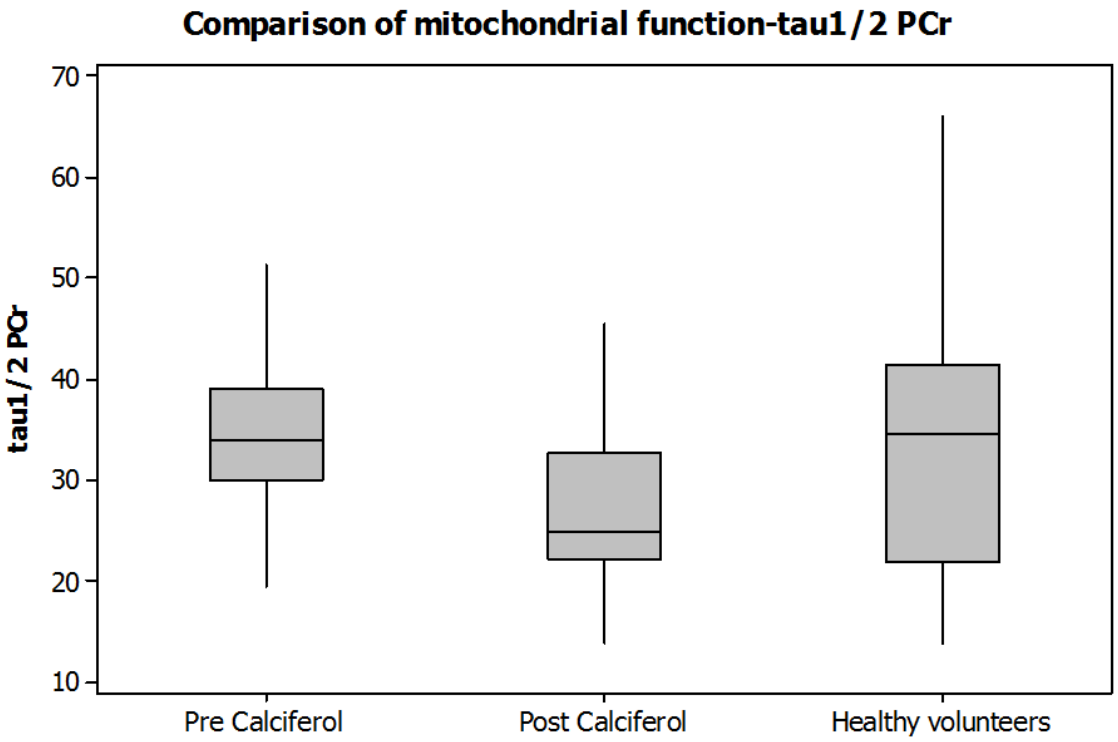

Figure 35 Comparisons of mitochondrial function before and after calciferol therapy and with healthy controls.

The boxplot above shows an improvement in mitochondrial function before and after calciferol therapy. The wide range of mitochondrial function in asymptomatic healthy controls as evident from the wide range of the box plot signifies the wide biological variability of this measure. It is important to note that vitamin D deficiency does not result in abnormal mitochondrial function but correction of vitamin D status leads to an enhancement in mitochondrial function.

It is also important to highlight that the vitamin D deficient subjects were assessed using 35% MVC whereas the healthy controls were assessed using 25% MVC. Importantly, however, both groups demonstrated similar %PCr depletion and end-exercise pH.

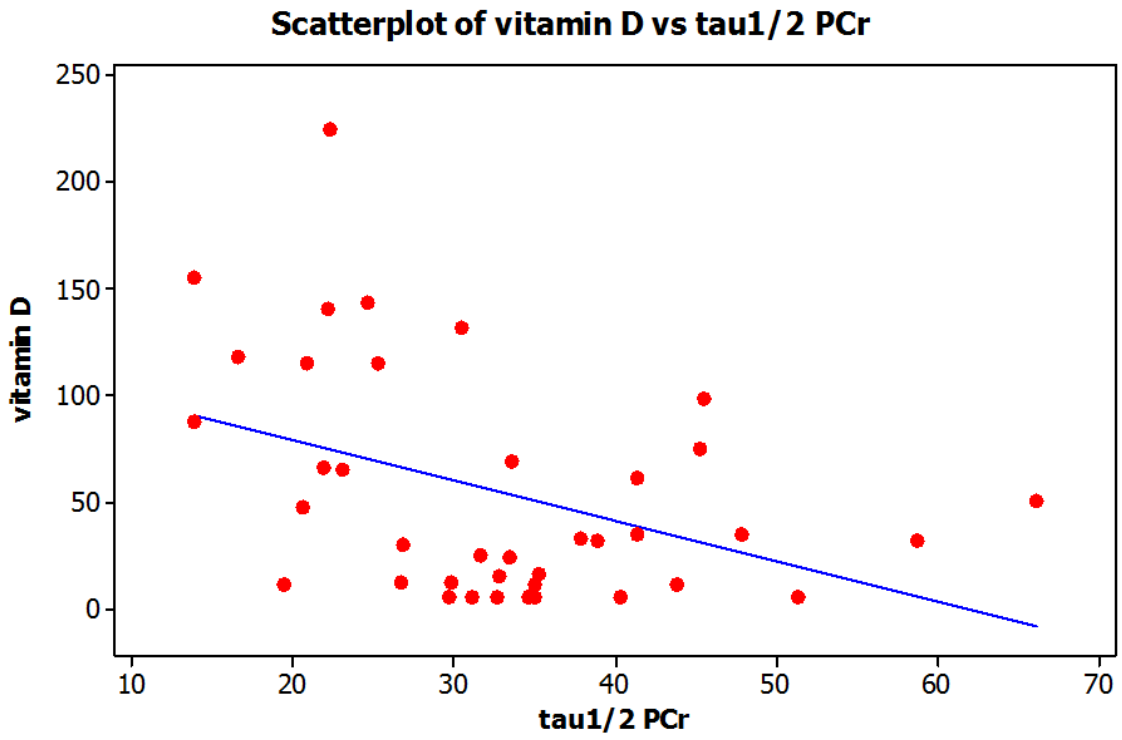

Figure 36 A regression model built by pooling data from serum 25OHD and $\tau_{1/2}$PCr of all the participants.

This model shows that lower vitamin D is associated with lower maximal mitochondrial oxidative phosphorylation. This model includes data from both pre and post vitamin D treatment subjects and healthy controls. (r=-0.41, r^2 adj=14.67%, p=0.009)

3.4 Conclusions

The maximal mitochondrial oxidative phosphorylation rate was enhanced ($\tau_{1/2}$PCr and $\tau_{1/2}$ADP recovery times were reduced) following cholecalciferol therapy in severely vitamin D deficient, symptomatic individuals. Correcting vitamin D deficiency was associated with an improvement in symptoms of myopathy and fatigue in all participants. There was no evidence of abnormal resting metabolic parameters often found in conditions with altered membrane permeability when compared with healthy controls (Kemp and Radda, 1994).

The relationship between vitamin D and muscle has been a subject of much interest recently with 363 articles being published on this topic in PubMed from Jan 2011-Feb 2013 (search terms: vitamin D +muscle). The present body of evidence suggests that treatment with vitamin D does not necessarily lead to an improvement in muscle strength in otherwise healthy vitamin D deficient adults (mean serum 25OHD 25nmol/L) (Gallagher, 2012). A recent meta-analysis concluded that vitamin D supplementation did not result in an improvement in muscle strength in vitamin D replete adults although there was evidence to suggest an increase in muscle strength in adults who had 25OHD ≤25nmol/L pre-treatment (Stockton *et al.*, 2011). A randomised controlled trial (RCT) investigated muscle strength and resting skeletal muscle energy metabolism in young (mean age 31.5yrs) vitamin D deficient Asian Indians (mean serum 25OHD 25nmol/L) and found improvements in muscle strength but no changes in resting skeletal muscle energy metabolism (Gupta *et al.*, 2010). However, the authors did not acquire dynamic phosphorus data whilst exercising and hence did not compute oxidative phosphorylation capacity ($\tau_{1/2}$PCr and $\tau_{1/2}$ADP). Besides the studied patient groups were neither symptomatic nor severely vitamin D deficient since they were apparently healthy volunteers. However, when the same group conducted a double-blinded, placebo-controlled RCT to assess the effect of vitamin D and/or calcium on muscle strength in young, asymptomatic Asian Indian females (mean age 21.7yrs) with vitamin D deficiency (mean serum 25OHD 23.3nmol/L) they found no improvements in skeletal muscle strength (Goswami *et al.*, 2012). This unexpected observation was surprising especially since the authors had used similar methods for the assessment of skeletal muscle strength and duration of supplementation, thus, minimising confounding variables.

Although the precise reasons for this discrepancy remain unclear, there could be several explanations. Firstly, as suggested by J.C. Gallagher in a recent editorial in the Journal of Clinical Endocrinology and Metabolism, improvements in 25OHD levels may not translate to improvements in 1,25OHD levels, the biologically active metabolite of vitamin D (Gallagher, 2012). 1,25OHD modulates calcium entry into the muscle (by its direct non-genomic action) and maybe important for muscle strength. However, Goswami *et al.* noted a similar increase in the serum 1,25OHD levels (7.1% in the double-supplementation group and 9.2% in the cholecalciferol/placebo group) as did Bischoff *et al.* when they reported a significant reduction in the frequency of falls after 12 weeks of calcium and vitamin D supplementation in elderly women (8% increase in 1,25OHD levels) (Bischoff *et al.*, 2003). Evidence for a possible direct action of 25OHD was also demonstrated by Birge *et al.* in 1975. They showed that administration of vitamin D to vitamin D deficient, phosphate depleted rats resulted in a significant increase in *in vitro* concentrations of phosphate metabolites in muscle cells followed by the stimulation of phosphate dependent metabolic processes including ATP synthesis in these cells. This effect was not reproduced by repletion of phosphates in the rats. The abolition of 1α hydroxylation following nephrectomy had no effect on 25OHD mediated phosphate uptake, thereby confirming a direct role of 25OHD in ATP synthesis (Birge and Haddad, 1975). Secondly, gender specific differences may account for the discrepancy in findings. Whereas Gupta *et al.* recruited young males and females, Goswami *et al*. recruited only young postmenarchal females. Vitamin D levels have been shown to be positively associated with testosterone levels (Nimptsch *et al.*, 2012) and therefore, vitamin D supplementation in adult males may lead to improvements in testosterone levels which subsequently may result in enhanced muscle strength.

The broad evidence is generally in favour of a role of vitamin D in the development and function of skeletal muscle (discussed in subchapter Vitamin D and skeletal muscle function). The strongest evidence comes from studies that report distinct morphological changes in the muscle of vitamin D deficient subject, those that describe impairments in the muscle function of the VDRKO mouse and molecular *in vitro* studies that have mapped out the various intracellular responses of cultured muscles to vitamin D (Sorensen *et al.*, 1979; Kalueff *et al.*, 2004; Boland, 2011). This is also corroborated by clinical studies where clinicians have reported resolution of myopathy and myalgia in

vitamin D deficient adults with vitamin D therapy (Plotnikoff and Quigley, 2003; Reginato and Coquia, 2003; Mouyis *et al.*, 2008; Badsha *et al.*, 2009).

In a broader context, the assessment of muscle function entails the measurement of strength as well as of efficiency. Exercise efficiency represents the chemical conversion of energy from nutrient to mechanical work and is a key factor in exercise performance. Using ^{31}P MRS to study the metabolic work-cost relationship of the vitamin D deficient muscle, I have demonstrated an improvement in skeletal muscle metabolism or efficiency with calciferol therapy.

Although my data did not show overt abnormalities of mitochondrial oxidative function in the vitamin D deficient subjects when compared to similar aged healthy controls, cholecalciferol therapy resulted in significant enhancement of mitochondrial maximal oxidative phosphorylation. It is also important to highlight that the vitamin D deficient subjects were assessed using 35% MVC whereas the healthy controls were assessed using 25% MVC. Importantly, however, both groups demonstrated similar %PCr depletion and end-exercise pH. The wide range of mitochondrial function in the healthy control data reflects the wide biological variability in mitochondrial function. $\tau_{1/2}$PCr is a widely accepted measure of maximal mitochondrial capacity. It is a function that reflects a composite of mechanisms including mitochondrial number, oxidative enzyme content, mitochondrial components and vascular supply of substrates and oxygen (Kemp *et al.*, 1993). Perturbations in any or all of the above mechanisms may lead to suboptimal mitochondrial oxidative function. Lower $\tau_{1/2}$PCr represents enhanced maximal mitochondrial oxidative phosphorylation. Long-term endurance training and ageing have been reported to positively and negatively modulate mitochondrial function respectively (McCully *et al.*, 1989; Conley *et al.*, 2000). A linear regression model confirmed the relationship between vitamin D and mitochondrial function. As vitamin D levels fall, mitochondrial recovery kinetics increase.

The precise basis for the muscle symptoms observed in vitamin D deficiency has been unclear until now. The described study indicates that some of these symptoms maybe mediated by the mitochondria in the skeletal muscle. Mitochondria play an important role in the cellular processes via regulation of intracellular Ca^{2+} concentrations (Ca^{2+}). Hormone responsive elements (HRE) are located at various sites within the mitochondria genome which in turn can upregulate cellular energy production through genomic and

non-genomic mechanisms (Psarra *et al.*, 2006). Mitochondria in skeletal muscle fibers can take up cytoplasmic Ca^{2+}released from the sarcoplasmic reticulum during twitch and tetanic responses (Rudolf *et al.*, 2004). Such Ca^{2+} uptake may stimulate mitochondrial ATP production, although it may not be essential (Bruton *et al.*, 2003). Experiments on vitamin D deficient chick muscles demonstrated alterations in oxidative phosphorylation and an inability of muscle mitochondria to retain Ca^{2+} (Mukherjee *et al.*, 1981). An established link between cytosolic and mitochondrial Ca^{2+} concentrations, identification of transport mechanisms, and the proximity of mitochondria to Ca^{2+} release sites supports the notion that Ca^{2+} can be an important signaling molecule in the energy metabolism interplay of the cytosol with the mitochondria (Figure 37) (Glancy and Balaban, 2012). Vitamin D through stimulation of the vitamin D receptor (VDR) may play a significant role in Ca^{2+} uptake by the mitochondria which in turn are involved in the orchestration of cellular metabolic homeostasis such as oxidative phosphorylation, cytosolic calcium signaling and apoptosis. Further understanding of this molecular pathway may also help to improve our understanding of the putative role of vitamin D in several extraskeletal diseases.

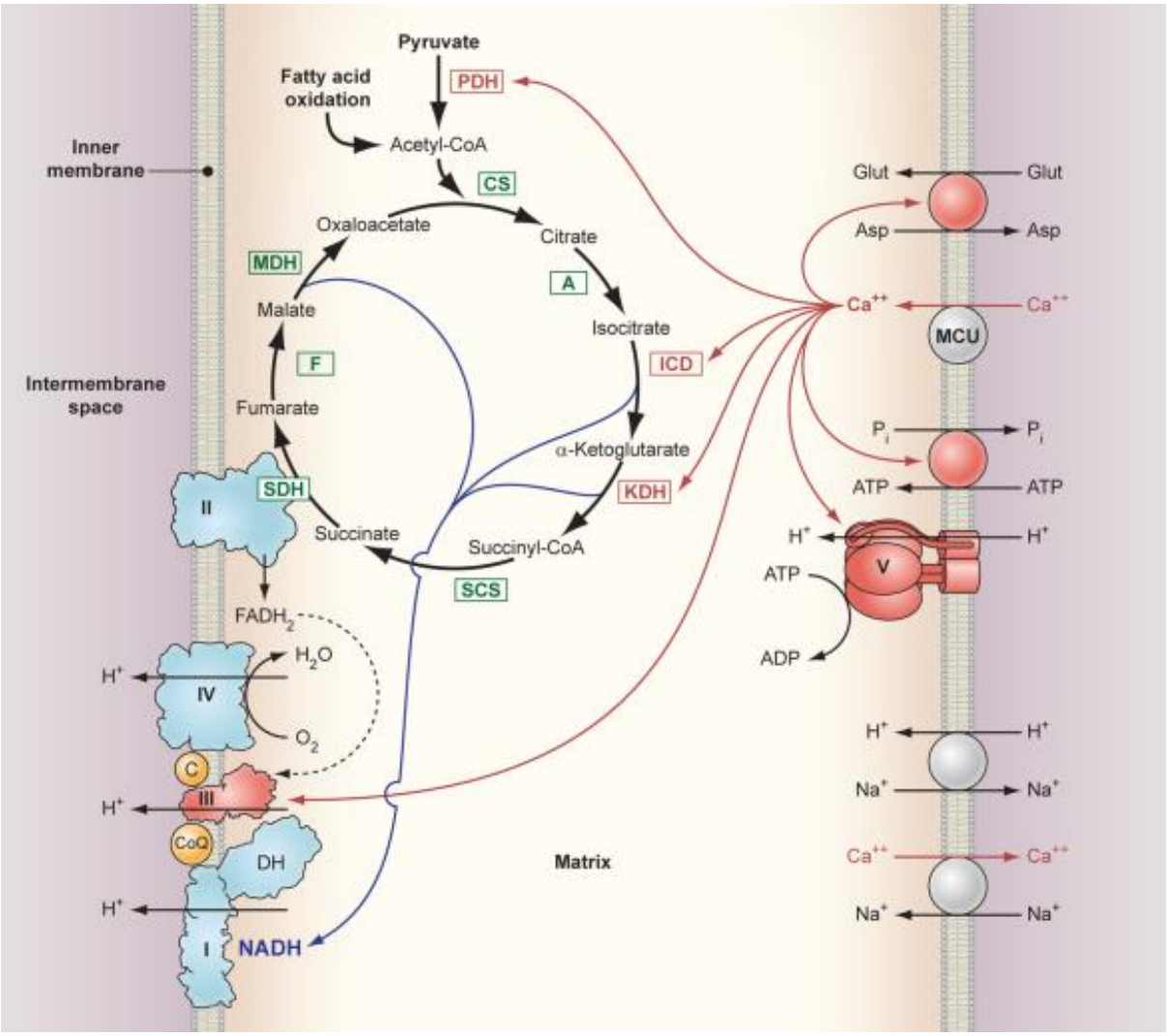

Figure 37 Schematic diagram of the interaction of matrix Ca^{2+} with the processes involved in oxidative phosphorylation

Enzyme abbreviations: PDH, pyruvate dehydrogenase; CS, citrate synthase; A, aconitase; ICD, isocitrate dehydrogenase; KDH, α-ketoglutarate dehydrogenase; SCS, succinyl- CoA synthetase; SDH, succinate dehydrogenase (also Complex II); F, fumarase; MDH, malate dehydrogenase; MCU, mitochondria calcium uniporter. The complexes of oxidative phosphorylation are labelled with Roman numerals (I–V). The DH on Complex 1 refers to the intrinsic and possibly associated NADH dehydrogenase activity. The red arrows from Ca2+ to the different interaction sites imply either a direct or indirect modulation of transport or enzymatic activity (Glancy and Balaban, 2012).

