The characterisation of growth hormone-related cardiac disease with magnetic resonance imaging & The effects of growth hormone dysregulation on adenosine monophosphate-activated protein kinase in cardiac tissue

Thomas, Julia Dominique Janine

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Julia Dominique Janine Thomas

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Abstract

Chronic growth hormone (GH) excess, acromegaly, causes a specific cardiomyopathy, which remains poorly understood. The pattern of hypertrophy is distinct from other forms of cardiac disease and begins to appear before hypertension or diabetes. GH deficiency (GHD) also causes cardiovascular problems, with reduced ability to mount a cardiovascular response to exercise. Acromegaly and GHD patients have increased cardiac mortality.

Cardiac magnetic resonance imaging (CMR) is the gold standard for assessment of cardiac mass and provides data on cardiac function, fibrosis, valve function and ischaemia. This study used CMR to assess 23 patients with acromegaly or GHD, before and after treatment of their GH disorder, and 23 healthy controls. Patients with acromegaly demonstrated increased left ventricular mass index (LVMi), end diastolic volume index, stroke volume index and cardiac index, which persisted at one year, despite treatment of underlying disease. Patients with GHD demonstrated LVMi at the bottom (males) or beneath (females) published normal references ranges, which increased with one year of GH replacement.

The mechanisms by which GH influences cardiac tissue are poorly understood. Adenosine monophosphate-activated protein kinase (AMPK) is an energy-regulator enzyme, which interacts with several metabolic hormones. Mutations in AMPK cause arrhythmias and cardiac hypertrophy. AMPK activation may be a mechanism by which GH causes some of its cardiac effects.

This study used primary cardiomyocytes and mouse and rat models of GH excess and deficiency to study the effects of GH on cardiac AMPK. Acute GH treatment increased AMPK activity in both *in vivo* and *in vitro* studies; acute IGF-I treatment had the opposite effect. In 2 and 8 month old bovine GH-overexpresing (bGH) and GH receptor knock out (GHRKO) mice, functional AMPK assay did not demonstrate any difference in cardiac AMPK activity between transgenics and controls. However, Western blotting for Threonine-172 phospho (p)AMPK levels, a marker of AMPK activity, demonstrated increased cardiac pAMPK in 2 month old bGH mice and a reduction in cardiac pAMPK levels in 8 month old animals. A trend towards the same findings was seen in GHRKO mice. This indicates that both GH and IGF-I interact with myocardial AMPK, apparently via different mechanisms.

Contents

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D.G.

Abbreviations

ATP	Adenosine triphosphate
ACC	Acetyl-CoA carboxylase
AMPK	Adeosine monophosphate-activated protein kinase
Ao-	Adult onset
bGH	Bovine growth hormone
BSA	Body surface area
b-SSFP	Balanced steady state free procession sequence
CMR	Cardiac magnetic resonance imaging
CI	Cardiac index
CO	Cardiac output
Co-	Childhood onset
ECG	Electrocardiogram
EDV(i)	End diastolic volume (index)
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
ESV(i)	End systolic volume (index)
GH	Growth hormone
GHD	Growth hormone deficiency
GHKRO	Growth hormone receptor knockout
GTT	Glucose tolerance test
hGH	Human growth hormone
IGF-I	Insulin-like growth factor
IGF-I R	IGF-I receptor
ip	Intraperitoneal
IR	Insulin receptor
ITT	Insulin tolerance test
LVM(i)	Left ventricular mass (index)
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
OSA	Obstructive sleep apnoea
pACC	Phospho-ACC
pAMPK	Phospho-AMPK

PCr:ATP	Phosphocreatine:ATP ratio
QoL	Quality of life
rGH	Recombinant human growth hormone
sc	Subcutaneously
Ser485	Serine 485
SV(i)	Stroke volume (index)
SSTR	Somatostatin receptor
Thr172	Threonine 172
TSS	Transsphenoidal surgery

1 Introduction

1.1 Heart function and structure

The heart is the first functional organ in the human embryo and begins beating approximately 21 days after conception. Less than 50% of cardiomyocytes will regenerate throughout life, so the majority of the cells developed in utero will need to function for the entirety of the life of the individual[1]. In order to determine the impact of a disease or its treatment on the heart, methods for measuring cardiac structure and function are required. In clinical practice, the patient examination, electrocardiogram (ECG) or chest radiograph provide initial information. However, the data that can be gathered from these are limited. Hence, several forms of cardiac imaging have been developed and cardiac volume and function surrogate end-points have been established to allow for further assessment[2].

One of the more established surrogate end-points of cardiac function is left ventricular ejection fraction (EF). This is the volume of blood pumped out to the ventricle as a percentage of the total blood in the ventricle when maximally full, per heartbeat, and is presented as a fraction. EF makes the assumption that the fraction of blood expelled from the left ventricle (LV) is proportional to the force generated at LV contraction[2]. Other common end-points taken from cardiac imaging are: end diastolic volume (EDV), the volume of blood in the ventricle at the end of diastole; end systolic volume (ESV), the volume of blood in the ventricle at the end of systole; stroke volume (SV), the volume of blood ejected from a ventricle per heart beat (= EDV – ESV); myocardial mass (LVM); cardiac output (CO), the volume of blood circulated in a minute. These measurements are influenced by height and weight and are therefore commonly indexed to body surface area. When this is done the parameter is suffixed with “i”. There are many imaging techniques available to assess these parameters and each has its own advantages and disadvantages[2].

There is evidence that the above parameters correlate with prognosis in a range of cardiac conditions, including myocardial infarction[3] and heart failure[4] and even correlate with cardiac prognosis in those without established heart disease[5]. A further benefit of using these parameters in research is that they are familiar to clinicians, assisting in the ready translation of research into clinical practice[2].

1.2 Growth hormone

Human growth hormone (GH) is a 191 amino acid single chain polypeptide, coded for by a 66 kb DNA locus on chromosome 17q22-q24[6] (Figure 1.1). It is secreted in a pulsatile manner by the somatomammotroph cells of the anterior pituitary gland in response to hypothalamic growth hormone releasing hormone. GH induces fetal and postnatal somatic growth and plays a role in the maintenance of normal metabolic status[7]. GH acts via its receptor (GHR), a constitutively active homodimer of the cytokine receptor type Ia family. When a GH molecule binds to the GHR dimer, a subtle rotational plane change occurs with consequential signalling via the janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway[8,9] (Figure 1.2).

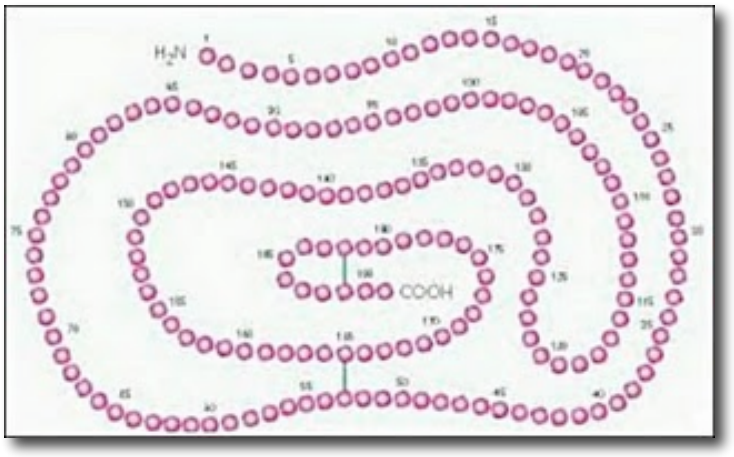

Figure 1.1 Schematic of the 2D structure of GH
(http://haileydawsonbiol4550.blogspot.com/)

Many of the effects of GH are mediated via insulin-like growth factor-I (IGF-I). IGF-I, previously known as somatomedin, is 70 amino acid peptide, coded for by a 90 kb DNA locus on chromosome 12[10]. Its receptor (IGF-IR) is a heterotetrameric glycoprotein that is 60% homologous with the insulin receptor (IR) and may form heterodimers with the IR[11]. As a consequence there are many crossovers between IGF-I and insulin systems[11].

GH treatment has been shown to have many positive cardiovascular effects. Acutely, it improves cardiac function and attenuates left ventricular (LV) remodelling in rats with myocardial infarction[12], and improves stroke volume, myocardial contractility and cardiac output in rats with heart failure[13,14]. Healthy individuals treated with IGF-I show an increase in LV ejection fraction and a trend towards increased heart rate, even in the absence of blood pressure changes, suggesting that IGF-I can have a direct effect on the heart[15].

