THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

The Role of Growth Hormone in the Regulation of the Anaerobic Energy System and Physical Function

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A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

School of Medicine

Abstract

Growth hormone (GH) regulates energy metabolism and body composition in adult life. Adults with GH deficiency (GHD) suffer from lack of energy and from impaired physical functioning. GH supplementation improves sprinting in recreational athletes, a performance measure dependent on the anaerobic energy system (AES). The AES underpins the initiation of all physical activities including those of daily living. The physiological and functional significance of GH in regulation of the AES is unknown.

This thesis tests the hypothesis that GH positively regulates the AES and aspects of physical functioning in adult life. The key objectives are to 1) investigate whether anaerobic capacity is impaired in adults with GHD and improved by GH replacement, ii) characterise facets of physical function that are AES-dependent and GH responsive and iii) identify GH-regulated genes governing anaerobic metabolism in skeletal muscle.

Exercise capacity, body composition, physical function and quality of life (QoL) were studied in 19 adults with GHD before and after GH replacement. Anaerobic capacity was assessed by the 30-second Wingate test, and aerobic capacity by the VO_2max test. Physical function was assessed by the stair-climb test, chair-stand test, and 7-day pedometry. QoL was assessed by a GHD-specific questionnaire. Lean body mass (LBM) was quantified by dual-energy x-ray absorptiometry. Muscle biopsies were obtained before and after 1 and 6 months of GH replacement. GH responsive genes were identified by microarray analysis.

In a cross-sectional study, anaerobic capacity and aerobic capacity were significantly reduced compared to age-, gender- and body mass index-matched normal adults. The duration of the stair climb test was longer, the number of chair stand repetitions and daily step counts were lower in

adults with GHD who had lower QoL scores. GH status was an independent predictor of anaerobic capacity, which significantly and independently correlated with stair climb performance and QoL.

One month of GH replacement (0.5 mg/day) did not significantly change anaerobic capacity nor any of the other outcome measures compared to placebo in adults with GHD. Six months of GH treatment significantly increased LBM, anaerobic capacity, chair-stand repetitions, daily step count, and QoL scores but not VO_2max. Improvement in anaerobic capacity significantly correlated with an improvement in the energy and vitality domains of QoL. GH treatment did not significantly affect the differential expression of metabolic genes in skeletal muscle.

In summary, anaerobic capacity, aerobic capacity, physical function and QoL were impaired in adults with GHD. GH replacement improved anaerobic capacity, LBM, chair-stand performance, daily step counts and QoL in a time-dependent manner without a concomitant improvement in aerobic capacity. GH at a replacement dose was insufficient to induce the detection of GH-regulated genes governing substrate metabolism and energy production in skeletal muscle.

In conclusion, GH positively regulates the AES, which improves selective aspects of physical function influencing QoL in adult humans.

Declaration by author

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Publications during candidature

Publications:

Chikani V, Cuneo RC, Hickman I, Ho KK. 2016 GH enhances anaerobic capacity: Impact on physical function and quality of life in adults with GH deficiency. Clin Endocrinol (Oxf). doi:10.1111/cen.13147

Chikani V, Cuneo RC, Hickman I, Ho KK. 2015 Impairment of anaerobic capacity in adults with growth hormone deficiency. J Clin Endocrinol Metab 100:1811-1818

Chikani V, Ho KK. 2014 Action of GH on skeletal muscle function: molecular and metabolic mechanisms. J Mol Endocrinol 52:R107-123

Conference Abstracts:

Chikani V, Cuneo RC, Hickman I, Ho KK. Effects of GH replacement on Anaerobic Capacity in Adults with GHD. Endocrine Society of Australia Annual Scientific Meeting, Adelaide 2015

Chikani V, Cuneo RC, Hickman I, Ho KK. Growth Hormone therapy improves Anaerobic Exercise Capacity and Physical Function in Adults with Growth Hormone Deficiency. The Endocrine Society Annual Scientific Meeting, San Diego, USA 2015; FRI-446

Chikani V, Cuneo RC, Hickman I, Ho KK. Impairment of Anaerobic Capacity in Adults with Growth Hormone Deficiency.' 7th International Congress of the GRS and IGF Society, Singapore 2014

Chikani V, Cuneo RC, Hickman I, Ho KK. Effects of Growth Hormone on Anaerobic Capacity in Adults with Growth Hormone Deficiency: A Double-Blind Placebo-Controlled Trial. Endocrine Society of Australia Annual Scientific Meeting, Melbourne 2014

Chikani V, Cuneo RC, Hickman I, Ho KK. Growth hormone regulation of muscle function: Role of the Anaerobic Energy System. The Endocrine Society Annual Scientific Meeting, Chicago, USA 2014; MON-673

Chikani V, Cuneo RC, Hickman I, Ho KK. Growth hormone action on muscle function: role of the anaerobic energy system. Endocrine Society of Australia Annual Scientific Meeting, Sydney 2013

Publications included in this thesis

No publications included.

Contributions by others to the thesis

I performed RNA extraction from muscle tissue under supervision of Dr. Johanna Barclay and Dr. Chris Morais. I performed qRT PCR experiments under supervision of Dr. Caroline Nelson. I performed statistical analysis of the data under guidance of Dr. Anne Bernard and bioinformatics analysis of microarray data under guidance of Dr. Stephen Rudd, Queensland Facility for Advanced Bioinformatics.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

I would like to sincerely thank my supervisor, Professor Ken Ho for his guidance and support through out this degree. He has been a great teacher and mentor to me. I'd also like to thank Dr Ross Cuneo, my secondary supervisor for always being available to help and provide advice whenever needed.

I would like to thank the Princess Alexandra Research Foundation for providing a postgraduate scholarship and NovoNordisk for providing growth hormone and placebo to undertake this research project.

I am indebted to my research nurse Ms Tracy Grierson, who helped me with the clinical studies. The assistance from the Dr Caroline Nelson with the laboratory techniques and Dr Anne Bernard with statistical analysis has been invaluable.

I am grateful to Associate Professors Tony Russell and Warrick Inder, for their continual encouragement and support throughout this period.

Finally, I would like to thank my wife and my parents, without their unconditional and tireless support this would never have been possible.

Keywords

Growth hormone deficiency, energy metabolism, anaerobic energy system, exercise capacity, muscle function

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110306, Endocrinology, 70%

ANZSRC code: 110602, Exercise Physiology, 30%

Fields of Research (FoR) Classification

FoR code: 1103 Clinical Sciences, 70%

FoR code: 1106, Human Movement and Sports Science, 30%

Abbreviations used in the thesis

ADP	adenosine diphosphate
AES	anaerobic energy system
ANOVA	analysis of variance
ATP	adenosine triphosphate
B2M	beta 2 microglobulin
BMI	body mass index
CT	computerized tomography
CTP I	carnitine palmitoyl transferase I
CYR61	cysteine-rich, angiogenic inducer 61
DXA	dual energy x-ray absorptiometry
DUSP1	dual specificity phosphatase 1
EGR1	early growth response 1
FA	fatty acid
FABP-3	fatty acid binding protein-3
FASN	fatty acid synthase
FOS	FBJ murine osteosarcoma viral oncogene homolog
G0S2	G0/G1 switch 2
GH	growth hormone
GHD	growth hormone deficiency
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
IGFI	insulin like growth factor I
IL32	interleukin 32
LBM	lean body mass
MT2A	metallothionein 2A
LDH	lactate dehydrogenase

MHC	myosin heavy chain
MRI	magnetic resonance imaging
NADH	reduced nicotinamide adenine dinucleotide
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PDK-4	pyruvate dehydrogenase kinase-4
QoL	quality of life
QoL-AGHDA	quality of life assessment of GHD in adults
RCAN1	regulator of calcineurin 1
SCD1	stearoyl-CoA desaturase 1
TCA	tricarboxylic acid
TFRC	transferrin factor
TSPAN8	tetraspanin 8
UCP3	uncoupling protein 3
VO_2max	maximal oxygen uptake

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1 INTRODUCTION

Growth hormone (GH) is the most abundant hormone in the adult pituitary gland. It is a major metabolic hormone regulating carbohydrate, lipid and protein homeostasis (1). Adult-onset GH deficiency (GHD) is mainly caused by tumours of pituitary gland or of the hypothalamic area or by their treatment (i.e. surgery or irradiation) (2). It results in abnormal body composition reduced muscle strength and endurance capacity, and in impaired quality of life (QoL) (3).

Energy derived from the metabolism of fuels is required to drive the function of all body tissues. The energy is derived from two metabolic pathways: aerobic (oxygen-dependent) and anaerobic (oxygen-independent) (4). Aerobic energy is derived from oxidative pathways in the mitochondria whereas anaerobic energy is available as preformed adenosine triphosphates (ATP) or ATP generated from glycolysis. Energy derived from aerobic metabolism is the form mainly used at rest and during endurance exercise. In contrast, energy drawn from anaerobic metabolism powers intensive physical activity of brief duration. Anaerobic energy underpins the initiation of all form of physical activity including activities of daily living such as climbing stairs and running for a bus (5). Impairment of anaerobic capacity leads to increased fatigue during the execution of ordinary activities of daily living, which is the most common symptom associated with GHD. The anaerobic energy system (AES) has been a neglected area of fitness research, which has heavily focussed on the aerobic energy system.

Strength and power are measures of muscle function. Strength is the force producing capacity of muscle and it relies on preformed ATP. Power is the ability to perform muscular work per unit of time (i.e. work rate). Muscle power relies on either aerobic or anaerobic energy system, depending on the intensity and duration of work. There is strong evidence that muscle strength and aerobic

capacity are significantly reduced in adults with GHD and restored with GH replacement over a period of few months (3, 6). The question as to whether GH regulates the AES, or whether anaerobic capacity is impaired in GHD, has not been investigated. Evidence pointing to a regulatory role of anaerobic metabolism by GH comes from two recent studies, one on metabolic gene expression in skeletal muscle (7) and the other on physical performance (8). In patients with GHD, GH treatment inhibited the expression of mitochondrial genes involved in oxidative energy production and stimulated those governing glycolysis, the major energy generating anaerobic pathway (7). In the other study involving recreational athletes, GH enhanced sprint capacity, a performance measure dependent on the AES (8). The physiological significance of these findings is unclear.

This thesis investigates GH regulation of the AES in adults with GHD and its significance. The literature review will provide an overview of the consequences of GHD and the effects of GH on body composition, substrate metabolism and muscle function and relate the information on function to muscle structure and bioenergetics.

1.1 GH effects on body composition

Body composition refers to the compartments of the body with differing chemical composition and functional properties. It describes the percentage of fat, muscle and bone in the body. GH plays a vital role in regulating body composition by stimulating lipolysis and protein synthesis.

Various techniques are used to estimate body composition. These include quantifying total body water by isotope dilution (9, 10), total body potassium by ^{40}K counting (10-12), dual-energy x-ray absorptiometry (DXA) (13-17), computerized tomography (CT) (11, 18-21) and magnetic resonance imaging (MRI) (22, 23). These methods rely on various assumptions such as constant hydration state of lean body mass (LBM), constant intracellular potassium concentration or a constant fat-free extracellular compartment (3). These assumptions may not necessarily apply to adults with GHD and hence, caution must be exercised while interpreting results of body composition measurements. Although, each method relies on different principles, similar results are obtained. These studies have reported that adults with GHD manifest an increase in fat mass, more pronounced in abdominal and visceral adipose depots and a reduction in LBM compared to healthy controls (9, 10, 16, 24-26). Majority of these studies report that adults with GHD have 7-8% reduction in LBM, corresponding to around 4kg LBM (12, 27-30) and 7% increase` in fat mass (10, 16, 24-27, 31).

GH replacement therapy restores body composition to normal. On average up to a 5 kg increase in LBM and an equivalent reduction in fat mass occurs after replacement therapy without a change in body weight (3, 6, 12, 20, 26, 27, 31-39). Long-term studies of up to 10 years report that the beneficial effects of GH on body composition are sustained (11, 14, 40). Men are more responsive to GH replacement therapy than women. The cause is unclear but likely to involve the divergent modulatory effects of oestrogen and androgens on the action of GH (41).

It is generally accepted that changes in LBM due to GH replacement reflect its effects on skeletal muscle. This has been demonstrated in studies showing a parallel increase in thigh cross-sectional area (28, 37). Most of the initial trials used higher doses of GH than currently recommended by guidelines (42). These higher doses were associated with fluid retention causing significant cardiovascular adverse effects (43, 44). A recent meta-analysis of placebo-controlled randomized

trials investigating GH effects on body composition found a dose-response relationship on LBM and fat mass (45). It showed a 3.93 ± 1.5 kg increase in LBM and a 3.93 ± 1.05 kg decrease in fat mass for each mg increase in GH dose.

In conclusion, body composition in adults with GHD is abnormal, as a consequence of a reduction in LBM and an increase in fat mass, changes that are reversed by GH replacement.

1.2 GH effects on muscle mass and strength

Muscle strength is defined as maximal force (in newtons, N) or torque (in newton-metres, N.m) that is generated by a muscle or a group of muscles during maximal voluntary contraction (46). This force is determined by fast twitch type II muscle fibres and relies on preformed ATP for energy (47). Muscle strength is commonly assessed by measuring the force or torque produced during isometric or isokinetic contraction. Isometric strength is the maximal voluntary contraction that can be developed against an immovable object without a change in joint angle, whilst isokinetic strength is a measure of torque/force through a range of motion in which limb is moving at a constant velocity (46).

Muscle strength is significantly reduced in adults with GHD (48, 49) (Table 1.1). The force generated is usually corrected for muscle area (cm^2) or volume (cm^3) to distinguish between the contributions of muscle mass and contractile quality. Janssen et al. reported a significant reduction in strength and volume of quadriceps muscle in adults with GHD compared to those of age- and height-matched controls (50). These findings suggest that diminished strength in GHD arise from reduced muscle mass rather than from reduced contractile function. Sartorio et al. found that the strength of quadriceps muscle in adults with GHD was reduced in proportion to a reduction in muscle mass (32). These results stand in contrast to those of Cuneo et al. who found that quadriceps

muscle force was reduced in adults with GHD when corrected for muscle area (51). These authors hypothesized that contractile properties, energy metabolism or neuromuscular function of skeletal muscle are impaired in the GH deficient state. Janssen et al. attributed the disagreement to the possible inaccurate muscle mass assessment from a single slice CT scan (51) as opposed to a more precise method from using multiple MRI slices in their study (50). Muscle biopsy studies in adults with GHD have failed to identify any qualitative differences in fibre types compared to healthy adults. Thus, it is likely that muscle strength in GHD is reduced from diminished mass rather than a change in contractile quality.

Table 1.1 Studies comparing muscle strength of adults with GHD with healthy controls

Study	Total No.	Mean Age years	Gender (M:F)	Type	Diagnostic Criteria	Control	Outcome
(48)	56	45±2	35:21	Mixed	Peak GH <1.7µg/L during insulin induced hypoglycaemia	Reference population of Goteborg. n=144, age 40-79 years matched for mean Ht and Wt	Lower isometric muscle strength in quadriceps and hamstring muscles. The peak handgrip strength was 83% and average 10-s handgrip strength was 81% of healthy control.
(49)	14	41.8±17.3	9:5	Mixed	Peak GH <6.0mU/L during insulin induced hypoglycaemia or oral clonidine	14 age and gender matched controls.	Lower isometric strength (84% of maximal predicted value for age gender and Ht).
(50)	28	49±2	28:0	Mixed	Peak GH <7.0mU/L during insulin induced hypoglycaemia	20 age and Wt matched controls	Lower maximal isometric strength. Maximal isokinetic strength tended to be lower (p=0.06)
(51)	24	39±2	16:8	AO	Peak GH <3.0mU/L during insulin induced hypoglycaemia	41 age, gender and Wt matched controls	Lower quadriceps force/body Wt (N/kg). Lower quadriceps force/quadriceps area (N/cm²).
(32)	8	29.6±3.4	8:0	CO	Peak GH < 5.0ng/ml to GHRH plus galanin & also to L-dopa plus propranolol	8 age and gender matched controls	Lower quadriceps isometric strength (63% of the controls)
(52)	6	29±3	3:3	CO	Peak GH < 3.4µg/L to insulin plus arginine stimulation test	Published normal values	Lower torque at speed of 30°/s and angular position of 45° during knee flexion/extension

Wt, weight; Ht, height; CO, childhood onset; AO, adult onset; N, Newton; GH, growth hormone; GHD, growth hormone deficiency; GHRH, growth hormone releasing hormone

Studies investigating the effects of GH replacement on muscle strength have provided conflicting results (Table 1.2 and 1.3). Jorgensen et al. observed that muscle strength did not change significantly after GH replacement for 4 months (20) but improved significantly after 12 months, with the improvement sustained at the end of 38 months of treatment (37, 53).

Similarly, a number of other investigators have observed a lack of effect in the short term but a significant increase in muscle strength after extended treatment (34, 54-56). In an open label prospective study of 109 adults with GHD, GH therapy normalized the strength of different muscle groups over 10 years of therapy (57). The majority of studies assessing GH effects beyond 12 months have reported a significant improvement in muscle strength (48-50, 57, 58), whereas trials of less than 6 months duration have not (20, 28, 34, 54-56, 59). The studies that show an increase in strength, also report a concomitant increase in muscle mass after long-term GH therapy (37, 50, 53). The collective findings indicate that GH replacement beyond 12 months is required to improve muscle strength in adults with GHD, reflecting the time taken to restore muscle mass towards normal. In summary, the collective evidence indicates that GH increases muscle strength by increasing muscle mass.

Table 1.2 The effects of GH on muscle strength in adults with GHD: Summary of Placebo-Controlled trials

Study	GHD patients	Diagnosis of GHD	Study Design	GH Dose	Effects of GH compared to placebo
(20)	n=22 M:F 14:8, CO Mean Age 23.8±1.2 years	Peak GH < 5µg/L after clonidine stimulation test	4 months DBPC crossover	2IU/m²	No significant change in isometric strength
(60)	n=55 M:F 31:24, AO Mean Age 49 years	Peak GH < 3µg/L to insulin induced hypoglycaemia	9 months DBPC crossover, 4 months washout	1.2IU/day for men 1.8IU/day for women	No significant change in isokinetic knee extensor strength
(54)	n=53 M:F 23:20, mixed Age 21-60 years	Peak GH < 5mg/L during insulin induced hypoglycaemia or GHRH stimulation test	6 months DBPC	0.125IU/kg/week for first week; thereafter 0.25IU/kg/week	No significant change in strength
(59)	n=28 M:F 15:13, AO Age 18-68 years	Peak GH <3.0µg/L during insulin induced hypoglycaemia	3 months DBPC crossover, 1 month washout	6.25µg/kg LBM for first month; 12.5µg/kg LBM thereafter	No significant change in isometric handgrip strength, isotonic arm, leg or chest press or isokinetic knee flexion / extension strength
(34)	n=40 M:F 19:21, mixed Age 19-67 years	Peak GH <6.0mU/L during insulin induced hypoglycaemia or oral clonidine	6 months DBPC	0.02 to 0.05IU/kg	No significant change in strength
(56)	n=30 M:F 10:20, mixed Age ~35 years	Peak GH <10.0mU/L after glucagon or insulin induced hypoglycaemia	6 months DBPC	0.125U/kg/week for first month; thereafter 0.25U/kg/week	No significant change in isometric quadriceps muscle strength
(21)	n=29 M:F 19:10, AO Mean Age 45.5±2 years	Peak GH <10.0µg/L during insulin induced hypoglycaemia	12 months DBPC	2IU/m²	No significant change in isometric quadriceps strength
(61)	n=24 M:F 16:8, AO Mean Age 39±2 years	Peak GH <3.0mU/L during insulin induced hypoglycaemia	6 months DBPC	0.07U/kg/day	Significant increase in hip flexion strength
(55)	n=35 M:F 18:17, mixed Mean Age 39.8 years	Peak GH <10.0mU/L after glucagon or insulin induced hypoglycaemia	6 months DBPC	0.125IU/kg/week for first 4 weeks; thereafter 0.25IU/kg/week	No significant change in isometric quadriceps strength
(28)	n=14 M:F 9:5, AO	Peak GH <7.0mU/L during insulin induced	6 months DBPC crossover with 1	0.5U/kg/week	No significant change isokinetic knee extension strength

Study	GHD patients	Diagnosis of GHD	Study Design	GH Dose	Effects of GH compared to placebo
	Mean Age 29.4±2.7 years	hypoglycaemia	month washout		
(52)	n=6 M:F 3:3, CO Mean Age 29±3 years	Peak GH < 3.4µg/L during insulin plus arginine stimulation test	3 months DBPC crossover with 3 month washout	0.5-0.6IU/kg/week	No significant change in isokinetic knee flexion and extension strength

DBPC, double blind placebo controlled; Wt, weight; Ht, height; CO, childhood onset; AO, adult onset; GH, growth hormone, GHD, growth hormone deficiency, IGFI, insulin like growth factor I; GHRH, growth hormone releasing hormone; LBM, lean body mass

Table 1.3 The effects of GH on muscle strength in adults with GHD: Summary of open label trials

Study	GHD patients	Diagnosis of GHD	Study Design	GH Dose	Effects of GH
(53)	n=13 M:F 9:4, CO Mean Age 24.4±1.7 years	Peak GH < 5µg/L after clonidine stimulation test	16 months open label	Median 2.9IU/m² (1.2-3.8IU/m²)	Significant increase in isometric strength of quadriceps
(37)	n=10 M:F 7:3, CO Mean Age 28.4±2.3 years	Peak GH < 5µg/L after clonidine stimulation test	37.6 months open label	2IU/m²	Significant increase in isometric strength of quadriceps
(58)	n=109 M:F 61:48, AO Mean Age 50 years	Peak GH < 3µg/L during insulin hypoglycaemia (n=95); 2 additional hormone deficiency plus 24h GH profile (n=9); 1 additional hormone deficiency plus 1 stimulation test (n=4)	5 years open label	First 80 patients, starting dose 0.25IU/kg/week and individualized when Wt based regimen abandoned. In other patients, individualized from the beginning.	Significant increase in isometric knee flexor strength, concentric knee flexor strength and right handgrip strength. No significant increase in isometric knee extensor strength, concentric knee extensor strength and left handgrip strength.
(32)	n=8 M:F 8:0, CO Mean Age 29.6±3.4 years	Peak GH < 5ng/ml during 2 stimulation tests, GHRH plus galanin and L-dopa plus propranolol	6 months open label	0.5IU/kg/week	Significant increase in isometric quadriceps strength.