3.4.1 Study Strengths

Mitochondrial oxidative phosphorylation measurement using ^{31}P MRS demonstrates low intra-individual variability with biological variability accounting for the vast majority of inter-individual variability (Layec *et al.*, 2009). An advantage of the study design lies in its ability to control for confounding variables. Earlier pilot work in our unit demonstrating an intra-individual CV of 9% in healthy controls is pertinent when interpreting the study findings. It is also important to highlight the fact that an 8 week study of increased physical activity in 10 healthy controls at our centre did not alter mitochondrial function as measured by $\tau_{1/2}$PCr (Trenell *et al.*, 2008). Moreover, our test-retest reproducibility is consistent with another centre that has cross-validated ^{31}P MRS by comparing oxidative capacity with high-resolution mitochondrial respirometry isolated from muscle biopsy tissue (Lanza *et al.*, 2011).

There are two major differences in my study compared to the recent experiments assessing the effects of vitamin D on muscle strength. All my subjects were symptomatic and presented to their General Practitioner with myalgia and myopathy. Secondly, to the best of my knowledge, no group has characterised skeletal muscle oxidative metabolism in vitamin D deficient adults using dynamic ^{31}P MRS.

3.4.2 Study Limitations

There are limitations to this study: the number of subjects studied was relatively small; the study was not placebo-controlled; and neither patient nor investigators (myself) were blinded during treatment. Comprehensive fatigue questionnaires were deliberately not used because of their low specificity to vitamin D deficiency, their intrusive nature and issues of language translation e.g. the Fatigue impact score (FIS) is constructed of items that require a graded response such as ‘I engage in less sexual activity’ and ‘I worry about how I look to other people’.

It also remains unknown whether vitamin D supplementation to asymptomatic individuals would result in enhancements in mitochondrial oxidative phosphorylation and there is much to learn about the precise relationship between vitamin D status and mitochondrial function. However, the presented data offers clear proof of principle that ^{31}P MRS is a valuable platform for future studies in this area.

3.4.3 Summary

In conclusion, these data show that cholecalciferol therapy in symptomatic, vitamin D deficient individuals results in improved skeletal muscle mitochondrial oxidative function as measured by ^{31}P MRS.

Chapter 4 Discussion

4.1 Summary

^{31}P MRS has emerged as a powerful technique for measuring skeletal muscle metabolism non-invasively and dynamically. It provides novel insights into the phosphate dependent metabolic processes (such as ATP synthesis) occurring within the skeletal muscle. Apart from quantifying oxidative muscle metabolism, it can measure cytosolic pH and thereby provide useful information about anaerobic metabolism within skeletal tissue. In other words ^{31}P MRS informs us of the metabolic work-cost relationship of the skeletal muscle for a given activity. Since skeletal muscles possess the distinctive ability to increase metabolic rate nearly 100-fold during the transition from resting basal state to near maximal contractile activity, it serves as an ideal candidate to interrogate. ^{31}P MRS has improved our understanding in muscle pathology in various diseases. The fact that the skeletal muscle is the largest effector organ for several hormones explains why endocrine myopathies are commonly observed in several endocrine disorders.

I have used ^{31}P MRS to examine skeletal muscle metabolism in two diverse endocrine conditions, namely growth hormone deficiency and vitamin D deficiency using two separate designs. The first study investigated skeletal muscle metabolism in GHD adults in a cross-sectional study design. It determined that untreated GHD adults do not express an abnormal bioenergetic 'footprint' in their skeletal muscle metabolism when compared to age and sex matched GH replaced GHD adults and healthy controls despite an enhanced perception of fatigue and impaired QoL in the untreated GHD group. This may indirectly suggest that the fatigue perceived by GHD patients may have a central component.

The second study investigated the effect of vitamin D therapy on skeletal muscle metabolism in vitamin D deficient adults using ^{31}P MRS in a longitudinal design. The results showed that oxidative mitochondrial function was enhanced with calciferol therapy in vitamin D deficient, symptomatic patients. This suggests that the fatigue experienced by these patients could be due to suboptimal oxidative phosphorylation in the mitochondria. Hence, I have established a link between vitamin D and mitochondrial function in humans using ^{31}P MRS. This work serves as a proof of concept study for

future work to further delineate the precise relationship between vitamin and muscle function.

Finally, given that the two studies were designed differently, one must be careful not to interpret the positive results of the vitamin D study as confirming the lack of effect seen in the GHD study.

4.2 The future

4.2.1 GH study

There are two implications of my work. Firstly, I attempted to examine the relationship between GH and skeletal muscle metabolism. Secondly, in a wider clinical context, I attempted to understand the aetiology of fatigue in GHD.

I have established that adults with GHD do not demonstrate any overt abnormalities in peripheral skeletal tissue metabolism. On the other hand they report increased fatigue compared to matched GH treated GHD adults and healthy controls, which therefore suggests a central determinant of fatigue.

However, several outstanding questions remain unanswered. Are there any short term subtler effects of GH on skeletal muscle metabolism which my study design could not detect? Could the underlying cause of GHD (survivors of cancer) and its resultant treatment (radiotherapy, chemotherapy) play a larger role in the aetiology of fatigue rather than GH *per se*? If central fatigue is an important cause of fatigue, what is the precise pathway by which GH/IGF-1 acts on the central nervous system? With the recent identification of a potential cellular mechanism for cellular fatigue (serotonin spillover), what implication does it have for GHD patients? (Florence Cotel, 2013). Do other hormones such as cortisol modulate central fatigue in GHD adults?

Future studies should be designed to address these unanswered questions. A trial of the effects of GH on peripheral muscle metabolism and cerebral metabolism in isolated GHD adults would help to answer many of the above questions. Apart from using ^{31}P MRS to study skeletal muscle metabolism, the use of functional MRI would shed more light on neural metabolism. This technique is based on undertaking specific tasks of executive memory which activate the brain. This increases cerebral blood flow because neural activity is enhanced. The results would be correlated with AGHDA-QoL scores to map any changes in neural metabolism and patient-reported benefits.

4.2.2 Vitamin D study

Using ^{31}P MRS, I have measured the metabolic work-cost relationship of the vitamin D deficient skeletal muscle and provided novel insights into the relationship between vitamin D and muscle exercise efficiency.

My study reports an enhancement in skeletal muscle mitochondrial oxidative function with vitamin D therapy in symptomatic vitamin D deficient individuals. This finding is corroborated by *in vitro* experiments on vitamin D deficient chick muscle demonstrating alterations in oxidative phosphorylation and an inability of muscle mitochondria to retain Ca^{2+} (Mukherjee *et al.*, 1981).

However, there are many unanswered questions. The precise mechanisms by which vitamin D modulates skeletal tissue mitochondrial function in human remains to be elucidated. Because of their central role in the regulation of energy transduction, mitochondria, the major site of oxidative processes within the cell, are considered likely subcellular targets for the action of steroid hormones. There is scope to clarify the molecular pathway by which vitamin D, a steroid hormone, affects the mitochondria. It also remains unclear whether this is modulated by 25OHD or its biologically active metabolite 1,25OHD. The debate continues regarding the true *in vivo* presence of VDR in skeletal tissue. It also remains uncertain whether there is any gender dimorphic effect of vitamin D on muscle strength.

An essential objective of any future study would be to attempt to connect the non-invasive measurements of skeletal muscle metabolism (using ^{31}P MRS) with true physical characteristics of the skeletal tissue (using biopsies) to provide a clear understanding of the measurements capability and enable accurate interpretation. These findings would be mapped to muscle function tests. In essence one would attempt to translate improved metabolic responses in the tissue to improved functional outcomes.

There is also considerable debate as to what constitutes optimum serum 25OHD levels. This partly stems from the absence of functional markers to corroborate serum 25OHD levels. ^{31}P MRS, therefore, offers the possibility to investigate oxidative mitochondrial function in the skeletal muscle in a dose dependent manner. This could be investigated simultaneously with *ex vivo* analyses of mitochondrial quality, quantity, and function using muscle biopsy samples. This would provide evidence regarding the direct mechanistic role of vitamin D on muscle mitochondrial function. Simultaneous

measurements of 1,25OHD should also be undertaken to verify its precise role. However, its short circulating half-life (~4hrs) may limit its reproducibility. The study should be powered to examine for any gender dimorphic effect since testosterone may potentiate the action of vitamin D on muscle strength. Mitochondria are not only critical for supplying 95% of the energy used by the cell through oxidative phosphorylation but also for their role in induction of cell death through apoptosis. These functions are mediated by intramitochondrial Ca^{2+} concentrations. Hence further understanding of the link between vitamin D and intramitochondrial Ca^{2+} may help us understand the purported role of vitamin D in several extraskeletal diseases as well.

Appendix

Appendix A AGHDA-QoL questionnaire *128*

Appendix B IPAQ physical activity questionnaire *129*

Appendix C Patient Information Sheet-GHD *130*

Appendix D Healthy control-GHD *135*

Appendix E Patient Information Sheet-Vitamin D *140*

Appendix F Consent form *145*

Appendix G Untreated GHD patient details *147*

Appendix H Treated GHD patient details *150*

Appendix A AGHDA-QoL questionnaire

(http://imperialendo.co.uk/AGHDA.pdf)

Appendix B IPAQ physical activity questionnaire

(http://www.ipaq.ki.se)

Appendix C Patient Information Sheet-GHD

REC reference number: 10/H0907/71

Committee; Newcastle and North Tyneside REC 1

Protocol version 4.3, 25/11/10

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Department of Endocrinology
Royal Victoria Infirmary
Newcastle upon Tyne NE1 4LP

Newcastle University

A study using Magnetic Resonance Spectroscopy (MRS) to assess changes in skeletal muscle in patients with growth hormone deficiency (GHD)

Patient Information Sheet

Why have I been chosen?

We are inviting you to take part in a research study because you have growth hormone deficiency (GHD). Before you accept or decline the invitation, it is important for you to understand why the research is being done and what it will involve. Please read the following information and feel free to discuss it with relatives, friends and your General Practitioner, if you wish. If there is anything that is not clear, or you have any further questions, please ask us (our contact details are at the end of this information sheet).

Take time to decide whether you would like to take part, or not. The study will not have any direct benefit to you, but will provide important information about the effects of growth hormone replacement therapy.

Who is taking part in the study?

We are asking patients with a diagnosis of growth hormone deficiency (GHD) to take part in this study. This includes patients with GHD who are on growth hormone replacement therapy as well as patients with GHD who are not on growth hormone therapy at the moment. Most patients will be asked to undergo one scan but some younger patients who have been on GH therapy until recently and who are thinking about whether to stay on GH may be asked to undergo a scan on 2 occasions.

What is the purpose of the study?

The aim of the study is to find out what happens to muscle when the levels of growth hormone are low. We will do this by taking a small sample of blood following an overnight fast (around 2 teaspoons in amount) and then by looking at the energy changes that take place within the muscle during a period of gentle foot exercise. The technique that is used to measure how the muscles are working is called Magnetic Resonance Spectroscopy (MRS). MRS allows doctors to obtain biochemical information about the tissues of the human body in a non-invasive way (without the need for a biopsy and without the need for X-rays).

The MRS scan involves lying in a special Magnetic Resonance Imaging (MRI) Scanner and then flexing and extending your ankle whilst measurements are taken. The procedure is not painful and the movement required is like depressing a clutch pedal on the car. You will be required to do this for 15-20 minutes.

What is a Magnetic Resonance Spectroscopy (MRS) scan?

MRS is a special technique that uses magnets, radio waves and computers to produce detailed images (or scans) of the inside of your body. MRS does not use X-rays and doesn’t cause pain or discomfort.

The scanning session takes approximately two hours and you will be need to lie on your back on a moveable table, which slides inside the cylinder shaped scanner. The scanner is open ended and as we are only scanning your leg, you will not be completely enclosed at any time.

A radiographer will operate the scanner from behind a window but will be able to hear you and see you during the scan. You will be given a call button to hold during the scan, which you can press to get the radiographer's attention, should you need to.

It can take several minutes for each image to be taken, and it is important to lie still and breath gently during the process. The machine is noisy and will make a loud knocking or buzzing sound throughout the scan. However, we will provide you with earplugs or headphones which will help block a lot of the sound out.

When the scans are complete, the table will be removed from the scanner, whilst you are on the table and then you will be able to get off once safely out. There will be someone to help you off the table if required.

What happens now?

If you are happy to participate in the study, we will ask you to sign a consent form indicating your willingness to take part. We will also arrange a time for you to come for your scan. The scanning session will take approximately two hours.

You will be able to eat and drink as usual and will not need to take any special precautions.

Are there any side effects to taking part in the study?

MRI scans are commonly performed and generally safe. However you need to be aware of the possible side-effects.

Some people are slightly claustrophobic in the scanner, however with the scan of your leg you will not be completely enclosed. The radiowaves used in the MRI can also cause metal and tissues to heat up and move and so you will need to inform us if you have any of the following:

Surgical clips,
A previous history of metal fragments in your eyes.
Any pacemakers or heart defibrillators,
Inner ear implant (a hearing aid),
Medicine infusion pump (insulin pump),
Neurostimulator,
Aneurysm clip (a metal clip on an artery),
Shunts (tubes) in the brain,
Joint replacements/large metal implants,
Stents (tubes) in the heart or arteries,
Eye, penis or breast implants,
An intrauterine contraceptive device or coil,
Any allergies.

You will also need to inform us if you have any body piercing, tattoos or transdermal patches, metal fragments anywhere in your body or gun/shrapnel wounds.

If you are pregnant or possibly pregnant please let us know, as you will not be able to be included in the study.

You may find that teeth fillings may tingle during the scan but they are safe.

Please note that there will be someone to help you onto and off the table if needed.

Since Magnetic Resonance Spectroscopy is primarily a research tool, we do not expect to detect any gross abnormalities. However, in the unlikely case that we detect any gross abnormalities, we shall discuss the findings with the relevant clinicians and contact you accordingly.

Confidentiality

All data collected will be anonymised – you will not be able to be identified from the information we collect. The results of the study are likely to be published in a medical journal, but again, you will not be able to be identified from this information.

Do I have to agree to take part in the study?

You do not have to agree to take part in this study. If you do not wish to take part, your future care will not be affected in any way. You may also wish to change your mind and withdraw from the study at any time. Again this will not affect your care in any way. If you choose to withdraw from the study, we will not use any of the data already collected on you.

If you have any further questions or concerns, please do not hesitate to telephone Dr Tim Cheetham on 0191 282 9562 or Dr Steve Ball on 0191 282 9094 or you can write to them:

Tim Cheetham (Department of Paediatric Endocrinology) or Dr Steve Ball (Department of Endocrinology), Royal Victoria Infirmary, Newcastle upon Tyne
NE1 4LP.

Thank you for reading this information sheet.