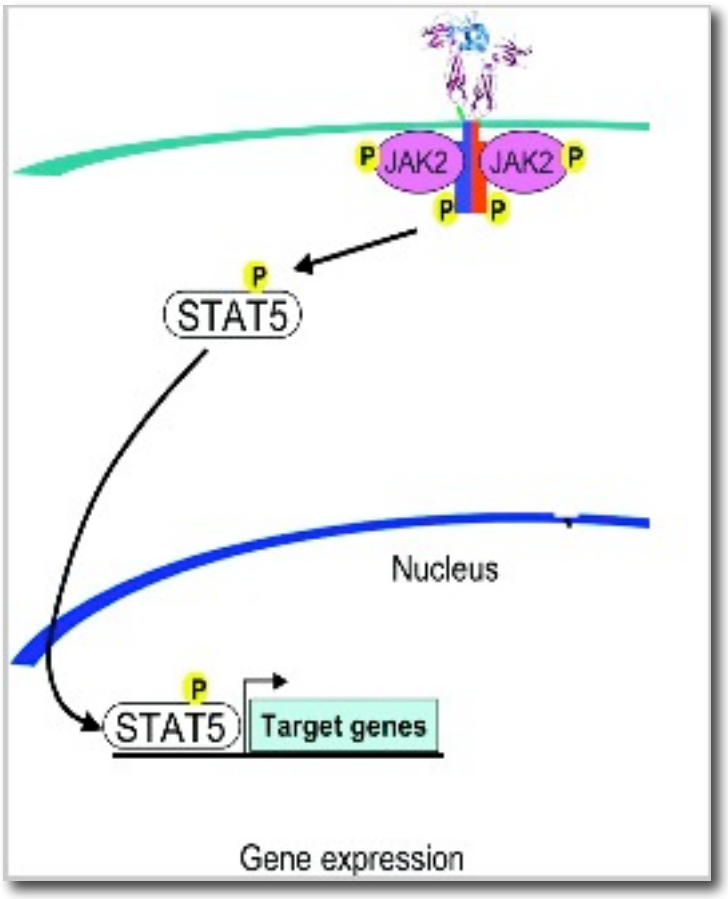

Figure 1.2 Simplified schematic of GHR activation: GH (blue) acting at its dimerised receptor (purple) to phosphorylate and activate the Jak-STAT pathway, resulting in transcription of target genes in the nucleus

1.3 Acromegaly

Acromegaly is caused by chronic GH excess, almost always as the result of a benign tumour of the pituitary somatomammotroph cells (Figure 1.3). It is a rare disease with an incidence of 3-4/million/year and a prevalence 50-80/million[16]. The GH excess results in increased soft tissue mass, with coarsening of facial features, an increase in size of the hands and feet and organomegaly. Other associated features include increased sweating, impaired glucose tolerance and type 2 diabetes mellitus, hypertension, salt and water retention, hirsutism and acne. Co-secretion of prolactin from the tumour may cause galactorrhoea, impotence or amenorrhoea. Local tumour growth may cause compression of the optic chiasm, resulting in visual loss in a bitemporal pattern, or destruction of the anterior or even, rarely, the posterior pituitary gland, with evidence of hypopituitarism and diabetes insipidus[17]. If GH excess begins prior to fusion of the epiphyses, the affected individual develops increased long bone length, and there is substantially increased height, a disease known as gigantism. Acromegaly is also associated with multi-nodular enlargement of the thyroid gland, which may produce thyroid hormone excess.

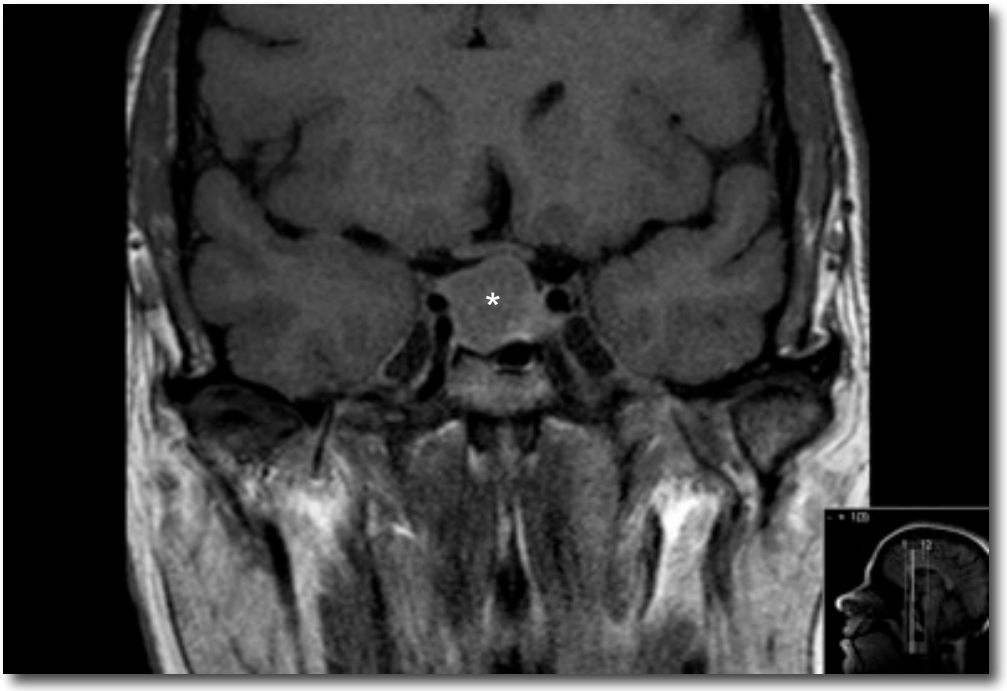

Figure 1.3 T_1-weighted MRI scan from a patient with acromegaly demonstrating a macroadenoma (*). The tumour touches the left side of the optic chiasm.

The first-line treatment for acromegaly is surgical removal of the tumour[17]. If there is evidence of visual impairment as a consequence of optic chiasm compression, this becomes a surgical emergency. Patients with larger tumours extending outside the sella may benefit from six month pre-treatment with somatostatin analogues prior to surgical intervention[18]. Hypothalamic somatostatin inhibits GH release and acts at the somatostatin receptor (SSTR1-5). In 1982, an analogue of somatostatin was created; due to its octopeptide structure it was named octreotide[19]. Octreotide continues to have a role in the management of acromegaly but its use is limited by the necessity of subcutaneous dosing three times a day. Slow-release somatostatin analogues, such as octreotide LAR, lanreotide LAR and pasireotide LAR, that are given by intramuscular or deep subcutaneous injection once a month, have been developed. These have been shown to be effective in reducing IGF-I levels and in improving the signs and symptoms of acromegaly[20]. They may also be effective in reducing tumour size to allow the surgeon the opportunity for maximum tumour clearance[18]. Somatostatin analogues are also used post-surgery to control residual disease or in the patient in whom surgery is inadvisable. These drugs have varying affinities for different somatostatin receptors, a characteristic that may be relevant in the targeting of treatment (Table 1.1). Alternative treatments for acromegaly include radiotherapy, which may be given as an adjuvant after surgery, and dopamine agonists such as cabergoline.

	SSTR1	*SSTR2*	*SSTR3*	*SSTR4*	*SSTR5*
Octreotide	280.0	0.38	7.10	>1000	6.30
Pasireotide	9.3	1.0	1.5	>1000	0.16

Table 1.1 Binding affinities of octreotide and pasireotide for human somatostatin receptors, shown as IC50, nmol/L. Pasireotide demonstrates broader effect but slightly lower SSTR2 affinity than octreotide[21].

In contrast to the beneficial cardiovascular effects of acute GH excess, acromegaly, is associated with distinct forms of cardiac disease[21] and increased cardiac mortality[22]. Most notably, patients exhibit cardiac enlargement but also demonstrate arterial hypertension, arrhythmias and heart failure. A specific acromegalic cardiomyopathy is described, consisting of biventricular hypertrophy, functional changes and fibrosis[23]. Such findings can only be partially explained by the systemic consequences of GH excess, such as hypertension, insulin resistance and increased preload[23].

1.4 Growth hormone deficiency

GH deficiency (GHD) may develop in childhood (co-GHD) or in adulthood (ao-GHD). Childhood-onset GHD may arise for many reasons (Table 1.2) and may be an isolated deficiency or associated with loss of other pituitary hormones. Incidence is estimated at 1:3,500 live births[24].