Study	GHD patients	Diagnosis of GHD	Study Design	GH Dose	Effects of GH
(54)	n=53 M:F 23:20, mixed Age 21-60 years	Peak GH < 5mg/L during insulin induced hypoglycaemia or GHRH stimulation test	6 months open label	0.125IU/kg/week for first week; thereafter 0.25IU/kg/week	Significant increase in knee extension and arm flexion strength.
(34)	n=40 M:F 19:21, mixed Age 19-67 years	Peak GH <6.0mU/L during insulin induced hypoglycaemia or oral clonidine	12 and 18 months open label	0.05IU/kg	12 months: Significant increase neck flexion, elbow flexion and extension, and hip flexion and extension 18 months: Significant increase in neck flexion and elbow extension
(49)	n=6 M:F 3:3, mixed Mean Age 41.8±17.3 years	Peak GH <6.0mU/L during insulin induced hypoglycaemia or oral clonidine	6 to 24 months open label	0.04±0.01IU/kg/day	Significant increase in maximal voluntary isometric strength.
(56)	n=30 M:F 10:20, mixed Age ~35 years	Peak GH <10.0mU/L after glucagon or insulin induced hypoglycaemia	6 months open label 2 years open label (n=12)	0.125U/kg/week for first month; thereafter 0.25U/kg/week	6 months: No significant change in isometric quadriceps muscle strength. 2 years: Significant improvement in quadriceps muscle strength
(50)	n=28 M:F 28:0, mixed Mean Age 49±2 years	Peak GH <7.0mU/L during insulin induced hypoglycaemia	12 months open label	First 24 week, 0.6, 1.2 or 1.8U and thereafter individualized dosing to normalize IGFI	Significant increase in isokinetic muscle strength. No change in isometric muscle strength.
(48)	n=56 M:F 35:21, mixed Mean Age 45±2 years	Peak GH <1.7µg/L during insulin induced hypoglycaemia	2 years open label	Individualized according to IGFI level. Mean GH dose 0.62±0.03mg/day	Significant increase in knee extension and flexion strength No change in handgrip strength.
(55)	n=35 M:F 18:17, mixed Mean Age 39.8 years	Peak GH <10.0mU/L after glucagon or insulin induced hypoglycaemia	6 months open label	0.125IU/kg/week for first 4 weeks; thereafter 0.25IU/kg/week	Significant increase in isometric quadriceps muscle strength

Wt, weight; Ht, height; CO, childhood onset; AO, adult onset; GH, growth hormone, GHD, growth hormone deficiency, IGFI, insulin like growth factor I; GHRH, growth hormone releasing hormone; LBM, lean body mass

Implicit in these studies of GH and muscle strength is the mediatory role of insulin like growth factor I (IGFI), which stimulates proliferation and differentiation of satellite cells into myoblasts and formation of new myofibres (62, 63). IGFI knockout mice exhibit muscle hypoplasia (64), whereas overexpression of IGFI leads to muscle hypertrophy (65) and accelerates muscle regeneration after disuse atrophy (66). Kim et al. observed a significantly increased muscle mass and stimulation of satellite cells and myofibre hypertrophy in skeletal muscle of wild type mice treated with GH, but these effects were absent in mice that lacked a functioning IGFI receptor in skeletal muscle (67). These studies indicate that the action of GH on muscle growth and strength are mediated via IGFI.

Only a few double blind placebo controlled studies have investigated the effect of GH on muscle strength in healthy adults (8, 68-71). A 6-week GH administration failed to demonstrate any effect on maximal muscle strength in 8 healthy males (68). Similarly, in a study of nearly 100 recreational athletes, muscle strength did not increase after 8-week of GH treatment (8). GH administration in 16 healthy men combined with resistance exercise did not further enhance muscle strength more than exercise alone after 3 months (69). Studies in healthy elderly subjects have also failed to observe any increase in muscle strength following 6 months of GH therapy (70, 71). These studies suggest that short-term GH therapy does not enhance muscle strength in healthy adults; however, the effects of long-term GH treatment are yet to be evaluated in this population.

In summary, GH increases muscle strength by increasing muscle mass in adults with GHD, an effect that is IGFI mediated. At present, there is no evidence to support a role of GH in the enhancement of contractile function of skeletal muscle.

1.3 GH regulation of functional muscle proteins and muscle fibre type distribution

Skeletal muscle is composed of fibres that are made up of different proteins with distinct properties. Actin and myosin are functional proteins that are responsible for the contractile function of muscle, whereas tropomyosin and troponin are structural proteins that keep the contractile proteins in proper alignment, and give muscle fibres elasticity and extensibility. Myosin protein consists of 2 heavy chains and 4 light chains. Muscle fibres are classified by myosin heavy chain (MHC) isoforms mainly into 2 types (72). Type I fibres, also known as slow twitch fibres, contain an abundance of mitochondria and rely on aerobic or oxidative pathways for energy production. These fibres determine the endurance capacity of muscle. In contrast, type II fibres, also known as fast twitch fibres, generate energy from anaerobic or glycolytic pathways due to their low mitochondrial content. These fibres have high contractile force, but easy fatigability. They subserve high intensity activities such as sprinting and weight lifting.

MHC isoforms are distinguished by various methods including myofibrillar adenosine triphosphatase staining (73), immunohistochemistry with specific MHC isoform antibodies (74) and electrophoretic isoform separation (75). Several factors determine fibre type distribution in skeletal muscle. These include age, exercise, functional usage, neural input and hormones (76). For example, ageing is associated with a reduction in type II fibres (77), whereas thyroid hormone excess leads to a reduction in type I fibres (78). The effects of GH on contractile muscle proteins have been investigated in rodents and humans by studying the consequences of GHD and GH treatment.

1.3.1 Animal Studies

Yamaguchi et al. reported a significant increase in type I fibres and decrease in type II fibres in rodents after hypophysectomy (79). These findings were supported by Roy et al., who observed a significant increase in fibres expressing MHC type I in hypophysectomized rats (80). A study investigating the long-term effects of hypophysectomy in rats reported a complete loss of type II fibres after 33 months (81). In contrast to these findings, Ayling et al. reported 50% reduction in type I fibres after hypophysectomy (82). Loughna et al. also observed a significant reduction of type I and an increase in type II MHC mRNA expression in hypophysectomized rats (83). In these studies, GH replacement almost completely reversed the changes observed after hypophysectomy (82, 83). However, some studies have reported no change in the composition of type I or type II fibres after GH replacement in hypophysectomized rats (80, 84). The reasons for these discrepancies are unclear. Possible explanations include the variable duration of GHD, which ranged between 21 days to 50 days, and the duration of GH therapy, which ranged from 7 days to 33 months. Most studies did not account for the effects of other pituitary hormone deficiencies on muscle fibre types, in particular thyroid hormone. When investigating the effects of GH in normal rats, Florini et al. observed no significant change in the number of type I or type II fibres after six months (85). These results in normal rats have been confirmed by other groups (86-88).

1.3.2 Human Studies

There are few human studies investigating GH regulation of muscle fibre composition, and most of these entail small numbers. Most studies in adult subjects with GHD have reported no significant difference in fibre type distribution from matched normal subjects (89-91). A time-dependent relationship between the duration of GHD and fibre type composition is unlikely from a comparison of findings between patients with childhood-onset and adult-onset GHD (89-91). Daugaard et al.

found no relationship between IGFI levels and MHC composition, suggesting that the severity of GHD does not influence MHC composition (92). Studies of GH replacement up to 6 months have reported no significant change in muscle fibre composition in adults with GHD (90-92). One of these studies reported an increase in muscle size and improvement in endurance capacity, but observed no change in the number of type I or II fibres (90). It is unclear from this study whether the relationship between the improvement in endurance and in type I fibre size is associative or causal. This study did not test muscle function reflective of type II fibre type that subserve high intensity contractile activity. There is insufficient evidence to support a role of GH in regulating type I or II fibres in human skeletal muscle, and more studies with larger numbers are required to determine whether GH regulates skeletal muscle fibre composition.

1.4 GH effects on protein metabolism

Protein turnover is defined as the continuing breakdown and synthesis of proteins, with recycling of amino acids. At a steady state, the rate of protein breakdown equals the rate of protein synthesis, and there is no net gain or loss of proteins. Amino acids released from protein breakdown are either reutilized in protein synthesis or irreversibly lost via oxidation. Over the last 2 decades, isotope tracer methods such as leucine turnover technique have made it possible to accurately measure these components of whole body protein metabolism by tracking the metabolic fate of a labeled amino acid (93).

LBM and muscle mass are reduced in adults with GHD suggesting that there is an underlying perturbation of protein metabolism (6). Hoffman et al. compared protein metabolism in 10 patients with GHD and healthy controls using labeled leucine and found that the rate of protein synthesis and breakdown were significantly reduced in GHD subjects (94). These results corroborate previous

findings of Beshyah et al. and suggest that the whole body protein turnover is reduced in adults with GHD (95).

GH replacement in adults with GHD improves protein balance by partitioning amino acids away from oxidative towards synthetic pathways (96-100). Russell-Jones et al. observed an increase in protein synthesis and a reduction in protein oxidation without any change in protein breakdown after 2 months of GH in subjects with GHD (99). The same findings were obtained by Shi et al. following 2 weeks, and by Binnerts et al. after 1 month of GH therapy in adults with GHD (27, 100). These anabolic effects of GH are also observed in healthy adults supplemented with GH. In healthy subjects, Copeland and Nair et al. observed an acute reduction in whole body protein oxidation following GH administration (101). Similar effects were reported by Horber and Haymond et al. in a study undertaken in a fasting and fed state following 7 days of GH treatment in healthy volunteers (102).

Long-term studies in adults with GHD have observed attenuation in anabolic effects after prolonged therapy (95, 100). Binnerts et al. observed that GH-induced reduction in protein oxidation was diminished after 6 months of GH therapy (27). In another study, the reduction of protein oxidation seen at 2 weeks of GH therapy had returned to baseline at 12 weeks, indicating a waning GH effect with time (103). Studies have shown that the reduction in protein oxidation is a predictor of a later increase in LBM, which occurs over the first few months (95, 103). Thus, GH causes a time-dependent change in whole body protein metabolism. In the early weeks, GH reduces the rate of protein oxidation, leading to an accrual of protein mass. However, as the increase in LBM begins to plateau, there is a gradual return in the rate of protein oxidation to baseline, protein balance reaches a new steady state. The metabolic mechanisms accounting for this adaptation are unknown.

Whole body protein turnover studies do not provide information regarding the site of protein synthesis although it is widely assumed that this is muscle. To address the direct effects of GH on skeletal muscle protein turnover, investigators have measured the arterio-venous difference of labeled and unlabeled amino acids across the forearm or leg (93). Using this technique, Fryburg et al. reported that GH induced an increase in protein synthesis without affecting the rate of protein breakdown in forearm muscles (104, 105). However, Copeland et al. found no significant stimulation of protein synthesis in the leg during GH infusion, despite observing a concomitant stimulation of whole body protein synthesis (101). The latter findings were corroborated by Yarasheski et al. who also failed to observe any effect on protein synthesis of quadriceps muscle following GH therapy (106). These observations suggest a greater proportion of whole body protein anabolism occurs in tissues and organs than in skeletal muscle. This could explain why the improvement by GH in muscle strength in GHD is slow, and the paucity of evidence supporting a beneficial effect in GH replete subjects.

According to the somatomedin hypothesis, the anabolic action of GH is mediated by circulating IGFI, which is mainly derived from the liver (107, 108). However, it is recognized that IGFI, produced locally in tissues under GH stimulation, mediate some of the growth promoting actions of GH. (62, 108). The extent to which circulating and local IGFI contributes to tissue growth has been the subject of great interest in the field and remains controversial. Human studies employing recombinant IGFI provide the strongest evidence that circulating IGFI is anabolic. IGFI enhances protein anabolism by reducing the rate of proteolysis, an action similar to that of insulin (109-111). When IGFI is infused in rats, it leads to a reduction in protein breakdown without any change in protein synthesis (110). Thus, the protein anabolic effects of systemic IGFI are similar to insulin and different from GH, which regulates the metabolic fate of amino acids from oxidative to synthesis pathways. These observations indicate that the effects of GH on amino acid fluxes are mediated by mechanisms in addition to those mediated by IGFI.

In summary, GH regulates protein anabolism via IGFI-dependent endocrine and paracrine mechanisms as well as IGFI independent pathways. The net effect of GH on whole body protein metabolism is the metabolic partitioning of amino acids towards synthesis and away from irreversible oxidative loss, but with tissue effects that differ between muscle and extra-muscular tissues.

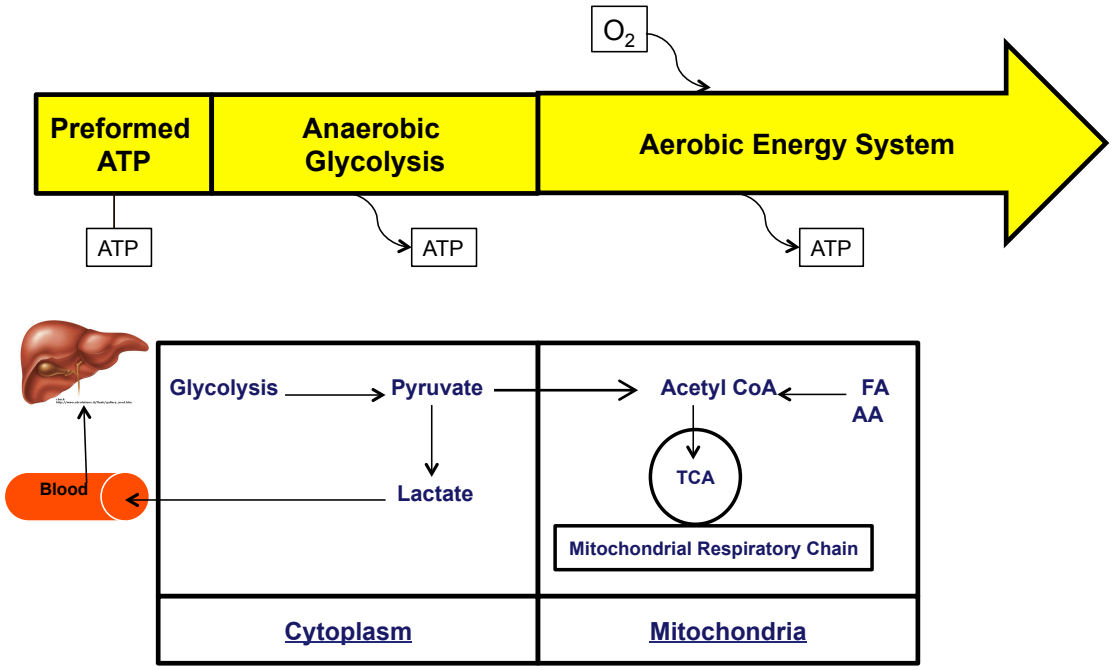
1.5 GH and substrate metabolism in skeletal muscle

1.5.1 Bioenergetics in skeletal muscle

1.5.1.1 The Energy Continuum

The contractile function of skeletal muscle relies on a constant supply of chemical energy. During muscle contraction, chemical energy is converted to mechanical energy that leads to movement. In humans, chemical energy is available in the form of ATP, which is generated by two energy systems: aerobic and anaerobic (4). The aerobic energy system relies on oxygen whereas the AES does not. Although, both systems function as a continuum, physical activity/exercise is described in terms of aerobic or anaerobic, depending on which system predominates. Figure 1.1 illustrates the metabolic processes involved in energy production in a muscle cell and the concept of energy continuum during physical activity.

Figure 1.1 Anaerobic and aerobic energy systems.
AA, amino acid; FA, fatty acid; TCA, tricarboxylic acid cycle



1.5.1.1.1 Glycolysis

Glycolysis, or glucose breakdown, is an essential pathway of energy metabolism in all tissues. Glycolysis leads to the formation of either pyruvate or lactate depending on the oxidative status of the cell (Figure 1.1). Pyruvate is the end product of glycolysis in cells with mitochondria and adequate oxygen supply. This series of reactions is called aerobic glycolysis because oxygen is required to reoxidize NADH (reduced nicotinamide adenine dinucleotide), an energy-rich coenzyme formed during the process. Pyruvate is then converted into Acetyl CoA by an enzyme located in mitochondrial matrix called pyruvate dehydrogenase (PDH). Acetyl CoA is a major source of fuel for oxidative energy production in mitochondria. In tissues with inadequate oxygen supply (for example, skeletal muscle during intense exercise) or cells that lack mitochondria (for

example, red blood cells), pyruvate is reduced by NADH in the presence of lactate dehydrogenase (LDH) to form lactate. This conversion of glucose to lactate is called anaerobic glycolysis because there is no net gain of NADH. Lactate is then released into the blood and converted back to glucose via gluconeogenesis in the liver.

Aerobic or anaerobic glycolysis to pyruvate or lactate yields 2 ATP. The subsequent metabolism of the 2 pyruvate molecules yields 34 ATP on complete oxidation via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondrial respiratory chain.

Stoichiometry of glucose metabolism

Anaerobic glycolysis:

$$\text{Glucose} + 2\ \text{Pi} + 2\ \text{ADP} \longrightarrow 2\ \text{Lactate} + 2\ \text{ATP} + 2\ H_2O$$

Aerobic glycolysis:

$$\text{Glucose} + 2\ \text{Pi} + 2\ NAD^+ + 2\ \text{ADP} \longrightarrow 2\ \text{Pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+ + H_2O$$

Complete oxidation of glucose:

$$\text{Glucose} + 6\ O_2 + 36\ \text{Pi} + 36\ \text{ADP} \longrightarrow 36\ \text{ATP} + 6\ CO_2 + 42\ H_2O$$

1.5.1.1.2 The aerobic energy system

The aerobic energy system relies on the generation of ATP from oxidation of Acetyl CoA in the mitochondria. Acetyl CoA is derived from the metabolism of substrates such as carbohydrates, lipids and protein. It undergoes a series of reactions in the TCA cycle where four pairs of electrons

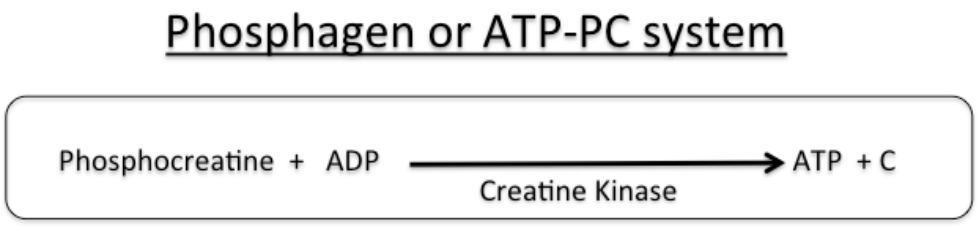
via energy-rich coenzymes are transported to the mitochondrial respiratory chain, generating 12 ATP from each molecule of Acetyl CoA oxidized.

The aerobic energy system provides energy at rest, as well as that required for sustaining long duration of physical activity such as prolonged walking at a comfortable pace. During the transition from rest to physical activity, oxygen consumption is increased to meet the increased energy demands. After a few minutes, the rate of oxygen consumption reaches a new steady state and oxidative energy production matches energy consumption. However, the body relies predominantly on anaerobic energy at the start of any physical activity, until aerobic energy metabolism increases to supply the new energy needs.

1.5.1.1.3 The anaerobic energy system

The AES is comprised of an alactic system and a lactic acid system (5). The alactic system (Figure 1.2) consists of preformed ATP and phosphocreatine (PCr), which donates a phosphate group to ADP (adenosine diphosphate) to form ATP instantaneously. The lactic acid system provides energy from anaerobic glycolysis. The alactic system provides energy for the first few seconds (~ 10 seconds) of physical activity; thereafter anaerobic glycolysis provides energy for the next 30-40 seconds, when aerobic metabolism begins to take over and provides energy for prolonged sustained activity (112).

Figure 1.2 Alactic anaerobic system.
ADP, adenosine diphosphate; ATP, adenosine triphosphate



1.5.2 GH effects on substrate metabolism in skeletal muscle

Muscle function is dependent on the availability of metabolic fuels and its capacity to synthesize ATP. The energy synthesis from substrate utilization in exercising muscle is regulated by nutritional, genetic, and hormonal factors as well as physical training. GH stimulates lipolysis at rest (113-115) and during exercise (116, 117), leading to an increase in plasma fatty acid (FA) levels. GH also increases plasma glucose concentration by various mechanisms including augmentation of glycogenolysis (118) and gluconeogenesis (119). Thus, GH may enhance muscle function by increasing availability of FA and glucose as metabolic fuels for energy production.