Appendix D Healthy control-GHD

REC reference number: 10/H0907/71

Committee; Newcastle and North Tyneside REC 2

Protocol version 4.3, 25/11/10

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Institute of Human Genetics
International Centre for Life
Central Parkway
Newcastle upon Tyne NE1 3BZ

Newcastle University

A study using Magnetic Resonance Spectroscopy (MRS) to assess changes in the skeletal muscle in patients with Growth hormone deficiency

Volunteer Information Sheet

Why have I been chosen?

As a healthy person we are inviting you to take part in a research study. Before you accept or decline the invitation, it is important for you to understand why the research is being done and what it will involve. Please read the following information and discuss it with relatives, friends and your General Practitioner, if you wish. If there is anything that is not clear, or you have any further questions, please ask us (our contact details are at the end of this information sheet). Take time to decide whether you would like to take

part, or not. This study will have no direct benefit to you, but will provide important information for possible future therapeutic trials.

Who is taking part in the study?

We are asking healthy volunteers as well as patients with growth hormone deficiency (GHD) to take part in this study.

What is the purpose of the study?

The aim of the study is to find out what happens to muscle and the way that muscles work when growth hormone levels are low. To do this we will be studying people who make growth hormone normally (healthy volunteers like you) as well as people who are unable to make growth hormone normally. We plan to take a small sample of blood at the start of the study in the morning following an overnight fast (around 2 teaspoons in amount). We will then look at the energy changes that take place within the muscle during exercise. The technique that is used to measure how the muscle is working is called Magnetic Resonance Spectroscopy (MRS). MRS allows doctors to obtain biochemical information about the tissues of the human body in a non-invasive way (without the need for a biopsy and without the need for X-rays). The MRS scan involves lying in a special Magnetic Resonance Imaging (MRI) Scanner and then flexing and extending your ankle whilst measurements are taken. The procedure is not painful and the movement required is like depressing a clutch pedal on the car. You will be required to do this for 15-25 minutes.

What is a Magnetic Resonance Imaging (MRI) scan?

MRI is a special technique that uses powerful magnets, radio waves and computers to produce detailed images (or scans) of the inside of your body. MRI does not use X-rays and doesn't cause pain or discomfort.

The scanning session takes approximately two hours and you will be need to lie on your back on a moveable table, which slides inside the cylinder shaped scanner. The scanner is open ended and as we are only scanning your leg, you will not be completely enclosed at any time.

A radiographer will operate the scanner from behind a window but will be able to hear you and see you during the scan. You will be given a call button to hold during the scan, which you can press to get the radiographer's attention, should you need to.

It can take several minutes for each image to be taken, and it is important to lie still and breath gently during the process. The machine is noisy and will make a loud knocking or buzzing sound throughout the scan. However, we will provide you with earplugs or headphones which will help block a lot of the sound out.

When the scans are complete, the table will be removed from the scanner, whilst you are on the table and then you will be able to get off once safely out. There will be someone to help you off the table if required.

What happens now?

If you are happy to participate in the study, we will ask you to sign a consent form indicating your willingness to take part. We will also arrange a time for you to come for your scan. The scanning session will take approximately two hours.

You will be able to eat and drink as usual and will not need to take any special precautions.

Are there any side effects to taking part in the study?

MRI scans are commonly performed and generally safe. However you need to be aware of the possible side-effects.

Some people are slightly claustrophobic in the scanner; however with the scan of your leg you will not be completely enclosed. The radiowaves used in the MRI can cause metal and tissues to heat up, you will therefore need to inform us if you have any of the following:

Surgical clips,

A previous history of metal fragments in your eyes.

Any pacemakers or heart defibrillators,

Inner ear implant (a hearing aid),
Medicine infusion pump (insulin pump),
Neurostimulator,
Aneurysm clip (a metal clip on an artery),
Shunts (tubes) in the brain,
Joint replacements/large metal implants,
Stents (tubes) in the heart or arteries,
Eye, penis or breast implants,
An intrauterine contraceptive device or coil,
Any allergies.

You will also need to inform us if you have any body piercing, tattoos or transdermal patches, metal fragments anywhere in your body or gun/shrapnel wounds.

If you are pregnant or possibly pregnant please let us know, as you will not be able to be included in the study.

You may find that teeth fillings may tingle during the scan but are safe.

Please note that there will be someone to help you onto and off the table if needed.

Since Magnetic Resonance Spectroscopy is primarily a research tool, we do not expect to detect any gross abnormalities. However, in the unlikely case that we detect any gross abnormalities, we shall discuss the findings with the relevant clinicians and contact you accordingly.

Confidentiality

All data collected will be anonymised – you will not be able to be identified from the information we collect. The results of the study are likely to be published, but again, you will not be able to be identified from this information.

Do I have to agree to take part in the study?

You do not have to agree to take part in this study. If you do not wish to take part, your future care will not be affected in any way. You may also wish to change your mind and withdraw from the study at any time. Again this will not affect your care in any way. If you choose to withdraw from the study, we will not use any of the data already collected on you.

If you have any further questions or concerns, please do not hesitate to telephone Dr Tim Cheetham on 0191 282 9562 or Dr Steve Ball on 0191 282 9094 or you can write to them:

Tim Cheetham (Department of Paediatric Endocrinology) or Dr Steve Ball (Department of Endocrinology), Royal Victoria Infirmary, Newcastle upon Tyne
NE1 4LP.

Thank you for reading this information sheet.

Appendix E Patient Information Sheet-Vitamin D

REC reference number: 10/H0907/71

Committee; Newcastle and North Tyneside REC 2

Protocol version 4.4, 18/08/11

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Institute of Human Genetics
International Centre for Life
Central Parkway
Newcastle upon Tyne NE1 3BZ

Newcastle University

A study using Magnetic Resonance Spectroscopy (MRS) to assess changes in the skeletal muscle in people with Vitamin D deficiency

Volunteer Information Sheet

Why have I been chosen?

As a healthy person with vitamin D deficiency we are inviting you to take part in a research study. Before you accept or decline the invitation, it is important for you to understand why the research is being done and what it will involve. Please read the following information and discuss it with relatives, friends and your General Practitioner, if you wish. If there is anything that is not clear, or you have any further questions,

please ask us (our contact details are at the end of this information sheet). Take time to decide whether you would like to take part, or not. This study will have no direct benefit to you, but will provide important information for possible future therapeutic trials.

Who is taking part in the study?

We are asking healthy volunteers who have Vitamin D deficiency to take part in this study.

What is the purpose of the study?

The aim of the study is to find out what happens to muscle and the way that muscles work when Vitamin D levels are low. Vitamin D deficiency is widespread and is a public health concern, especially in certain high risk groups such as dark skinned people, the elderly and pregnant mothers. To do this we will be studying people who are Vitamin D deficient. We plan to take a small sample of blood at the start of the study. We will then look at the energy changes that take place within the muscle during exercise. The technique that is used to measure how the muscle is working is called Magnetic Resonance Spectroscopy (MRS). MRS allows doctors to obtain biochemical information about the tissues of the human body in a non-invasive way (without the need for a biopsy and without the need for X-rays). The MRS scan involves lying in a special Magnetic Resonance Imaging (MRI) Scanner and then flexing and extending your ankle whilst measurements are taken. The procedure is not painful and the movement required is like depressing a clutch pedal on the car. You will be required to do this for 15-25 minutes.

We shall then invite you for a repeat MRS scan and a repeat blood test 8-12 weeks later when your vitamin D deficiency has been corrected (as per the local guidelines).

What is a Magnetic Resonance Imaging (MRI) scan?

MRI is a special technique that uses powerful magnets, radio waves and computers to produce detailed images (or scans) of the inside of your body. MRI does not use X-rays and doesn’t cause pain or discomfort.

The scanning session takes approximately two hours and you will be need to lie on your back on a moveable table, which slides inside the cylinder shaped scanner. The scanner is open ended and as we are only scanning your leg, you will not be completely enclosed at any time.

A radiographer will operate the scanner from behind a window but will be able to hear you and see you during the scan. You will be given a call button to hold during the scan, which you can press to get the radiographer's attention, should you need to.

It can take several minutes for each image to be taken, and it is important to lie still and breath gently during the process. The machine is noisy and will make a loud knocking or buzzing sound throughout the scan. However, we will provide you with earplugs or headphones which will help block a lot of the sound out.

When the scans are complete, the table will be removed from the scanner, whilst you are on the table and then you will be able to get off once safely out. There will be someone to help you off the table if required.

What happens now?

If you are happy to participate in the study, we will ask you to sign a consent form indicating your willingness to take part. We will also arrange a time for you to come for your scan. The scanning session will take approximately two hours.

You will be able to eat and drink as usual and will not need to take any special precautions.

Are there any side effects to taking part in the study?

MRI scans are commonly performed and generally safe. However you need to be aware of the possible side-effects.

Some people are slightly claustrophobic in the scanner; however with the scan of your leg you will not be completely enclosed. The radiowaves used in the MRI can cause metal and tissues to heat up, you will therefore need to inform us if you have any of the following;

Surgical clips,
A previous history of metal fragments in your eyes.
Any pacemakers or heart defibrillators,
Inner ear implant (a hearing aid),
Medicine infusion pump (insulin pump),
Neurostimulator,
Aneurysm clip (a metal clip on an artery),
Shunts (tubes) in the brain,
Joint replacements/large metal implants,
Stents (tubes) in the heart or arteries,
Eye, penis or breast implants,
An intrauterine contraceptive device or coil,
Any allergies.

You will also need to inform us if you have any body piercing, tattoos or transdermal patches, metal fragments anywhere in your body or gun/shrapnel wounds.

If you are pregnant or possibly pregnant please let us know, as you will not be able to be included in the study.

You may find that teeth fillings may tingle during the scan but are safe.

Please note that there will be someone to help you onto and off the table if needed.

Since Magnetic Resonance Spectroscopy is primarily a research tool, we do not expect to detect any gross abnormalities. However, in the unlikely case that we detect any gross abnormalities, we shall discuss the findings with the relevant clinicians and contact you accordingly.

Confidentiality

All data collected will be anonymised – you will not be able to be identified from the information we collect. The results of the study are likely to be published, but again, you will not be able to be identified from this information.

Do I have to agree to take part in the study?

You do not have to agree to take part in this study. If you do not wish to take part, your future care will not be affected in any way. You may also wish to change your mind and withdraw from the study at any time. Again this will not affect your care in any way. If you choose to withdraw from the study, we will not use any of the data already collected on you.

If you have any further questions or concerns, please do not hesitate to telephone Dr Tim Cheetham on 0191 282 9562 or Dr Steve Ball on 0191 282 9094 or you can write to them:

Tim Cheetham (Department of Paediatric Endocrinology) or Dr Steve Ball (Department of Endocrinology), Royal Victoria Infirmary, Newcastle upon Tyne
NE1 4LP.

Thank you for reading this information sheet.

Appendix F Consent form

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: MRS assessment of skeletal muscle function in GHD

Name of Researchers: Dr A Sinha, Dr S Ball and Dr T Cheetham

Please initial box

1. I confirm that I have read and understand the information sheet dated 25/11/10 (version 4.3) regarding the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the University of Newcastle or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

[1 for patient; 1 for researcher; 1 to be kept with hospital notes]

Appendix G Untreated GHD patient details

GHD ID no	Age	Sex	Rx	Cx	Sx	Overall Treatment	Thyrotrophs	Corticotrophs	Gonadotrophs	Posterior Pituitary	MPHD	Childhood onset	Hypothalamic	Pituitary
1	23.6	1	1	1	1	Medulloblastoma	1	2	2	2	1	1	1	2
3	29.9	2	2	2	2	Empty sella syndrome	1	1	1	2	1	1	2	1
14	26.6	1	2	2	2	MPHD partial	2	2	1	2	1	1	2	1
18	25.1	2	1	2	1	Craniopharyngioma	1	1	1	1	1	1	1	2
22	49.6	1	1	2	1	NFPT	1	1	1	2	1	2	2	1
24	20.5	1	1	1	2	Nasopharyngeal tumour	2	2	2	2	2	1	2	1
25	17.1	2	1	1	2	Parameningeal rhabdomyosarcoma	2	2	2	2	2	1	2	1
27	17.	1	1	1	1	Medulloblastoma	1	2	2	2	1	1	2	1

	6													
33	17.0	1	1	1	2	Ependymoma	2	2	2	2	2	1	2	1
34	27.2	2	2	2	1	Craniopharyngioma	1	1	1	1	1	1	1	2
35	38.2	1	2	2	1	Craniopharyngioma	1	1	1	2	1	1	2	1
38	45.8	1	1	2	1	Prolactinoma	1	1	1	1	1	2	1	2
40	21.4	1	1	1	1	Medulloblastoma	1	2	2	2	1	1	2	1
41	27.2	2	1	2	1	Medulloblastoma	1	2	2	2	1	1	2	1
46	25.8	2	1	2	1	Germinoma	1	1	1	1	1	2	1	2
47	33.0	2	1	2	1	Astrocytoma	2	2	2	2	2	1	2	1
49	24.6	2	2	2	2	Isolated GHD	2	2	2	2	2	1	2	1
50	21.7	1	1	2	2	Medulloblastoma	1	2	2	2	1	1	2	1
51	33.0	1	1	2	1	Craniopharyngioma	1	1	1	1	1	1	1	2
5	4	2	2	2	1	Cranioph	1	1	1	1	1	2	1	2

2	4.6					aryngioma								
56	23.6	1	1	2	1	NFPT	1	1	2	1	1	1	1	2
57	19.9	1	1	2	2	Germinoma	1	1	1	1	1	1	1	2

1=Yes, 2=No

Rx- radiotherapy

Cx-chemotherapy

Sx-Surgery

MPHD-multiple pituitary hormone deficiency

Appendix H Treated GHD patient details

GHD ID no	Age	Sex	Rx	Cx	Sx	Overall Treatment	Thyrotrophs	Corticotrophs	Gonadotrophs	Posterior Pituitary	MPHD	Childhood onset	Hypothalamic	Pituitary
7	40.3	2	1	2	1	MPHD-NFPT	1	1	1	2	1	2	2	1
8	19.9	1	2	2	2	MPHD-Empty sella syndrome	1	1	1	2	1	1	2	1
9	17.4	2	2	2	2	MPHD-Empty sella syndrome	1	1	1	2	1	1	2	1
13	28.2	2	2	2	1	Craniopharyngioma	1	1	1	1	1	1	1	2
15	40.6	1	1	2	1	optic glioma	1	1	2	2	1	1	2	1
17	25.7	1	2	2	2	Isolated GHD	2	2	2	2	2	1	2	1
19	39.3	1	1	2	1	Prolactinoma	1	1	1	2	1	2	2	1
20	24	1	2	2	2	MPHD-Empty	1	1	2	2	1	1	2	1

	.1					sella syndrome								
23	45.4	1	2	2	2	Prolactinoma	2	2	1	2	1	2	2	1
26	17.4	2	1	1	1	Isolated GHD	2	2	2	2	2	1	2	1
29	17.8	1	*	*	*	Medulloblastoma	1	1	2	2	1	1		
31	21.4	2	2	2	2	trauma	1	2	1	2	1	1	2	1
32	35.4	2	2	2	2	trauma	1	1	1	1	1	1	1	2
36	33.9	2	*	*	*	Craniopharyngioma	1	1	1	1	1			
37	42.1	2	2	2	1	Craniopharyngioma	1	1	1	1	1	2	1	2
39	34.2	1	1	2	2	Dysgerminoma	1	2	2	1	1	1	1	2
42	25.1	1	2	2	2	Prolactinoma	1	2	1	2	1	2	2	1
44	37.3	1	1	2	2	NHL	2	2	2	2	2	2	2	1
45	17.	1	2	2	1	Craniopharyngioma	1	1	1	1	1	1	1	2

	0													
48	39.3	2	2	2	1	Craniopharyngioma	1	1	1	1	1	2	1	2
54	21.6	1	1	2	2	Pineal dysgerminoma	1	1	1	1	1	1	1	2
58	16.2	1	2	2	2	Isolated GHD+thyroxine def	1	2	2	2	1	1	2	1
59	35.6	1	1	2	1	MPHD-NFPT	1	1	1	2	1	2	2	1

1=Yes, 2=No

Rx- radiotherapy

Cx-chemotherapy

Sx-Surgery

MPHD-multiple pituitary hormone deficiency

Appendix I Publication-Improving the vitamin D status of vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle

Appendix J Publication-Prevention and treatment of vitamin D deficiency

References:

Abs, R., Bengtsson, B.A., Hernberg-Stahl, E., Monson, J.P., Tauber, J.P., Wilton, P. and Wuster, C. (1999) 'GH replacement in 1034 growth hormone deficient hypopituitary adults: demographic and clinical characteristics, dosing and safety', *Clin Endocrinol (Oxf)*, 50(6), pp. 703-13.

Abs, R., Feldt-Rasmussen, U., Mattsson, A.F., Monson, J.P., Bengtsson, B.A., Goth, M.I., Wilton, P. and Koltowska-Haggstrom, M. (2006) 'Determinants of cardiovascular risk in 2589 hypopituitary GH-deficient adults - a KIMS database analysis', *Eur J Endocrinol*, 155(1), pp. 79-90.