Idiopathic	
Genetic	Pit-1 defects Prop-1 defects
Congenital	*Associated with structural defects:* Agenesis of corpus callosum Septo-optic dysplasia Holoprosencephaly Arachnoid cyst *Associated with midline facial defects:* Single central incisor Cleft lip/palate Nasal dimple
Hypothalamic or pituitary tumours	Craniopharyngioma Glioma/astrocytoma Germinoma Histocytosis Lymphoma
Trauma	Perinatal trauma Postnatal trauma
Infection	Tuberculosis Other Central nervous system infections
Cranial irradiation	
Transient	Psychosocial deprivation

Table 1.2 Causes of childhood GHD, adapted from Camacho-Hübner, Disorders of growth hormone in childhood [24]

The most common cause of GHD in adults is pituitary destruction due to the direct effect of a pituitary tumour or its treatment[25]. Isolated GHD is difficult to diagnose outside of childhood and the presence of multiple pituitary hormone deficiencies makes the diagnosis easier. GHD produces tiredness, reduced muscle strength and poor exercise tolerance, and results in a detrimental body composition, with increased fat mass and reduced lean body mass[26]. Recombinant human (h)GH is licensed for the treatment of both GHD and short stature in children. In adults its use is restricted to those displaying evidence of severe GHD[27]. This is assessed through a combination of dynamic testing of GH reserve and an assessment of the clinical and psychological impact of GHD, using the quality of life - assessment of GH deficiency in adults (AGHDA) questionnaire[28,29].

GHD causes cardiac dysfunction. Patients with GHD develop a hypokinetic syndrome with loss of cardiac response to exercise, which may be ameliorated by GH replacement[30,31]. Childhood-onset GHD (co-GHD) results in reduced cardiac mass[32]. Findings are less consistent in adult-onset disease (ao-GHD); however, the ability to accurately determine the impact of ao-GHD on cardiac mass has been limited by historical methods of assessment[33]. GHD is also associated with an atherogenic biochemical profile, with raised low density lipoprotein (LDL)-cholesterol, reduced high density lipoprotein (HDL)-cholesterol and increased inflammatory markers[26,34,35]. Thus, both chronic GH excess and deficiency may result in cardiac disease.

1.5 Basic studies: GH & the heart

The GH and IGF-I receptors are expressed in both human and rat myocardium[36-39]. GH administration increases cardiac IGF-I content[40] and induces cardiac IGF-I expression[41]. To date, most of the direct actions of the GH/IGF-I axis in the heart appear to be mediated via IGF-I action. IGF-I, but not GH, directly stimulates cardiomyocyte growth[42], induces muscle gene expression[42] and increases cardiac contractility[43]. IGF-I treatment is able to increase contractility of neonatal rat cardiomyocytes[44], isolated rat hearts[43] and ferret papillary muscles[43] by increasing calcium ion sensitivity, but not calcium concentration, and may be prevented by blocking the phosphatidylinositol 3-kinase (PI3K) pathway. Increased calcium sensitivity has also seen in *in vivo* models of chronic GH excess[45].

IGF-I expression is increased in rat myocardium following both pressure and volume overload, suggesting a role for local IGF-I in the development of compensatory hypertrophy even in the absence of GH excess[46-48].

IGF-I induced cardiomyocyte growth can be inhibited by the addition of IGF-binding protein 3 (IGFBP3)[42]. IGFBP3 is the major circulating carrier for IGF-I and is raised in patients with acromegaly[49]. IGFBP3 has been shown to promote apoptosis in prostate and breast cancer cells[50,51] and it has been suggested that IGFBP3 provides a counterbalance to the growth induction of IGF-I[52]. Therefore, the distinctive cardiac phenotype seen in GH excess is likely to result from interactions between both IGF-I induced protein synthesis and IGFBP3 induced programmed cell death. Furthermore, low levels of IGFBP3 are associated with increased risk of coronary events suggesting a cardioprotective role for IGFBP3[53]. However, as IGFBP3-overexpressing mice demonstrate cardiac hypertrophy the action of IGFBP3 in the development of the cardiac changes of GH excess remains unclear[54]. Both GH and IGF-I have been shown to increase endothelial nitric oxide synthase (eNOS) activation through phosphorylation[55,56](Figure 1.3). The eNOS system induces vasodilatation and has been shown to be involved in cardiovascular protection.

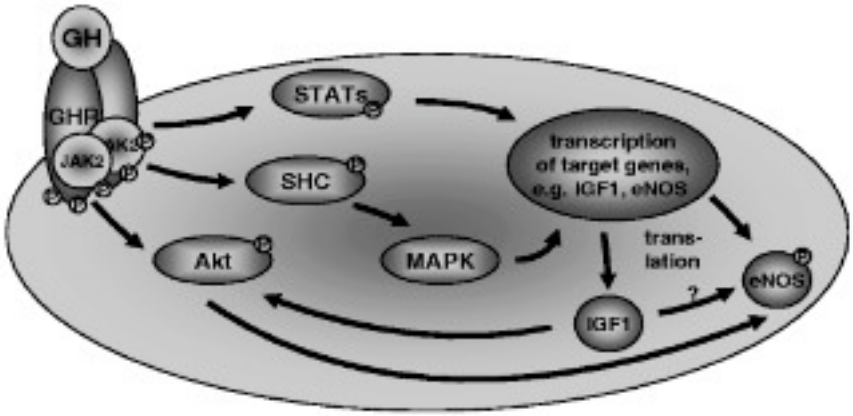

Figure 1.3 Potential signalling pathways in GH-mediated activation of eNOS. MAPK = mitogen-activated protein kinse; P = phosphorylation; SHC = Src=homology/collagen. *From Thum and Bauersachs*[67]

The systemic effects of GH combined with the complexities of the GH/IGF-I system, with both paracrine and endocrine effects, necessitate the use of animal models in the investigation of GH excess and deficiency. The oldest model of acromegaly, other than simple injection of GH into an animal, a situation which poorly mimics disease, is the GH3-implanted rat[57]. GH3 is a rat pituitary tumour cell line which, when injected subcutaneously into the flank of a rat, will grow to form a GH-secreting tumour. This model is not completely true to acromegaly, animals do not display true cardiac hypertrophy as their cardiac enlargement remains proportionate to body enlargement, but has been able to provide a wealth of *in vivo* data regarding the effects of GH on the heart[23]. Transgenic mice provide an alternative method of studying chronic GH excess. Most commonly, the bovine (b)GH gene is inserted into the mouse genotype resulting in ubiquitous expression of the peptide in heterozygous offspring. This model demonstrates marked rises in both GH and IGF-I levels (Table 1.3).

	Non-transgenic littermates	*bGH transgenic mice*
GH (ng/ml)	1.6 ± 0.5	636.3 ± 136
IGF-I (ng/ml)	492.5 ± 20.1	868.4 ± 25.1

Table 1.3 Fasting serum GH and IGF-I levels in the bGH transgenic mice, demonstrating both are significantly raised in transgenic mice ($p<0.05$)[58]

Conversely, transgenic mice with GHD have been developed, including those with a knocked out (KO) or disrupted GH receptor gene and those that produce non-functional GH (Figure 1.4)[59]. A spontaneously arising mutation in a colony of AS rats led to the establishment of the *dw/dw* dwarf rat. These have a paucity of somatotroph cells in the pituitary and produce low amounts of GH throughout life. The remainder of the pituitary hormones are within normal limits for the GHD state[60]. The *dw/dw* rat demonstrates low serum IGF-I levels, reduced LV mass to tibial length ratio, reduced cardiomyocyte area and reduced myocardial contractility and distensibility, which are fully reversible with human GH (hGH) treatment[61].

Figure 1.4 Transgenic mice. From left to right: bGH-overexpressing, wild type, GH antagonist, GH receptor knock out (from Kopchick *et al.*[60])

1.6 Acute GH excess in humans

There are limited studies to show the acute effect of IGF-I and GH in humans. IGF-I infusion (60 µg/kg) given over four hours has been shown to increase cardiac output (+37.3% ± 9% from baseline, $p<0.01$), heart rate (13% ± 2%, $p<0.01$) and stroke volume (+27% ± 7%, $p<0.005$) when measured by impedance cardiography in healthy volunteers[62]. IGF-I given subcutaneously (60 µg/kg) also improves cardiac output and stroke volume as well as significantly increasing ejection fraction as measured by echocardiography, with no significant change in blood pressure[63]. Even low dose subcutaneous IGF-I injection (20 µg/kg), achieving circulating IGF-I levels in the upper part of the normal range, was able to induce a small, but significant, improvement in LV ejection fraction when measured by 99mTc-radionuclide angiography[15]. In these studies, volunteers exhibited the predictable fall in circulating GH levels that occurs after exogenous IGF-I administration indicating, and confirming *in vitro* findings[42], that it is IGF-I rather than GH that causes these direct cardiac effects. Similar acute cardiovascular improvements are also seen in chronic heart failure patients treatment treated with an IGF-I infusion (60 µg/kg)[64].

Studies in healthy volunteers demonstrate direct GH action on the vascular system. GH infusion increases local forearm blood flow in the absence of significant change in plasma IGF-I levels, muscle IGF-I mRNA expression and muscle Akt phosphorylation[65,66]. Vascular effects are presumed to be mediated through increased eNOS action and suggest that GH directly influences this system. IGF-I is also known to be potent inducer of eNOS but this occurs through phosphorylation of Akt in the P13K pathway, which was absent in this experiment. The complex interactions of GH and IGF-I with eNOS and vascular resistance may be tissue-specific as well as mediated through multiple pathways, the details of which are not yet fully understood. The topic has been reviewed by Thum and Bauersachs[67].