GH stimulates whole body lipid oxidation and reduces carbohydrate utilization in healthy adults (113, 120, 121) and in adults with GHD (122-124). Given that LBM accounts for the majority of substrate metabolism in the body (125), and muscle comprises almost 50% of total LBM, it is widely assumed that an increase in whole body lipid oxidation is a reflection of its action on skeletal muscle, however, very limited evidence is available to support this assumption. This traditional thinking was recently challenged by studies in rodents as well as humans, indicating that

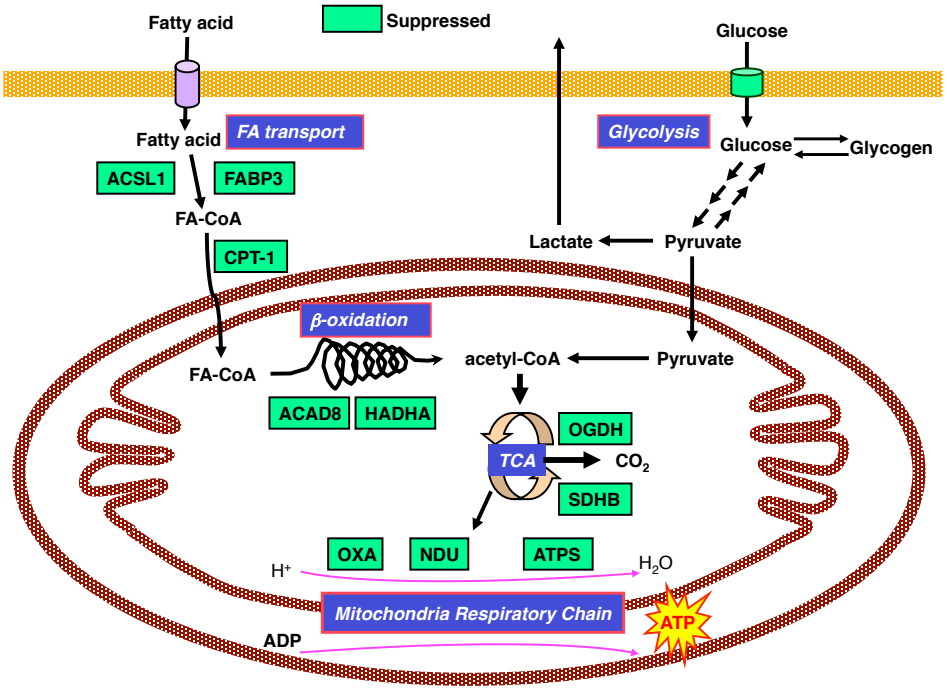
GH action is rather tissue specific. These studies used DNA microarray technology as a platform to study GH effects on global changes in metabolic gene expression in the target tissues.

Tollet-Egnell et al. studied effects of GH on liver and skeletal muscle of rats (126). In the liver, GH reduced expression of genes for lipid synthesis, such as fatty acid synthase and stearyl-CoA desaturase, but increased genes for fat oxidation including carnitine palmitoyl transferase I (CPT I), a key enzyme for the transfer of FA from cytosol into the mitochondrial matrix. Whereas in skeletal muscle, genes for FA uptake were reduced, and glycolysis were increased upon GH treatment. These data indicate that GH action on substrate metabolism is tissue specific.

Sjogren et al. studied effects of GH replacement on metabolic gene expression in skeletal muscle of adults with GHD (7). These authors found a striking difference between the whole body and skeletal muscle substrate metabolism following GH treatment. GH stimulated whole body resting energy expenditure, lipid oxidation and reduced carbohydrate oxidation. In skeletal muscle, however, GH downregulated key genes governing lipid oxidation, TCA cycle activity and mitochondrial respiration as well as genes that suppress glucose utilization (Figure 1.3). Expression of genes involved in FA transport such as fatty acid binding protein-3 (FABP-3) and CTP-I fell by 50%. Genes for several enzymes that mediate β-oxidation of FA and ATP synthesis via oxidative pathway in the mitochondria were also suppressed in skeletal muscle following GH therapy. For example, the expression of oxoglutarate dehydrogenase and succinate dehydrogenase complex B in the TCA cycle and ATP synthase and NADH dehydrogenase in the mitochondrial respiratory chain were reduced by up to 40%. GH also reduced the transcripts levels of pyruvate dehydrogenase kinase-4 (PDK-4) and glycogen synthase-I in muscle. The latter has been shown in previous studies in healthy (127) as well as adults with GHD (128). PDK-4 is an inhibitor PDH, the key enzyme that

converts pyruvate to Acetyl CoA for oxidation in mitochondria (129). These findings indicate that GH favours glucose as a metabolic fuel over lipid in skeletal muscle.

Figure 1.3 GH effects on metabolic gene expression in skeletal muscle.
Schematic diagram of changes in the expression of key genes in skeletal muscle governing the oxidative metabolism of fatty acids and glucose after GH therapy [adapted from the study by Sjogren et al. (7)]. Metabolic genes that were down regulated by GH in skeletal muscle are boxed in green with the abbreviated names are expanded below. FA, Fatty acid; TCA, tricarboxylic acid cycle; ADP, adenosine diphosphate; ATP, adenosine triphosphate. Lipid metabolism: FABP3, Fatty acid-binding protein-3; ACSL, Acyl-CoA synthetase, long chain; CPTI, Carnitinepalmitoyltransferase I; ACAD8, Acyl-CoA dehydrogenase, family member 8; HADHA, Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase; TCA cycle: OGHD, Oxoglutarate dehydrogenase; SDHB, Succinate dehydrogenase complex B; Mitochondrial chain: OXA, Oxidase; NDU, NADH dehydrogenase; ATPS, ATP synthase



Assuming that the transcriptional changes reflect effects on protein expression, these findings suggest that GH inhibits oxidative metabolism of substrates and may favour non-oxidative (anaerobic) pathways for ATP synthesis in skeletal muscle. This was a small uncontrolled study conducted in only 5 subjects with GHD administered GH for two weeks. However the findings are supported by a study in trained cyclists, in whom GH administration increased plasma lactate levels

during intense exercise compared to placebo, consistent with an increased rate of anaerobic disposal of pyruvate (130).

In summary, the effects of GH on substrate metabolism are tissue specific. GH may promote non-oxidative or anaerobic substrate metabolism in skeletal muscle for ATP synthesis, findings contrary to its effects on whole body metabolism. Further work is required to confirm gene expression findings in a larger cohort in a placebo-controlled design.

1.6 GH effects on muscle power

Muscle power is defined as work performed per unit of time and is expressed in joules per second or watts. It is described in terms of aerobic and anaerobic power, depending on which energy source is predominantly utilized to do the work. Thus, muscle power can be assessed by measuring aerobic exercise capacity and anaerobic exercise capacity.

1.6.1 Aerobic exercise capacity

Aerobic exercise capacity is a measure of endurance i.e. the muscle's ability to sustain work for a prolonged period with energy provided principally from oxidation of carbohydrates or lipids in the mitochondria. In the athletic world, it determines performance in sports such as distance running and swimming, while in day-to-day life, it supports activities such as walking. Aerobic exercise capacity is a stronger predictor of mortality in men than any other established risk factors for cardiovascular disease such as hypertension, smoking, and diabetes (131). It is determined by the measurement of maximal oxygen uptake (VO_2max) in L/min or ml/kg/min or maximal aerobic power output in watts or kilojoules during an incremental exercise test on a cycle ergometer or a treadmill (132).

Studies in subjects with GHD have provided strong evidence that GH is a significant positive regulator of aerobic exercise capacity. Cuneo et al. reported a mean reduction of 28% in VO_2max in adults with GHD compared to their maximum predicted value based on age, weight and height (133). Many studies have reported a similar degree of impairment in aerobic exercise capacity in these individuals (28, 134, 135).

Numerous double blind placebo controlled trials have investigated GH effects on aerobic exercise capacity in adults with GHD (Table 1.4). In a study of 22 adults with GHD, aerobic exercise capacity increased significantly after 4 months of GH therapy and was sustained for up to 38 months of GH treatment (20, 37). Cuneo et al. observed a near normalization of VO_2max over a period of 6 months with GH replacement in a study involving 24 adults with GHD (133). Most of these studies show an improvement in VO_2max and/or maximal aerobic power output following GH therapy for 4 to 12 months (20, 21, 28, 37, 53, 60, 133, 134). A few studies failed to show a positive effect of GH on aerobic exercise capacity in comparison to placebo (43, 52, 59). This is likely due to a type II statistical error arising from the small number of participants in these trials.

Table 1.4 The effects of GH on aerobic exercise capacity in adults with GHD: Summary of Placebo-Controlled trials

Study	GHD Patients	Age	Diagnosis of GHD	Study Design	GH Dose	Method	Effects of GH compared to placebo
(20)	n=22, CO M:F 14:8	23.8±1.2 years	Peak GH < 5µg/L after clonidine stimulation test	4 months DBPC crossover	2IU/m²	Cycle Ergometer	Increase in exercise capacity
(133)	n=24, AO M:F 16:8	39±2 years	Peak GH <3.0mU/L during insulin induced hypoglycaemia	6 months DBPC	0.07U/kg/day	Cycle Ergometer	Increase in VO_2max and maximal power output
(21)	n=29, AO M:F 19:10	45.5±2 years	Peak GH <10.0µg/L during insulin induced hypoglycaemia	12 months DBPC	2IU/m²	Cycle Ergometer	Increase in exercise capacity
(135)	n=20, AO M:F 15:5	~45 years	Peak GH < 2ng/ml during insulin induced hypoglycaemia	6 months DBPC	12.5µg/kg/day	Cycle Ergometer	No change in VO_2max Increase in maximal power output
(43)	n=10, AO M:F 9:1	47 years	Peak GH <5.0mU/L during insulin induced hypoglycaemia	6 months DBPC crossover	0.5U/kg/week	Cycle Ergometer	No change in maximal power output
(28)	n=14, AO M:F 9:5	29.4±2.7 years	Peak GH <7.0mU/L during insulin induced hypoglycaemia	6 months DBPC crossover with 1 month washout	0.5U/kg/week	Cycle Ergometer	Increase in exercise capacity and VO_2max
(55)	n=35, mixed M:F 18:17	39.8 years	Peak GH <10.0mU/L after glucagon or insulin induced hypoglycaemia	6 months DBPC	0.125IU/kg/week for first 4 weeks; thereafter 0.25IU/kg/week	Treadmill	No change in VO_2max
(60)	n=55, AO M:F 31:24	49 years	Peak GH < 3µg/L to insulin hypoglycaemia (<2.2mmol/L)	9 months DBPC crossover, 4 months washout	1.2IU/day for men 1.8IU/day for women	Treadmill	Increase in VO_2max
(59)	n=28, AO M:F 15:13	18-68 years	Peak GH <3.0µg/L during insulin	3 months DBPC crossover, 1	6.25µg/kg LBM for first month;	Treadmill	No change in VO_2max

Study	GHD Patients	Age	Diagnosis of GHD	Study Design	GH Dose	Method	Effects of GH compared to placebo
			induced hypoglycaemia	month washout	12.5µg/kg LBM thereafter		
(34)	n=40, mixed M:F 19:21	19-67 years	Peak GH <6.0mU/L during insulin induced hypoglycaemia or oral clonidine	6 months DBPC	0.02 to 0.05IU/kg	Treadmill	No change in exercise capacity

DBPC, double blind placebo controlled; VO_2max, maximal oxygen uptake; CO, childhood onset; AO, adult onset; GH, growth hormone, GHD, growth hormone deficiency; LBM, lean body mass; W, watts; kJ, kilojoules

The underlying mechanisms responsible for the improvement in aerobic performance during GH replacement are multifactorial. Oxygen delivery to exercising muscles depends on cardiac function, lung capacity and oxygen carrying capacity of the blood (136). Adults with GHD have impaired cardiac function (137), diminished lung capacity (138) and reduced red cell mass (139). These deficits are restored with GH replacement. In adults with GHD, GH replacement increases i) cardiac output, which arises from enhancement of heart rate and stroke volume (20, 133, 135, 140); ii) lung capacity by increasing respiratory muscle strength and lung volumes (135, 138); and iii) red cell mass, which determines oxygen carrying capacity of the blood (139, 141, 142). As discussed previously, biopsy data in humans do not provide evidence that GH increases the number of oxidative type I muscle fibres. However, studies uniformly show that the increase in muscle mass is associated with an increase in oxygen consumption during GH replacement (28, 135). These observations are consistent with the delivery of a greater amount of oxygen to an increased muscle mass as a result of GH replacement in adults with GHD, leading to an increase in aerobic capacity of exercising muscles.

Several studies have failed to show any significant effects of GH on VO_2max in healthy adults (143). Berggren et al. observed no significant increase in VO_2max following 28 days of low dose (0.033 mg/kg/day) and high dose (0.067 mg/kg/day) GH in a double blind placebo controlled trial of 30 healthy adults (144). These findings were supported by the lack of improvement in VO_2max of 96 recreational athletes following 8 weeks of GH administration (2mg/kg/day) (8). Thus, GH does not enhance aerobic exercise capacity in healthy adults.

Collectively, these results indicate that GH enhances aerobic exercise capacity in subjects with GHD, but not in healthy adults. The improvement can be explained by effects on muscle mass, cardiorespiratory function and haematological parameters.

1.6.2 Anaerobic exercise capacity

Anaerobic exercise capacity is defined as the total amount of work during a maximal exhausting exercise of a short duration, which is underpinned by anaerobic ATP supply (145). This work is executed by fast twitch type II muscle fibres. Various exercise tests have been used to assess anaerobic exercise capacity (146). In sporting activities that involve short-term high intensity physical activity, such as sprinting, baseball, gymnastics etc., the main energy source is anaerobic ATP. All physical activities including activities of daily living also depend on anaerobic energy upon initiation, for the first few seconds, before aerobic metabolism becomes the predominant energy source (5, 147). Thus, it is conceivable that a suboptimal AES impairs muscle function leading to easy fatigue in patients and diminished performance in athletes.

Factors other than physical training that regulate anaerobic exercise capacity are largely unknown (5). So far only one study has investigated the effects of GH on anaerobic exercise capacity (8). This double blind placebo controlled study in a large group of recreational athletes reported a significant improvement in anaerobic exercise capacity after GH therapy for 8 weeks, as assessed by the Wingate test. GH did not increase body cell mass, the functional compartment of LBM that is predominantly composed of muscle, nor standard measures of muscle strength (dynamometry) and power (jump height) (Figure 1.4). These findings suggest that muscle anabolism is unlikely to explain the improvement in the Wingate test. Jump height represents instantaneous work whereas the Wingate test involves all-out intensive exercise on a cycle ergometer for 30 seconds. Although both tests measure anaerobic power, the energy required for jumping is drawn from PCr stores while that for the longer Wingate test, from PCr stores and that derived from glycolysis. A likely explanation is a GH effect on energy supply stimulating ATP production from glycolysis, leading to an increase in anaerobic exercise capacity in skeletal muscle. As the effects were assessed in GH-

replete individuals treated with a supraphysiological dose of GH, the physiological and functional relevance of these findings remain unknown.

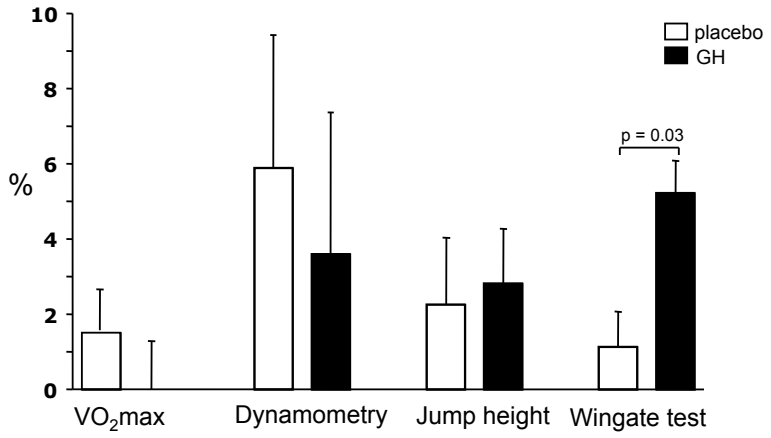


Figure 1.4 GH effects on physical performance in recreational athletes.
[Adapted from Meinhardt et al. (8)]. This figure illustrates the percent change after GH or placebo treatments in four measures of physical performance: VO_2max, strength (dynamometry), jump height and Wingate test.

In summary, the AES provides energy for initiation of all biological activities including activities of daily living and powers short-term high intensity physical activity (5, 147). Hence, the finding that GH may regulate the AES has potential therapeutic implications not only in the GH deficient population but also possibly in accelerating physical rehabilitation and improving physical function in the frail elderly. It also provides further justification of GH prohibition in sports.

1.7 GH effects on quality of life

Many investigators have assessed QoL in adults with GHD. There is good evidence to suggest that GHD leads to impaired QoL (148-150).(151) Amongst the various domains of QoL, energy and vitality are the most affected areas in adults with GHD with easy fatigue a prominent symptom (152, 153).

The question as to whether GH replacement improves QoL in adults with GHD has been investigated in many double-blind placebo-controlled and open label trials. Table 1.5 summarizes the findings of placebo-controlled trials investigating the effects of GH replacement on QoL in adults with GHD. The majority of these trials report a significant improvement (56, 149, 154-161). In a double-blind placebo-controlled trial involving 21 patients with GHD, a significant improvement in QoL occurred following 9 months of GH replacement; the domains that showed the great improvement were energy, emotions and cognitive function (148). The spouses who were also blinded to the intervention, observed a marked improvement in energy, mood and behavior in the subjects during GH therapy. Improvement of QoL is observed within first few months of starting the GH therapy. Some studies have reported an improvement as early as 3 months (151, 153, 162) although most occurs within the first year of GH replacement (6, 42). These benefits are sustained several years after starting the GH therapy (163, 164) and lost as early as 3 months after the withdrawal of GH replacement (157). The response to GH therapy is proportional to the baseline QoL deficit in patients with GHD (153, 165). This means that patients with a low QoL at the outset are likely to benefit most from GH replacement.

The mechanisms that lead to impairment of QoL and restoration after GH replacement are unclear. Although, it seems reasonable to assume anabolic effects of GH on body composition, muscle strength and aerobic exercise capacity may play a role in restoration of QoL in adults with GHD, no

relationship has been found between QoL and these parameters (6, 165, 166). It is not known whether improvement in these anabolic performance measures lead to an improvement in physical function, which is likely to have a significant bearing on their QoL. Many studies have failed to detect a relationship between QoL and serum IGFI levels (164, 167). Also, aerobic exercise capacity and muscle strength improve only after long-term therapy (> 12 months), in contrast to improvement in QoL, which is seen within a few months, raising a possibility of GH effects other than muscle anabolism affects QoL in adults with GHD such as energy metabolism. Short term GH therapy improves anaerobic exercise capacity in healthy adults (8). If a similar effect occurs in GH deficient population, the stimulation of the AES could explain the early improvement in QoL of adults with GHD by increasing the availability of energy and reducing fatigue.

In summary, adults with GHD experience impaired QoL, which improves within first few months after initiation of GH replacement and sustained long-term with continuation of GH therapy. Despite extensive research in this field, the mechanisms of QoL impairment in GHD and its restoration with GH replacement are not well understood.

Table 1.5 The effects of GH on quality of life in adults with GHD: Summary of Placebo-Controlled trials

Study	GHD patients	Study Design	GH dose	QoL measure	Effects of GH
(149)	N=24 Age 18-55 years	6-month DBPC	0.07 U/kg	NHP	Improvement in overall score compared to baseline and in energy compared to placebo
				PGWB	Improvement in only mood subsection compared to baseline
				GHQ	Improvement in overall scores compared to baseline
(148)	N= 36 M:F 27:36 Age 34.9 years	21-month cross-over DBPC	0.25IU/kg/week	NHP	Improvement in overall score compared to baseline
				HSCL	Total score improved in both, placebo and GH groups. Cognitive impairment improved significantly in GH group compared to placebo.
				PGWB	Improvement in overall score compared to baseline
(154)	N=40 males	18-month DBPC	4 ± 2 mcg/kg	NHP	No difference
				PGWD	No difference
				GHQ	No difference
				MMPI-2	No difference, On the Hysteria scale, a small increase in report of adverse symptoms in the GH group when compared with the placebo group
(156)	N=26 M:F 12:14 Age 51 years	6-month DBPC	3.7mcg/kg	KSQ	No difference
				HDS	Improvement in GH group compared to baseline
(15)	N=166 M:F 86:80	12-month DBPC	0.0125mg/kg	BDI	No difference
				NHP	No difference
				PGWB	No difference
				PQ	No difference
				NHIS	No difference
				TMT	No difference
(56)	N=30 M:F 10:20	6-month DBPC	0.25U/kg/week	NHP	Improvement in Energy score in GH group compared to baseline
				HAS	No difference
				Self-esteem	Improvement in placebo group
				MFQ	No difference
				Impact scale	Improvement in GH group compared to baseline
				Life fulfilment	No difference

Study	GHD patients	Study Design	GH dose	QoL measure	Effects of GH
(168)	N=50 males	6-month DBPC	1.7 ± 0.8 IU/m^2	HSCL	No difference
				POMS	No difference
				STAI	No difference
(160)	N=9	6-month	0.25 IU/kg/week	HDS	Improvement in GH group compared to placebo
	M:F 6:3	DBPC		BDI	Improvement in GH group compared to placebo
(169)	N=19	12-month	3.6 IU/day	GHQ	No difference
	M:F 13:6	DBPC			
(158)	N=165	6-month	0.25 IU/kg/week	NHP	Improvement in energy and emotional reaction in both groups and in social isolation in placebo group
	M:F 99:66	DBPC			
				QoL-AGHDA	Improvement in GH group compared to baseline
(155)	N=38	6-month	0.025mg/kg and	NHP	Improvement in GH group compared to baseline
	M:F 21:17	DBPC	0.012mg/kg	PGWB	Improvement in both groups
(170)	N=166	6-month	0.25 IU/kg/week	NHP	No difference
	M:F 91:72	DBPC		GHDQ	No difference
(161)	N=203	6-month	0.07 IU/kg	NHP	Improvement in GH group compared to placebo
	M:F 100:103	DBPC		PGWB	Improvement in mood only in GH group compared to baseline
				GHQ	Improvement in psychological distress in GH group compared to baseline
(60)	N=55	9-month	1.8 IU in females,	HSCL-58	No difference
	M:F 31:24	DBPC	1.2 IU in males	SF-36	No difference
(157)	N=21	3-month	0.125-0.25	HDQoL	Deterioration in physical activity in placebo group
	M:F 10:11	DBPC	IU/kg/week	GWBI	No difference
				W-BQ12	Deterioration in energy in placebo group
				SF-36	Deterioration in general health in placebo group
				NHP	No difference
				GHQ	No difference

DBPC, double-blind placebo-controlled; QoL, quality of life; HSCL-58, Hopkins symptom checklist 58; SF-36, short form 36; AGHDA, adult GH deficiency assessment; NHP, Nottingham health profile; PGWB, psychological general well-being index; GHQ, general health questionnaire; MMPI-2, Minnesota Multiphasic Personality Inventory-2; POMS, profile of mood scales; STAI, state trait anxiety inventory; KSQ, Kellner symptom questionnaire; HDS, Hamilton depression scale; BDI, Beck depression inventory; HAS, hospital anxiety and depression scale; SE, self esteem scale; MFQ, mental fatigue questionnaire; PQ, Paffenbarger Questionnaire; NHIS, National Health Interview Survey; TMT, Trail Making Tests; HDQoL, Hormone Deficiency-Dependent quality of life questionnaire; GWBI, general well-being questionnaire; W-BQ 12, the well-being questionnaire

1.8 Statement of aims

This chapter provides an overview of the consequences of GHD in adults and the effects of GH replacement in ameliorating these changes via its metabolic and anabolic properties. GHD leads to detrimental changes in body composition causing diminished muscle strength and endurance capacity (6). Strength and endurance are measures of muscle function that depend on muscle size, muscle fibre composition and the availability of energy to support the exercising muscle. This energy is available as ATP which is produced by two complementary energy systems: anaerobic and aerobic (4).