Agha, A. and Monson, J.P. (2007) 'Modulation of glucocorticoid metabolism by the growth hormone - IGF-1 axis', *Clin Endocrinol (Oxf)*, 66(4), pp. 459-65.

Agha, A., Walker, D., Perry, L., Drake, W.M., Chew, S.L., Jenkins, P.J., Grossman, A.B. and Monson, J.P. (2007) 'Unmasking of central hypothyroidism following growth hormone replacement in adult hypopituitary patients', *Clin Endocrinol (Oxf)*, 66(1), pp. 72-7.

Ahmad, A.M., Hopkins, M.T., Thomas, J., Ibrahim, H., Fraser, W.D. and Vora, J.P. (2001) 'Body composition and quality of life in adults with growth hormone deficiency; effects of low-dose growth hormone replacement', *Clin Endocrinol (Oxf)*, 54(6), pp. 709-17.

Akin, F., Yaylali, G.F., Turgut, S. and Kaptanoglu, B. (2009) 'Growth hormone/insulin-like growth factor axis in patients with subclinical thyroid dysfunction', *Growth Horm IGF Res*, 19(3), pp. 252-5.

Alatzoglou, K.S. and Dattani, M.T. (2009) 'Genetic forms of hypopituitarism and their manifestation in the neonatal period', *Early Hum Dev*, 85(11), pp. 705-12.

Alatzoglou, K.S., Turton, J.P., Kelberman, D., Clayton, P.E., Mehta, A., Buchanan, C., Aylwin, S., Crowne, E.C., Christesen, H.T., Hertel, N.T., Trainer, P.J., Savage, M.O., Raza, J., Banerjee, K., Sinha, S.K., Ten, S., Mushtaq, T., Brauner, R., Cheetham, T.D., Hindmarsh, P.C., Mullis, P.E. and Dattani, M.T. (2009) 'Expanding the spectrum of mutations in GH1 and GHRHR:

genetic screening in a large cohort of patients with congenital isolated growth hormone deficiency', *J Clin Endocrinol Metab*, 94(9), pp. 3191-9.

Allen, D.G., Lamb, G.D. and Westerblad, H. (2008) 'Skeletal muscle fatigue: cellular mechanisms', *Physiol Rev*, 88(1), pp. 287-332.

Aloia, J.F. (2011) 'Clinical Review: The 2011 report on dietary reference intake for vitamin D: where do we go from here?', *J Clin Endocrinol Metab*, 96(10), pp. 2987-96.

Amato, G., Carella, C., Fazio, S., La Montagna, G., Cittadini, A., Sabatini, D., Marciano-Mone, C., Sacca, L. and Bellastella, A. (1993) 'Body composition, bone metabolism, and heart structure and function in growth hormone (GH)-deficient adults before and after GH replacement therapy at low doses', *J Clin Endocrinol Metab*, 77(6), pp. 1671-6.

Annweiler, C., Schott, A.M., Berrut, G., Fantino, B. and Beauchet, O. (2009) 'Vitamin D-related changes in physical performance: a systematic review', *J Nutr Health Aging*, 13(10), pp. 893-8.

Argov, Z., Renshaw, P.F., Boden, B., Winokur, A. and Bank, W.J. (1988) 'Effects of thyroid hormones on skeletal muscle bioenergetics. In vivo phosphorus-31 magnetic resonance spectroscopy study of humans and rats', *J Clin Invest*, 81(6), pp. 1695-701.

Arwert, L.I., Deijen, J.B., Muller, M. and Drent, M.L. (2005a) 'Long-term growth hormone treatment preserves GH-induced memory and mood improvements: a 10-year follow-up study in GH-deficient adult men', *Horm Behav*, 47(3), pp. 343-9.

Arwert, L.I., Deijen, J.B., Witlox, J. and Drent, M.L. (2005b) 'The influence of growth hormone (GH) substitution on patient-reported outcomes and cognitive functions in GH-deficient patients: a meta-analysis', *Growth Horm IGF Res*, 15(1), pp. 47-54.

Arwert, L.I., Veltman, D.J., Deijen, J.B., van Dam, P.S., Delemarre-van deWaal, H.A. and Drent, M.L. (2005c) 'Growth hormone deficiency and memory functioning in adults visualized by functional magnetic resonance imaging', *Neuroendocrinology*, 82(1), pp. 32-40.

Arwert, L.I., Veltman, D.J., Deijen, J.B., van Dam, P.S. and Drent, M.L. (2006) 'Effects of growth hormone substitution therapy on cognitive functioning in growth hormone deficient patients: a functional MRI study', *Neuroendocrinology*, 83(1), pp. 12-9.

Attanasio, A.F., Bates, P.C., Ho, K.K., Webb, S.M., Ross, R.J., Strasburger, C.J., Bouillon, R., Crowe, B., Selander, K., Valle, D. and Lamberts, S.W. (2002a) 'Human growth hormone replacement in adult hypopituitary patients: long-term effects on body composition and lipid status--3-year results from the HypoCCS Database', *J Clin Endocrinol Metab*, 87(4), pp. 1600-6.

Attanasio, A.F., Howell, S., Bates, P.C., Frewer, P., Chipman, J., Blum, W.F. and Shalet, S.M. (2002b) 'Body composition, IGF-I and IGFBP-3 concentrations as outcome measures in severely GH-deficient (GHD) patients after childhood GH treatment: a comparison with adult onset GHD patients', *J Clin Endocrinol Metab*, 87(7), pp. 3368-72.

Attanasio, A.F., Lamberts, S.W., Matranga, A.M., Birkett, M.A., Bates, P.C., Valk, N.K., Hilsted, J., Bengtsson, B.A. and Strasburger, C.J. (1997) 'Adult growth hormone (GH)-deficient patients demonstrate heterogeneity between childhood onset and adult onset before and during human GH treatment. Adult Growth Hormone Deficiency Study Group', *J Clin Endocrinol Metab*, 82(1), pp. 82-8.

Badsha, H., Daher, M. and Ooi Kong, K. (2009) 'Myalgias or non-specific muscle pain in Arab or Indo-Pakistani patients may indicate vitamin D deficiency', *Clin Rheumatol*, 28(8), pp. 971-3.

Bangsbo, J., Johansen, L., Quistorff, B. and Saltin, B. (1993) 'NMR and analytic biochemical evaluation of CrP and nucleotides in the human calf during muscle contraction', *J Appl Physiol*, 74(4), pp. 2034-9.

Bangsbo, J., Madsen, K., Kiens, B. and Richter, E.A. (1996) 'Effect of muscle acidity on muscle metabolism and fatigue during intense exercise in man', *J Physiol*, 495 (Pt 2), pp. 587-96.

Baum, H.B., Katznelson, L., Sherman, J.C., Biller, B.M., Hayden, D.L., Schoenfeld, D.A., Cannistraro, K.E. and Klibanski, A. (1998) 'Effects of physiological growth hormone (GH)

therapy on cognition and quality of life in patients with adult-onset GH deficiency', *J Clin Endocrinol Metab*, 83(9), pp. 3184-9.

Beguinot, F., Kahn, C.R., Moses, A.C. and Smith, R.J. (1985) 'Distinct biologically active receptors for insulin, insulin-like growth factor I, and insulin-like growth factor II in cultured skeletal muscle cells', *J Biol Chem*, 260(29), pp. 15892-8.

Beshyah, S.A., Freemantle, C., Shahi, M., Anyaoku, V., Merson, S., Lynch, S., Skinner, E., Sharp, P., Foale, R. and Johnston, D.G. (1995a) 'Replacement treatment with biosynthetic human growth hormone in growth hormone-deficient hypopituitary adults', *Clin Endocrinol (Oxf)*, 42(1), pp. 73-84.

Beshyah, S.A., Freemantle, C., Thomas, E., Rutherford, O., Page, B., Murphy, M. and Johnston, D.G. (1995b) 'Abnormal body composition and reduced bone mass in growth hormone deficient hypopituitary adults', *Clin Endocrinol (Oxf)*, 42(2), pp. 179-89.

Bigland-Ritchie, B., Rice, C.L., Garland, S.J. and Walsh, M.L. (1995) 'Task-dependent factors in fatigue of human voluntary contractions', *Adv Exp Med Biol*, 384, pp. 361-80.

Biller, B.M., Samuels, M.H., Zagar, A., Cook, D.M., Arafah, B.M., Bonert, V., Stavrou, S., Kleinberg, D.L., Chipman, J.J. and Hartman, M.L. (2002) 'Sensitivity and specificity of six tests for the diagnosis of adult GH deficiency', *J Clin Endocrinol Metab*, 87(5), pp. 2067-79.

Binzoni, T., Ferretti, G., Schenker, K. and Cerretelli, P. (1992) 'Phosphocreatine hydrolysis by 31P-NMR at the onset of constant-load exercise in humans', *J Appl Physiol*, 73(4), pp. 1644-9.

Birge, S.J. and Haddad, J.G. (1975) '25-hydroxycholecalciferol stimulation of muscle metabolism', *J Clin Invest*, 56(5), pp. 1100-7.

Bischoff-Ferrari, H.A., Shao, A., Dawson-Hughes, B., Hathcock, J., Giovannucci, E. and Willett, W.C. (2010) 'Benefit-risk assessment of vitamin D supplementation', *Osteoporos Int*, 21(7), pp. 1121-32.

Bischoff, H.A., Stahelin, H.B., Dick, W., Akos, R., Knecht, M., Salis, C., Nebiker, M., Theiler, R., Pfeifer, M., Begerow, B., Lew, R.A. and Conzelmann, M. (2003) 'Effects of vitamin D and

calcium supplementation on falls: a randomized controlled trial', *J Bone Miner Res*, 18(2), pp. 343-51.

Bjork, S., Jonsson, B., Westphal, O. and Levin, J.E. (1989) 'Quality of life of adults with growth hormone deficiency: a controlled study', *Acta Paediatr Scand Suppl*, 356, pp. 55-9; discussion 60, 73-4.

Bland, J.M. and Altman, D.G. (1986) 'Statistical methods for assessing agreement between two methods of clinical measurement', *Lancet*, 1(8476), pp. 307-10.

Boger, R.H., Skamira, C., Bode-Boger, S.M., Brabant, G., von zur Muhlen, A. and Frolich, J.C. (1996) 'Nitric oxide may mediate the hemodynamic effects of recombinant growth hormone in patients with acquired growth hormone deficiency. A double-blind, placebo-controlled study', *J Clin Invest*, 98(12), pp. 2706-13.

Boland, R., de Boland, A.R., Marinissen, M.J., Santillan, G., Vazquez, G. and Zanello, S. (1995) 'Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D3', *Mol Cell Endocrinol*, 114(1-2), pp. 1-8.

Boland, R.L. (2011) 'VDR activation of intracellular signaling pathways in skeletal muscle', *Mol Cell Endocrinol*, 347(1-2), pp. 11-6.

Bondy, C.A. and Lee, W.H. (1993) 'Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. Functional implications', *Ann N Y Acad Sci*, 692, pp. 33-43.

Booth, F.W. and Thomason, D.B. (1991) 'Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models', *Physiol Rev*, 71(2), pp. 541-85.

Booth, M.L. (2002). Available at: www.ipaq.ki.se.

Borina, E., Pellegrino, M.A., D'Antona, G. and Bottinelli, R. (2010) 'Myosin and actin content of human skeletal muscle fibers following 35 days bed rest', *Scand J Med Sci Sports*, 20(1), pp. 65-73.

Boska, M. (1994) 'ATP production rates as a function of force level in the human gastrocnemius/soleus using 31P MRS', *Magn Reson Med*, 32(1), pp. 1-10.

Brasitus, T.A. and Dudeja, P.K. (1988) 'Effect of hypothyroidism on the lipid composition and fluidity of rat colonic apical plasma membranes', *Biochim Biophys Acta*, 939(2), pp. 189-96.

Brill, K.T., Weltman, A.L., Gentili, A., Patrie, J.T., Fryburg, D.A., Hanks, J.B., Urban, R.J. and Veldhuis, J.D. (2002) 'Single and combined effects of growth hormone and testosterone administration on measures of body composition, physical performance, mood, sexual function, bone turnover, and muscle gene expression in healthy older men', *J Clin Endocrinol Metab*, 87(12), pp. 5649-57.

Brody, H. and Miller, F.G. (2011) 'Lessons from recent research about the placebo effect--from art to science', *JAMA*, 306(23), pp. 2612-3.

Bruton, J.D., Dahlstedt, A.J., Abbate, F. and Westerblad, H. (2003) 'Mitochondrial function in intact skeletal muscle fibres of creatine kinase deficient mice', *J Physiol*, 552(Pt 2), pp. 393-402.

Burman, P., Broman, J.E., Hetta, J., Wiklund, I., Erfurth, E.M., Hagg, E. and Karlsson, F.A. (1995) 'Quality of life in adults with growth hormone (GH) deficiency: response to treatment with recombinant human GH in a placebo-controlled 21-month trial', *J Clin Endocrinol Metab*, 80(12), pp. 3585-90.

Cantor, F. (2010) 'Central and peripheral fatigue: exemplified by multiple sclerosis and myasthenia gravis', *PM R*, 2(5), pp. 399-405.

Carroll, P.V., Christ, E.R., Bengtsson, B.A., Carlsson, L., Christiansen, J.S., Clemmons, D., Hintz, R., Ho, K., Laron, Z., Sizonenko, P., Sonksen, P.H., Tanaka, T. and Thorne, M. (1998) 'Growth hormone deficiency in adulthood and the effects of growth hormone replacement: a review. Growth Hormone Research Society Scientific Committee', *J Clin Endocrinol Metab*, 83(2), pp. 382-95.

Carroll, P.V., Littlewood, R., Weissberger, A.J., Bogalho, P., McGauley, G., Sonksen, P.H. and Russell-Jones, D.L. (1997) 'The effects of two doses of replacement growth hormone on the biochemical, body composition and psychological profiles of growth hormone-deficient adults', *Eur J Endocrinol*, 137(2), pp. 146-53.

Carter, G.D., Carter, C.R., Gunter, E., Jones, J., Jones, G., Makin, H.L. and Sufi, S. (2004a) 'Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation', *J Steroid Biochem Mol Biol*, 89-90(1-5), pp. 467-71.

Carter, G.D., Carter, R., Jones, J. and Berry, J. (2004b) 'How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme', *Clin Chem*, 50(11), pp. 2195-7.

Cea, G., Bendahan, D., Manners, D., Hilton-Jones, D., Lodi, R., Styles, P. and Taylor, D.J. (2002) 'Reduced oxidative phosphorylation and proton efflux suggest reduced capillary blood supply in skeletal muscle of patients with dermatomyositis and polymyositis: a quantitative 31P-magnetic resonance spectroscopy and MRI study', *Brain*, 125(Pt 7), pp. 1635-45.

Ceglia, L., Chiu, G.R., Harris, S.S. and Araujo, A.B. (2011) 'Serum 25-hydroxyvitamin D concentration and physical function in adult men', *Clin Endocrinol (Oxf)*, 74(3), pp. 370-6.

Ceglia, L., da Silva Morais, M., Park, L.K., Morris, E., Harris, S.S., Bischoff-Ferrari, H.A., Fielding, R.A. and Dawson-Hughes, B. (2010) 'Multi-step immunofluorescent analysis of vitamin D receptor loci and myosin heavy chain isoforms in human skeletal muscle', *J Mol Histol*, 41(2-3), pp. 137-42.

Chance, B., Eleff, S. and Leigh, J.S., Jr. (1980) 'Noninvasive, nondestructive approaches to cell bioenergetics', *Proc Natl Acad Sci U S A*, 77(12), pp. 7430-4.

Chaudhuri, A. and Behan, P.O. (2004) 'Fatigue in neurological disorders', *Lancet*, 363(9413), pp. 978-88.

Chihara, K., Kato, Y., Kohno, H., Takano, K., Tanaka, T., Teramoto, A. and Shimatsu, A. (2006) 'Efficacy and safety of growth hormone (GH) in the treatment of adult Japanese patients with GH deficiency: a randomised, placebo-controlled study', *Growth Horm IGF Res*, 16(2), pp. 132-42.

Chrisoulidou, A., Kousta, E., Beshyah, S.A., Robinson, S. and Johnston, D.G. (1998) 'How much, and by what mechanisms, does growth hormone replacement improve the quality of life in GH-deficient adults?', *Baillieres Clin Endocrinol Metab*, 12(2), pp. 261-79.

Christ, E.R., Cummings, M.H., Westwood, N.B., Sawyer, B.M., Pearson, T.C., Sonksen, P.H. and Russell-Jones, D.L. (1997) 'The importance of growth hormone in the regulation of erythropoiesis, red cell mass, and plasma volume in adults with growth hormone deficiency', *J Clin Endocrinol Metab*, 82(9), pp. 2985-90.

Cleare, A.J. (2003) 'The neuroendocrinology of chronic fatigue syndrome', *Endocr Rev*, 24(2), pp. 236-52.

Coculescu, M. (1999) 'Blood-brain barrier for human growth hormone and insulin-like growth factor-I', *J Pediatr Endocrinol Metab*, 12(2), pp. 113-24.