There have been attempts to use GH to improve cardiac function in patients with chronic heart failure, a disease associated with relative GH-resistance[68]. Doses of GH used have often been high, resulting in unacceptable side effects, the patient numbers small and studies unrandomised, so that conclusions that can be drawn are limited. Some studies have still managed to show benefits in exercise capacity, LV function, vascular reactivity and quality of life with six months treatment, whereas others report no improvement[69,70]. Large scale, randomised control trials are needed to address this more fully[71].

1.7 Heart disease in acromegaly

The diagnosis of acromegaly is often made after many years of symptoms[72] making it hard to establish the early impact of GH on the cardiovascular system. Even patients who are diagnosed relatively early usually have cardiovascular changes[73]. These include increased plasma volume[74], hyperkinesis[75] and cardiac hypertrophy in the absence of hypertension[76]. In a study of ten subjects with less than five years clinical evidence of acromegaly (mean duration 3.2 ± 1.2 years), patients exhibited cardiac hypertrophy as determined by increased LV mass index (LVMi) compared to age-matched controls (110 ± 20 v. 81 ± 16 g/m^2; p<0.005) and hyperkinesis, as evidenced by increased stroke volume index (39 ± 6 v. 33 ± 2 ml/m^2, p<0.01) and increased cardiac index (2.85 ± 0.57 v. 2.30 ± 0.34 L/min/m^2, p<0.001)[77]. There were no differences in diastolic function between the two groups. Twenty, young patients with less than 5 years acromegaly studied with echo by Colao *et al.* confirmed these findings, demonstrating increased LVMi and increased left ventricular end systolic and end diastolic volumes compared to age-matched controls[78].

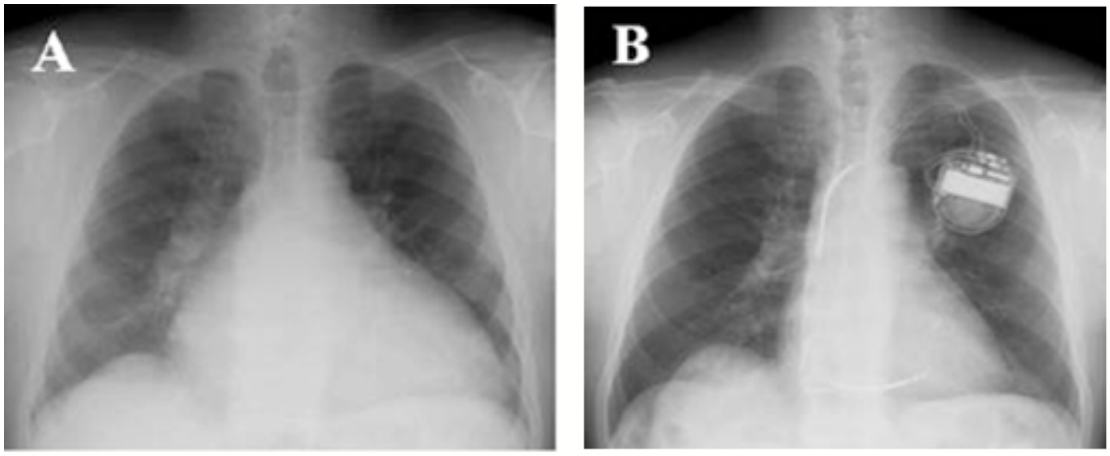

Figure 1.5 Chest radiographs (A) Patient with undiagnosed acromegaly presenting with heart failure and ventricular tachycardia (B) Same patient after treatment for acromegaly with normalisation of GH and IGF-I levels (from Yakota *et al.*[95])

As acromegaly progresses diastolic dysfunction, disrupted ventricular filling and loss of cardiac response to exercise develop[76,79,80]. Aortic root diameter increases when compared to controls[81]. With time, systolic dysfunction occurs and may progress to congestive heart failure. Ventricular tachycardia occurs with increased frequency, and possibly increased severity,

compared to control populations[79,82]. Atrial fibrillation is reported as occurring with increased frequency, although there are no studies demonstrating this. Such arrhythmias are thought to arise from interruption of myocardial electrophysiological pathways by interstitial fibrous tissue[23].

The cardiac hypertrophy that occurs in acromegaly consists of concentric thickening of both the ventricular walls, due to cardiomyocyte size increase, without cavity enlargement[83] (Figure 1.6)[84]. However, with time cavity dilatation occurs and fulminant heart failure develops. Severity of hypertrophy correlates with disease duration and patient age but not with IGF-I levels, and is worsened by the presence of hypertension[83,85]. Post-mortem studies show high levels of mitral and aortic valve abnormalities and extensive interstitial fibrosis[86]. Cardiac biopsies demonstrate high levels of myocyte apoptosis (495-fold when compared to samples from patients without acromegaly) and collagen deposition consistent with previous myocyte necrosis. Apoptosis magnitude and collagen accumulation both correlated negatively with ejection fraction suggesting that apoptosis and necrosis each play a role in the development of acromegalic cardiomyopathy[87].

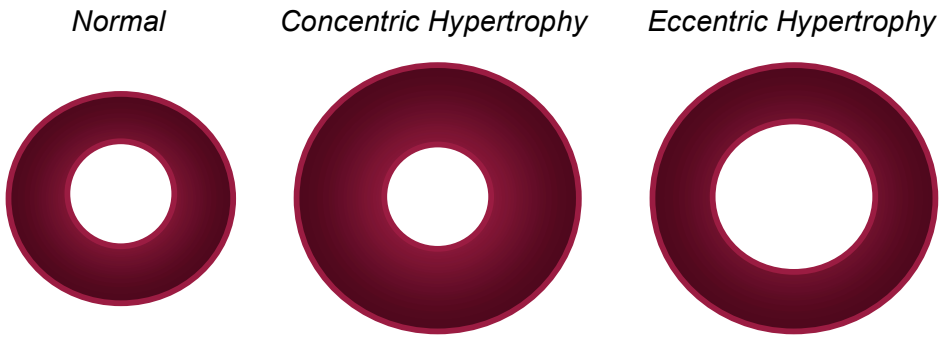

Figure 1.6 Schematic demonstrating the LV wall and cavity changes in different forms of cardiac hypertrophy. Concentric hypertrophy, as seen in early acromegaly, involves enlargement of the ventricular wall thickness with no change in the cavity size, while with time cavity dilatation occurs (adapted from Mihl *et al.*[84])

There is good evidence that many of the cardiac findings in acromegaly occur as a consequence of direct GH/IGF-I action. *In vitro* experiments demonstrate that IGF-I treatment induces cardiomyocyte hypertrophy[42,88]. *In vivo* studies with the heart-specific IGF-I transgenic mouse demonstrate cardiac hypertrophy and improved systolic function with short-term cardiac IGF-I excess but systolic dysfunction and fibrosis with chronic exposure, mirroring acromegalic heart disease[89].

It is probable that secondary effects of GH excess also contribute towards acromegalic cardiomyopathy. Usually, cardiac hypertrophy is a compensatory mechanism for chronic work overload, arising as a result of increased pressure overload (raised afterload) or volume overload (raised preload). Acromegaly is associated with both increased afterload, as a result of hypertension, and increased preload, due to sodium and water retention[90]. However, the pattern of acromegalic cardiac hypertrophy is distinctive from hypertensive cardiomyopathy and increased LV mass precedes the development of hypertension in young patients with acromegaly. Furthermore, although salt and water retention occur in acromegaly, GH also causes a reduction in ventricular wall tension, counteracting the stretch force on cardiomyocytes that increased preload should cause. The secondary consequences of GH excess are inadequate in themselves to explain the hypertrophy seen. For reviews of this topic see Sacca, Colao and Clayton[23,73,91].

The cardiac changes of acromegaly improve with treatment of the underlying disease. Studies with somatostatin analogues demonstrate that lowering GH and IGF-I levels results in a rapid reduction of ventricular mass and wall thickness[92,93] and an improvement diastolic function[93], although it should be considered that these drugs may have a direct effect on the heart. Retrospective analysis of a cohort of treated acromegaly patients shows that after five years of hormonal inactivity ventricular mass does not vary from control levels[94]. Figure 1.5 shows chest radiographs from a patient presenting with heart failure and ventricular tachycardia, who was later diagnosed with acromegaly. Successful treatment of acromegaly resulted in a marked improvement in cardiac function and a drastic reduction in heart size[95].