The aerobic energy system supports endurance exercise whereas the AES powers intensive activity of short-term duration. The AES underpins the initiation of all physical activities including activities of daily living (5). Thus it is conceivable that impairment of anaerobic capacity increases fatigue, impairing the activities of daily living, a symptom commonly observed in adults with GHD (152).

Muscle strength and aerobic capacity are impaired in GHD and restored by GH replacement over a period of few months (3, 6). Whether anaerobic capacity is impaired in GHD and improved with GH replacement has not been studied. A recent study reported that GH improves anaerobic capacity in healthy adults within few weeks of treatment, indicating an effect mediated via energy metabolism, rather anabolism (8). Sjogren et al. reported that GH suppressed genes in skeletal muscle governing lipid oxidation and stimulated genes promoting glycolysis in skeletal muscle. Although this gene expression study provides evidence supporting a role of GH in stimulating the AES, the results were derived from a small uncontrolled study (7, 8). The relationship between

anaerobic capacity, physical function and health-related QoL in adults with GHD, and the impact of GH replacement have not been investigated.

The major aims of this thesis are to investigate whether:

1. anaerobic capacity is impaired in adults with GHD and is improved with GH replacement
2. there is a relationship between anaerobic capacity, and physical function and QoL
3. GH alters the expression profiles of skeletal muscle genes in favour of anaerobic metabolism

The hypotheses to be tested are:

1. Anaerobic capacity is impaired in adults with GHD and improved with GH replacement.
2. Anaerobic capacity positively regulates physical function and QoL.
3. GH represses genes regulating oxidative metabolism in skeletal muscle

2 METHODS

The major procedures undertaken for this body of work were:

i) Physical performance tests

ii) Physical function tests

iii) QoL assessment

iv) Body composition assessment

v) Gene expression profiling of skeletal muscle

All protocols were approved by the Metro South Human Research Ethics Committee, Brisbane (HREC Reference number**:** HREC/12/QPAH/126) and carried out in accordance with Good Clinical Practice guidelines. This study is registered in The Australian New Zealand Clinical Trials Registry (number: ACTRN12615000046505).

All procedures were performed in the Endocrine Testing Area of Princess Alexandra Hospital, with assistance of research nurses Kevin Carter and Tracy Grierson. I completed a clinical Bone Densitometry course to satisfy the requirements of radiation safety legislation for undertaking and analyzing DXA for body composition assessment. I performed the RNA extraction from muscle tissues under guidance of Dr. Johanna Barclay and Dr. Chris Morais at the School of Medicine, University of Queensland. Gene expression profiling of the muscle biopsy samples was undertaken at the Australian Genome Research Facility (AGRF), Melbourne and the University of Queensland Diamantina Institute, Brisbane. I performed statistical analysis of the data under guidance of Dr Anne Bernard and the analysis of gene expression data under guidance of Dr Stephen Rudd, Queensland Facility for Advanced Bioinformatics. I performed qRT-PCR experiments under guidance of Dr. Caroline Nelson at the School of Medicine, University of Queensland.

2.1 Subjects

19 adults with GHD were recruited from Endocrine clinics at the Princess Alexandra Hospital, Brisbane. GHD was established by a peak GH response < 3μg/L to insulin-induced hypoglycemia or 3 or more pituitary hormone deficiencies with a subnormal IGF-I level (171). Subjects with severe cardio-respiratory, liver or renal impairment, or malignancy were excluded. The majority of patients had coexisting anterior pituitary hormone deficiencies and they were placed on stable replacement therapies for at least 6 months prior to enrollment in the study. 13 healthy controls matched for age-, gender-, and body mass index (BMI) to the first 13 adults with GHD were recruited through advertisement in the hospital and university newsletter.

2.2 Clinical trial material and randomization

Recombinant human GH (Norditropin) and placebo were provided by Novo Nordisk, Australia. The random allocation sequences were computer-generated and concealed until the time of allocation. Novo Nordisk generated the allocation sequences for GH and placebo in identical matched packaging labeled with the allocation number. Dr. Johanna Barclay, who was not involved in measuring study outcomes or analyzing data, randomized participants to GH or placebo. Participants and trial staff were blinded to the interventions all times.

2.3 Assessments

The following assessments were undertaken over two consecutive days. After an overnight fast, subjects arrived at the test venue at 0800h. After arrival, height, weight, pulse and blood pressure were measured. Blood was collected for urea and electrolytes, full blood count, thyroid function

test, oestradiol or testosterone. 5mL blood was centrifuged and plasma was stored at -20°C for later measurement of IGF-I after unblinding. The reproducibility of Wingate, VO_2max, Stair climb and Chair stand tests, and pedometry were assessed by measuring the CV and ICC in 5 healthy volunteers who undertook these tests on two separate occasions (see below).

2.3.1 Physical performance tests

2.3.1.1 Wingate Test

The Wingate anaerobic test was used to assess anaerobic capacity. It is one of the most widely used tests and has been validated in various groups ranging from athletes to individuals with chronic disease (172). The test-retest reliability values (*r*) for the Wingate test have ranged between 0.89 and 0.99 (173). It requires pedaling for 30 seconds at maximal speed against a constant force based on body weight. Figure 2.1 illustrates the power output profile over 30 seconds. Two variables are calculated: peak anaerobic power and mean anaerobic power. Peak anaerobic power is the highest mechanical power elicited during the test, and typically occurs within the first few seconds. Mean anaerobic power is defined as the average power sustained throughout the 30-second period. Peak anaerobic power is thought to reflect alactic process and mean anaerobic power, anaerobic glycolysis.

For this thesis, the Wingate test was performed after an overnight fast as described by Bar-Or et al. (172) using a Monark ergometer (Monark Exercise AB, Vansbro, Sweden). The force was calculated as 0.075 kp per kg body weight. A 3-minute warm-up followed by a 5-minute rest period preceded the actual test. The actual testing consisted of subject performing a 15-second countdown phase, a 30-second all-out pedaling phase and a 2-minute active cooldown phase.

Setup

Seat height is adjusted so that no more than 5 degree of knee flexion present when the leg is fully extended to ensure optimal riding condition. Stirrups are applied so that a pushing or pulling force can be exerted on the pedal throughout the full cycle. A polar heart rate monitor is placed around subject's chest for heart rate recording.

Warm up

Each subject is given a 3-minute warm-up period on the Monark ergometer against light resistance interspersed with two 5-second 'all-out sprints' for the subjects to get a feel for the actual test.

The actual test

The conduct of the full test is controlled by the Monark software, with a 15-second countdown prior to test initiation and subsequent data collection. The participants pedal as fast as possible to achieve maximum cadence three seconds before the resistance is applied. All subjects are verbally encouraged to continue to pedal as fast as possible for the entire 30 seconds.

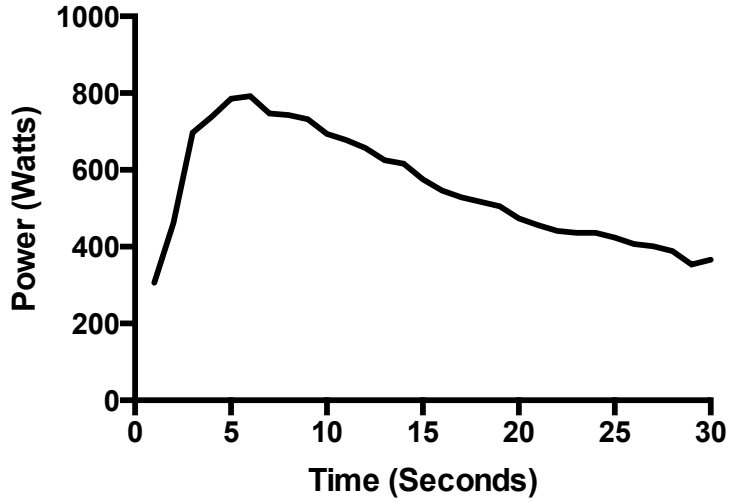
Cool down phase

This involves a 2-minute of pedaling (cooldown phase) against light resistance immediately after the test.

Data collection and analysis

Power output per second is recorded by the Monark software. Peak anaerobic power and anaerobic capacity (mean anaerobic power) are calculated and recorded in watts (W).

Figure 2.1 Power output over 30 seconds during the Wingate test



The reproducibility of the Wingate test was assessed in 5 healthy volunteers. The data are shown in table 2.1.

Table 2.1 Reproducibility of measures of the Wingate test.

Subject	1	2	3	4	5
Peak anaerobic power (Watts)					
Week 1	792	460	463	591	652
Week 2	817	486	480	629	696
Mean	805	473	472	610	674
SD	18	18	12	27	31
CV (%)	2	4	3	4	5
Mean CV (%)	4				
ICC	0.99				
Mean anaerobic power (Watts)					
Week 1	551	308.7	327.7	423	375
Week 2	573	328	315	428	394
Mean	562	318	321	426	385
SD	16	14	9.0	3.5	13
CV (%)	3	4	3	1	3
Mean CV (%)	3				
ICC	0.99				

2.3.1.2 VO_2max test

The VO_2max test measures aerobic capacity. VO_2max or maximal oxygen consumption is an objective measure of endurance (174). It measures the body's maximum capacity to transport and utilize oxygen during exercise for energy production (175). In the laboratory, aerobic capacity is assessed by measuring the peak oxygen uptake during an incremental exercise test. This test can be performed on a treadmill or cycle ergometer and oxygen uptake is measured by a metabolic monitor.

Exercise testing guidelines (176, 177) recommend adapting a protocol to the study participants and adjusting the work increment so that the total duration of exercise test is maintained between 8-12 minutes.

For this thesis, VO_2max was determined using a Monark ergometer. Oxygen consumption (VO_2), carbon dioxide production (VCO_2) and ventilatory volumes are measured continuously by a metabolic monitor (ParvoMedics, UT, USA). This machine uses infrared CO_2 and paramagnetic O_2 analyzers and a pneumotachograph. Calibration against standard gases (16% O_2 and 4% CO_2), volume (3 litre), operating temperature, humidity and barometric pressure is performed immediately before each test.

Toe stirrups are fitted and seat height is adjusted as described in the section 'Wingate test'. Polar heart rate monitor is placed around the chest of the subject. A work rate is set at 40W and increased by 10W every minute until exhaustion. Subjects are encouraged verbally to maximize effort. VO_2 values are averaged for each 15-second period and highest value is recorded as VO_2max. It is expressed in absolute values (L/min) and also in ml/min/kg.

The reproducibility of the VO_2max test in 5 healthy volunteers is shown in table 2.2.

Table 2.2 Reproducibility of VO_2max Test.

Subject	**1**	**2**	**3**	**4**	**5**
Week 1 (ml/kg/min)	47	32	33	43	29
Week 2 (ml/kg/min)	50	30	36	42	29
Mean	48	31	34	42	29
SD	2.6	1.6	2	0.3	0.4
CV (%)	5	5	6	1	1
Mean CV (%)	4				
ICC	0.96				

2.3.2 Physical function tests

The functional significance of anaerobic capacity was evaluated by the stair climb test, chair stand test and aerobic capacity by 7-day pedometry.

2.3.2.1 Stair climb test

The stair climb test is widely used for the assessment of physical function and leg muscle power (178-180). Various protocols have been developed for different populations, to measure the time to ascend a set number of stairs or the step count for a set period of time (179).

The stair climb test in this study measures the time to ascend 4 flights of stairs totaling 48 steps (step height 17cm). The time is recorded to the nearest 0.001 second, using a switch mat timing system (TAG Heuer HL440 Minitimer, TAGHeuer Professional Timing, La Chaux-De-Fonds, Switzerland). The participants are instructed to climb the stairs as fast as possible one step at a time. The test is performed three times with each occasion separated by a rest period of 5 minutes. The best of three readings is included for data analysis.

The reproducibility of the stair climb test in 5 healthy volunteers is shown in table 2.3.

Table 2.3 Reproducibility of the stair climb test.

Subjects	1	2	3	4	5
Week 1 (sec)	13	13	18	13	16
Week 2 (sec)	12	13	18	13	15
Mean	12	13	18	13	15
SD	0.5	0.4	0.6	0.4	0.9
CV (%)	4	3	4	3	6
Mean CV (%)	4				
ICC	0.97				

2.3.2.2 Chair stand test

The chair stand test is a validated test to assess functional performance (181). It measures the number of times one can stand up and sit down in 30 seconds from a fully seated position in a chair.

Participants are seated in the middle of chair with their arms crossed and held against the chest. The chair dimensions are 43cm height and 47.5 cm depth, with a fixed 4-legged base. It is placed against the wall to restrict movement during the test. The time is recorded by a stopwatch and repetitions are counted by an electronic counter. The participants are instructed to rise to a full stand with knees fully extended and then return back to a fully seated position as many times as possible within 30-second period. The total number of stands executed correctly within 30-second period is recorded (more than halfway up at the end of 30 second is counted as a full stand). The test is repeated three times separated by a 5-min rest period and the best reading out of three is used for data analysis.

The reproducibility of the chair stand test in 5 healthy volunteers is shown in table 2.4.

Table 2.4 Reproducibility of the chair stand test.

Subject	1	2	3	4	5
Week 1 (Repetitions)	32	39	17	22	18
Week 2 (Repetitions)	39	42	22	27	19
Mean	36	41	20	25	19
SD	4.6	2.1	3.5	3.5	0.7
CV (%)	14	5	18	14	4
Mean CV (%)	11				
ICC	0.95				

2.3.2.3 Pedometry

Pedometry is a simple, inexpensive and user-friendly research tool, for the objective assessment of habitual physical activity in healthy and in patients with chronic disease (182). It measures the number of steps per day (183). In this project Kenz activity monitor (Kenz Lifecorder, Suzuken Co., Ltd., Nagoya, Aichi, Japan) was used to measure daily step count for 7 consecutive days. It has an intramodel reliability of 0.998 and absolute accuracy of +/- 3% (184). Participants are instructed to wear the Kenz activity monitor on their waist belt continuously for 7 days, which is sufficient to provide a reliable estimate of monthly activity in adults (185). The total daily physical activity (i.e. steps) data are downloaded to a computer using the Kenz Lifecorder software and the average is reported as the daily step count.

The reproducibility of 7-day pedometry in 5 healthy volunteers is shown in table 2.5.

Table 2.5 Reproducibility of 7-day pedometry.

Subject	1	2	3	4	5
Week 1 (Steps)	68429	74369	39718	21998	70069
Week 2 (Steps)	62975	67702	46556	23506	70820
Mean	65702	71035.5	43137	22752	70444.5
SD	3857	4714	4835	1066	531
CV (%)	6%	7%	11%	5%	1%
Mean CV (%)	6				
ICC	0.98				

2.3.3 Quality of life assessment

QoL was assessed by QoL-AGHDA (Quality of Life Assessment of GH Deficiency in Adults) questionnaire, a research tool specifically validated in adults with GHD, to determine whether there was an association between anaerobic capacity and QoL in adults with GHD (186, 187). The QoL-AGHDA questionnaire contains 25 items with a 'yes/no' response format. Each positive (yes) response is given a score of 1, allowing scores to range from 0 to 25. High scores indicate poor QoL. Out of 25 questions in the AGHDA questionnaire, 8 questions directed at energy and vitality domains are used in a sub-analysis to assess GH effects on energy metabolism. The remaining 17 questions directed at QoL-domains such as social isolation, memory and concentration, emotional reactions and sleep are grouped together as non-energy and vitality domain and analyzed separately.

2.3.4 Body composition assessment

Body composition was assessed by DXA using a Hologic absorptiometer (Model QDR 4500A, S/N 45432, Software version 12.6) before and after GH therapy to allow correction of anaerobic power and VO_2max for increases in LBM that were expected to occur.

2.3.5 Muscle biopsy in GHD group

Muscle biopsies were undertaken in adults with GHD using 5mm Bergstrom needle. The biopsy is obtained from vastus lateralis 12cm above the upper border of patella. The skin and subcutaneous tissue is infiltrated with 1% lignocaine without adrenaline. The depth of fascia overlying muscle is defined using a 25-gauge needle tip. A size 11 scalpel is used to puncture the skin, subcutaneous tissue and muscle fascia. Using a suction technique, Bergstrom needle is used to yield 100-150mg of muscle. Muscle tissue is immediately frozen in liquid nitrogen and stored at -80°C.

2.3.6 RNA extraction

During the 1-month placebo controlled trial, total RNA was isolated from snap-frozen muscle biopsies using the TRIzol Reagent (Life Technologies Inc., Gaithersburg, Maryland 20884, USA) according to the manufacturer's instructions as follows:

1. Homogenizing samples and phase separation

1ml of TRIzol reagent is added to 50-80mg of muscle tissue and the sample is homogenized with a rotor stator. Homogenized sample is incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. 0.2mL of chloroform is added and the sample is centrifuged at 12000x g for 15 minutes. The mixture separates into a lower red phenol-chloroform

phase, an interphase, and a colorless upper aqueous phase, which contains RNA. The upper aqueous is removed and placed in a separate tube for RNA precipitation.

2. RNA precipitation

0.5mL of 100% isopropanol is added to the aqueous phase. The mixture is incubated at room temperature for 10 minutes followed by centrifugation at 12000x g for 10 minutes. The RNA forms a pellet on the side and bottom of the tube.

3. RNA wash

The supernatant from the tube is removed, leaving only the RNA pellet. The pellet is washed with 1mL 75% ethanol and centrifuged at 7500x g for 5 minutes. The wash is discarded and the RNA pellet is air dried for 5-10 minutes.

4. RNA resuspension

The RNA pellet is resuspended in 30µL of RNase-free water and incubated in a heat block set at 55°C for 10 minutes. The RNA is stored at -80°C.

5. RNA quality assessment

The quality of the RNA samples is determined using the Agilent RNA 6000 Nano Kit on the 2100 Bioanalyzer (Agilent, Amstelveen, The Netherlands). Only total RNA samples with a RNA integrity number higher than seven are included for further analysis.

During the open label study, total RNA was isolated from snap-frozen muscle biopsies using RNeasy fibrous tissue extraction kit (Qiagen, Valencia, USA) according to the manufacturer's instructions as follows:

1. 30mg muscle is disrupted and homogenized with rotor-stator in the presence of 300µl of Buffer RLT.
2. 590µl RNase-free water is added to the lysate. Then 10µl of proteinase K solution is added and mixed thoroughly.
3. Lysate is incubated at 55°C for 10 minutes and then centrifuged for 3 minutes at 10,000xg.

4. The supernatant is transferred into a new microcentrifuge tube.
5. 0.5 volume of 100% ethanol (usually 450µl) is added to the cleared lysate and mixed well by pipetting.
6. 700µl of the sample is transferred to an RNeasy Mini spin column placed in a 2ml collection tube and centrifuged for 15 s at ≥ 8000xg. The flow-through is discarded. This step is repeated using the remainder of the sample.
7. 350µl Buffer RW1 is added to the RNeasy Spin column and centrifuged for 15 s ≥8000xg to wash the membrane. The flow-through is discarded.
8. 10µl DNase I stock solution is added to 70µl Buffer RDD, mixed by gently inverting the tube, and centrifuged briefly to collect residual liquid from the sides of the tube.
9. The DNase I incubation mix (80µl) is added directly to the RNeasy spin column membrane, and placed on the benchtop for 15 min.
10. 350µl Buffer RW1 is added to the RNeasy spin column and centrifuged for 15 s at ≥8000xg at 20–25°C. The flow-through is discarded.
11. 500µl Buffer RPE is added to the RNeasy spin column and centrifuged at 20–25°C for 15 s at ≥8000xg to wash the membrane. The flow-through is discarded.
12. 500 µl Buffer RPE is added to the RNeasy spin column and centrifuged at 20–25°C for 2 min at ≥8000xg to wash the membrane.
13. The RNeasy spin column is placed in a new 1.5 ml collection tube. 30µl RNase-free water is added directly to the RNeasy spin column membrane and centrifuged for 1 min at ≥8000xg to elute the RNA. The RNA is stored at -80°C.
14. The quality of the RNA samples is determined using the Agilent RNA 6000 Nano Kit on the 2100 Bioanalyzer (Agilent, Amstelveen, The Netherlands). Only total RNA samples with a RNA integrity number higher than seven are included for further analysis.

2.3.7 Gene expression analysis

Microarray experiments were undertaken separately for the 1 month and 6 month studies. Samples for the 1-month study were processed at the Australian Genome Research Facility (Melbourne, Australia) and for the 6-month study at the University of Queensland Diamantina Institute (Brisbane, Australia). For both experiments, the RNA samples were biotinylated and amplified using the Illumina® TotalPrep™ RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with a standardized input amount of 500ng.