Colao, A., Marzullo, P., Di Somma, C. and Lombardi, G. (2001) 'Growth hormone and the heart', *Clin Endocrinol (Oxf)*, 54(2), pp. 137-54.

Conley, K.E., Esselman, P.C., Jubrias, S.A., Cress, M.E., Inglin, B., Mogadam, C. and Schoene, R.B. (2000) 'Ageing, muscle properties and maximal O(2) uptake rate in humans', *J Physiol*, 526 Pt 1, pp. 211-7.

Conley, K.E., Kemper, W.F. and Crowther, G.J. (2001) 'Limits to sustainable muscle performance: interaction between glycolysis and oxidative phosphorylation', *J Exp Biol*, 204(Pt 18), pp. 3189-94.

'Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency', (1998) *J Clin Endocrinol Metab*, 83(2), pp. 379-81.

Copinschi, G., Nedeltcheva, A., Leproult, R., Morselli, L.L., Spiegel, K., Martino, E., Legros, J.J., Weiss, R.E., Mockel, J. and Van Cauter, E. (2010) 'Sleep disturbances, daytime sleepiness, and quality of life in adults with growth hormone deficiency', *J Clin Endocrinol Metab*, 95(5), pp. 2195-202.

Costa, E.M., Blau, H.M. and Feldman, D. (1986) '1,25-dihydroxyvitamin D3 receptors and hormonal responses in cloned human skeletal muscle cells', *Endocrinology*, 119(5), pp. 2214-20.

Craig, C.L., Marshall, A.L., Sjostrom, M., Bauman, A.E., Booth, M.L., Ainsworth, B.E., Pratt, M., Ekelund, U., Yngve, A., Sallis, J.F. and Oja, P. (2003) 'International physical activity questionnaire: 12-country reliability and validity', *Med Sci Sports Exerc*, 35(8), pp. 1381-95.

Cuneo, R.C., Judd, S., Wallace, J.D., Perry-Keene, D., Burger, H., Lim-Tio, S., Strauss, B., Stockigt, J., Topliss, D., Alford, F., Hew, L., Bode, H., Conway, A., Handelsman, D., Dunn, S., Boyages, S., Cheung, N.W. and Hurley, D. (1998) 'The Australian Multicenter Trial of Growth Hormone (GH) Treatment in GH-Deficient Adults', *J Clin Endocrinol Metab*, 83(1), pp. 107-16.

Cuneo, R.C., Salomon, F., Wiles, C.M., Hesp, R. and Sonksen, P.H. (1991) 'Growth hormone treatment in growth hormone-deficient adults. II. Effects on exercise performance', *J Appl Physiol*, 70(2), pp. 695-700.

Cuneo, R.C., Salomon, F., Wiles, C.M., Round, J.M., Jones, D., Hesp, R. and Sonksen, P.H. (1992) 'Histology of skeletal muscle in adults with GH deficiency: comparison with normal muscle and response to GH treatment', *Horm Res*, 37(1-2), pp. 23-8.

Cuneo, R.C., Salomon, F., Wiles, C.M. and Sonksen, P.H. (1990) 'Skeletal muscle performance in adults with growth hormone deficiency', *Horm Res*, 33 Suppl 4, pp. 55-60.

Dahlstedt, A.J., Katz, A. and Westerblad, H. (2001) 'Role of myoplasmic phosphate in contractile function of skeletal muscle: studies on creatine kinase-deficient mice', *J Physiol*, 533(Pt 2), pp. 379-88.

Darzy, K.H., Aimaretti, G., Wieringa, G., Gattamaneni, H.R., Ghigo, E. and Shalet, S.M. (2003) 'The usefulness of the combined growth hormone (GH)-releasing hormone and arginine stimulation test in the diagnosis of radiation-induced GH deficiency is dependent on the post-irradiation time interval', *J Clin Endocrinol Metab*, 88(1), pp. 95-102.

Davidson, M.B. (1987) 'Effect of growth hormone on carbohydrate and lipid metabolism', *Endocr Rev*, 8(2), pp. 115-31.

Dawson-Hughes, B., Heaney, R.P., Holick, M.F., Lips, P., Meunier, P.J. and Vieth, R. (2005) 'Estimates of optimal vitamin D status', *Osteoporos Int*, 16(7), pp. 713-6.

de Boer, H., Blok, G.J., Popp-Snijders, C. and van der Veen, E.A. (1994) 'Diagnosis of growth hormone deficiency in adults', *Lancet*, 343(8913), pp. 1645-6.

De Boer, H., Blok, G.J., Voerman, H.J., De Vries, P.M. and van der Veen, E.A. (1992) 'Body composition in adult growth hormone-deficient men, assessed by anthropometry and bioimpedance analysis', *J Clin Endocrinol Metab*, 75(3), pp. 833-7.

De Feyter, H.M., van den Broek, N.M., Praet, S.F., Nicolay, K., van Loon, L.J. and Prompers, J.J. (2008) 'Early or advanced stage type 2 diabetes is not accompanied by in vivo skeletal muscle mitochondrial dysfunction', *Eur J Endocrinol*, 158(5), pp. 643-53.

Debono, M., Ross, R.J. and Newell-Price, J. (2009) 'Inadequacies of glucocorticoid replacement and improvements by physiological circadian therapy', *Eur J Endocrinol*, 160(5), pp. 719-29.

Deijen, J.B., de Boer, H., Blok, G.J. and van der Veen, E.A. (1996) 'Cognitive impairments and mood disturbances in growth hormone deficient men', *Psychoneuroendocrinology*, 21(3), pp. 313-22.

Deijen, J.B., de Boer, H. and van der Veen, E.A. (1998) 'Cognitive changes during growth hormone replacement in adult men', *Psychoneuroendocrinology*, 23(1), pp. 45-55.

DeLuca, H.F. (2004) 'Overview of general physiologic features and functions of vitamin D', *Am J Clin Nutr*, 80(6 Suppl), pp. 1689S-96S.

Drincic, A.T., Armas, L.A., Van Diest, E.E. and Heaney, R.P. (2012) 'Volumetric Dilution, Rather Than Sequestration Best Explains the Low Vitamin D Status of Obesity', *Obesity (Silver Spring)*.

Endo, I., Inoue, D., Mitsui, T., Umaki, Y., Akaike, M., Yoshizawa, T., Kato, S. and Matsumoto, T. (2003) 'Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors', *Endocrinology*, 144(12), pp. 5138-44.

Falleti, M.G., Maruff, P., Burman, P. and Harris, A. (2006) 'The effects of growth hormone (GH) deficiency and GH replacement on cognitive performance in adults: a meta-analysis of the current literature', *Psychoneuroendocrinology*, 31(6), pp. 681-91.

Farrar, M.D., Kift, R., Felton, S.J., Berry, J.L., Durkin, M.T., Allan, D., Vail, A., Webb, A.R. and Rhodes, L.E. (2011) 'Recommended summer sunlight exposure amounts fail to produce sufficient vitamin D status in UK adults of South Asian origin', *Am J Clin Nutr*, 94(5), pp. 1219-24.

Ferrando, A.A., Sheffield-Moore, M., Yeckel, C.W., Gilkison, C., Jiang, J., Achacosa, A., Lieberman, S.A., Tipton, K., Wolfe, R.R. and Urban, R.J. (2002) 'Testosterone administration to older men improves muscle function: molecular and physiological mechanisms', *Am J Physiol Endocrinol Metab*, 282(3), pp. E601-7.

Fitts, R.H. (1994) 'Cellular mechanisms of muscle fatigue', *Physiol Rev*, 74(1), pp. 49-94.

Florence Cotel, R.E., Stephanie J. Cragg, and Jean-Francois Perrier (2013) 'Serotonin spillover onto the axon initial segment of motoneurons induces central fatigue by inhibiting action potential initiation', *Proceedings of the National Academy of Sciences*, 10.1073/pnas.1216150110.

Florini, J.R., Ewton, D.Z. and Roof, S.L. (1991) 'Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression', *Mol Endocrinol*, 5(5), pp. 718-24.

Florini, J.R., Nicholson, M.L. and Dulak, N.C. (1977) 'Effects of peptide anabolic hormones on growth of myoblasts in culture', *Endocrinology*, 101(1), pp. 32-41.

Forlenza, M.J., Hall, P., Lichtenstein, P., Evengard, B. and Sullivan, P.F. (2005) 'Epidemiology of cancer-related fatigue in the Swedish twin registry', *Cancer*, 104(9), pp. 2022-31.

Frayn, K.N. (2009) *Metabolic regulation: A human persepctive*. John Wiley and Sons.

Frick, G.P., Tai, L.R., Baumbach, W.R. and Goodman, H.M. (1998) 'Tissue distribution, turnover, and glycosylation of the long and short growth hormone receptor isoforms in rat tissues', *Endocrinology*, 139(6), pp. 2824-30.

Fryburg, D.A., Gelfand, R.A. and Barrett, E.J. (1991) 'Growth hormone acutely stimulates forearm muscle protein synthesis in normal humans', *Am J Physiol*, 260(3 Pt 1), pp. E499-504.

Fryer, M.W., Owen, V.J., Lamb, G.D. and Stephenson, D.G. (1995) 'Effects of creatine phosphate and P(i) on Ca2+ movements and tension development in rat skinned skeletal muscle fibres', *J Physiol*, 482 (Pt 1), pp. 123-40.

Fukuda, K., Straus, S.E., Hickie, I., Sharpe, M.C., Dobbins, J.G. and Komaroff, A. (1994) 'The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group', *Ann Intern Med*, 121(12), pp. 953-9.

Gallagher, J.C. (2012) 'Vitamin D deficiency and muscle strength: are they related?', *J Clin Endocrinol Metab*, 97(12), pp. 4366-9.

Gandevia, S.C. (2001) 'Spinal and supraspinal factors in human muscle fatigue', *Physiol Rev*, 81(4), pp. 1725-89.

Gelfand, R.A. and Barrett, E.J. (1987) 'Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man', *J Clin Invest*, 80(1), pp. 1-6.

Gerdhem, P., Ringsberg, K.A., Obrant, K.J. and Akesson, K. (2005) 'Association between 25-hydroxy vitamin D levels, physical activity, muscle strength and fractures in the prospective population-based OPRA Study of Elderly Women', *Osteoporos Int*, 16(11), pp. 1425-31.

Giannoulis, M.G., Sonksen, P.H., Umpleby, M., Breen, L., Pentecost, C., Whyte, M., McMillan, C.V., Bradley, C. and Martin, F.C. (2006) 'The effects of growth hormone and/or testosterone in healthy elderly men: a randomized controlled trial', *J Clin Endocrinol Metab*, 91(2), pp. 477-84.

Gibney, J., Wolthers, T., Johannsson, G., Umpleby, A.M. and Ho, K.K. (2005) 'Growth hormone and testosterone interact positively to enhance protein and energy metabolism in hypopituitary men', *Am J Physiol Endocrinol Metab*, 289(2), pp. E266-71.

Gilchrist, F.J., Murray, R.D. and Shalet, S.M. (2002) 'The effect of long-term untreated growth hormone deficiency (GHD) and 9 years of GH replacement on the quality of life (QoL) of GH-deficient adults', *Clin Endocrinol (Oxf)*, 57(3), pp. 363-70.

Girgis, C.M., Clifton-Bligh, R.J., Hamrick, M.W., Holick, M.F. and Gunton, J.E. (2012) 'The Roles of Vitamin D in Skeletal Muscle: Form, Function, and Metabolism', *Endocr Rev*.

Glancy, B. and Balaban, R.S. (2012) 'Role of mitochondrial Ca2+ in the regulation of cellular energetics', *Biochemistry*, 51(14), pp. 2959-73.

Gleeson, H.K., Gattamaneni, H.R., Smethurst, L., Brennan, B.M. and Shalet, S.M. (2004) 'Reassessment of growth hormone status is required at final height in children treated with growth hormone replacement after radiation therapy', *J Clin Endocrinol Metab*, 89(2), pp. 662-6.

Glerup, H., Mikkelsen, K., Poulsen, L., Hass, E., Overbeck, S., Andersen, H., Charles, P. and Eriksen, E.F. (2000) 'Hypovitaminosis D myopathy without biochemical signs of osteomalacic bone involvement', *Calcif Tissue Int*, 66(6), pp. 419-24.

Goswami, R., Vatsa, M., Sreenivas, V., Singh, U., Gupta, N., Lakshmy, R., Aggarwal, S., Ganapathy, A., Joshi, P. and Bhatia, H. (2012) 'Skeletal Muscle Strength in Young Asian Indian Females after Vitamin D and Calcium Supplementation: A Double-Blind Randomized Controlled Clinical Trial', *J Clin Endocrinol Metab*, 97(12), pp. 4709-16.

Gotherstrom, G., Elbornsson, M., Stibrant-Sunnerhagen, K., Bengtsson, B.A., Johannsson, G. and Svensson, J. (2009) 'Ten years of growth hormone (GH) replacement normalizes muscle strength in GH-deficient adults', *J Clin Endocrinol Metab*, 94(3), pp. 809-16.

Graaf, R.A.d. (2007) *In Vivo NMR Spectroscopy: Principles and Techniques*. Second edition edn. Wiley.

Greene-Finestone, L.S., Berger, C., de Groh, M., Hanley, D.A., Hidiroglou, N., Sarafin, K., Poliquin, S., Krieger, J., Richards, J.B. and Goltzman, D. (2011) '25-Hydroxyvitamin D in Canadian adults: biological, environmental, and behavioral correlates', *Osteoporos Int*, 22(5), pp. 1389-99.

Gupta, R., Sharma, U., Gupta, N., Kalaivani, M., Singh, U., Guleria, R., Jagannathan, N.R. and Goswami, R. (2010) 'Effect of cholecalciferol and calcium supplementation on muscle strength and energy metabolism in vitamin D-deficient Asian Indians: a randomized, controlled trial', *Clin Endocrinol (Oxf)*, 73(4), pp. 445-51.

Hamilton, G., Patel, N., Forton, D.M., Hajnal, J.V. and Taylor-Robinson, S.D. (2003) 'Prior knowledge for time domain quantification of in vivo brain or liver 31P MR spectra', *NMR Biomed*, 16(3), pp. 168-76.

Harper, J.M., Soar, J.B. and Buttery, P.J. (1987) 'Changes in protein metabolism of ovine primary muscle cultures on treatment with growth hormone, insulin, insulin-like growth factor I or epidermal growth factor', *J Endocrinol*, 112(1), pp. 87-96.

Harper, M.E. and Seifert, E.L. (2008) 'Thyroid hormone effects on mitochondrial energetics', *Thyroid*, 18(2), pp. 145-56.

Hazem, A., Elamin, M.B., Bancos, I., Malaga, G., Prutsky, G., Domecq, J.P., Elraiyah, T.A., Abu Elnour, N.O., Prevost, Y., Almandoz, J.P., Zeballos-Palacios, C., Velasquez, E.R., Erwin, P.J., Natt, N., Montori, V.M. and Murad, M.H. (2012) 'Body composition and quality of life in adults treated with GH therapy: a systematic review and meta-analysis', *Eur J Endocrinol*, 166(1), pp. 13-20.

Heaney, R.P., Dowell, M.S., Hale, C.A. and Bendich, A. (2003) 'Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D', *J Am Coll Nutr*, 22(2), pp. 142-6.

Hickok, J.T., Morrow, G.R., Roscoe, J.A., Mustian, K. and Okunieff, P. (2005) 'Occurrence, severity, and longitudinal course of twelve common symptoms in 1129 consecutive patients during radiotherapy for cancer', *J Pain Symptom Manage*, 30(5), pp. 433-42.

Hilding, A., Hall, K., Wivall-Helleryd, I.L., Saaf, M., Melin, A.L. and Thoren, M. (1999) 'Serum levels of insulin-like growth factor I in 152 patients with growth hormone deficiency, aged 19-82 years, in relation to those in healthy subjects', *J Clin Endocrinol Metab*, 84(6), pp. 2013-9.

Hochachka, P.W. and McClelland, G.B. (1997) 'Cellular metabolic homeostasis during large-scale change in ATP turnover rates in muscles', *J Exp Biol*, 200(Pt 2), pp. 381-6.

Hoffman, D.M., Nguyen, T.V., O'Sullivan, A.J., Baxter, R.C. and Ho, K.K. (1994) 'Diagnosis of growth hormone deficiency in adults', *Lancet*, 344(8920), pp. 482-3.

Hoffman, D.M., O'Sullivan, A.J., Freund, J. and Ho, K.K. (1995) 'Adults with growth hormone deficiency have abnormal body composition but normal energy metabolism', *J Clin Endocrinol Metab*, 80(1), pp. 72-7.

Hoffman, D.M., Pallasser, R., Duncan, M., Nguyen, T.V. and Ho, K.K. (1998) 'How is whole body protein turnover perturbed in growth hormone-deficient adults?', *J Clin Endocrinol Metab*, 83(12), pp. 4344-9.

Hofman, M., Ryan, J.L., Figueroa-Moseley, C.D., Jean-Pierre, P. and Morrow, G.R. (2007) 'Cancer-related fatigue: the scale of the problem', *Oncologist*, 12 Suppl 1, pp. 4-10.