Almost all patients with active acromegaly have evidence of cardiac disease[76] and ~60% will die from cardiac-related deaths[96]. Despite the increased prevalence of traditional cardiovascular risk factors in these patients, such as hypertension and diabetes, ischaemia alone cannot explain the distinct functional and morphological findings seen[23]. Indeed, it appears that GH

may even exert a degree of protection against atherosclerosis[97]. This may arise from eNOS activation, although data demonstrating this are limited and some studies suggest that acromegaly may be associated with depressed endothelial function due to reduced nitric oxide bioavailability[98]. A greater understanding of both the direct and indirect effects of the GH/IGF-I axis on the heart is required to help unravel this disease further.

1.8 Heart disease in GHD

Although less extensively studied, GHD is also associated with cardiac disease. Hypopituitary patients, receiving conventional hormone replacement therapy (thyroxine, glucocorticoids and sex steroids) demonstrate excessive cardiac mortality which is thought to be attributable, at least in part, to GHD[99,100]. This is confirmed by observational databases of hypopituitary patients which show an improvement in standardised mortality ratio (SMR) back to national baseline with recombinant human GH (hGH) replacement (mean length of treatment, 60 months)[101]. However, data are limited and the duration of treatment needed to see changes, along with widespread use of GH-replacement therapy in adults with GHD, precludes randomised control trials.

GHD causes cardiac hypokinesis, with loss of response to exercise as demonstrated by reduced workload achieved, reduced exercise capacity and by patient reporting[23]. GH replacement ameliorates these findings and may do so even in the absence of increases in cardiac mass, demonstrating that these improvements are not simply mechanical[102,103].

Interpretation of the effect of GHD on cardiac mass is often confounded by the inclusion of both ao- and co-GHD patients in studies. However, studies that separate these two groups find consistently reduced cardiac muscle mass in co-GHD[104,105], yet variable results in ao-GHD[33,106-108]. As IGF-I correlates with LVMi in patients with GHD[109], the finding of reduced LVMi in co-GHD patients may simply indicate more severe GHD in childhood-onset disease. These studies used echocardiography to assess LV mass. Cardiac MRI has now superseded echo as the most accurate method of assessment of LV mass and can identify subtle changes in cardiac mass and volume that are harder to identify by echo[110].

It is clear that GH replacement causes an improvement in exercise tolerance[31] but it remains hard to extrapolate to what extent this results from improvements in cardiac function, changes in circulating volume or increases in cardiac mass. An additional reason for GH replacement in adults is to reduce cardiovascular risk[26]. Observational databases of patients on GH replacement demonstrate significant improvements in the total cholesterol, LDL-cholesterol and HDL-cholesterol with normalisation of IGF-I[111]. Other biochemical markers of increased atherogenesis, such as C-reactive protein[112], interleukin-6[112] and homocysteine[113], and morphological markers, such as intima media thickness[114], have also been shown to improve with GH replacement.

1.9 Cardiac volume and mass measurement

LV volume and mass has traditionally been measured by echocardiography (echo). Echo uses ultrasound to define the cardiac borders and collect data on cardiac volume and function. LV volume is determined using geometric modelling to account for the complex shape of the heart. This is most commonly done using Simpson's rule[115]. This assumes that the volume of an object equates the sum of the volumes of multiple slices through it (Figure 1.7).

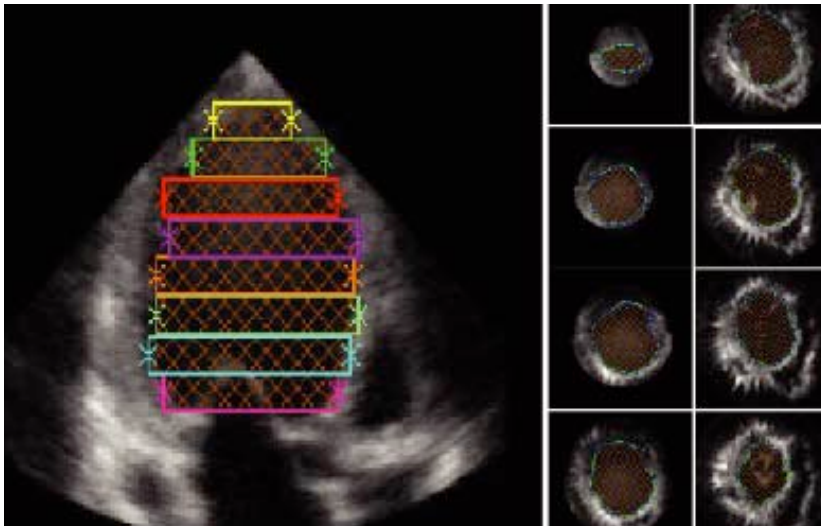

Figure 1.7 LV volume measurement with echo, using Simpson's rule. An end-diastolic long-axis view (left image) is selected as a reference image. The left ventricle is automatically sliced by the paraplane method into eight equidistant parallel short-axis slices spanning the left ventricular cavity from the apex to mitral annulus. The endocardial border of the left ventricle is traced manually in each of short-axis slices 1-8 (right panel). (http://www.hmc.org.qa/hmc/heartviews/h-v-v3%20n1/3-b.htm)

As echo looks at the heart from two directions, two planes can be used in the calculation, X and Y (Figure 1.8). The biplane Simpson's rule assumes each slice through the heart is an ellipse, with a major axis determined in one plane and a minor axis determined in the other. The area of each disc is multiplied by its thickness to provide its volume[2].

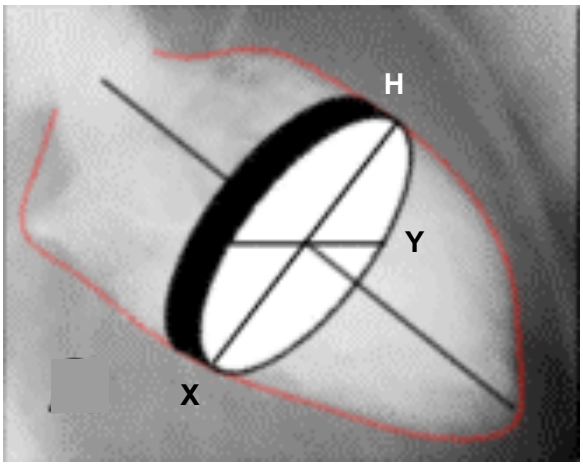

Figure 1.8 Simpson's rule. The heart is viewed from two aspects, allowing two planes (X and Y) drawn through each slice. H indicates slice height, the oblique line indicates the plane for measurement of LV length. The formula assumes each ventricular slice to be elliptical (http://www.sandersdata.com/QLVATech.htm)

To estimate LV volume measurements gathered are entered into a formula, most commonly, the modified Simpson's biplane formula:

$$V = (\pi/4) \times (LVL/n) \sum_{i=1} DiX \times DiY$$

Where, V = volume, LVL = length of LV, n = number of slices, DiX = diameter in plane X and slice i, DiY = diameter in plane Y and slice i[115].

LV mass is calculated from the LV volume by multiplying it with myocardial density. Volume calculated in this manner refers to the total LV volume and should not be confused with end diastolic and systolic volumes, which refer to the volume of blood in the ventricle at the end of diastole and systole respectively. Therefore, the term "volumetric indices", which includes EDV, ESV and SV, refers to blood volume, representing the volume of cardiac chamber, rather than myocardial muscle volume.

The weaknesses of echo are image quality, geometric assumptions regarding LV volumes taken from two dimensions and a tendency for echo formulae to overestimate LV mass when compared to cardiac magnetic resonance imaging[116,117]. Foreshortening may also occur, when the ultrasound plane is not positioned through the true apex of the LV.

1.10 Cardiac magnetic resonance imaging (MRI)

Clinical studies to date have mainly used traditional methods of cardiac assessment: echocardiography (echo) and radionuclide imaging. However, cardiac MRI (CMR) is rapidly becoming a routine imaging modality in modern cardiological practice and provides a more accurate measurement of muscle mass than echo along with excellent soft tissue resolution[110].

MRI utilises the polarity of hydrogen nuclei (each a single proton) within the water, fat and other body components. The application of a magnetic force causes the protons to act as magnets, aligning themselves with an applied magnetic field and spinning around the axis of this field. The frequency of the spin (precession) is related to the strength of the field applied, so that for a 1.5 Tesla magnet, the hydrogen nucleus spins at 63 MHz, a frequency that is within the radiofrequency range. When radiowaves are applied the net magnetisation vector of the nucleus temporarily rotates before returning to its previous position. While a component of the magnetisation is perpendicular to the applied magnetic field, energy is transmitted as a radio signal. This is picked up a receiver coil placed on the patient's chest, which transmits the information to a computer. A pulse sequence, or timed series of radiowaves and magnetic field gradients, is used. This provides the raw data, known as K-space, for the computer software to generate an image.