3 ANAEROBIC CAPACITY IN ADULTS WITH GROWTH HORMONE DEFICIENCY

3.1 Introduction

Energy derived from the aerobic energy system is used at rest and during endurance exercise. Whereas the AES powers intensive activity of short-term duration and it is also important for the initiation of all biological activities including activities of daily living such as rising from a chair, climbing stairs, rushing for a bus (5). Thus it is conceivable that impairment of anaerobic capacity leads to the perception of increased fatigue during the execution of ordinary activities of daily living, a symptom commonly observed in adults with GHD (152).

There is strong evidence from studies in adults with GHD that muscle strength and aerobic capacity are impaired in GHD and restored to near normal with GH replacement over a period of few months (3, 6). Whether anaerobic capacity is impaired in GHD or GH regulates the AES has not been studied. There is emerging evidence that GH may have a regulatory role in anaerobic metabolism (7, 8). In a gene expression study involving adults with GHD, GH therapy led to changes that may favour non-oxidative glycolysis for ATP production in skeletal muscle (7). In a study involving recreational athletes, short duration GH therapy significantly improved anaerobic capacity (8). However, this study was undertaken in GH sufficient healthy adults using a supraphysiological dose of GH and the physiological significance of these findings is unclear.

The aims of this section of work were to assess whether anaerobic exercise capacity is impaired in adults with GHD and to evaluate its functional relevance.

3.2 Methods

3.2.1 Subjects

13 hypopituitary adults with GHD and 13 age-, gender-, and BMI-matched normal adults were recruited. The causes of pituitary deficiencies and hormone replacements are summarized in the Table 3.1. None of the hypopituitary patients had been previously replaced with GH except for two subjects. In these patients, GH therapy was stopped at least 3 months before recruitment.

3.2.2 Study design

This was a cross-sectional comparison of anaerobic capacity, aerobic capacity, physical function, body composition and QoL. Subjects attended the test venue on 2 consecutive days for the assessments after an overnight fast.

Table 3.1 Causes of pituitary deficiency and pituitary hormone replacement therapies in 13 subjects with GHD.
H, hydrocortisone; C, cortisone acetate; P, prednisone; T4, thyroxine; E_2, estradiol; T, testosterone; D, desmopressin

Patient No.	Age	Gender	Diagnosis	Hormone Replacement
1	38	F	Cushing's disease	H, T4, E_2, D
2	24	F	Septo-optic dysplasia	H, T4, E_2
3	38	F	Macroprolactinoma	C, T4, E_2, D
4	46	F	Silent corticotroph macroadenoma	H, T4
5	60	F	Panhypopituitarism, Empty sella	C, T4
6	57	F	Non-functioning macroadenoma	T4
7	52	F	Cushing's disease	C, T4, E_2
8	58	M	Cushing's disease	C, T, T4
9	49	F	Macroprolactinoma	H, T4, E_2, D
10	39	F	Glioma	P, T4, E_2
11	46	F	Cushing's disease	P, T4, E_2, D
12	48	F	Non-functioning macroadenoma	H
13	58	F	Rathke's cleft cyst	C, T4

3.2.3 Assessments

The detailed protocols of assessments are described in the Chapter 2. Anaerobic and aerobic capacities were assessed by the Wingate test and VO_2max test respectively. The stair climb test and chair stand test were used as the functional assessments of anaerobic capacity, and 7-day pedometry as of aerobic capacity. DXA scan was performed to measure LBM and fat mass. QoL was assessed by QoL-AGHDA questionnaire.

3.3 Statistical analysis

Statistical analysis was performed with IBM SPSS statistics software (Version 22, Chicago, IL, USA). Results are reported as mean ± SEM. Comparisons between normal and GH deficient groups were performed with unpaired t test. Associations were explored with simple linear regressions. Multiple linear regression analyses were used to determine the independent predictors among age, gender, LBM and GHD of peak and mean anaerobic power, and VO_2max; and independent predictors among peak and mean anaerobic power, VO_2max, age, gender, LBM and GHD of functional measures. A p value of less than 0.05 was considered statistically significant.

3.4 Results

Age, gender and the BMI of 13 normal and 13 adults with GHD are shown in Table 3.2. There were no significant differences in age and BMI between the two groups. Mean fat mass tended to be higher and LBM lower in adults with GHD but the differences were not statistically significant.

LBM significantly correlated with peak and mean anaerobic power, and VO_2max in the combined groups of normal and adults with GHD (Figure 3.1). For data analysis, peak and mean anaerobic power, and VO_2max are corrected for LBM and expressed as watts.kgLBM^{-1} and ml.kgLBM^{-1}.min^{-1} respectively.

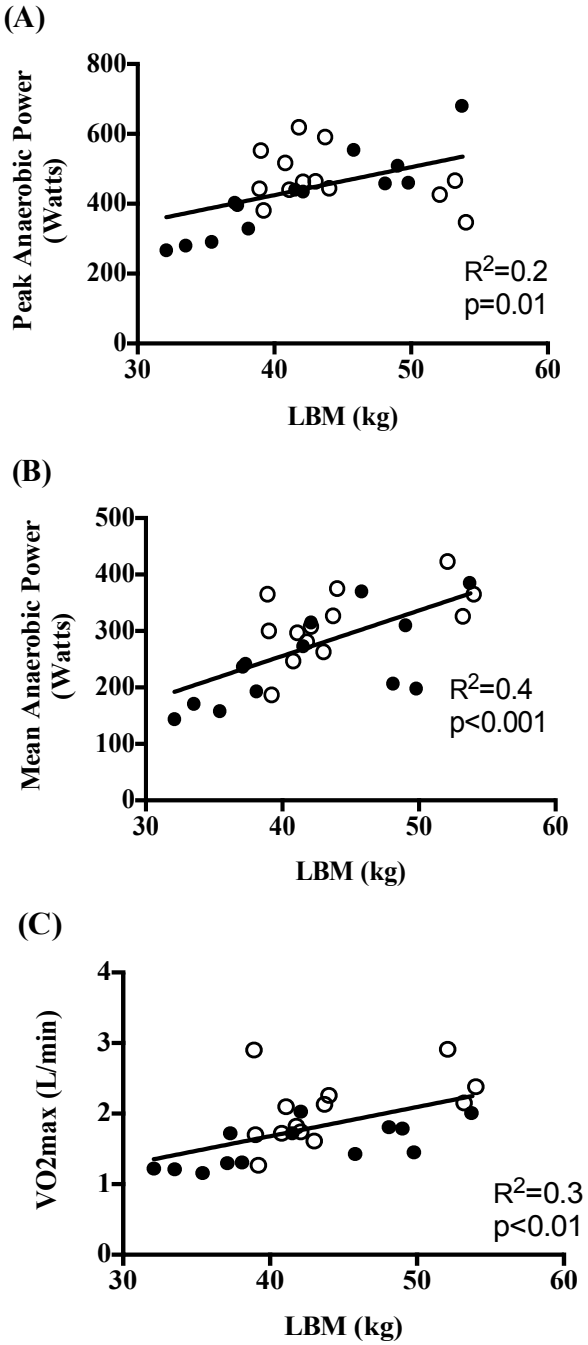
3.4.1 Physical Performance (Table 3.2)

Mean anaerobic power ($p<0.05$) and VO_2max (<0.01) were lower in adults with GHD compared to normal adults. These measures remained significantly lower when corrected for LBM in adults with GHD ($p<0.05$ and $p<0.01$ respectively). The mean anaerobic power and VO_2max were 18% and 20% lower in GH deficient adults respectively.

Table 3.2 Baseline characteristics, performance tests, functional measures and quality of life in 13 adults with GHD and 13 normal subjects.
All values are shown as the mean ± SEM. NS, Not significant.

	GHD	Normal	p value
M:F	1:12	1:12	
Age (years)	45.3 ± 3.2	43.7 ± 3.1	NS
BMI ($kg.m^{-2}$)	27.0 ± 1.4	26.0 ± 1.1	NS
LBM (kg)	41.8 ± 1.9	44.1 ± 1.5	NS
Fat mass (kg)	26.8 ± 2.3	21.9 ± 2.1	NS
Peak anaerobic power (W)	424 ± 33	473 ± 22	NS
Peak anaerobic power ($W.kgLBM^{-1}$)	10.0 ± 0.4	10.7 ± 0.3	NS
Mean anaerobic power (W)	246 ± 22	312 ± 17	<0.05
Mean anaerobic power ($W.kgLBM^{-1}$)	5.8 ± 0.4	7.1 ± 0.3	<0.05
VO_2max ($L.min^{-1}$)	1.6 ± 0.1	2.1 ± 0.1	<0.01
VO_2max ($ml.min^{-1}.kgLBM^{-1}$)	37.2 ± 1.5	46.7 ± 2.9	<0.01
Stair climb test (Seconds)	19.4 ± 0.7	16.5 ± 0.7	<0.01
Chair stand test (Repetitions)	19.2 ± 1.2	25.5 ± 2.1	<0.01
Pedometry (Steps per day)	6655 ± 634	9565 ± 668	<0.01
QoL-AGHDA score	16.6 ± 1.6	2.1 ± 0.8	<0.0001

Figure 3.1 Relationship between LBM and peak anaerobic power (Panel A), mean anaerobic power (Panel B) and VO_2max (Panel C) in the combined groups of 13 adults with GHD (●) and matched normal subjects (O).



3.4.2 Physical function (Table 3.2)

The functional relevance of anaerobic capacity was assessed by tasks that were completed over 15 to 30 seconds, the time course of physical activity that is mainly subserved by the AES. In the stair climb test, adults with GHD took a significantly longer time ($p<0.01$) to complete the task of climbing 4-flights of stairs than normal adults. In the chair stand test, number of repetitions performed in 30 seconds was approximately 30% lower ($p<0.01$) in adults with GHD. In order to assess the functional relevance of aerobic capacity, 7-day pedometry was performed. The average daily step count was in adults with GHD was approximately 30% lower ($p<0.01$) compared to normal subjects.

3.4.3 Quality of life (Table 3.2)

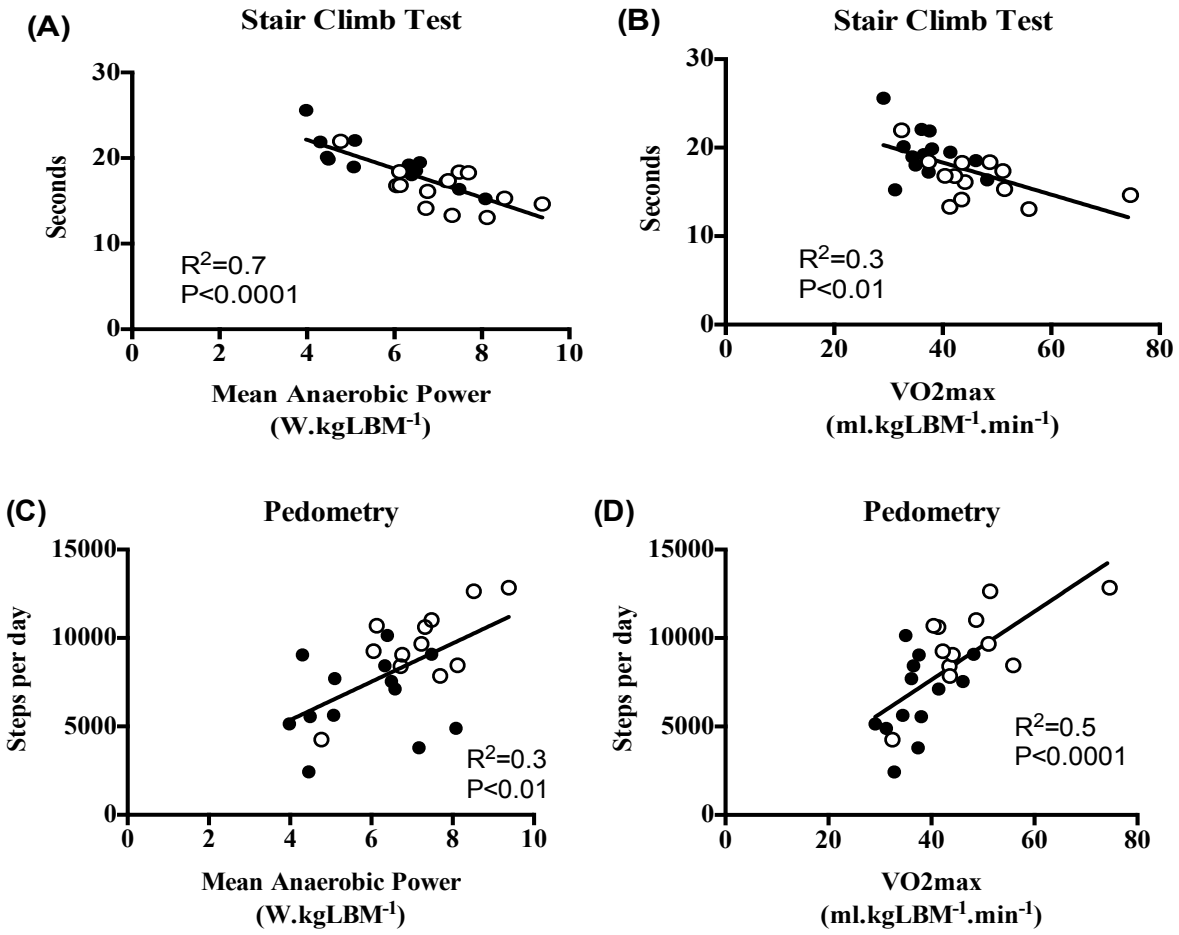
Mean QoL-AGHDA scores were significantly higher in GH-deficient adults, indicating a worse QoL ($p<0.0001$).

3.4.4 Relationship between physical performance measures, functional measures and quality of life (Figure 3.2)

Relationships between performance measures, functional measures and QoL were analyzed by simple linear regression. Mean anaerobic power significantly correlated with the stair climb performance (R^2= 0.7, p<0.0001, Figure 3.2 Panel A) and pedometry (R^2= 0.3, p<0.01, Figure 3.2 Panel C). VO_2max correlated significantly with the stair climb performance (R^2= 0.3, p<0.01, Figure 3.2 Panel B) and pedometry (R^2= 0.5, p<0.0001, Figure 3.2 Panel D). The relationship between stair climb performance and mean anaerobic power was stronger than with VO_2max. Mean anaerobic power accounted for 70% while the VO_2max only 30% of variation in the stair climb performance.

The relationship between pedometry and VO_2max was stronger than with mean anaerobic power. VO_2max accounted for 50% while the mean anaerobic power only 30% of variation in pedometry. The relationship between mean anaerobic power and chair stand performance was not significant as was the case for VO_2max. Both mean anaerobic power (R^2= 0.3, p<0.01) and VO_2max (R^2= 0.2, p<0.05) were inversely related with QoL-AGHDA score.

Figure 3.2 Relationship between stair climb performance and anaerobic power (Panel A) and VO_2max (Panel B), and between daily step counts quantified by pedometry and anaerobic power (Panel C) and VO_2max (Panel D) in the combined groups of 13 adults with GHD (●) and matched normal subjects (O).



3.4.5 Predictors of physical performance measures, functional measures and quality of life

Multiple regression analyses were performed to determine significant predictors of physical performance measures, functional measures and QoL. For mean anaerobic power, GH status, age, gender and LBM were significant independent determinants ($p<0.05$) while only LBM significantly and independently predicted peak anaerobic power ($p<0.05$) (Table 3.3 and 3.4). These four variables were also significant determinants of VO_2max ($p<0.05$) (Table 3.5).

For stair climbing, with peak and mean anaerobic power, VO_2max, GH status, age, gender and LBM as independent variables, only mean anaerobic power ($p<0.01$) emerged as the significant determinant (Table 3.6). For chair stand performance, GH status ($p<0.05$) was an independent predictor (Table 3.7); while for pedometry, only VO_2max ($p=0.05$) was an independent predictor at a borderline level of significance (Table 3.8).

For QoL, with peak and mean anaerobic power, VO_2max, GH status, age, gender and LBM as independent variables, GH status ($p<0.001$) and mean anaerobic power ($p<0.05$) emerged as significant determinants (Table 3.9).

Table 3.3 Linear regression model of predictors of mean anaerobic power

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	169	125.6		1.4	.19
GH Status	44.7	19.6	.30	2.3	.03
Age	-2.7	1.0	-.39	-2.8	.01
Gender	-86.8	39.5	-.31	-2.2	.04
LBM	7.6	1.7	.61	4.6	.00

a. Dependent Variable: Mean anaerobic power

Table 3.4 Linear regression model of predictors of peak anaerobic power

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	-19.3	122		-.16	.88
GH Status	13.7	19.1	.07	.72	.48
Age	-1.9	.94	-.22	-2.1	.05
Gender	-44.9	38.6	-.12	-1.2	.26
LBM	14.4	1.6	.88	8.9	.00

a. Dependent Variable: Peak anaerobic power

Table 3.5 Linear regression model of predictors of VO_2max

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	1.5	.81		1.8	.08
GH Status	.40	.13	.43	3.2	.01
Age	-.01	.01	-.31	-2.1	.05
Gender	-.63	.26	-.36	-2.5	.02
LBM	.04	.01	.46	3.3	.004

a. Dependent Variable: VO_2max

Table 3.6 Linear regression model of predictors of stair climb test

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	22.092	5.1		4.3	.00
GH Status	-.780	.87	-.14	-.90	.38
Age	.011	.04	.04	.26	.80
Gender	.035	1.6	.003	.02	.98
LBM	.019	.15	.04	.13	.90
Peak Power	.022	.01	.74	1.6	.14
Mean Power	-.056	.02	-1.5	-3.7	.002
VO_2max	1.000	1.5	.16	.70	.50

a. Dependent Variable: Stair climb test

Table 3.7 Linear regression model of predictors of chair stand test

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	14.7	17.6		0.83	0.42
GH Status	6.8	3.0	0.51	2.3	0.04
Age	-0.11	0.14	-0.18	-0.77	0.45
Gender	0.88	5.6	0.04	0.16	0.88
LBM	0.68	0.50	0.62	1.4	0.19
Peak Power	-0.08	0.05	-1.2	-1.7	0.10
Mean Power	0.04	0.05	0.42	0.70	0.50
VO_2max	-0.99	5.0	-0.07	-0.20	0.85

a. Dependent Variable: Chair stand test

Table 3.8 Linear regression model of predictors of pedometry

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	-127	6230		-.02	.98
GH Status	750	1120	.14	.67	.51
Age	-19.2	48.7	-.08	-.39	.70
Gender	1917	1982	.20	.97	.35
LBM	17.3	177	.04	.10	.92
Peak Power	-14.9	17.0	-.57	-.88	.39
Mean Power	12.5	18.7	.37	.67	.51
VO_2max	3691	1796	.66	2.1	.05

a. Dependent Variable: Pedometry

Table 3.9 Linear regression model of predictors of quality of life

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	53.3	13.0		4.1	.001
GH Status	-12.5	2.3	-.73	-5.6	.000
Age	-.13	.10	-.17	-1.3	.21
Gender	-6.7	4.8	-.16	-1.4	.19
LBM	-.20	.37	-.14	-.5	.60
Peak Power	.05	.04	.60	1.4	.18
Mean Power	-.10	.04	-.89	-2.6	.02
VO_2max	4.7	3.8	.23	1.2	.24

a. Dependent Variable: Quality of life

3.5 Discussion

This study compared physical performance measures, functional measures and QoL between adults with GHD and age-, gender-, and BMI-matched normal adults. GH-deficient adults had lower anaerobic and aerobic capacities, functional capacities and QoL compared to normal adults. Mean anaerobic power and VO_2max significantly correlated with stair climb performance, daily step count and QoL. GH status was an independent predictor of mean anaerobic power, VO_2max and QoL. Mean anaerobic power independently predicted stair climb performance and QoL, while VO_2max predicted daily step count.

Adults with GHD had a lower LBM and higher fat mass. Although these findings did not reach statistical significance, they are consistent with the current literature (188) and likely to reflect the relatively small sample size. LBM was a positive determinant of peak and mean anaerobic power, and VO_2max; hence these measures were corrected for LBM and expressed as watts.kgLBM^{-1} and ml.min^{-1}.kgLBM^{-1} respectively. In this study, anaerobic and aerobic capacities were reduced in adults with GHD even after correcting for LBM. These findings indicate that the quantity and quality of skeletal muscle are reduced in GHD.

This study provides the first evidence that anaerobic capacity of adults with GHD is subnormal. The observation that GH status is an independent predictor of anaerobic capacity suggests that GH regulates the AES. This finding supports a finding in recreational athletes that anaerobic sprint capacity improves after 8 weeks of GH therapy (8). Anaerobic capacity is dependent on strength and energy required for work performance. The improvement of anaerobic sprint capacity in athletes occurred without an increase in muscle strength (8) suggesting mediation by metabolic rather than anabolic mechanisms. The metabolic energy requirement during a 30-second Wingate test is predominantly met by the phosphocreatine stores (pre-formed ATP) for the first 10 seconds

and by anaerobic glycolysis thereafter (189). The authors did not report a sub-analysis of the Wingate profile. It is therefore unclear whether the improvement in sprint capacity occurred by an increase in the power output throughout the 30-second period or the peak component. In the present cross-sectional study, adults with GHD exhibited lower mean anaerobic power, but not the peak anaerobic power. These findings suggest that the improvement in anaerobic capacity observed in athletes following GH treatment was related to the stimulation of anaerobic glycolysis.