Holick, M.F. (2007) 'Vitamin D deficiency', *N Engl J Med*, 357(3), pp. 266-81.

Holick, M.F. (2009) 'Vitamin D status: measurement, interpretation, and clinical application', *Ann Epidemiol*, 19(2), pp. 73-8.

Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H. and Weaver, C.M. (2011) 'Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline', *J Clin Endocrinol Metab*, 96(7), pp. 1911-30.

Hollingsworth, K.G., Newton, J.L., Taylor, R., McDonald, C., Palmer, J.M., Blamire, A.M. and Jones, D.E. (2008) 'Pilot study of peripheral muscle function in primary biliary cirrhosis: potential implications for fatigue pathogenesis', *Clin Gastroenterol Hepatol*, 6(9), pp. 1041-8.

Holmes SJ, M.S., Doward LD, Hunt SM & Shalet SM. (1995) 'Development of a questionnaire to assess the quality of life of adults with GHD', *Endocrinology and Metabolism*, 2, pp. 63-69.

Hoult, D.I., Busby, S.J., Gadian, D.G., Radda, G.K., Richards, R.E. and Seeley, P.J. (1974) 'Observation of tissue metabolites using 31P nuclear magnetic resonance', *Nature*, 252(5481), pp. 285-7.

Houston, D.K., Cesari, M., Ferrucci, L., Cherubini, A., Maggio, D., Bartali, B., Johnson, M.A., Schwartz, G.G. and Kritchevsky, S.B. (2007) 'Association between vitamin D status and physical performance: the InCHIANTI study', *J Gerontol A Biol Sci Med Sci*, 62(4), pp. 440-6.

Hoybye, C., Jonsson, P., Monson, J.P., Koltowska-Haggstrom, M., Hana, V., Geffner, M. and Abs, R. (2007) 'Impact of the primary aetiology upon the clinical outcome of adults with childhood-onset GH deficiency', *Eur J Endocrinol*, 157(5), pp. 589-96.

Hussain, M.A., Schmitz, O., Mengel, A., Glatz, Y., Christiansen, J.S., Zapf, J. and Froesch, E.R. (1994) 'Comparison of the effects of growth hormone and insulin-like growth factor I on substrate oxidation and on insulin sensitivity in growth hormone-deficient humans', *J Clin Invest*, 94(3), pp. 1126-33.

Jacobsen, P.B. (2004) 'Assessment of fatigue in cancer patients', *J Natl Cancer Inst Monogr*, (32), pp. 93-7.

Janssen, Y.J., Doornbos, J. and Roelfsema, F. (1999) 'Changes in muscle volume, strength, and bioenergetics during recombinant human growth hormone (GH) therapy in adults with GH deficiency', *J Clin Endocrinol Metab*, 84(1), pp. 279-84.

Jeneson, J.A., Wiseman, R.W. and Kushmerick, M.J. (1997) 'Non-invasive quantitative 31P MRS assay of mitochondrial function in skeletal muscle in situ', *Mol Cell Biochem*, 174(1-2), pp. 17-22.

Jessen, N., Djurhuus, C.B., Jorgensen, J.O., Jensen, L.S., Moller, N., Lund, S. and Schmitz, O. (2005) 'Evidence against a role for insulin-signaling proteins PI 3-kinase and Akt in insulin

resistance in human skeletal muscle induced by short-term GH infusion', *Am J Physiol Endocrinol Metab*, 288(1), pp. E194-9.

Johannsson, G., Grimby, G., Sunnerhagen, K.S. and Bengtsson, B.A. (1997) 'Two years of growth hormone (GH) treatment increase isometric and isokinetic muscle strength in GH-deficient adults', *J Clin Endocrinol Metab*, 82(9), pp. 2877-84.

Johansen, L. and Quistorff, B. (2003) '31P-MRS characterization of sprint and endurance trained athletes', *Int J Sports Med*, 24(3), pp. 183-9.

Jones, D.E., Hollingsworth, K.G., Taylor, R., Blamire, A.M. and Newton, J.L. (2010) 'Abnormalities in pH handling by peripheral muscle and potential regulation by the autonomic nervous system in chronic fatigue syndrome', *J Intern Med*, 267(4), pp. 394-401.

Jones, H.L., Jr., Wetzel, F.E. and Black, B.K. (1948) 'Sickle cell anemia with striking electrocardiographic abnormalities and other unusual features, with autopsy', *Ann Intern Med*, 29(5), pp. 928-35.

Jones, N.L. and Ehrsam, R.E. (1982) 'The anaerobic threshold', *Exerc Sport Sci Rev*, 10, pp. 49-83.

Jorgensen, A.P., Fougner, K.J., Ueland, T., Gudmundsen, O., Burman, P., Schreiner, T. and Bollerslev, J. (2011a) 'Favorable long-term effects of growth hormone replacement therapy on quality of life, bone metabolism, body composition and lipid levels in patients with adult-onset growth hormone deficiency', *Growth Horm IGF Res*, 21(2), pp. 69-75.

Jorgensen, A.P., Fougner, K.J., Ueland, T., Gudmundsen, O., Burman, P., Schreiner, T. and Bollerslev, J. (2011b) 'Favorable long-term effects of growth hormone replacement therapy on quality of life, bone metabolism, body composition and lipid levels in patients with adult-onset growth hormone deficiency', *Growth Horm IGF Res*.

Jorgensen, J.O. (1999) 'Two hot topics in the field of GH therapy: quality of life in hypopituitary adults, and clinical implications of the somatopause. Report from an interactive discussion', *J Endocrinol Invest*, 22(5 Suppl), pp. 150-6.

Jorgensen, J.O., Pedersen, S.A., Thuesen, L., Jorgensen, J., Ingemann-Hansen, T., Skakkebaek, N.E. and Christiansen, J.S. (1989) 'Beneficial effects of growth hormone treatment in GH-deficient adults', *Lancet*, 1(8649), pp. 1221-5.

Kalueff, A.V., Lou, Y.R., Laaksi, I. and Tuohimaa, P. (2004) 'Impaired motor performance in mice lacking neurosteroid vitamin D receptors', *Brain Res Bull*, 64(1), pp. 25-9.

Kaminsky, P., Robin-Lherbier, B., Brunotte, F., Escanye, J.M., Walker, P., Klein, M., Robert, J. and Duc, M. (1992) 'Energetic metabolism in hypothyroid skeletal muscle, as studied by phosphorus magnetic resonance spectroscopy', *J Clin Endocrinol Metab*, 74(1), pp. 124-9.

Katznelson, L., Atkinson, J.L., Cook, D.M., Ezzat, S.Z., Hamrahian, A.H., Miller, K.K. and American Association of Clinical, E. (2011) 'American Association of Clinical Endocrinologists medical guidelines for clinical practice for the diagnosis and treatment of acromegaly--2011 update', *Endocr Pract*, 17 Suppl 4, pp. 1-44.

Kemp, G.J. and Radda, G.K. (1994) 'Quantitative interpretation of bioenergetic data from 31P and 1H magnetic resonance spectroscopic studies of skeletal muscle: an analytical review', *Magn Reson Q*, 10(1), pp. 43-63.

Kemp, G.J., Roberts, N., Bimson, W.E., Bakran, A. and Frostick, S.P. (2002) 'Muscle oxygenation and ATP turnover when blood flow is impaired by vascular disease', *Mol Biol Rep*, 29(1-2), pp. 187-91.

Kemp, G.J., Taylor, D.J., Thompson, C.H., Hands, L.J., Rajagopalan, B., Styles, P. and Radda, G.K. (1993) 'Quantitative analysis by 31P magnetic resonance spectroscopy of abnormal mitochondrial oxidation in skeletal muscle during recovery from exercise', *NMR Biomed*, 6(5), pp. 302-10.

Kemp, G.J., Thompson, C.H., Sanderson, A.L. and Radda, G.K. (1994) 'pH control in rat skeletal muscle during exercise, recovery from exercise, and acute respiratory acidosis', *Magn Reson Med*, 31(2), pp. 103-9.

Kemp, G.J., Thompson, C.H., Taylor, D.J. and Radda, G.K. (1997) 'Proton efflux in human skeletal muscle during recovery from exercise', *Eur J Appl Physiol Occup Physiol*, 76(5), pp. 462-71.

Kent-Braun, J.A. and Ng, A.V. (2000) 'Skeletal muscle oxidative capacity in young and older women and men', *J Appl Physiol*, 89(3), pp. 1072-8.

Koltowska-Haggstrom, M., Mattsson, A.F., Monson, J.P., Kind, P., Badia, X., Casanueva, F.F., Busschbach, J., Koppeschaar, H.P. and Johannsson, G. (2006) 'Does long-term GH replacement therapy in hypopituitary adults with GH deficiency normalise quality of life?', *Eur J Endocrinol*, 155(1), pp. 109-19.

Koob, G.F., Heinrichs, S.C., Pich, E.M., Menzaghi, F., Baldwin, H., Miczek, K. and Britton, K.T. (1993) 'The role of corticotropin-releasing factor in behavioural responses to stress', *Ciba Found Symp*, 172, pp. 277-89; discussion 290-5.

Kopelman, P.G., White, N., Pilkington, T.R. and Jeffcoate, S.L. (1979) 'Impaired hypothalamic control of prolactin secretion in massive obesity', *Lancet*, 1(8119), pp. 747-50.

Kuhl, C.K., Layer, G., Traber, F., Zierz, S., Block, W. and Reiser, M. (1994) 'Mitochondrial encephalomyopathy: correlation of P-31 exercise MR spectroscopy with clinical findings', *Radiology*, 192(1), pp. 223-30.

Kushmerick, M.J., Moerland, T.S. and Wiseman, R.W. (1992) 'Mammalian skeletal muscle fibers distinguished by contents of phosphocreatine, ATP, and Pi', *Proc Natl Acad Sci U S A*, 89(16), pp. 7521-5.

Kutsuzawa, T., Shioya, S., Kurita, D., Haida, M. and Yamabayashi, H. (2001) 'Effects of age on muscle energy metabolism and oxygenation in the forearm muscles', *Med Sci Sports Exerc*, 33(6), pp. 901-6.

Kyrdalen, A.E., Dahl, A.A., Hernes, E., Cvancarova, M. and Fossa, S.D. (2010) 'Fatigue in hormone-naive prostate cancer patients treated with radical prostatectomy or definitive radiotherapy', *Prostate Cancer Prostatic Dis*, 13(2), pp. 144-50.

Lai, Z., Roos, P., Zhai, O., Olsson, Y., Fholenhag, K., Larsson, C. and Nyberg, F. (1993) 'Age-related reduction of human growth hormone-binding sites in the human brain', *Brain Res*, 621(2), pp. 260-6.

Lai, Z.N., Emtner, M., Roos, P. and Nyberg, F. (1991) 'Characterization of putative growth hormone receptors in human choroid plexus', *Brain Res*, 546(2), pp. 222-6.

Lanza, I.R., Bhagra, S., Nair, K.S. and Port, J.D. (2011) 'Measurement of human skeletal muscle oxidative capacity by 31P-MR spectroscopy: a cross-validation with in vitro measurements', *J Magn Reson Imaging*, 34(5), pp. 1143-50.

Larson-Meyer, D.E., Newcomer, B.R., Hunter, G.R., Hetherington, H.P. and Weinsier, R.L. (2000) '31P MRS measurement of mitochondrial function in skeletal muscle: reliability, force-level sensitivity and relation to whole body maximal oxygen uptake', *NMR Biomed*, 13(1), pp. 14-27.

Layec, G., Bringard, A., Le Fur, Y., Vilmen, C., Micallef, J.P., Perrey, S., Cozzone, P.J. and Bendahan, D. (2009) 'Reproducibility assessment of metabolic variables characterizing muscle energetics in vivo: A 31P-MRS study', *Magn Reson Med*, 62(4), pp. 840-54.

Lee, H.C. and Wei, Y.H. (2005) 'Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress', *Int J Biochem Cell Biol*, 37(4), pp. 822-34.

Lee, K.A., Hicks, G. and Nino-Murcia, G. (1991) 'Validity and reliability of a scale to assess fatigue', *Psychiatry Res*, 36(3), pp. 291-8.

Leung, K.C. and Ho, K.K. (1997) 'Stimulation of mitochondrial fatty acid oxidation by growth hormone in human fibroblasts', *J Clin Endocrinol Metab*, 82(12), pp. 4208-13.

Leung, K.C., Johannsson, G., Leong, G.M. and Ho, K.K. (2004) 'Estrogen regulation of growth hormone action', *Endocr Rev*, 25(5), pp. 693-721.

Lijffijt, M., Van Dam, P.S., Kenemans, J.L., Koppeschaar, H.P., de Vries, W.R., Drent, M.L., Wittenberg, A. and Kemner, C. (2003) 'Somatotropic-axis deficiency affects brain substrates

of selective attention in childhood-onset growth hormone deficient patients', *Neurosci Lett*, 353(2), pp. 123-6.

Lips, P. (2010) 'Worldwide status of vitamin D nutrition', *J Steroid Biochem Mol Biol*, 121(1-2), pp. 297-300.

Lloyd, A.R. (1998) 'Chronic fatigue and chronic fatigue syndrome: shifting boundaries and attributions', *Am J Med*, 105(3A), pp. 7S-10S.

Lodi, R., Cooper, J.M., Bradley, J.L., Manners, D., Styles, P., Taylor, D.J. and Schapira, A.H. (1999a) 'Deficit of in vivo mitochondrial ATP production in patients with Friedreich ataxia', *Proc Natl Acad Sci U S A*, 96(20), pp. 11492-5.

Lodi, R., Kemp, G.J., Muntoni, F., Thompson, C.H., Rae, C., Taylor, J., Styles, P. and Taylor, D.J. (1999b) 'Reduced cytosolic acidification during exercise suggests defective glycolytic activity in skeletal muscle of patients with Becker muscular dystrophy. An in vivo 31P magnetic resonance spectroscopy study', *Brain*, 122 (Pt 1), pp. 121-30.

Lodi, R., Tonon, C., Valentino, M.L., Iotti, S., Clementi, V., Malucelli, E., Barboni, P., Longanesi, L., Schimpf, S., Wissinger, B., Baruzzi, A., Barbiroli, B. and Carelli, V. (2004) 'Deficit of in vivo mitochondrial ATP production in OPA1-related dominant optic atrophy', *Ann Neurol*, 56(5), pp. 719-23.

Losa, M., Scavini, M., Gatti, E., Rossini, A., Madaschi, S., Formenti, I., Caumo, A., Stidley, C.A. and Lanzi, R. (2008) 'Long-term effects of growth hormone replacement therapy on thyroid function in adults with growth hormone deficiency', *Thyroid*, 18(12), pp. 1249-54.

Louwerens, M., Appelhof, B.C., Verloop, H., Medici, M., Peeters, R.P., Visser, T.J., Boelen, A., Fliers, E., Smit, J.W. and Dekkers, O.M. (2012) 'Fatigue and fatigue-related symptoms in patients treated for different causes of hypothyroidism', *Eur J Endocrinol*, 167(6), pp. 809-15.

Lukaski, H.C., Johnson, P.E., Bolonchuk, W.W. and Lykken, G.I. (1985) 'Assessment of fat-free mass using bioelectrical impedance measurements of the human body', *Am J Clin Nutr*, 41(4), pp. 810-7.

Ma°rdh G, L.K., Borg G, Jonsson B & Lindeberg A on behalf of and investigators, t. (1994) 'Growth hormone replacement therapy in adult
hypopituitary patients with growth hormone deficiency: combined
data from 12 European placebo-controlled clinical trials.', *Endocrinology and Metabolism*, 1((Supplement A)), pp. 43-49.

Maghnie, M., Salati, B., Bianchi, S., Rallo, M., Tinelli, C., Autelli, M., Aimaretti, G. and Ghigo, E. (2001) 'Relationship between the morphological evaluation of the pituitary and the growth hormone (GH) response to GH-releasing hormone Plus arginine in children and adults with congenital hypopituitarism', *J Clin Endocrinol Metab*, 86(4), pp. 1574-9.

Mainwood, G.W. and Alward, M. (1982) 'Evidence for an extracellular mechanism for the action of H+ on recovery of muscles following fatigue', *Can J Physiol Pharmacol*, 60(12), pp. 1720-4.

Mainwood, G.W. and Renaud, J.M. (1985) 'The effect of acid-base balance on fatigue of skeletal muscle', *Can J Physiol Pharmacol*, 63(5), pp. 403-16.

Maiter, D., Abs, R., Johannsson, G., Scanlon, M., Jonsson, P.J., Wilton, P. and Koltowska-Haggstrom, M. (2006) 'Baseline characteristics and response to GH replacement of hypopituitary patients previously irradiated for pituitary adenoma or craniopharyngioma: data from the Pfizer International Metabolic Database', *Eur J Endocrinol*, 155(2), pp. 253-60.