The amount of signal produced is mainly dependent on two relaxation times: T_1 and T_2. T_1 is the time constant that refers to the return of longitudinal magnetisation to baseline; T_2 refers to the return of transverse magnetisation. The T_1 and T_2 of each proton is tissue dependent, Water has a long T_1 and a short T_2, whereas fat has a short T_1 and a long T_2. Therefore, on T_1 imaging, water appears dark, fat appears bright and the myocardium appear grey, or iso-intense[118]. To provide further information, additional radiofrequency pulses are applied in a pulse sequence.

The information received from the coil is converted into digital images, which may be displayed as static, cine (moving) or multi-planar reconstructions. In order to coordinate the images to the correct phase of the cardiac cycle, the images are linked to an ECG recording (ECG-gating). To reduce artefact, patients are asked to hold their breath during the acquisition of certain images, usually for no more than 20 seconds[110].

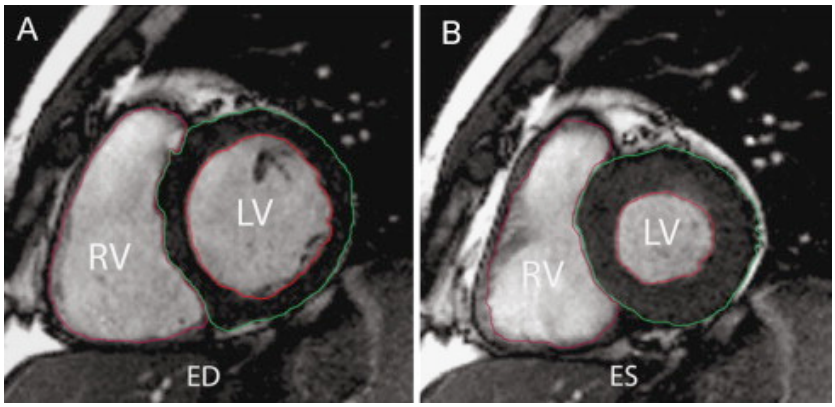

Figure 1.9 MRI image of a mid-ventricular short axis slice in end-diastole (A) and end-systole (B) with traced left ventricular endocardial (red) and epicardial contours (green) and right ventricular endocardial contour (red). (from Teo *et al.*[119])

CMR takes a three dimensional approach to measurement of ventricular volume. As in echo, this is based on Simpson's rule. Epicardial and endocardial borders are drawn on multiple ventricular short axis slices, taken at the end of diastole (Figure 4.9)[119]. Computer software then calculates the area of the slice, which is then multiplied by slice depth or the distance between slices to produce volume data. This is then multiplied by myocardial density to achieve mass[2]. This method does not make any assumptions regarding the uniformity of the ventricular shape and so there improved accuracy and lower standard deviations when using CMR rather then echo to measure LV volumes and mass. This accuracy is further increased by better contrast between blood and myocardium on CMR. There is also less inter- and intra-observer variability. In 24 hypertensive subjects LV mass was shown to be 319 ± 21g using echo but only 232 ± 11g when mass was calculated using MRI[120].

CMR images provide details of cardiac function and valve integrity. Gadolinium contrast is used to assess coronary artery flow and to determine the presence of myocardial scarring and fibrosis. Gadolinium is a water-based tracer which freely distributes in extracellular water but cannot cross intact cell membranes and so accumulates in areas of expanded extracellular space, such as fibrosis, protein infiltration and fibre disarray[121]. Such areas demonstrate evidence of gadolinium uptake longer than healthy myocardium, a finding known as late gadolinium enhancement. The combination of late gadolinium enhancement with a regional wall movement abnormality indicates the presence of previous ischaemic damage.

In order to examine for reversible myocardial ischaemia, pharmacological stress is used to increase blood flow to the myocardium. Areas of myocardium supplied by coronary arteries with flow-limiting atheroma demonstrate reduced contrast enhancement when compared to adequately perfused tissues. Dobutamine, adenosine and dipyridamole are the most commonly used agents used in perfusion CMR[122]. Dobutamine infusion results in increased rate of myocardial contraction, adenosine and dypridamole cause vasodilation and hence hyperaemia. Myocardial images are inspected for inducible wall motion abnormalities and for areas that do not demonstrate an increase in contrast agent with the induction of hyperaemia. Both these findings are considered to represent reversible myocardial ischaemia.

Magnetic resonance spectroscopy (MRS) is used to detect signals from naturally occurring isotopes ^{31}P, ^{23}Na and ^{13}C as well as from hydrogen nuclei, providing data regarding the metabolic state of the heart[123]. Most commonly, ^{31}P MRS is used to measure the ratio between cardiac phosphocreatine and ATP (PCr:ATP) a marker of future viability and function[124]. A reduced cardiac PCr:ATP ratio has been shown in dilated cardiomyopathy[125] and correlates with severity of heart failure arising from ischaemic heart disease[126]. At present, MRS remains a research technique.

CMR is a safe technique and no long-term ill effects have been noted[110]. A small percentage (about 2%) of patients find claustrophobia prevents them from tolerating the process. MRI must be avoided in patients with ferromagnetic metal implants such as pacemakers, although most valves, prostheses and sutures are safe. Gadolinium injection is avoided in patients with allergy and in anyone with stage 4 chronic kidney disease (estimated glomerular filtration rate $<30ml/min/1.73m^2$) or worse because of the association with nephrogenic systemic fibrosis[127]. However, CMR has its limitations: RV mass assessment is limited due to difficulty identifying the plane of the tricuspid valve[118] and at present assessment of diastolic dysfunction is limited. There is also need for careful assessment of fibrosis. CMR identifies fibrosis by assessing for differences in the quantity of gadolinium visualised between healthy and fibrosed tissues. If diffuse fibrosis exists, such as may be seen in advanced acromegalic cardiomyopathy[86], then there is a possibility that it may be missed and hence under reported.

1.11 Cardiac MRI and growth hormone-related heart disease

To date, there are few CMR data reviewing the impact of GH deficiency and excess on the heart. There is only one study, published in 2011, using CMR to assess patients with adult GHD[128]. This study found patients had smaller LV EDVi (p=0.032) and LV ESVi (p=0.038) than controls but no difference in LVMi or EF. After one year of GH replacement there were no significant changes in volume, mass or function indices, despite a good IGF-I response to GH replacement. In addition, CMR has been used to track cardiac changes in patients with dilated cardiomyopathy treated with GH[129].

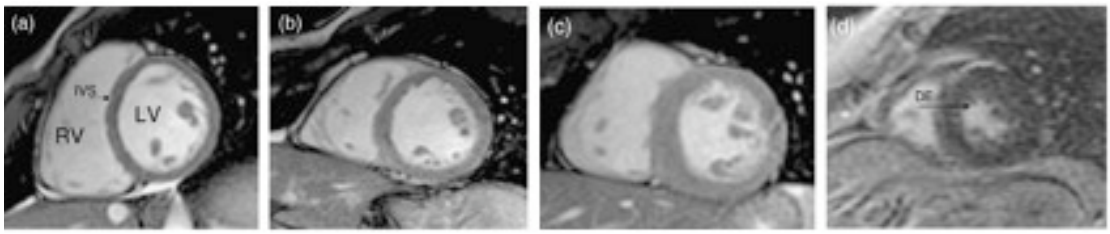

Figure 1.10 CMR imaging of: (a) normal subject, (b) patient with acromegaly and moderate LV hypertrophy, (c) patient with acromegaly and marked LV hypertrophy, (d) patient with acromegaly and mild late gadolinium enhancement (indicated by arrow). RV, right ventricle; DE, late gadolinium enhancement; the dark ring delimiting LV corresponds to the walls of the LV, which, at the boundary with the RV, is the interventricular septum (IVS). (from Bogzzi *et al.*[130])

One study performed CMR on 14 patients with untreated acromegaly (Figure 1.10)[130]. This found a greater prevalence of cardiac hypertrophy (LVMi) when measured by CMR (72%) as compared to echo (36%) and noted the absence of cardiac fibrosis, previously considered to be a key feature of acromegalic cardiomyopathy[86]. A second study used T_2-weighted imaging to assess cardiac oedema before and 8-10 days after treatment for acromegaly. An improvement was demonstrated in this parameter but the study only addressed this very narrow time window and made no effort to correlate cardiac findings with true extent of acromegaly or its cure[131]. A final study compared eight patients with acromegaly to matched controls[132]. Patients were found to have increased LVMi and ESVi compared to controls at baseline. After three months of treatment for acromegaly, patients had a significant increase in ESVi but no change in any other cardiac parameters. All patients were treated with medical therapies, some in addition to surgery, and six out of eight received somatostatin analogues. The size of this study, short duration, failure to normalise IGF-I levels in patients make interpretation of these results difficult.

Additionally, there is some evidence that somatostain analogues may also have an impact on cardiac function over and above their effect on GH levels[133].