GH stimulates whole body lipid oxidation and resting energy expenditure in healthy subjects (113, 120, 121) and in adults with GHD (122-124). It has been assumed that these whole body effects reflect the actions of GH in skeletal muscle. However, there is emerging evidence that metabolic actions of GH are tissue specific and whole body changes may not reflect those in skeletal muscle. Tollet-Egnell et al. reported down regulation of genes governing lipid oxidation in skeletal muscle of rats following GH treatment (126). Sjogren et al. found that GH down regulated genes in skeletal muscle involved in lipid metabolism, the TCA cycle activity and the mitochondrial respiration (7). These findings suggest that GH inhibits aerobic metabolism in favor of non-oxidative (anaerobic) pathways for ATP synthesis. These findings provide a plausible explanation for the loss of anaerobic capacity and function in GH deficiency.

The present study confirms previous findings that aerobic capacity is impaired in adults with GHD (6, 28, 133-135). The underlying mechanisms responsible for the impaired aerobic capacity are multifactorial. Oxygen delivery to muscles depends on cardiac function, lung capacity and oxygen carrying capacity of blood. Adults with GHD have impaired cardiac function (137), diminished lung capacity (138) and reduced red cell mass (139), factors that collectively reduce oxygen delivery to exercising muscles. These deficits lead to a decrease in oxygen supply to a reduced muscle mass explaining impaired aerobic capacity in adults with GHD.

A novel aspect of this study is the investigation of functional relevance of anaerobic and aerobic capacities in adults with GHD. There are no previous studies investigating the relationship between physical performance and functional assessment nor their relationships to QoL in GHD. Because anaerobic energy initiates all movement and powers short burst of intense activities, it was postulated that its physical functional relevance could be reflected in the stair climb test and chair stand test performed over a brief duration. In contrast, the functional relevance of aerobic energy system, which supports low intensity prolonged activities, would be reflected in daily pedometry. Adults with GHD on average took 18% longer to climb 4 flights of stairs, performed 30% less number of chair stand repetitions and had 30% lower daily step count. In a multiple regression analysis, mean anaerobic power independently predicted the stair climb performance supporting the hypothesis that stair climbing relies predominantly on the AES. There was no significant relationship observed between chair stand performance and mean anaerobic power or VO_2max. In contrast VO_2max was the major predictor of weekly pedometry, suggesting that daily walking is dependent on the aerobic energy system. These data support results by Izawa et al., who reported a positive correlation between daily walking activity and aerobic capacity in patients with chronic heart failure (190). Thus the anaerobic and aerobic energy systems subserve distinct aspects of physical function of daily living that are regulated by the GH.

QoL-AGHDA scores were significantly higher in adults with GHD compared to normal adults, indicating a worse QoL, in agreement with previous studies (150, 152, 153, 191). Both anaerobic power and VO_2max were inversely related with QoL-AGHDA score. However, in a multivariate analysis, only GH status and anaerobic power independently predicted QoL. Although these findings do not prove causality, they suggest a significant role of the AES in maintaining a normal QoL in adults with GHD.

This study has a number of limitations. It was undertaken in a group of subjects who were predominantly women. It is likely that the finding of impaired anaerobic capacity in GHD apply to

both men and women. Thyroid and adrenal hormones are among several hormones that influence the AES in addition to GH (192-194). In this study, most of the subjects with GHD harbored coexisting anterior pituitary hormone deficiencies with four subjects having a history of Cushing's disease. These subjects were replaced with thyroid, adrenal and gonadal hormones in accordance with standard endocrine practice. Although the groups were matched for gender, age and BMI, a possibility that impairment of anaerobic capacity arose from suboptimal replacement therapies, the consequences of chronic hypercortisolism or non-hormone related factors can not be ruled out. One such confounder is the impact of a chronic suboptimal level of health on the performance variables. A prospective GH intervention study is required to confirm whether GH improves anaerobic capacity in adults with GHD.

In summary, anaerobic capacity, aerobic capacity, stair climb and chair stand performance, weekly pedometry and QoL are reduced in adults with GHD. GH status is an independent determinant of anaerobic and aerobic capacity. Anaerobic capacity independently predicts stair climb performance but not daily step count, which is determined by aerobic capacity. GH status and anaerobic capacity are independent determinants of QoL.

In conclusion, GH regulates the AES. Stair climb is a functional measure of anaerobic capacity. The AES is a significant contributor to QoL in patients with GHD.

4 EFFECTS OF GH ON ANAEROBIC CAPACITY AND PHYSICAL FUNCTION

4.1 Introduction

There is emerging evidence that GH plays a role in the regulation of the AES. In adults with GH deficiency, Sjogren et al. found that short-term GH replacement repressed genes regulating aerobic metabolic pathways in skeletal muscle, favoring anaerobic energy production (7). In recreational athletes, Meinhardt et al. reported that GH treatment improved sprint capacity without affecting muscle strength suggesting facilitation by energy metabolism rather by muscle anabolism (8). This study however, was undertaken in GH-sufficient subjects treated with a supraphysiological dose of GH. As reported in Chapter 3, anaerobic capacity is reduced in adults with GHD compared to age, gender and BMI matched control subjects. As the GHD group comprised of subjects with organic hypopituitarism, subnormal anaerobic capacity may have arisen from suboptimal replacement of other pituitary hormone therapies such as thyroid or adrenal hormone or concurrent chronic suboptimal health rather than GHD alone. This study investigated whether physiological replacement of GH improves anaerobic capacity and physical function dependent on the AES in adults with GHD.

4.2 Material and Methods

4.2.1 Subjects

19 hypopituitary adults (15 females and 4 males, mean ± SD age: 46.2 ± 10.3 (range 24-60)) with GHD were recruited. The causes of pituitary deficiencies and hormone replacements are summarized in Table 4.1. None of the hypopituitary patients had been previously replaced with GH except for three subjects. In one patient, GH therapy was stopped 3 months before the recruitment. The other two patients had not received GH for five years.

Table 4.1 Causes of pituitary deficiency and pituitary hormone replacement therapies in 19 subjects with GHD.
F, female; M, male; H, hydrocortisone; C, cortisone acetate; P, prednisone; T4, thyroxine; E_2, estradiol; T, testosterone; D, desmopressin

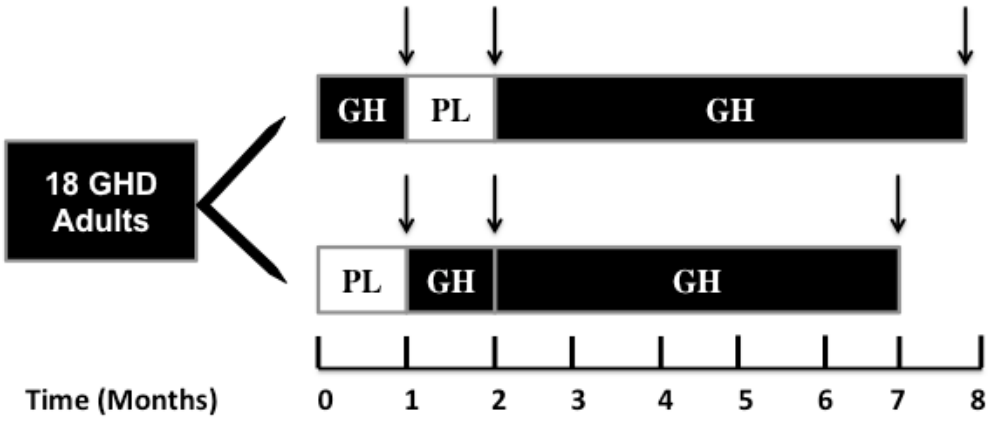
Patient No.	Age	Gender	Diagnosis	Surgery/ Irradiation	Hormone Replacement
1	38	F	Cushing's disease	Yes/No	H, T4, E_2, D
2	24	F	Septo-optic dysplasia	No/No	H, T4, E_2
3	38	F	Macroprolactinoma	Yes/No	C, T4, E_2, D
4	46	F	Silent corticotroph macroadenoma	Yes/No	H, T4
5	60	F	Panhypopituitarism, Empty sella	No/No	C, T4
6	57	F	Non-functioning	Yes/No	T4

			macroadenoma		
7	59	M	Non-functioning macroadenoma	Yes/Yes	C, T4, T, D
8	58	M	Cushing's disease	Yes/Yes	C, T, T4
9	49	F	Macroprolactinoma	Yes/No	H, T4, E_2, D
10	39	F	Glioma	Yes/Yes	P, T4, E_2
11	46	F	Cushing's disease	Yes/Yes	P, T4, E_2, D
12	48	F	Non-functioning macroadenoma	Yes/No	H
13	58	F	Rathke's cleft cyst	Yes/No	C, T4
14	38	F	Non-functioning macroadenoma	Yes/No	T4
15	41	F	Craniopharyngioma	Yes/No	H, T4, E_2
16	32	M	Non-functioning macroadenoma	Yes/No	H, T4, T, D
17	44	M	Non-functioning macroadenoma	Yes/No	H, T4, T
18	58	F	Macroprolactinoma	Yes/Yes	P, T4
19	52	F	Cushing's disease	Yes/Yes	C, T4, E_2

4.2.2 Study design

This is a two-phase design involving (a) a 2-month double-blind placebo-controlled GH with a crossover at 1-month, followed by (b) an open-label active treatment phase of 6 months (Figure 4.1). GH effects on energy metabolism in skeletal muscle are detected as early as 2 weeks (7), however, an increase in muscle mass is seen only after several months of treatment (3). The rationale for the 1-month and 6-month GH treatment is to separate the early effects of GH on the AES from the later anabolic effects on muscle mass and strength.

Figure 4.1 Study design: 2-month double blind placebo controlled GH study with a crossover at 1-month, followed by an open label active treatment for 6 months.
Assessments of aerobic capacity, anaerobic capacity, physical function, body composition and quality of life are undertaken at time points indicated by arrows. GHD, growth hormone deficiency; GH, growth hormone; PL, placebo



During the placebo-controlled study, GH (Norditropin; NovoNordisk, Denmark) replacement was initiated at a dose of 0.2mg/day for the first week, increased to 0.4 mg for the second week and to 0.5 mg, the target dose for the last two weeks. The gradual stepwise regimen was implemented to

reduce side effects. During the open-label study, the GH dose was titrated individually to maintain serum IGFI concentrations in the adult normal reference range (9 to 33 nmol/L). Assessments for anaerobic capacity, aerobic capacity, physical function, body composition and QoL were undertaken at baseline and at the end of each treatment phase. Subjects attended the test venue on two consecutive days on each occasion after an overnight fast.

4.2.3 Assessments

The detailed protocols of assessments are described in the section 2. Anaerobic and aerobic capacities were assessed by the Wingate test and VO_2max test respectively. The stair climb test and chair stand test were used as the functional assessments of anaerobic capacity, and 7-day pedometry as of aerobic capacity. DXA scan was performed to measure LBM and fat mass. QoL was assessed by QoL-AGHDA questionnaire.

4.3 Statistical analysis

This study was powered to detect a significant change in anaerobic and aerobic capacity. The sample size was based on anaerobic capacity data from previous study in recreational athletes (8) and aerobic performance data from Cuneo et al. in adults with GHD (133). A sample of 15 is required to show a 5% improvement in Wingate power and 18 for a 15% improvement in VO_2max at a 0.05 significance level with 80% power. Statistical analysis was performed with IBM SPSS Statistics Software (Version 22, Chicago, IL, USA). Results are reported as mean ± SEM. In the 2-month placebo controlled study, comparison between GH and placebo groups were performed with analysis of variance for repeated measures (ANOVA). The data were analysed for carryover effects using a linear mixed model analysis by studying the effect of interaction between the treatment order (GH/Placebo or Placebo/GH) and treatment itself on outcome measures. In the 6-month open

label study, the effects of GH on outcome measures were compared with the baseline measurements and 1-month placebo treatment using a paired t test based on a review of controlled GH trials that contain a placebo arm in which functional (physical function and QoL) outcomes were measured on more than two occasions. These trials showed that a placebo effect if present at the first evaluation does not change significantly on repeat evaluation (8, 148, 195). Based on the results of the cross-section study described in Chapter 3 (196) and directional hypothesis of this trial, one-tailed t-test was used to assess the effects of GH. Multiple regression analysis was performed to assess independent predictors of QoL amongst age, gender, LBM, measures of physical performance and physical function. A p value of less than 0.05 was considered statistically significant.

4.4 Results

Of the 19 subjects enrolled, one withdrew during the open-label study due to GH related side effects (see below). One patient lost significant weight of 13kg during the study from lifestyle change. By contrast the mean change in body weight for the remaining study cohort (n=18) during the trial was 1.3 ± 0.5 kg. This patient was not included in the analysis, which comprised 18 of the subjects recruited into the study.

4.4.1 IGFI concentration (Table 4.2)

Mean IGFI concentration rose significantly ($p<0.0001$) in response to GH administration during the placebo-controlled and open phase. During the open phase, the GH dose was titrated to maintain the IGF-I concentration in the adult normal range and in accordance with side effects (see below), resulting in a fall of the mean GH dose from 0.5 to 0.37 mg/day.

Table 4.2 Effects of 1-month and 6-month GH replacement on IGFI concentrations, body composition, physical performance, physical function and QoL.

LBM, lean body mass; VO_2max, maximal oxygen consumption; QoL, quality of life, NS, not significant. All values are shown as the mean ± SEM. [a]GH vs. Placebo during placebo-controlled study, [b]GH vs. Baseline during open label study.

		1-month placebo-controlled study			6-month open label study	
	Baseline	**Placebo**	**GH**	**p[a]**	**GH**	**p[b]**
IGF1 (nmol/L) (Reference range 7-32)	11.5 ± 1.7	11.4 ± 1.3	32.9 ± 2.9	<0.0001	28.3 ± 2.0	<0.0001
Body Composition						
LBM (kg)	44.6 ± 1.9	44.4 ± 1.9	45.3 ± 1.8	NS	45.2 ± 2.0	0.06
Fat mass (kg)	29.5 ± 2.2	29.8 ± 2.2	29.2 ± 2.1	NS	28.0 ± 2.2	NS
Physical performance						
Anaerobic Power (W)	263 ± 17	265 ± 16	266 ± 17	NS	278 ± 16	<0.05
Anaerobic Power (W/kg LBM)	5.9 ± 0.2	6.0 ± 0.3	5.9 ± 0.2	NS	6.1 ± 0.2	NS
VO_2max (L/min)	1.58 ± 0.1	1.63 ± 0.1	1.65 ± 0.1	NS	1.63 ± 0.1	NS
VO_2max (mL/min/kg LBM)	35.8 ± 1.4	37.4 ± 1.8	36.7 ± 1.5	NS	36.2 ± 1.5	NS

Physical function						
Stair climb test (seconds)	20.0 ± 0.8	19.1 ± 0.7	18.8 ± 0.6	NS	18.9 ± 0.7	<0.01
Chair stand test (Repetitions)	19.5 ± 1.2	21.4 ± 1.2	21.7 ± 1.1	NS	24.8 ± 1.2	<0.0001
Pedometry (Steps/day)	6593 ± 563	6295 ± 493	6323 ± 595	NS	7780 ± 1044	<0.05
Quality of life						
QoL-AGHDA score (out of 25)	16.1 ± 1.6	12.7 ± 1.8	13.2 ± 1.6	NS	8.8 ± 1.4	<0.001
Energy and vitality score (out of 8)	5.8 ± 0.6	4.3 ± 0.7	4.7 ± 0.7	NS	2.7 ± 0.6	<0.001
Non-energy and vitality score (out of 17)	10.5 ± 1.1	8.6 ± 1.2	8.4 ± 1.1	NS	6.2 ± 1.0	<0.01

4.4.2 Side effects

There were no significant side effects observed during the placebo-controlled study that warranted any blinded-dose adjustment. 10 subjects reported side effects related to fluid retention including peripheral edema, joint stiffness, arthralgia and myalgia during the open label GH trial. These symptoms improved or resolved with a reduction in GH dose. One patient experienced significant peripheral edema and exacerbation of a preexisting carpal tunnel syndrome leading to withdrawal from the trial.

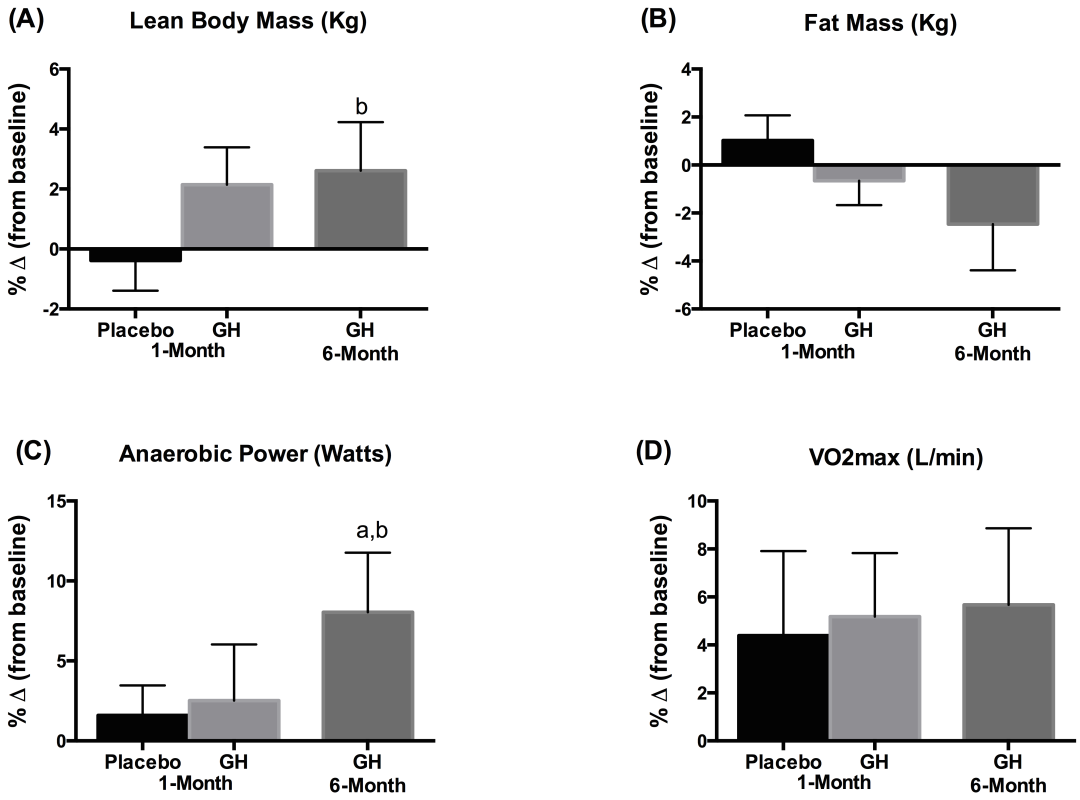
4.4.3 2-month placebo-controlled crossover study

The data were analysed for carryover effects using a linear mixed model analysis. No significant carryover effects were identified in any outcome measures.

4.4.3.1 Body composition (Table 4.2)

There was no significant change in LBM or fat mass during placebo or GH treatment. (Figure 4.2, panel A and B).

Figure 4.2 Percent change from baseline in LBM (Panel A), fat mass (Panel B), anaerobic power (Panel C) and VO_2max (Panel D) following 1 month of placebo and 1 month of GH (randomized controlled study), and 6 months of GH (open label study).
LBM, lean body mass; VO_2max, maximal oxygen consumption. 'a' indicates $p < 0.05$ vs. baseline; 'b' indicates $p < 0.05$ vs. 1-month placebo treatment.



4.4.3.2 Physical performance (Table 4.2)

There was no significant change in anaerobic power during placebo or GH treatment (Figure 4.2, panel C). Similarly, no significant change in VO_2max occurred during placebo or GH treatment (Figure 4.2, panel D).

4.4.3.3 Physical function (Table 4.2)

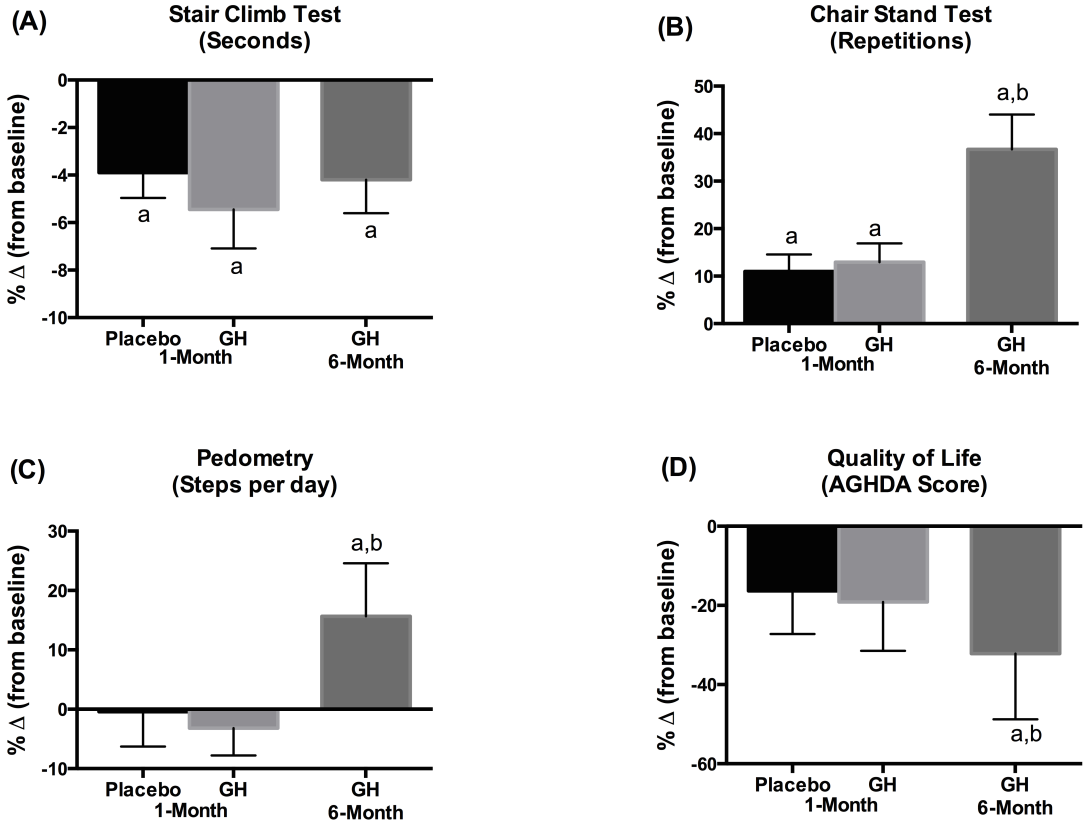
There was an equivalent mean reduction in stair climb duration during GH ($p=0.03$) and placebo ($p=0.02$) treatments (Figure 4.3, panel A). The number of chair stand repetitions increased significantly during GH ($p=0.03$) and placebo ($p<0.05$) treatments. However, the increase in chair

stand repetitions was not significantly different between GH and placebo treatments (Figure 4.3, panel B). Daily step count did not change significantly during placebo or GH treatments (Figure 4.3, panel C). In summary, placebo effects were observed for stair climb and chair stand performance, which were similar to those observed during GH treatment.