Makimura, H., Stanley, T.L., Sun, N., Hrovat, M.I., Systrom, D.M. and Grinspoon, S.K. (2011) 'The association of growth hormone parameters with skeletal muscle phosphocreatine recovery in adult men', *J Clin Endocrinol Metab*, 96(3), pp. 817-23.

Mantovani, G., Maghnie, M., Weber, G., De Menis, E., Brunelli, V., Cappa, M., Loli, P., Beck-Peccoz, P. and Spada, A. (2003) 'Growth hormone-releasing hormone resistance in pseudohypoparathyroidism type ia: new evidence for imprinting of the Gs alpha gene', *J Clin Endocrinol Metab*, 88(9), pp. 4070-4.

Mateika, J.H. and Duffin, J. (1994) 'Coincidental changes in ventilation and electromyographic activity during consecutive incremental exercise tests', *Eur J Appl Physiol Occup Physiol*, 68(1), pp. 54-61.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F. and Turner, R.C. (1985) 'Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28(7), pp. 412-9.

McCully, K.K., Boden, B.P., Tuchler, M., Fountain, M.R. and Chance, B. (1989) 'Wrist flexor muscles of elite rowers measured with magnetic resonance spectroscopy', *J Appl Physiol*, 67(3), pp. 926-32.

McCully, K.K., Forciea, M.A., Hack, L.M., Donlon, E., Wheatley, R.W., Oatis, C.A., Goldberg, T. and Chance, B. (1991) 'Muscle metabolism in older subjects using 31P magnetic resonance spectroscopy', *Can J Physiol Pharmacol*, 69(5), pp. 576-80.

McGauley, G.A., Cuneo, R.C., Salomon, F. and Sonksen, P.H. (1990) 'Psychological well-being before and after growth hormone treatment in adults with growth hormone deficiency', *Horm Res*, 33 Suppl 4, pp. 52-4.

McKenna, S.P., Doward, L.C., Alonso, J., Kohlmann, T., Niero, M., Prieto, L. and Wiren, L. (1999) 'The QoL-AGHDA: an instrument for the assessment of quality of life in adults with growth hormone deficiency', *Qual Life Res*, 8(4), pp. 373-83.

Medicine, I.o. (2011) 'Dietary Reference Intakes for Calcium and Vitamin D'.

Merrill, G.F., Florini, J.R. and Dulak, N.C. (1977) 'Effects of multiplication stimulating activity (MSA) on AIB transport into myoblast and myotube cultures', *J Cell Physiol*, 93(2), pp. 173-82.

Meyer, V. and Jones, H.G. (1957) 'Patterns of cognitive test performance as functions of the lateral localization of cerebral abnormalities in the temporal lobe', *J Ment Sci*, 103(433), pp. 758-72.

Misra, M., Pacaud, D., Petryk, A., Collett-Solberg, P.F., Kappy, M., Drug and Therapeutics Committee of the Lawson Wilkins Pediatric Endocrine, S. (2008) 'Vitamin D deficiency in

children and its management: review of current knowledge and recommendations', *Pediatrics*, 122(2), pp. 398-417.

Mithal, A., Wahl, D.A., Bonjour, J.P., Burckhardt, P., Dawson-Hughes, B., Eisman, J.A., El-Hajj Fuleihan, G., Josse, R.G., Lips, P. and Morales-Torres, J. (2009) 'Global vitamin D status and determinants of hypovitaminosis D', *Osteoporos Int*, 20(11), pp. 1807-20.

Molitch, M.E. (2011) 'Growth Hormone Treatment in Adults with Growth Hormone Deficiency: The Transition', *J Endocrinol Invest*.

Molitch, M.E., Clemmons, D.R., Malozowski, S., Merriam, G.R. and Vance, M.L. (2011) 'Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline', *J Clin Endocrinol Metab*, 96(6), pp. 1587-609.

Moller, N. and Norrelund, H. (2003) 'The role of growth hormone in the regulation of protein metabolism with particular reference to conditions of fasting', *Horm Res*, 59 Suppl 1, pp. 62-8.

Moon, R.B. and Richards, J.H. (1973) 'Determination of intracellular pH by 31P magnetic resonance', *J Biol Chem*, 248(20), pp. 7276-8.

Moore, J.S., Monson, J.P., Kaltsas, G., Putignano, P., Wood, P.J., Sheppard, M.C., Besser, G.M., Taylor, N.F. and Stewart, P.M. (1999) 'Modulation of 11beta-hydroxysteroid dehydrogenase isozymes by growth hormone and insulin-like growth factor: in vivo and in vitro studies', *J Clin Endocrinol Metab*, 84(11), pp. 4172-7.

Moorkens, G., Wynants, H. and Abs, R. (1998) 'Effect of growth hormone treatment in patients with chronic fatigue syndrome: a preliminary study', *Growth Horm IGF Res*, 8 Suppl B, pp. 131-3.

Mouyis, M., Ostor, A.J., Crisp, A.J., Ginawi, A., Halsall, D.J., Shenker, N. and Poole, K.E. (2008) 'Hypovitaminosis D among rheumatology outpatients in clinical practice', *Rheumatology (Oxford)*, 47(9), pp. 1348-51.

Mukherjee, A., Tolhurst-Cleaver, S., Ryder, W.D., Smethurst, L. and Shalet, S.M. (2005) 'The characteristics of quality of life impairment in adult growth hormone (GH)-deficient

survivors of cancer and their response to GH replacement therapy', *J Clin Endocrinol Metab*, 90(3), pp. 1542-9.

Mukherjee, A., Zerwekh, J.E., Nicar, M.J., McCoy, K. and Buja, L.M. (1981) 'Effect of chronic vitamin D deficiency on chick heart mitochondrial oxidative phosphorylation', *J Mol Cell Cardiol*, 13(2), pp. 171-83.

Mulder, R.L., Kremer, L.C., van Santen, H.M., Ket, J.L., van Trotsenburg, A.S., Koning, C.C., Schouten-van Meeteren, A.Y., Caron, H.N., Neggers, S.J. and van Dalen, E.C. (2009) 'Prevalence and risk factors of radiation-induced growth hormone deficiency in childhood cancer survivors: a systematic review', *Cancer Treat Rev*, 35(7), pp. 616-32.

Murray, R.D., Bidlingmaier, M., Strasburger, C.J. and Shalet, S.M. (2007) 'The diagnosis of partial growth hormone deficiency in adults with a putative insult to the hypothalamo-pituitary axis', *J Clin Endocrinol Metab*, 92(5), pp. 1705-9.

Murray, R.D., Skillicorn, C.J., Howell, S.J., Lissett, C.A., Rahim, A., Smethurst, L.E. and Shalet, S.M. (1999) 'Influences on quality of life in GH deficient adults and their effect on response to treatment', *Clin Endocrinol (Oxf)*, 51(5), pp. 565-73.

Nass, R., Huber, R.M., Klauss, V., Muller, O.A., Schopohl, J. and Strasburger, C.J. (1995) 'Effect of growth hormone (hGH) replacement therapy on physical work capacity and cardiac and pulmonary function in patients with hGH deficiency acquired in adulthood', *J Clin Endocrinol Metab*, 80(2), pp. 552-7.

NICE (2003) 'TA64 Growth hormone deficiency (adults) - human growth hormone: guidance'.

Nimptsch, K., Platz, E.A., Willett, W.C. and Giovannucci, E. (2012) 'Association between plasma 25-OH vitamin D and testosterone levels in men', *Clin Endocrinol (Oxf)*, 77(1), pp. 106-12.

Nishida, M., Nishijima, H., Yonezawa, K., Sato, I., Anzai, T., Okita, K. and Yasuda, H. (1992) 'Phosphorus-31 magnetic resonance spectroscopy of forearm flexor muscles in student

rowers using an exercise protocol adjusted for differences in cross-sectional muscle area', *Eur J Appl Physiol Occup Physiol*, 64(6), pp. 528-33.

Nyberg, F. and Burman, P. (1996) 'Growth hormone and its receptors in the central nervous system--location and functional significance', *Horm Res*, 45(1-2), pp. 18-22.

Park, J.H., Niermann, K.J., Ryder, N.M., Nelson, A.E., Das, A., Lawton, A.R., Hernanz-Schulman, M. and Olsen, N.J. (2000) 'Muscle abnormalities in juvenile dermatomyositis patients: P-31 magnetic resonance spectroscopy studies', *Arthritis Rheum*, 43(10), pp. 2359-67.

Park, J.H., Vital, T.L., Ryder, N.M., Hernanz-Schulman, M., Partain, C.L., Price, R.R. and Olsen, N.J. (1994) 'Magnetic resonance imaging and P-31 magnetic resonance spectroscopy provide unique quantitative data useful in the longitudinal management of patients with dermatomyositis', *Arthritis Rheum*, 37(5), pp. 736-46.

Patti, M.E. and Corvera, S. (2010) 'The role of mitochondria in the pathogenesis of type 2 diabetes', *Endocr Rev*, 31(3), pp. 364-95.

Pearce, S.H. and Cheetham, T.D. (2010) 'Diagnosis and management of vitamin D deficiency', *BMJ*, 340, p. b5664.

Pedersen, B.K. and Febbraio, M.A. (2008) 'Muscle as an endocrine organ: focus on muscle-derived interleukin-6', *Physiol Rev*, 88(4), pp. 1379-406.

Pfeifer, M., Verhovec, R., Zizek, B., Prezelj, J., Poredos, P. and Clayton, R.N. (1999) 'Growth hormone (GH) treatment reverses early atherosclerotic changes in GH-deficient adults', *J Clin Endocrinol Metab*, 84(2), pp. 453-7.

Phinney, K.W. (2008) 'Development of a standard reference material for vitamin D in serum', *Am J Clin Nutr*, 88(2), pp. 511S-512S.

Phinney, K.W., Bedner, M., Tai, S.S., Vamathevan, V.V., Sander, L.C., Sharpless, K.E., Wise, S.A., Yen, J.H., Schleicher, R.L., Chaudhary-Webb, M., Pfeiffer, C.M., Betz, J.M., Coates, P.M. and Picciano, M.F. (2012) 'Development and certification of a standard reference material for vitamin d metabolites in human serum', *Anal Chem*, 84(2), pp. 956-62.

Place, N., Bruton, J.D. and Westerblad, H. (2009) 'Mechanisms of fatigue induced by isometric contractions in exercising humans and in mouse isolated single muscle fibres', *Clin Exp Pharmacol Physiol*, 36(3), pp. 334-9.

Plotnikoff, G.A. and Quigley, J.M. (2003) 'Prevalence of severe hypovitaminosis D in patients with persistent, nonspecific musculoskeletal pain', *Mayo Clin Proc*, 78(12), pp. 1463-70.

Poehlman, E.T., Melby, C.L., Badylak, S.F. and Calles, J. (1989) 'Aerobic fitness and resting energy expenditure in young adult males', *Metabolism*, 38(1), pp. 85-90.

Porretti, S., Giavoli, C., Ronchi, C., Lombardi, G., Zaccaria, M., Valle, D., Arosio, M. and Beck-Peccoz, P. (2002) 'Recombinant human GH replacement therapy and thyroid function in a large group of adult GH-deficient patients: when does L-T(4) therapy become mandatory?', *J Clin Endocrinol Metab*, 87(5), pp. 2042-5.

Priemel, M., von Domarus, C., Klatte, T.O., Kessler, S., Schlie, J., Meier, S., Proksch, N., Pastor, F., Netter, C., Streichert, T., Puschel, K. and Amling, M. (2010) 'Bone mineralization defects and vitamin D deficiency: histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients', *J Bone Miner Res*, 25(2), pp. 305-12.

Prince, R.L., Austin, N., Devine, A., Dick, I.M., Bruce, D. and Zhu, K. (2008) 'Effects of ergocalciferol added to calcium on the risk of falls in elderly high-risk women', *Arch Intern Med*, 168(1), pp. 103-8.

Psarra, A.M., Solakidi, S. and Sekeris, C.E. (2006) 'The mitochondrion as a primary site of action of steroid and thyroid hormones: presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells', *Mol Cell Endocrinol*, 246(1-2), pp. 21-33.

Quinn, L.S., Steinmetz, B., Maas, A., Ong, L. and Kaleko, M. (1994) 'Type-1 insulin-like growth factor receptor overexpression produces dual effects on myoblast proliferation and differentiation', *J Cell Physiol*, 159(3), pp. 387-98.

Quistorff, B., Johansen, L. and Sahlin, K. (1993) 'Absence of phosphocreatine resynthesis in human calf muscle during ischaemic recovery', *Biochem J*, 291 (Pt 3), pp. 681-6.

Raben, M.S. (1962) 'Growth hormone. 1. Physiologic aspects', *N Engl J Med*, 266, pp. 31-5.

Rapkin, B.D. and Schwartz, C.E. (2004) 'Toward a theoretical model of quality-of-life appraisal: Implications of findings from studies of response shift', *Health Qual Life Outcomes*, 2, p. 14.

Reginato, A.J. and Coquia, J.A. (2003) 'Musculoskeletal manifestations of osteomalacia and rickets', *Best Pract Res Clin Rheumatol*, 17(6), pp. 1063-80.

Robinson, P.D., Hogler, W., Craig, M.E., Verge, C.F., Walker, J.L., Piper, A.C., Woodhead, H.J., Cowell, C.T. and Ambler, G.R. (2006) 'The re-emerging burden of rickets: a decade of experience from Sydney', *Arch Dis Child*, 91(7), pp. 564-8.

Rosen, T., Johannsson, G., Johansson, J.O. and Bengtsson, B.A. (1995) 'Consequences of growth hormone deficiency in adults and the benefits and risks of recombinant human growth hormone treatment. A review paper', *Horm Res*, 43(1-3), pp. 93-9.

Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-Arvizu, R.A., Gallagher, J.C., Gallo, R.L., Jones, G., Kovacs, C.S., Mayne, S.T., Rosen, C.J. and Shapses, S.A. (2011) 'The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know', *J Clin Endocrinol Metab*, 96(1), pp. 53-8.

Ross, B.D., Radda, G.K., Gadian, D.G., Rocker, G., Esiri, M. and Falconer-Smith, J. (1981) 'Examination of a case of suspected McArdle's syndrome by 31P nuclear magnetic resonance', *N Engl J Med*, 304(22), pp. 1338-42.

Rudolf, R., Mongillo, M., Magalhaes, P.J. and Pozzan, T. (2004) 'In vivo monitoring of Ca(2+) uptake into mitochondria of mouse skeletal muscle during contraction', *J Cell Biol*, 166(4), pp. 527-36.

Ryan, M.M. and Jones, H.R., Jr. (2004) 'Delayed neurophysiologic abnormalities in Charcot-Marie-Tooth disease type 1A', *Muscle Nerve*, 30(1), pp. 123-5.

S Iotti, R.L., C Frassineti, P Zaniol, and B Barbiroli (1993) 'In Vivo Assessment of Mitochondrial Functionality in Human Gastrocnemius Muscle by 31P MRS: The role of pH in the Evaluation of Phosphocreatine and Inorganic Phosphate Recoveries from Exercise', *NMR in Biomedicine*, 6, pp. 248-253.

Sachan, A., Gupta, R., Das, V., Agarwal, A., Awasthi, P.K. and Bhatia, V. (2005) 'High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India', *Am J Clin Nutr*, 81(5), pp. 1060-4.

Sahlin, K., Tonkonogi, M. and Soderlund, K. (1998) 'Energy supply and muscle fatigue in humans', *Acta Physiol Scand*, 162(3), pp. 261-6.

Sahota, O., Mundey, M.K., San, P., Godber, I.M., Lawson, N. and Hosking, D.J. (2004) 'The relationship between vitamin D and parathyroid hormone: calcium homeostasis, bone turnover, and bone mineral density in postmenopausal women with established osteoporosis', *Bone*, 35(1), pp. 312-9.

Salomon, F., Cuneo, R.C., Hesp, R. and Sonksen, P.H. (1989) 'The effects of treatment with recombinant human growth hormone on body composition and metabolism in adults with growth hormone deficiency', *N Engl J Med*, 321(26), pp. 1797-803.

Sartorio, A., Molinari, E., Riva, G., Conti, A., Morabito, F. and Faglia, G. (1995) 'Growth hormone treatment in adults with childhood onset growth hormone deficiency: effects on psychological capabilities', *Horm Res*, 44(1), pp. 6-11.

Sathiavageeswaran, M., Burman, P., Lawrence, D., Harris, A.G., Falleti, M.G., Maruff, P. and Wass, J. (2007) 'Effects of GH on cognitive function in elderly patients with adult-onset GH deficiency: a placebo-controlled 12-month study', *Eur J Endocrinol*, 156(4), pp. 439-47.

Sato, Y., Iwamoto, J., Kanoko, T. and Satoh, K. (2005) 'Low-dose vitamin D prevents muscular atrophy and reduces falls and hip fractures in women after stroke: a randomized controlled trial', *Cerebrovasc Dis*, 20(3), pp. 187-92.

Scheller, K. and Sekeris, C.E. (2003) 'The effects of steroid hormones on the transcription of genes encoding enzymes of oxidative phosphorylation', *Exp Physiol*, 88(1), pp. 129-40.