Three dimensional methods of LV volume estimation are particularly important in conditions where a non-uniform ventricular shape is likely to be present, such as heart failure or cardiomyopathy, hence the suitability of CMR for the assessment of acromegalic cardiomyopathy[134]. Furthermore, the high levels of accuracy in assessing volume and mass and low inter-observer variability make it particularly suitable for answering questions regarding subtle cardiac mass changes that may occur in ao-GHD.

CMR provides an opportunity to gather a broad range of cardiac data in patients with one test; furthermore, it does not involve exposure to ionising radiation. This makes it an ideal tool for endocrinologists and cardiologists in the management of their patients, both at diagnosis and during follow up. Further studies are needed in this field to validate this test in these patient groups and to answer several ongoing clinical questions, including the accurate assessment of adult heart mass in ao-GHD and the presence of fibrosis in acromegalic heart disease[135].

1.12 AMPK

The effect of GH on the heart is likely to occur via many mechanisms: through direct GH action and through IGF-I; through local paracrine and hepatic endocrine IGF-I; through direct action at GH receptors, causing rapid effects, and more slowly via altered gene transcription and expression. One potential mechanism of GH action may be via activation of adenosine monophosphate-activated protein kinase (AMPK).

The enzyme AMPK was first discovered in the 1970s[136,137]. It plays a key role in energy homeostasis and cell response to stress. Increased ATP consumption or reduced ATP production increases the AMP:ATP ratio causing AMPK activation. A switch from anabolism to catabolism then occurs, ATP production is increased and energy-utilising pathways, such as cell division and growth, are reduced (Figure 4.12)[138,139].

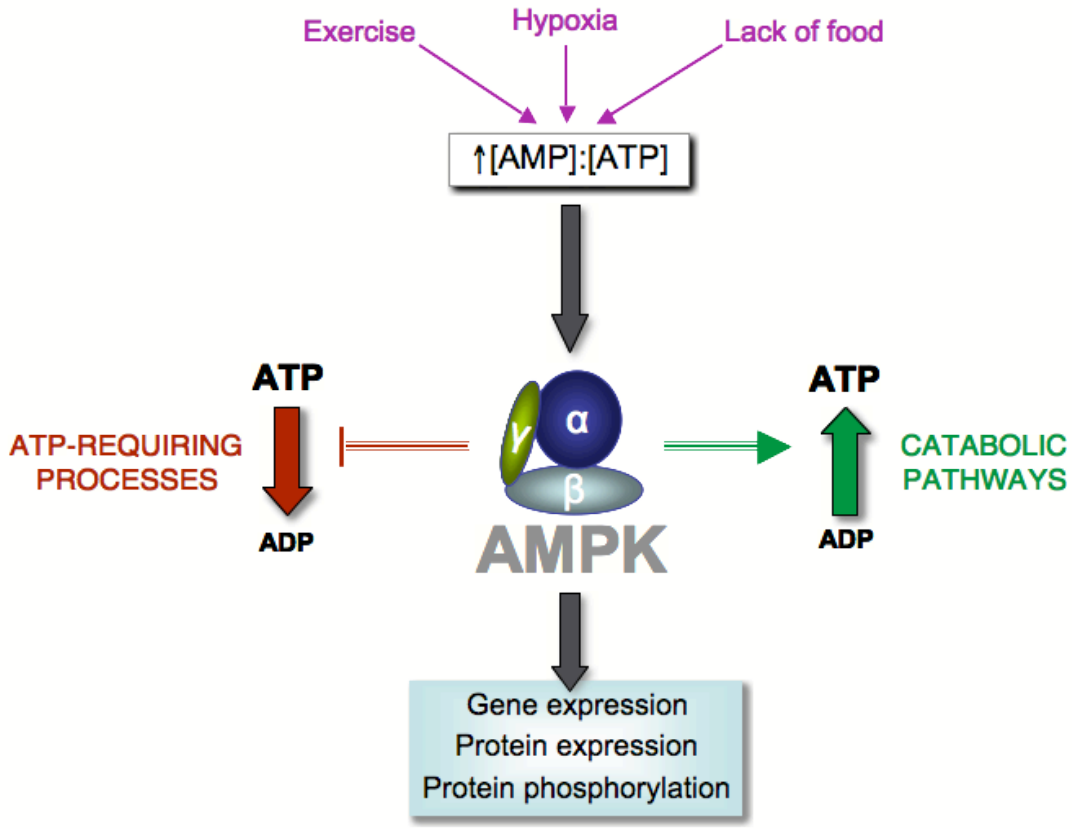

Figure 1.11 AMPK activation through physical and metabolic stress acts to conserve ATP and through a range of actions.

AMPK consists of a catalytic α subunit and regulatory β and γ subunits. The α subunit contains the kinase domain and an autoinhibitory region, the β subunit contains a glycogen domain and provides the scaffold for the complex, while the γ subunit contains AMP-binding sites (Bateman or cystathione domains)[138,140-142]. There are three AMP-binding sites: two that bind AMP (activating AMPK) or Mg•ATP (inactivating AMPK) and one that contains a tightly-bound, non-exchangeable AMP that is not involved in AMP:ATP sensing ratio. It has been suggested that AMP binding at the two exchangeable sites allows inter-subunit interactions that cannot occur when ATP is bound. The consequential structure changes increase kinase domain activity and reduce AMPK dephosphorylation, sustaining activation. Binding studies indicate that under physiological conditions AMPK mainly exists in its inactive form, complexed to Mg•ATP[143].

Each subunit is encoded by two (α) or three (β, γ) separate genes resulting twelve potential heterotrimeric combinations[141]. AMPK isoforms are expressed in tissue-specific manner, with different subunit isoforms having preponderance in different tissues. The isoforms α1, β1 and γ1 are expressed ubiquitously, although to varying amounts. In cardiac and skeletal muscle α2 complexes account for 70-80% of total AMPK activity, while α1 complexes account for the remaining 20-30%[144].

	Skeletal muscle	*Cardiac muscle*	*Adipose tissue*	*Liver*	*β cells*
Insulin	↔	↓	↓		↔
Adipoectin	↑	↑	↑	↑	↑
Leptin	↑	↓	↑		↔
Glucagon				↑	
Noradrenaline			↑		
Ghrelin	↔	↑	↓	↓	

Table 1.4 The tissue-specific effects of hormones on AMPK activity. ↑ increased, ↓ reduced, ↔ no change. Adapted from Kola *et al.*[145]

AMPK may be activated through both physiological and pathological mechanisms. Hypoglycaemia, hypoxia, heat shock, metabolic poisons or ischaemia all reduce ATP production. ATP consumption is physiologically increased through skeletal muscle

contraction[140]. The metabolic hormones leptin, adiponectin, insulin, adrenaline and ghrelin all interact with the AMPK system and do so in a tissue-specific manner[142,145] (Table 1.4)[145]. The diabetic drugs metformin and the thiazolidinediones activate AMPK, thiazolidinediones through increasing the AMP:ATP ratio[146], metformin through increasing cytosolic AMP concentration in the absence of any changes in AMP:ATP ratio[147].

AMPK is activated by two main mechanisms, by AMP binding to the γ subunit, resulting in allosteric changes, and by phosphorylation of the α subunit at threonine (Thr) 172 by upstream kinases. The combined impact of AMP-binding and Thr172 phosphorylation is that AMPK activity increases 1000-fold[148]. It appears that AMP binding renders the enzyme a better target for kinase activity and reduces dephosphorylation[149], although the mechanism by which this occurs is not understood[150]. Kinases that have been shown to phosphorylate Thr172 include LKB1 (a tumour suppresser gene), CaMKK2 (calmodulin-dependent protein kinase kinase 2) and TAK1 (mammalian transforming growth factor β-activated kinase)[151], and possibly MLCK (myosin light chain kinase)[152]. LKB1 has been shown to be constitutively active [153], as well as present in the majority of tissues. It may be that, *in vivo*, the regulation of AMPK activity is achieved by changes in dephosphorylation, rather than by kinase action on AMPK itself.

With activation of AMPK, glucose uptake is stimulated via glucose transporter (GLUT)-4 translocation to the cell surface[154] and glycolysis occurs via 6-phosphofructo-2-kinase (PFK2) activation[155]. Acetyl-CoA carboxylase (ACC) is inactivated through phosphorylation (pACC), resulting in a reduction in malonyl-CoA and a consequential increase in carnitine parmitoyltransferase I (CPT-1), which in turn increases fatty acid oxidation[156]. Activation of these pathways increase ATP availability for the cell. Other pathways that utilise ATP are suppressed. Protein synthesis is inhibited through tuberous sclerosis protein (TSC) 1/2 complex mediated inhibition of the mammalian target of rapamycin (mTOR)[157] and eukaryotic elongation factor 2 kinase (eEF2K) mediated inhibition of eukaryotic elongation factor 2 (eEF2)[158]. Cell growth is also reduced through p53 accumulation[159]. For a summary of some of the major effects of AMPK activation, see Figure 1.12.