4.4.3.4 Quality of Life (Table 4.2)

QoL-AGHDA score (Figure 4.3, panel D), energy and vitality score or non-energy and vitality score did not change significantly during placebo or GH treatment.

Figure 4.3 Percent change from baseline in stair climb duration (Panel A), chair stand repetitions (Panel B), pedometry (Panel C), and QoL-AGHDA score (Panel D) following 1 month of placebo and 1 month of GH (randomized controlled study), and 6 months of GH (open label study).
'a' indicates $p < 0.05$ vs. baseline; 'b' indicates $p < 0.05$ vs. 1-month placebo treatment



4.4.4 6-month open label GH study

Because significant placebo effects occurred with some of the outcome measures (stair climb and chair stand performance), the effects of 6 months of GH therapy were compared with the change observed with placebo therapy during the double-blind phase. These effects were also compared with the baseline measurements. The results were similar except for stair climb duration, which improved significantly if a placebo effect was not accounted for.

4.4.4.1 Body composition (Table 4.2)

The mean LBM increased during 6 months of GH treatment with the change significantly greater than that observed after 1-month of placebo treatment ($p=0.007$) (Figure 4.2, panel A). FM fell during GH treatment, but the change did not reach statistical significance compared to that after 1-month of placebo treatment ($p=0.05$) (Figure 4.2, panel B).

4.4.4.2 Physical performance (Table 4.2)

Mean anaerobic power increased at the end of 6 months of GH replacement ($p<0.05$). This increase was significantly greater than that observed after placebo treatment ($p=0.04$) (Figure 4.2, panel C). Increase in anaerobic power lost significance when corrected for changes in LBM (W/kg LBM). In contrast, the change in VO_2max in absolute values (L/min) or corrected for LBM (mL/min/kg LBM) during 6 months of GH replacement was not significantly different from baseline or that observed after placebo treatment during the blinded phase (Figure 4.2, panel D).

4.4.4.3 Physical function (Table 4.2)

The time to complete the stair climb test fell after 6 months of GH replacement ($p<0.01$). However, the reduction in stair climb duration was not significantly different from that observed after placebo treatment during the blinded phase (Figure 4.3, panel A). In the chair stand test, the number of repetitions performed in 30 seconds increased during 6 months of GH, a change that was significantly greater than that observed after placebo treatment ($p<0.0001$) (Figure 4.3, panel B). The average daily step count increased significantly after 6 months of GH replacement compared to that observed after placebo treatment ($p=0.04$) (Figure 4.3, panel C).

4.4.4.4 Quality of Life (Table 4.2)

Total QoL-AGHDA scores fell after 6 months of GH when compared to changes observed after placebo treatment during the blinded phase ($p=0.04$) (Figure 4.3, panel D). A sub-analysis revealed that scores for energy and vitality domains and for the non-energy and vitality related domains fell significantly ($p<0.05$) during GH treatment.

4.4.5 Predictors of QoL

The factors that significantly improved QoL during 6 months of GH replacement were identified using multiple regression analysis. This included age, gender, and improvements in LBM, anaerobic power, chair stand and stair climb performance, and daily step count as independent variables, the improvement in anaerobic power emerged as a significant predictor of the improvement in QoL energy and vitality scores ($p=0.03$) (table 4.3). However, none of the variables

including anaerobic power was a significant independent predictor for overall QoL and non-energy and vitality related QoL domains.

Table 4.3 Linear regression model of predictors of energy and vitality domains of QoL

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	5.7	6.0		.95	.37
Delta_LBM	-.05	.31	-.05	-.17	.87
Delta_anaerobic power	-.07	.03	-.78	-2.7	.03
Age	-.01	.08	-.03	-.11	.92
Gender	-4.6	2.5	-.63	-1.9	.09
Delta_chair stand repetitions	-.09	.18	-.13	-.51	.62
Delta_daily steps	.00	.00	-.23	-.94	.37

a. Dependent Variable: Delta_energy and vitality scores

4.5 Discussion

This study investigated whether GH replacement improves anaerobic capacity, physical function and QoL in adults with GHD in a two-phase study comprising a 1-month double-blind placebo-controlled phase followed by a 6-month open-label phase. During the blinded phase, GH did not improve any of the outcome measures compared to placebo, which induced significant improvements in stair climb and chair stand performance. In the open-label 6-month study, GH therapy significantly improved anaerobic power, chair stand performance, daily step count and QoL compared to placebo changes observed during the blinded phase. GH therapy did not improve

aerobic capacity. The increase in anaerobic capacity was significantly associated with improvement in energy and vitality QoL domains.

A major aim was to determine whether GH improves anaerobic performance, which was assessed by the Wingate test. Anaerobic capacity is determined by energy availability and by contractile strength of muscle. To distinguish between these two mechanisms, the study was designed to assess the effects of GH treatment after 1 and 6 months. It was postulated that a mechanism enhancing energy provision would manifest early whereas that improving muscle strength through protein anabolism would take several months of therapy (197). This study found that anaerobic capacity did not change after 1 month but improved after 6 months of GH treatment. The loss of statistical significance in anaerobic capacity when corrected for changes in LBM, suggests that the improved anaerobic performance was in part due to increased LBM and muscle mass. These findings suggest that improvement in anaerobic capacity by GH may occur via an anabolic rather than a metabolic mechanism.

The present finding that short-term GH treatment failed to improve anaerobic capacity in adults with GHD stand in contrast to a study in recreational athletes (8). In the latter study, short-term GH treatment significantly increased sprint capacity, which returned to baseline after cessation of treatment. The reasons for conflicting findings between the two studies are unclear but could arise from difference in dose, duration and subjects. The latter study was undertaken in GH replete recreational athletes who were administered a supraphysiological dose of 2 mg/day for 8 weeks. In contrast, the current study employed a daily dose of only 0.5mg for 4 weeks in subjects who were GH deficient. It is possible that the difference reflects the physiological and pharmacological effect of GH on the AES.

A second study aim was to determine the functional relevance of the anaerobic and aerobic energy systems. This was addressed by investigating whether a relationship is present between these energy systems and selected measures of daily activity. Activities such as rising from a chair or climbing stairs are supported mainly by the AES. Walking is a regular component of daily habitual activity. As walking consists of an initiation phase and a maintenance phase, both anaerobic and aerobic systems likely to contribute to daily walking capacity, which was estimated from weekly pedometry measurement. After 6 months of GH therapy, an improvement in anaerobic capacity was observed along with an improvement in chair stand performance and daily step counts. There was no change observed in aerobic capacity with or without LBM correction. A likely reason that limits walking in patients with GHD is fatigue that develops quickly on initiation of physical activity because of subnormal anaerobic capacity. In real life, early fatigue may discourage the patient to continue further even though the physical activity of walking is far below the VO_2max. There is a positive relationship between regular physical activity and health (198). Regular physical activity, including walking in healthy adults is associated with a reduction in coronary events (199), cerebrovascular events (200), cognitive dysfunction (201), and an improvement in health related QoL (202). Adults with hypopituitarism and GHD are an increased risk of premature cardiovascular mortality (6, 42). Thus, an improvement in daily habitual activity such as walking from GH replacement may have a beneficial long term impact on metabolic and cardiovascular health of adults with GHD.

There was no significant improvement observed in aerobic capacity or body composition after GH replacement for up to 6 months. These findings stand in contrast with previous studies reporting an improvement in aerobic capacity (20, 21, 28, 37, 53, 133, 134, 197) and body composition (12, 20, 32-34) in GHD after GH therapy. However, these studies employed much higher doses of GH compared to the current study dose. In a metaanalysis, Widdowson et al. observed that studies showing an improvement in aerobic capacity used a GH dose ranging between 0.86 and 2.2 mg per

day compared to 0.37mg per day in this study (203). Similarly, a separate metaanalysis of placebo-controlled randomized trials found a clear dose response relationship for changes in LBM and fat mass (45). Hence, the modest changes in aerobic capacity and body composition are likely due to the lower doses of GH used in this study compared to the previous studies.

A significant improvement in QoL was observed after 6 months of GH treatment in agreement with previous placebo-controlled studies (148, 149, 154, 157, 158). Further analysis revealed an improvement in the energy and vitality domain, which is the worst affected in GHD and exhibits the greatest improvement during GH replacement (153). The mechanisms explaining the impairment of QoL and its improvement after GH replacement are unclear. Although it seems reasonable to assume that anabolic effects of GH on muscle mass, and improvement in aerobic capacity may play a role in restoring QoL in adults with GHD, no correlation has been found between QoL and these parameters (6, 165, 166). It is conceivable that energy systems required to drive physical activity and function are likely factors influencing QoL. This study showed that during GH replacement, QoL improved in parallel with an improvement in anaerobic capacity, chair stand and daily walking capacity and in the absence of any improvement in aerobic capacity. Furthermore, within this global change, the improvement in the energy and vitality domains was independently influenced by anaerobic capacity. These results suggest that an improvement in the anaerobic but not the aerobic energy system underlies the improvement in energy and vitality during GH replacement, in turn facilitating physical activities such as rising from a chair and walking.

This study has a number of limitations. The 6-month GH replacement study was not placebo-controlled. This was address by comparing the outcome measures to those observed in response to placebo therapy in the 1-month blinded phase. It was a carefully planned study in the face of the feasibility and ethics of conducting a 6-month placebo-controlled trial for GH, already an approved replacement therapy for adults with GH deficiency involving daily injections. In planning this

study, a review of placebo-controlled GH trials was undertaken, in which functional (physical function and QoL) outcomes were measured on more than two occasions during the placebo arm. These trials showed that a placebo effect if present at the first evaluation does not change significantly on repeat evaluation (8, 148, 195). In these trials, the magnitude of change from the first evaluation during placebo phase to a later time point, usually after 3 to 6 months, was less than 1.5%. The inclusion of a 1-month placebo controlled phase in this study allowed for the quantification of a placebo effect. Based on this evidence, the comparison of 6-month GH treatment outcomes with 1-month placebo measurements is valid and it was interpreted accordingly, but with some caution. This study was undertaken in a group of subjects who were predominantly women, who are less responsive to GH than men (13, 41). The higher female preponderance may have contributed to the modest changes observed in the outcome measures. Because this study addresses the energy systems, it was investigated whether change in the energy system is accompanied by change in a health-related QoL domain that assesses energy-related symptoms. However, the use of subsections of the AGHDA questionnaire in this way requires further validation.

This is the first study investigating the functional relevance of the AES in adults with GHD. 6 months of GH replacement in adults with GHD increased anaerobic capacity, chair-stand performance, daily step counts and QoL but not aerobic capacity. Improvement in QoL occurred without an improvement in aerobic capacity during GH replacement. The improvement in anaerobic capacity independently predicted the improvement in energy and vitality of patients with GHD during GH replacement.

In conclusion, GH replacement improves anaerobic capacity in a time-dependent manner. Chair stand and daily walking capacity are likely functional measures of the AES. Improvement in the anaerobic but not aerobic energy system explains in part the improvement in physical function and QoL in adults with GHD during GH replacement.

5 EFFECTS OF GH ON METABOLIC GENE EXPRESSION IN SKELETAL MUSCLE

5.1 Introduction

The AES powers intense physical activity of brief duration as well as the initiation of all physical movement including activities of daily living (5). Thus, the AES plays a vital role in daily life. Impairment to the AES leads to early fatigue and loss of vitality, symptoms commonly experienced by adults with GHD (153).

Early work from this thesis revealed that anaerobic capacity, the measure of AES, is impaired in adults with GHD compared to age, gender and BMI matched healthy adults (196). An intervention trial showed that GH replacement improves anaerobic capacity of adults with GHD in a time dependent manner (204). The mechanisms underlying the increase in anaerobic capacity following GH remain unclear. Anaerobic capacity depends on muscle mass and availability of energy to skeletal muscle. GH increases muscle mass in adults with GHD, but its effects on anaerobic energy metabolism in skeletal muscle are not known.

GH is a major regulator of substrate metabolism and energy production. It increases plasma concentration of free FA by stimulating lipolysis (113-117), and glucose by stimulating gluconeogenesis and glycogenolysis (118, 119). GH effects on metabolism of these energy fuels have been extensively studied at a whole body level. The studies have shown that GH stimulates whole body FA oxidation and reduces glucose utilization in healthy adults (113, 120, 121) as well as adults with GHD (122-124). Because LBM accounts for the majority of substrate metabolism, it

is widely assumed that these effects reflect its action on skeletal muscle, however, the evidence to support this assumption is lacking.

There is emerging evidence that GH action on substrate metabolism is tissue specific. Tollet-Egnell et al. reported that the effects of GH on liver and skeletal muscle in rodents may be disparate (126). In the liver, GH reduced expression of genes governing FA synthesis with an increase in genes for FA oxidation; where in muscle, genes for FA uptake were reduced and glycolysis were increased upon GH treatment. Similarly, in a small study of four adults with GHD, Sjogren at el. found that GH downregulated key genes governing lipid oxidation, TCA cycle activity and mitochondrial respiration as well as genes that suppress glucose utilization in skeletal muscle (7). These findings suggest that GH inhibits oxidative metabolism of substrates favoring non-oxidative glycolytic pathways for energy synthesis in skeletal muscle, opposite to that is seen at the whole body level.

The aim of this study was to investigate whether GH alters the expression profiles of skeletal muscle genes in favour of anaerobic metabolism. This study was undertaken to identify differentially expressed genes involved in substrate metabolism in skeletal muscle following GH replacement in adults with GHD employing microarray technology.

5.2 Material and Method

5.2.1 Subjects

10 out of 19 adults with GHD from the intervention trial described in chapter 4 participated in this study. The baseline characteristics, pituitary deficiencies and hormone replacements are summarized in chapter 4, Table 4.1 (Patient No. 2, 3, 4, 5, 6, 9, 10, 11, 13 and 19).

5.2.2 Study design

10 adults with GHD underwent a 1-month double blind placebo controlled crossover study followed by 6-month open phase (Chapter 4). To understand the biochemical mechanisms underlying the improvement in anaerobic capacity, microarray experiments were performed to identify the differentially expressed genes in skeletal muscle after 1-month. These experiments were repeated after 6-month of GH treatment to investigate whether the short-term GH effects on metabolic gene expression are sustained with the continuation of GH treatment.

Subjects attended the test venue after an overnight fast for a muscle biopsy of vastus lateralis after 1-month of placebo and 1-month of GH treatment (placebo controlled phase), and at the end of 6-month open label treatment with GH. Muscle samples collected during placebo treatment were considered as control samples. During the placebo-controlled study, GH (Norditropin; NovoNordisk, Denmark) replacement was initiated at a dose of 0.2mg/day for the first week, increased to 0.4 mg for the second week and to 0.5 mg, the target dose for the last two weeks. The gradual stepwise regimen was implemented to reduce side effects. During the open-label study, the GH dose was titrated individually to maintain serum IGFI concentrations in the age-adjusted adult normal reference range.

Microarray experiments were performed and analyzed in two parts. The first part compared the gene expression in skeletal muscle in 6-subjects after 1-month of GH and placebo treatments (Patient No. 2, 3, 4, 5, 6 and 11; Chapter 4, Table 4.1). The second part compared gene expression in skeletal muscle after 1-month placebo with that after 6-month GH replacement in 9 subjects (Patient No. 2, 3, 4, 5, 9, 10, 11, 13 and 19; five out of nine patients participated in the 1-month study). The study was carried out in accordance with Good Clinical Practice guidelines, approved

by the Metro South Human Research Ethics Committee, and signed informed consent was obtained from all subjects. This study is registered in The Australian New Zealand Clinical Trials Registry (number: ACTRN12615000046505).

5.2.3 Muscle biopsy

Muscle biopsies were obtained using 5mm Bergstrom needle from vastus lateralis muscle as described in the Chapter 2. Using a suction technique, Bergstrom needle was used to yield 100-150mg of muscle. Muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C.

5.2.4 RNA isolation

Total RNA was isolated from snap-frozen muscle biopsies using the TRIzol Reagent (Life Technologies Inc., Gaithersburg, Maryland 20884, USA) during the placebo controlled study and RNeasy fibrous tissue extraction kit (Qiagen, Valencia, USA) during the open label study according to the manufacturer's instructions as described in chapter 2 and stored at -80°C. The concentration of the RNA was assessed by NanoDrop spectrophotometer and the quality was determined using the Agilent RNA 6000 Nano Kit on the 2100 Bioanalyzer (Agilent, Amstelveen, The Netherlands). Only total RNA samples with a RNA integrity number (RIN) higher than seven were included for further analysis.

5.2.5 Microarray hybridization

Microarray experiments were undertaken separately for the 1 month and 6 month studies. Samples for the 1-month study were processed at the Australian Genome Research Facility (Melbourne, Australia) and for the 6-month study at the University of Queensland Diamantina Institute (Brisbane, Australia). For both experiments, the RNA samples were biotinylated and amplified using the Illumina® TotalPrep™ RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with a standardized input amount of 500ng.

Briefly, the procedure consists of reverse transcription of total RNA to first strand cDNA, which then undergoes second strand synthesis and purification to become a template for transcription. In vitro transcription of dsDNA was used to synthesize cRNA labelled with biotin-16-uridine-5-triphosphate (UTP). The labelled probes were hybridized to Illumina HumanHT-12 v4 microarrays (San Diego, CA) including approximately 47,000 probes as per manufacturer's instructions and whole-genome transcriptional profiling was performed. The beadchips were scanned using Illumina BeadStation, and the signal was extracted by using Illumina BeadStudio software.

5.2.6 qRT-PCR verification

Reverse transcription PCR was performed to validate the microarray findings using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA was used for real-time quantitative PCR, which was performed in triplicate on a ViiA^{TM} Real Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression assays (Applied Biosystems), with the assay IDs as follows: Fatty acid synthase (FASN) (Hs01005622_ml), Stearoyl-CoA desaturase 1 (SCD) (Hs01682761_ml), Dual specificity phosphatase 1 (DUSP1) (Hs00610256_ml), G0/G1 switch 2 (G0S2) (Hs00274783_ml), 3-hydroxy-3-methylglutaryl-CoA

synthase 2 (HMGCS2) (Hs00985427_ml)), Uncoupling protein 3 (UCP3) (Hs01106052_ml), Beta-2-microglobulin (B2M) (Hs00187842_ml). Relative mRNA levels were calculated by the comparative threshold cycle method using B2M as the housekeeping gene.

5.3 Bioinformatics and statistical analysis

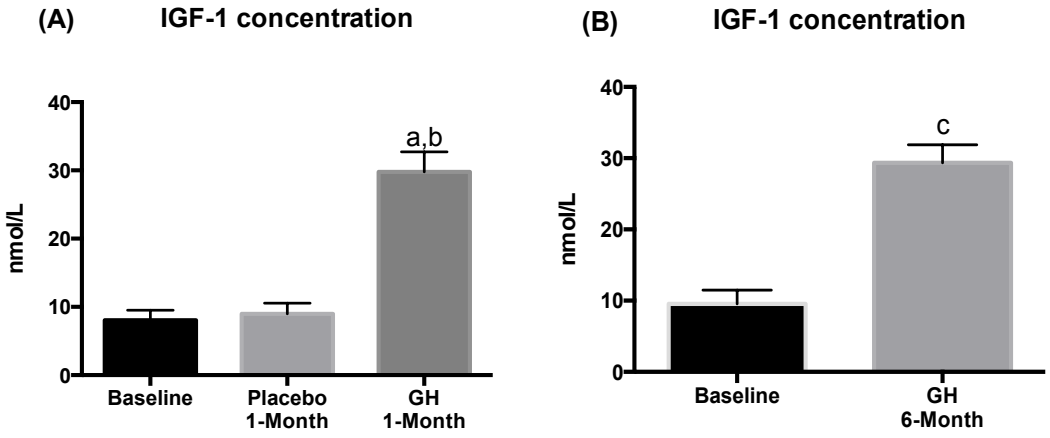
The data were analyzed using statistical programming language R and quality control steps were performed using Bioconductor packages lumi (205) and limma (206). To correct the variation between the Genechips, Microarray data of .idat files of Illumina beadchips were log2 transformed and normalized using quantile normalization. The unsupervised technique principal component analysis (PCA) multilevel was used to explore the data and identify a potential natural separation between patients before and after treatment as well as potential confounder effects. After logarithmic transformation, the results for baseline samples and GH treated samples (1-month and 6-month) were analyzed using paired t-test. The P value was adjusted for multiple testing, using the Benjamini and Hochberg method (207) set at a false discovery rate (FDR) of 5%. A gene was considered regulated if there was a fold change of ≥1.4 or ≤0.7 in response to GH, criteria similar to those previously published (7). Because the GHD patients were treated with a physiological replacement dose of GH, only a modest effect on gene expression was expected. qRT-PCR method was used to validate the microarray results.

5.4 Results

5.4.1 IGFI concentration

Mean IGFI concentration rose significantly in response to GH administration during the placebo-controlled ($p<0.01$) and open phase ($p<0.0001$) (Figure 5.1). During the open phase, the GH dose was titrated to maintain the IGF-I concentration in the adult normal range (9 to 33 nmol/L), resulting in a reduction of the mean GH dose from 0.5 to 0.43 mg/day.