Scheuermann-Freestone, M., Madsen, P.L., Manners, D., Blamire, A.M., Buckingham, R.E., Styles, P., Radda, G.K., Neubauer, S. and Clarke, K. (2003) 'Abnormal cardiac and skeletal muscle energy metabolism in patients with type 2 diabetes', *Circulation*, 107(24), pp. 3040-6.

Schrauwen-Hinderling, V.B., Kooi, M.E., Hesselink, M.K., Jeneson, J.A., Backes, W.H., van Echteld, C.J., van Engelshoven, J.M., Mensink, M. and Schrauwen, P. (2007) 'Impaired in vivo mitochondrial function but similar intramyocellular lipid content in patients with type 2 diabetes mellitus and BMI-matched control subjects', *Diabetologia*, 50(1), pp. 113-20.

Schroder, L., Schmitz, C. and Bachert, P. (2005) 'Cumulative "roof effect" in high-resolution in vivo 31P NMR spectra of human calf muscle and the Clebsch-Gordan coefficients of ATP at 1.5 T', *J Magn Reson*, 174(1), pp. 68-77.

Schwartz, C.E. and Sprangers, M.A. (1999) 'Methodological approaches for assessing response shift in longitudinal health-related quality-of-life research', *Soc Sci Med*, 48(11), pp. 1531-48.

Sedrani, S.H., Elidrissy, A.W. and El Arabi, K.M. (1983) 'Sunlight and vitamin D status in normal Saudi subjects', *Am J Clin Nutr*, 38(1), pp. 129-32.

Sesmilo, G., Biller, B.M., Llevadot, J., Hayden, D., Hanson, G., Rifai, N. and Klibanski, A. (2000) 'Effects of growth hormone administration on inflammatory and other cardiovascular risk markers in men with growth hormone deficiency. A randomized, controlled clinical trial', *Ann Intern Med*, 133(2), pp. 111-22.

Sesmilo, G., Miller, K.K., Hayden, D. and Klibanski, A. (2001) 'Inflammatory cardiovascular risk markers in women with hypopituitarism', *J Clin Endocrinol Metab*, 86(12), pp. 5774-81.

Shah, M., Salhab, N., Patterson, D. and Seikaly, M.G. (2000) 'Nutritional rickets still afflict children in north Texas', *Tex Med*, 96(6), pp. 64-8.

Short, K.R., Moller, N., Bigelow, M.L., Coenen-Schimke, J. and Nair, K.S. (2008) 'Enhancement of muscle mitochondrial function by growth hormone', *J Clin Endocrinol Metab*, 93(2), pp. 597-604.

Short, K.R., Nygren, J. and Nair, K.S. (2007) 'Effect of T(3)-induced hyperthyroidism on mitochondrial and cytoplasmic protein synthesis rates in oxidative and glycolytic tissues in rats', *Am J Physiol Endocrinol Metab*, 292(2), pp. E642-7.

Sinha, A., Cheetham, T.D. and Pearce, S.H. (2012) 'Prevention and Treatment of Vitamin D Deficiency', *Calcif Tissue Int*.

Sirikul, B., Gower, B.A., Hunter, G.R., Larson-Meyer, D.E. and Newcomer, B.R. (2006) 'Relationship between insulin sensitivity and in vivo mitochondrial function in skeletal muscle', *Am J Physiol Endocrinol Metab*, 291(4), pp. E724-8.

Sorensen, O.H., Lund, B., Saltin, B., Lund, B., Andersen, R.B., Hjorth, L., Melsen, F. and Mosekilde, L. (1979) 'Myopathy in bone loss of ageing: improvement by treatment with 1 alpha-hydroxycholecalciferol and calcium', *Clin Sci (Lond)*, 56(2), pp. 157-61.

Sprangers, M.A. and Schwartz, C.E. (1999) 'Integrating response shift into health-related quality of life research: a theoretical model', *Soc Sci Med*, 48(11), pp. 1507-15.

Stanley, T.L., Zanni, M.V., Johnsen, S., Rasheed, S., Makimura, H., Lee, H., Khor, V.K., Ahima, R.S. and Grinspoon, S.K. (2011) 'TNF-alpha antagonism with etanercept decreases glucose and increases the proportion of high molecular weight adiponectin in obese subjects with features of the metabolic syndrome', *J Clin Endocrinol Metab*, 96(1), pp. E146-50.

Stockton, K.A., Mengersen, K., Paratz, J.D., Kandiah, D. and Bennell, K.L. (2011) 'Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis', *Osteoporos Int*, 22(3), pp. 859-71.

Strasser, F., Palmer, J.L., Schover, L.R., Yusuf, S.W., Pisters, K., Vassilopoulou-Sellin, R., DeGracia, B., Willey, J.S. and Bruera, E. (2006) 'The impact of hypogonadism and autonomic dysfunction on fatigue, emotional function, and sexual desire in male patients with advanced cancer: a pilot study', *Cancer*, 107(12), pp. 2949-57.

Stump, C.S., Short, K.R., Bigelow, M.L., Schimke, J.M. and Nair, K.S. (2003) 'Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts', *Proc Natl Acad Sci U S A*, 100(13), pp. 7996-8001.

Taivassalo, T., De Stefano, N., Argov, Z., Matthews, P.M., Chen, J., Genge, A., Karpati, G. and Arnold, D.L. (1998) 'Effects of aerobic training in patients with mitochondrial myopathies', *Neurology*, 50(4), pp. 1055-60.

Tarnopolsky, M.A. and Parise, G. (1999) 'Direct measurement of high-energy phosphate compounds in patients with neuromuscular disease', *Muscle Nerve*, 22(9), pp. 1228-33.

Tauber, M., Moulin, P., Pienkowski, C., Jouret, B. and Rochiccioli, P. (1997) 'Growth hormone (GH) retesting and auxological data in 131 GH-deficient patients after completion of treatment', *J Clin Endocrinol Metab*, 82(2), pp. 352-6.

Taylor, D.J., Bore, P.J., Styles, P., Gadian, D.G. and Radda, G.K. (1983) 'Bioenergetics of intact human muscle. A 31P nuclear magnetic resonance study', *Mol Biol Med*, 1(1), pp. 77-94.

Taylor, D.J., Kemp, G.J., Thompson, C.H. and Radda, G.K. (1997) 'Ageing: effects on oxidative function of skeletal muscle in vivo', *Mol Cell Biochem*, 174(1-2), pp. 321-4.

Taylor, D.J., Rajagopalan, B. and Radda, G.K. (1992) 'Cellular energetics in hypothyroid muscle', *Eur J Clin Invest*, 22(5), pp. 358-65.

Thambyrajah, J., Landray, M.J., McGlynn, F.J., Jones, H.J., Wheeler, D.C. and Townend, J.N. (2000) 'Abnormalities of endothelial function in patients with predialysis renal failure', *Heart*, 83(2), pp. 205-9.

Toogood, A.A., O'Neill, P.A. and Shalet, S.M. (1996) 'Beyond the somatopause: growth hormone deficiency in adults over the age of 60 years', *J Clin Endocrinol Metab*, 81(2), pp. 460-5.

Trenell, M.I., Hollingsworth, K.G., Lim, E.L. and Taylor, R. (2008) 'Increased daily walking improves lipid oxidation without changes in mitochondrial function in type 2 diabetes', *Diabetes Care*, 31(8), pp. 1644-9.

Tschop, M., Lahner, H., Feldmeier, H., Grasberger, H., Morrison, K.M., Janssen, O.E., Attanasio, A.F. and Strasburger, C.J. (2000) 'Effects of growth hormone replacement

therapy on levels of cortisol and cortisol-binding globulin in hypopituitary adults', *Eur J Endocrinol*, 143(6), pp. 769-73.

Turkalj, I., Keller, U., Ninnis, R., Vosmeer, S. and Stauffacher, W. (1992) 'Effect of increasing doses of recombinant human insulin-like growth factor-I on glucose, lipid, and leucine metabolism in man', *J Clin Endocrinol Metab*, 75(5), pp. 1186-91.

Unterluggauer, H., Hutter, E., Viertler, H.P. and Jansen-Durr, P. (2008) 'Insulin-like growth factor-induced signals activate mitochondrial respiration', *Biotechnol J*, 3(6), pp. 813-6.

Valk, N.K., vd Lely, A.J., de Herder, W.W., Lindemans, J. and Lamberts, S.W. (1994) 'The effects of human growth hormone (GH) administration in GH-deficient adults: a 20-day metabolic ward study', *J Clin Endocrinol Metab*, 79(4), pp. 1070-6.

van Dam, P.S., de Winter, C.F., de Vries, R., van der Grond, J., Drent, M.L., Lijffijt, M., Kenemans, J.L., Aleman, A., de Haan, E.H. and Koppeschaar, H.P. (2005) 'Childhood-onset growth hormone deficiency, cognitive function and brain N-acetylaspartate', *Psychoneuroendocrinology*, 30(4), pp. 357-63.

van Tienen, F.H., Praet, S.F., de Feyter, H.M., van den Broek, N.M., Lindsey, P.J., Schoonderwoerd, K.G., de Coo, I.F., Nicolay, K., Prompers, J.J., Smeets, H.J. and van Loon, L.J. (2012) 'Physical activity is the key determinant of skeletal muscle mitochondrial function in type 2 diabetes', *J Clin Endocrinol Metab*, 97(9), pp. 3261-9.

Vanhamme, L., Van Huffel, S., Van Hecke, P. and van Ormondt, D. (1999) 'Time-domain quantification of series of biomedical magnetic resonance spectroscopy signals', *J Magn Reson*, 140(1), pp. 120-30.

Veech, R.L., Lawson, J.W., Cornell, N.W. and Krebs, H.A. (1979) 'Cytosolic phosphorylation potential', *J Biol Chem*, 254(14), pp. 6538-47.

Verhelst, J., Abs, R., Vandeweghe, M., Mockel, J., Legros, J.J., Copinschi, G., Mahler, C., Velkeniers, B., Vanhaelst, L., Van Aelst, A., De Rijdt, D., Stevenaert, A. and Beckers, A. (1997) 'Two years of replacement therapy in adults with growth hormone deficiency', *Clin Endocrinol (Oxf)*, 47(4), pp. 485-94.

Verhelst, J., Kendall-Taylor, P., Erfurth, E.M., Price, D.A., Geffner, M., Koltowska-Haggstrom, M., Jonsson, P.J., Wilton, P. and Abs, R. (2005) 'Baseline characteristics and response to 2 years of growth hormone (GH) replacement of hypopituitary patients with GH deficiency due to adult-onset craniopharyngioma in comparison with patients with nonfunctioning pituitary adenoma: data from KIMS (Pfizer International Metabolic Database)', *J Clin Endocrinol Metab*, 90(8), pp. 4636-43.

Vieth, R., Bischoff-Ferrari, H., Boucher, B.J., Dawson-Hughes, B., Garland, C.F., Heaney, R.P., Holick, M.F., Hollis, B.W., Lamberg-Allardt, C., McGrath, J.J., Norman, A.W., Scragg, R., Whiting, S.J., Willett, W.C. and Zittermann, A. (2007) 'The urgent need to recommend an intake of vitamin D that is effective', *Am J Clin Nutr*, 85(3), pp. 649-50.

Vorgerd, M. and Zange, J. (2002) 'Carbohydrate oxidation disorders of skeletal muscle', *Curr Opin Clin Nutr Metab Care*, 5(6), pp. 611-7.

Wallymahmed, M.E., Foy, P., Shaw, D., Hutcheon, R., Edwards, R.H. and MacFarlane, I.A. (1997) 'Quality of life, body composition and muscle strength in adult growth hormone deficiency: the influence of growth hormone replacement therapy for up to 3 years', *Clin Endocrinol (Oxf)*, 47(4), pp. 439-46.

Wang, X.S. (2008) 'Pathophysiology of cancer-related fatigue', *Clin J Oncol Nurs*, 12(5 Suppl), pp. 11-20.

Wang, Y. and DeLuca, H.F. (2011) 'Is the vitamin d receptor found in muscle?', *Endocrinology*, 152(2), pp. 354-63.

Ward, K.A., Das, G., Berry, J.L., Roberts, S.A., Rawer, R., Adams, J.E. and Mughal, Z. (2009) 'Vitamin D status and muscle function in post-menarchal adolescent girls', *J Clin Endocrinol Metab*, 94(2), pp. 559-63.

Ward, K.A., Das, G., Roberts, S.A., Berry, J.L., Adams, J.E., Rawer, R. and Mughal, M.Z. (2010) 'A randomized, controlled trial of vitamin D supplementation upon musculoskeletal health in postmenarchal females', *J Clin Endocrinol Metab*, 95(10), pp. 4643-51.

Webb, A.R., Kift, R., Durkin, M.T., O'Brien, S.J., Vail, A., Berry, J.L. and Rhodes, L.E. (2010) 'The role of sunlight exposure in determining the vitamin D status of the U.K. white adult population', *Br J Dermatol*, 163(5), pp. 1050-5.

Webb, E.A., O'Reilly, M.A., Clayden, J.D., Seunarine, K.K., Chong, W.K., Dale, N., Salt, A., Clark, C.A. and Dattani, M.T. (2012) 'Effect of growth hormone deficiency on brain structure, motor function and cognition', *Brain*, 135(Pt 1), pp. 216-27.

Webb, S.M., Strasburger, C.J., Mo, D., Hartman, M.L., Melmed, S., Jung, H., Blum, W.F. and Attanasio, A.F. (2009) 'Changing patterns of the adult growth hormone deficiency diagnosis documented in a decade-long global surveillance database', *J Clin Endocrinol Metab*, 94(2), pp. 392-9.

Weitzel, J.M., Iwen, K.A. and Seitz, H.J. (2003) 'Regulation of mitochondrial biogenesis by thyroid hormone', *Exp Physiol*, 88(1), pp. 121-8.

Westerblad, H., Allen, D.G. and Lannergren, J. (2002) 'Muscle fatigue: lactic acid or inorganic phosphate the major cause?', *News Physiol Sci*, 17, pp. 17-21.

Whitehead, H.M., Boreham, C., McIlrath, E.M., Sheridan, B., Kennedy, L., Atkinson, A.B. and Hadden, D.R. (1992) 'Growth hormone treatment of adults with growth hormone deficiency: results of a 13-month placebo controlled cross-over study', *Clin Endocrinol (Oxf)*, 36(1), pp. 45-52.

Whitehead, H.M., Gilliland, J.S., Allen, I.V. and Hadden, D.R. (1989) 'Growth hormone treatment in adults with growth hormone deficiency: effect on muscle fibre size and proportions', *Acta Paediatr Scand Suppl*, 356, pp. 65-7; discussion 68, 73-4.

Widdowson, W.M. and Gibney, J. (2008) 'The effect of growth hormone replacement on exercise capacity in patients with GH deficiency: a metaanalysis', *J Clin Endocrinol Metab*, 93(11), pp. 4413-7.

Widdowson, W.M. and Gibney, J. (2010) 'The effect of growth hormone (GH) replacement on muscle strength in patients with GH-deficiency: a meta-analysis', *Clin Endocrinol (Oxf)*, 72(6), pp. 787-92.

Wiren, L., Bengtsson, B.A. and Johannsson, G. (1998) 'Beneficial effects of long-term GH replacement therapy on quality of life in adults with GH deficiency', *Clin Endocrinol (Oxf)*, 48(5), pp. 613-20.

Wong, R., Lopaschuk, G., Zhu, G., Walker, D., Catellier, D., Burton, D., Teo, K., Collins-Nakai, R. and Montague, T. (1992) 'Skeletal muscle metabolism in the chronic fatigue syndrome. In vivo assessment by 31P nuclear magnetic resonance spectroscopy', *Chest*, 102(6), pp. 1716-22.

Woodhouse, L.J., Asa, S.L., Thomas, S.G. and Ezzat, S. (1999) 'Measures of submaximal aerobic performance evaluate and predict functional response to growth hormone (GH) treatment in GH-deficient adults', *J Clin Endocrinol Metab*, 84(12), pp. 4570-7.

Woodhouse, L.J., Mukherjee, A., Shalet, S.M. and Ezzat, S. (2006) 'The influence of growth hormone status on physical impairments, functional limitations, and health-related quality of life in adults', *Endocr Rev*, 27(3), pp. 287-317.

Wu, J.S., Buettner, C., Smithline, H., Ngo, L.H. and Greenman, R.L. (2011) 'Evaluation of skeletal muscle during calf exercise by 31-phosphorus magnetic resonance spectroscopy in patients on statin medications', *Muscle Nerve*, 43(1), pp. 76-81.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C. and Spiegelman, B.M. (1999) 'Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1', *Cell*, 98(1), pp. 115-24.

Zenker, S., Haverkamp, F. and Klingmuller, D. (2002) 'Growth hormone deficiency in pituitary disease: relationship to depression, apathy and somatic complaints', *Eur J Endocrinol*, 147(2), pp. 165-71.

Ziambaras, K. and Dagogo-Jack, S. (1997) 'Reversible muscle weakness in patients with vitamin D deficiency', *West J Med*, 167(6), pp. 435-9.