Figure 5.1 Mean IGF concentration during 1-month placebo controlled study (Panel A) and 6-month open label study (Panel B) in six and nine adults with GHD respectively.
'a' indicates $p < 0.01$ vs. placebo, 'b' indicates $p < 0.01$ vs. baseline; 'c' indicates $p < 0.0001$ vs. baseline.



5.4.2 Effect of GH on gene expression

5.4.2.1 Effect of 1-month of GH

1-month GH replacement did not induce any significant changes in gene expression in skeletal muscle of adults with GHD at an FDR of 5%. When data were reanalyzed using an unadjusted P value without setting an FDR threshold, no differentially expressed genes were identified compared to the control samples. As described in Chapter 4, no significant changes in aerobic or anaerobic capacity were observed between GH and placebo treatments.

5.4.2.2 Effect of 6-month of GH

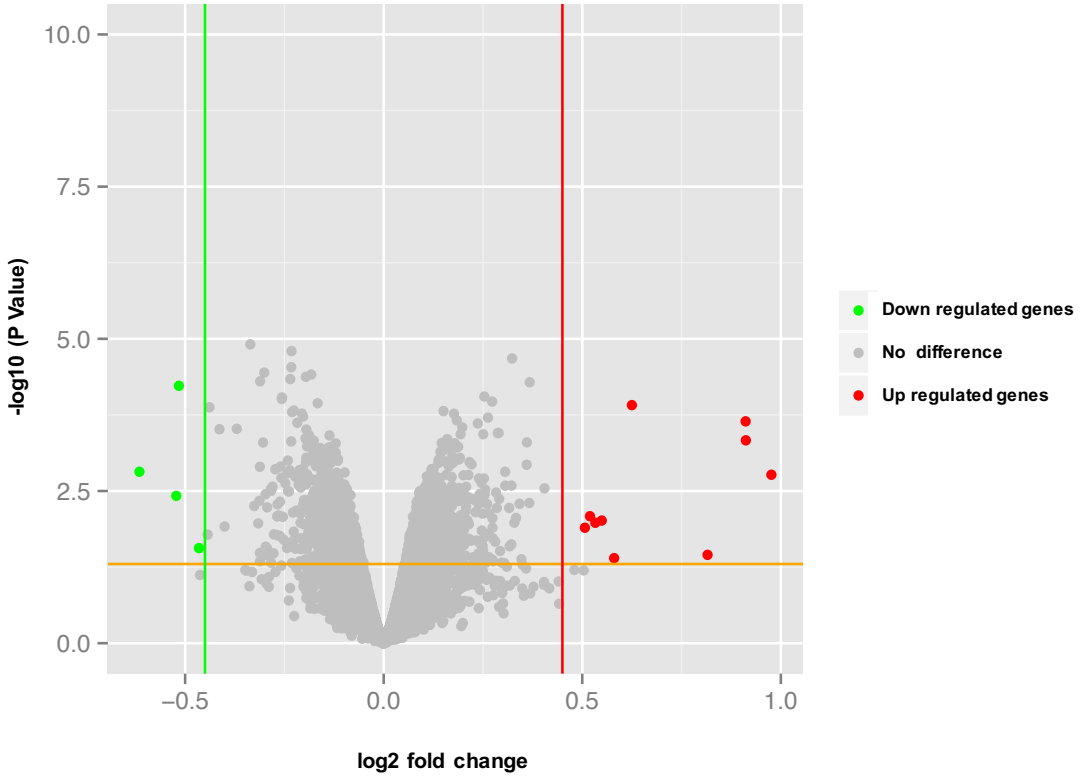
A significant improvement occurred in anaerobic capacity after six months of GH replacement. No significant changes in gene expression of skeletal muscle were detected at an FDR of 5%. However, when an unadjusted P value was used, 14 differentially expressed genes were identified. Amongst these genes, 10 were upregulated and 4 downregulated (Table 5.1). The relationship between P value and log fold change for all probes is represented by a volcano plot in figure 5.2, showing the 14 differentially expressed genes using an unadjusted P value of 0.05 (horizontal orange line) and the fold change of 0.7 and 1.4 by vertical green and red lines respectively.

Table 5.1 GH responsive genes in skeletal muscle

Gene title	Gene symbol	Fold change*	p value
Upregulated genes			
Fatty acid synthase	FASN	1.5	0.04
Stearoyl-CoA desaturase 1 (delta-9-desaturase)	SCD1	1.8	0.04
Dual specificity phosphatase 1	DUSP1	1.5	0.01
G0/G1 switch 2	G0S2	1.9	0.0002
Interleukin 32	IL32	1.5	0.0001
Early growth response 1	EGR1	1.9	0.0005
FBJ murine osteosarcoma viral oncogene homolog	FOS	2.0	0.002
Cysteine-rich, angiogenic inducer 61	CYR61	1.4	0.008
Regulator of calcineurin 1	RCAN1	1.6	0.01
Transferrin receptor	TFRC	1.4	0.01
Downregulated genes			
3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	HMGCS2	0.7	0.03
Uncoupling protein 3 (mitochondrial)	UCP3	0.7	0.002
Metallothionein 2A	MT2A	0.7	0.004
Tetraspanin 8	TSPAN8	0.7	<0.0001

*6-month GH-treated relative to baseline samples

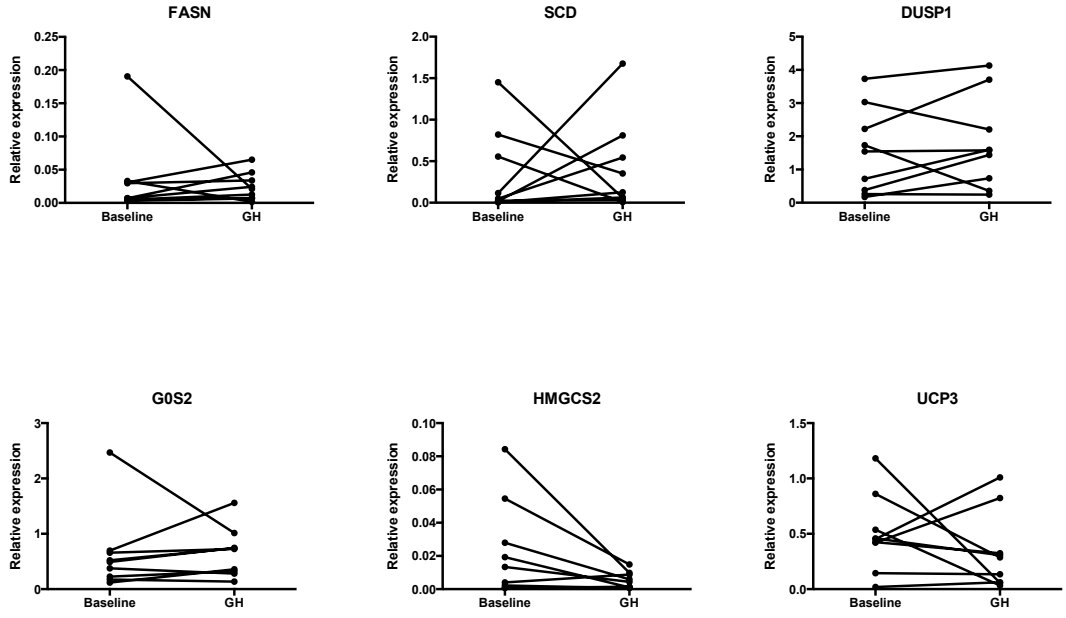
Figure 5.2 Relationship between p value and log fold change for all probes on microarray in 9 GH treated subjects at 6 months.
The horizontal line represents threshold p value of <0.05, whereas vertical lines indicate log2 fold change of 0.45.



5.4.3 Verification of Microarray results

To verify the microarray data, the transcript levels of six genes that play a major role in substrate metabolism (FASN, SCD1, DUSP1, G0S2, HMGCS2 and UCP3) were quantified by qRT-PCR. Analysis was performed on individual samples, with B2M included as an internal control. As shown in Figure 5.3, none of the transcripts quantified by qRT-PCR changed significantly during GH treatment.

Figure 5.3 qRT-PCR verification of six GH responsive genes.
Results are relative mRNA expression adjusted for the expression of B2M of baseline and 6-month GH treated samples. There was no significant change observed in the expression of any of these six genes after 6-month of GH treatment.



5.5 Discussion

This study investigated GH effects on the AES by studying the gene expression profile of skeletal muscle from adults with GHD in a two-phase study comprising a 1-month double-blind placebo-controlled phase followed by a 6-month open-label phase. During GH treatment, serum IGFI concentrations increased into the normal adult range. Neither one month or six months of GH replacement induced any significant change in expression of genes governing energy metabolism in skeletal muscle of adults with GHD.

These results stand in contrast with previous studies in rodents as well as in human adults with GHD, which provided evidence that GH suppresses oxidative metabolism in muscle and favours non-oxidative energy production via glycolysis (7, 126). Tollet-Egnell et al. reported that GH increased expression of 4 genes promoting glycolysis and reduced 2 transcripts involved in FA

uptake in skeletal muscle of rats (126). Sjogren et al. reported that GH suppressed genes governing lipid oxidation, TCA cycle activity and mitochondrial respiration as well as genes that suppress glucose utilization in skeletal muscle of adults with GHD (7). Expression of genes involved in FA transport such as fatty acid binding protein-3 (FABP-3) and CTP-I fell by 50%. Genes for several enzymes that mediate β-oxidation of FA and ATP synthesis via oxidative pathway in the mitochondria were also suppressed in skeletal muscle following GH therapy. For example, the expression of oxoglutarate dehydrogenase and succinate dehydrogenase complex B in the TCA cycle and ATP synthase and NADH dehydrogenase in the mitochondrial respiratory chain were reduced by up to 40%. GH also reduced the transcripts levels of pyruvate dehydrogenase kinase-4 (PDK-4) and glycogen synthase-I in muscle. The latter has been reported in previous studies in healthy (127) as well as adults with GHD (128). PDK-4 is an inhibitor PDH, the key enzyme that converts pyruvate to Acetyl CoA for oxidation in mitochondria (129). These findings validated by qRT-PCR indicate that GH favours glucose as a metabolic fuel over lipid in skeletal muscle.

The reasons for discrepant findings between the current study and that of Sjogren et al. are unclear. The current study used a dose of GH (0.5mg/ day) similar to the Sjogren study. A key difference between the two studies is the gender of subjects. The study by Sjogren et al. was undertaken in 4 men with GHD, as compared to women in the current study. Women with GHD are less responsive to GH as compared to men and require a higher replacement dose of GH than men (208-210). Testosterone enhances the anabolic responses to GH. Testosterone co administered with GH result in a significantly higher IGFI levels and rate of whole body protein synthesis compared with GH alone (8, 122). Thus, GH effects in the Sjogren study may have been more pronounced due to the concomitant administration of testosterone, and a higher dose of GH may be required to achieve similar effects in women.

Other explanation for the lack of significant change in gene expression to GH may be technical. Methodological issues may stem from quality of RNA, the amount of RNA included in the RNA labeling reaction, differences in efficiency of the biochemical transformations, and steps within the RNA labelling itself including reverse transcription, dye incorporation and fluorescent label detection. However, each sample was carefully assessed by a Bioanalyser and only the samples with a RIN above 7 were included in the experiments to ensure good quality of RNA. Robust quality control analyses were undertaken for the normalization of raw microarray data using the appropriate statistical software to control for other non-biological variations.

Subjects received a small physiological replacement dose of GH and hence only a modest change in gene expression was expected. In constraining false positives, setting a FDR at 5% may have been too stringent for detecting small effect of GH. When a FDR was not set at all, 14 differentially expressed genes were identified based on the ranking by fold change. Eight of these genes (RCAN1, EGR1, FOS, TFRC, CYR61, IL32, MT2A, TSPAN8) are involved in growth, cell proliferation and differentiation, apoptosis and inflammation, but their role in skeletal muscle substrate metabolism is unknown. The remaining six genes are known to play a significant role in substrate metabolism, 4 of which were upregulated (FASN, SCD1, DUSP1 and G0S2) and 2 downregulated (HMGCS2 and UCP3).

Among the upregulated genes, FASN is a GH-regulated gene that stimulates FA synthesis in adipose tissue and liver. Reduction in FASN expression in adipose tissue and liver leads to increased lipid oxidation (211, 212). Increased FASN expression in adipose tissue increases lipogenesis (213). Hence, it is conceivable that increased expression of FASN in muscle is also likely to increase lipogenesis and reduce lipid oxidation. SCD1 catalyzes the final rate-limiting step in de novo lipogenesis. SCD1 knock out mice manifest an increase in FA oxidation in liver, muscle and brown adipose tissue and a reduction in de novo lipid synthesis (214, 215), suggesting GH

induced increase in SCD1 expression is likely to inhibit lipid oxidation in muscle. DUSP1 is a key regulator of PGC1α. PGC1α drives mitochondrial biogenesis and function in skeletal muscle and maintains oxidative fibre type distribution (216, 217). DUSP1 overexpression leads to reduction in mitochondrial phosphorylation, lipid oxidation and proportion of oxidative fires in skeletal muscle (216, 218). G0S2 inhibits adipose triglyceride lipase, a rate-limiting enzyme in lipolysis, and causes reduction in lipid oxidation and increase in glucose uptake in skeletal muscle (219). Among the downregulated genes, HMGCS2 is an important enzyme in ketone body formation and reduction of this enzyme leads to an increase in glucose utilization for ATP synthesis (220, 221). Suppression of UCP3 expression causes a reduction in lipid oxidation in skeletal muscle (222). Collectively these results indicate that GH reduces lipid oxidation and mitochondrial function, and increases glucose utilization for ATP synthesis in skeletal muscle. However, these results could not be validated by qRT-PCR.

Apart from the availability of anaerobic energy, anaerobic capacity also depends on anabolic properties of skeletal muscle including muscle mass, strength and the proportion of fast twitch type II muscle fibres that determines the contractile force of muscle (5, 223). In this study, GH treatment did not significantly change the expression of genes involved in the regulation of muscle anabolism or fibre type distribution such as muscle IGFI receptor (IGFIR), myogenic differentiation 1 (MYOD1), myostatin (MSTN) and myosin heavy chain isoforms (MYH1, MYH 2).

In summary, GH replacement in a group of predominantly female patients with GHD was associated with minor changes in metabolic gene expression that failed to reach statistical significance and validation by qRT-PCR. In conclusion, studies with a larger sample size with a balanced gender distribution are required to identify the GH-responsive genes that underlie the regulation of anaerobic energy metabolism in skeletal muscle.

6 SUMMARY AND CONCLUSIONS

6.1 Introduction

This thesis investigated the role of GH in the regulation of the AES. The first aim was to investigate whether anaerobic capacity is impaired in adults with GHD and if so, its functional significance. This was assessed in a cross-sectional comparison of anaerobic capacity, physical function and QoL between adults with GHD and matched healthy controls, and reported in Chapter 3.

The second aim was to investigate whether GH replacement improves anaerobic capacity in adults with GHD and whether this is associated with changes in physical function and in QoL. Described in Chapter 4, this was addressed by a 1-month double-blind placebo controlled crossover study, followed by a 6-month open-label active treatment phase.

The third aim was to evaluate the effects of GH on anaerobic substrate metabolism in skeletal muscle of adults with GHD. Reported in the chapter 5, this was assessed by studying the changes in metabolic gene expression in muscle biopsy tissue obtained after 1-month and 6-month of GH treatment.

The methods used for this body of work, described in Chapter 2 included physical performance tests, physical function tests, body composition measurement, QoL assessment, RNA isolation, gene expression profiling using microarray technology and qRT-PCR. Anaerobic capacity was assessed by the Wingate test, and aerobic capacity by the VO_2max test. Physical function was assessed by the stair-climb test, chair-stand test, and 7-day pedometry. QoL was assessed by a GHD-specific questionnaire. The reproducibility of the physical performance and function tests were established by studying 5 healthy volunteers on two separate occasions. The microarray

experiments were performed at AGRF, Melbourne, Australia (1-month placebo controlled study) and UQ Diamantina Institute, Brisbane, Australia (6-month open-label study).

6.2 Summary and conclusions

The cross-sectional study showed that anaerobic power was nearly 20% lower in adults with GHD compared to control subjects, a deficit similar to that observed for VO_2max. Stair climb capability, chair stand performance and daily step counts, measures of physical performance were significantly impaired as was QoL. GH status of the subjects was an independent determinant of anaerobic capacity. Anaerobic capacity independently predicted stair climb performance and QoL.

One-month of GH replacement did not improve any of the outcome measures compared to placebo treatment. Six months of GH replacement increased anaerobic capacity, LBM, chair-stand performance, daily step count and QoL, but not aerobic capacity compared to baseline and to changes observed during placebo treatment for 1-month. QoL improved without a corresponding improvement in aerobic capacity. The improvement in anaerobic capacity independently predicted the improvement in energy and vitality domains of QoL in adults with GHD.

GH replacement was associated with minor changes in some metabolic genes in skeletal muscle that did not reach statistical significance and failed validation by qRT-PCR.

In conclusion, GH regulates the AES. In adults, a lack of GH impairs anaerobic capacity, adversely affecting physical function and QoL. GH replacement improves anaerobic capacity in a time-dependent manner. Chair stand and daily walking capacity are likely functional indicators of the anaerobic energy system. Improvement in the anaerobic but not aerobic energy system explains in part the improvement in physical function and QoL in adults with GHD during GH replacement.

The molecular and cellular mechanisms underlying GH mediated stimulation of the AES in skeletal muscle remain unclear.

6.3 Future directions

This body of work provides the first evidence for a regulatory role of GH on muscle function dependent on the AES and its functional relevance to daily living by studying adults with GHD. The work tested the hypothesis that GH replacement improves anaerobic capacity within a few weeks and this effect is sustained. However, GH improved anaerobic capacity only after six months of treatment without significant changes in metabolic gene expression in skeletal muscle. These findings stand in contract to a previous study in recreational athletes showing an enhancement of anaerobic capacity after 8 weeks (8) and a study in a small group of GH-deficient males reporting a significant change in metabolic gene expression in muscle after only 2 weeks of GH treatment (7). The reason for discrepant findings between the current and latter study (7) despite using the same dose and similar treatment duration is likely the gender of participants who were entirely women in the current study and men in the latter study. The preponderance of females in the current study arose by chance and the difficulty encountered in recruiting equal numbers of male participants with GHD. Women with GHD are less responsive to GH and require a higher replacement dose of GH than men (208-210). Hence, a larger sample size may be required to show a similar effect in a study involving only women. Although Meinhardt et al. (8) reported an improvement in anaerobic capacity in a pooled group of normal women and men after eight weeks of GH treatment, no significant effect occurred in women alone despite a 4 times higher GH dose used in this trial, again emphasizing gender-specific differences in GH sensitivity. The improvement in anaerobic capacity at 6 months in the current study likely arose from an anabolic effect on muscle mass and strength that occurs after long-term GH therapy (32, 49, 50, 61). To minimize sample size, future studies to

identify GH-regulated metabolic genes in skeletal muscle that improve anaerobic capacity should be undertaken using a similar replacement dose in men with GHD.

Microarray is a powerful research method that allows the study of expression patterns of tens of thousands of genes in a tissue sample. However, the results are based on an assumption that transcriptional changes reflect the effects on protein expression. Future studies investigating GH effects on substrate metabolism in skeletal muscle should also assess the changes in enzyme activities governing anaerobic and aerobic metabolism by analyzing protein expression by Western Blot or quantify biochemical pathway changes of glycolysis. The feasibility of such studies in humans is difficult due to the invasive procedure and large amount of tissue sample requirement. Such studies investigating tissue enzyme and biochemical changes should be conducted in animals.

The mechanisms underlying the impairment in QoL and its improvement after GH replacement in adults with GHD are poorly understood. Previous studies have not found any relationship between QoL and changes in aerobic capacity or other anabolic parameters during GH replacement therapy. Future studies should explore further the role the AES in influencing QoL in adults with GHD using validated research tools specifically designed to assess energy and vitality domains of QoL, such as Nottingham Health Profile questionnaire and Well-being Questionnaire 12 (224, 225).

The age-related decline in physical and function capacity has been attributed to a progressive fall in GH secretion with advancing age. Several well conducted GH trials have not observed any beneficial physical outcomes leading to considerable doubt as to the value in GH supplementation for older people (70, 71, 195, 226-228). However, these trials have focused on GH effects on muscle strength and aerobic capacity. None has assessed effects on the AES and related functional measures. A study investigating the effects of capromorelin a GH secretagogue, reported an improvement in tandem walk test and power stair climb test (195); both these tests assess the first

10-30 seconds of physical exertion, that is predominantly powered by the AES. The authors did not link this improvement in physical function to the AES or assessed its impact on QoL or on functional aspects of daily living. The current study provided strong evidence that daily walking capacity is a function of the AES, which improved after GH treatment. Daily walking capacity is associated with better cardiovascular outcomes, cognitive function and health related QoL in older adults (200-202). The findings of the current study call for a reassessment of GH supplementation on physical function relevant to the AES in older adults. This can be assessed by pedometry, Timed Up and Go test and Gait Speed measurement, and correlating these with the changes in their energy and vitality domains of QoL (179, 229). The Timed Up and Go test is a useful predictor of risk of falls in frail elderly (230), a cause of significant morbidity in this population. An improvement in gait speed predicts a significant reduction morbidity such as disability, institutionalization, falls, and mortality in older people (231, 232). A similar approach should be used to investigate whether GH accelerates physical mobilization and rehabilitation after major surgery or trauma by stimulating anaerobic capacity

The AES is an ignored area of fitness research, which has focused heavily on aerobic capacity. This body of work provides the first evidence for the hormonal regulation of the AES and its relevance in daily life. This information can be exploited in designing future research trials combating frailty associated with chronic diseases and ageing.

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