With longer activation there is increased expression of GLUT-4 and metabolic enzymes involved in the TCA cycle and the respiratory chain[140]. Micro-array analysis of muscle biopsy from mice with a muscle-specific overexpression of dominant negative AMPK-α2 showed up-

regulation of 234 genes and down-regulation of 130 genes compared to controls, demonstrating the immense breadth of interactions this system has[160].

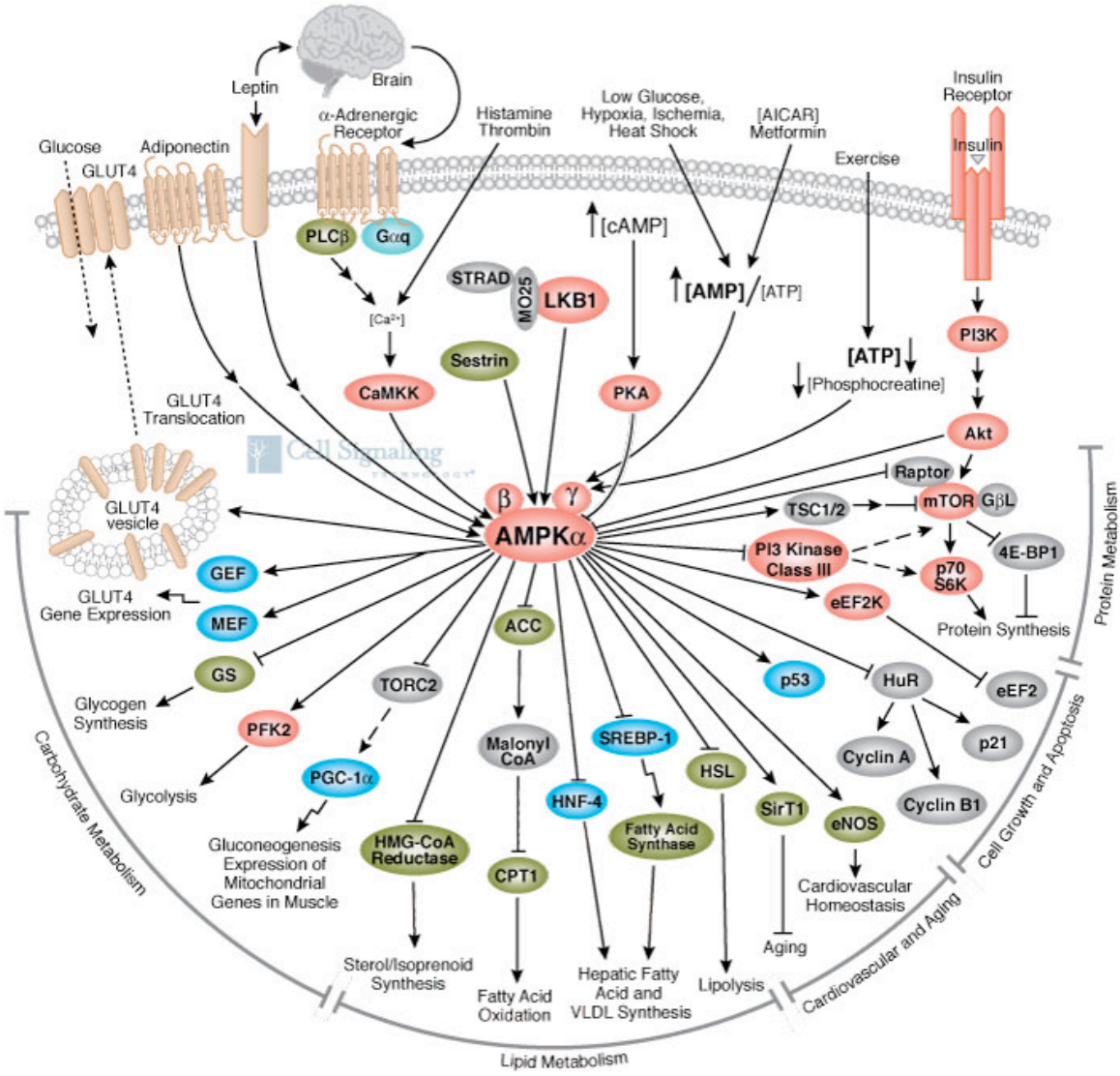

Figure 1.12 Schematic demonstrating some of the major known effects of AMPK activation. Red = kinase, green = enzyme, blue = transcription factor, dashed lines indicate tentative pathways. (http://www.cellsignal.com/reference/pathway/AMPK.html)

In the laboratory, AMPK activation can be measured in a range of ways. AMPK activity levels can be measured using a radioactive assay to measure the incorporation of ^{32}P into SAMS, a synthetic target peptide for AMPK. Western blotting can be used to blot for AMPK

phosphorylated at Threonine 172. To ensure that this value does not simply reflect a general upregulation of AMPK, it is corrected to total AMPK, which requires a second Western blot. Alternatively, a downstream marker of AMPK can measured on Western blot. Most commonly this is ACC, which again is presented as phosphorylated ACC as a percentage of total ACC. ACC is a large protein of 280 kDa, which can make it difficult to run a simultaneous housekeeping protein on the same gel.

1.13 AMPK and cardiac ischaemia

The α2 AMPK subunit isoform appears to mediate metabolic function in the heart. Alpha-1 knock out (KO) models fail to show any significant metabolic dysfunction[151]. However, blocking AMPK α2 using tissue-targeted dominant negative increases ATP depletion reduces glucose uptake and glycolysis during ischaemia and reduces fatty acid uptake and recovery of LV function in reperfusion[161,162]. The protective effects of α2 activation require the presence of functional LKB1, whereas α1 may be activated, albeit at a mildly reduced level, in the absence of LKB1[163].

The AMPK system is the sensor of cardiac energy metabolism. The heart preferentially produces ATP though β-oxidation of fatty acids, with approximately 60-90% of its energy arriving from this source[139]. Under ischaemic conditions the AMP:ATP ratio rises resulting in AMPK activation. This drives glucose uptake via GLUT-1 and GLUT-4 and increases glycolysis via PFK-2 phosphorylation and increased PFK-1 activtiy, to increase ATP availability to the myocardium and protecting against the consequences of ischaemia (see Figure 1.13, Figure 1.14 and Figure 1.14[151]).

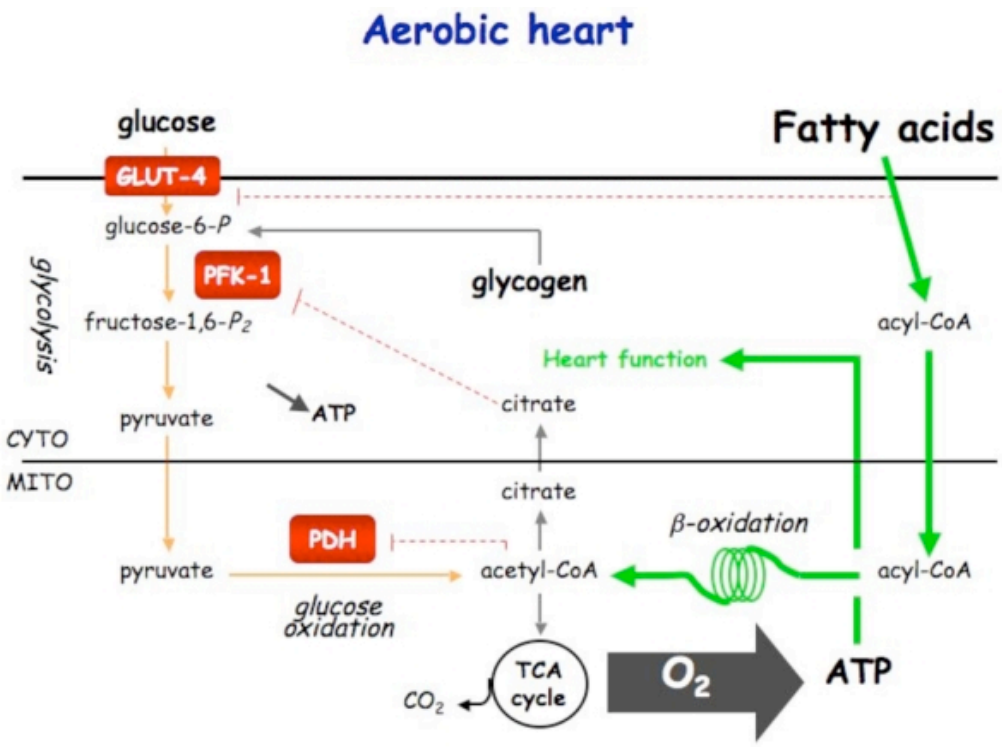

Figure 1.13 Cardiac metabolism during normoxia. ATP production from fatty acids (~70%) and glucose (~30%) From Viollet *et al.*[151]

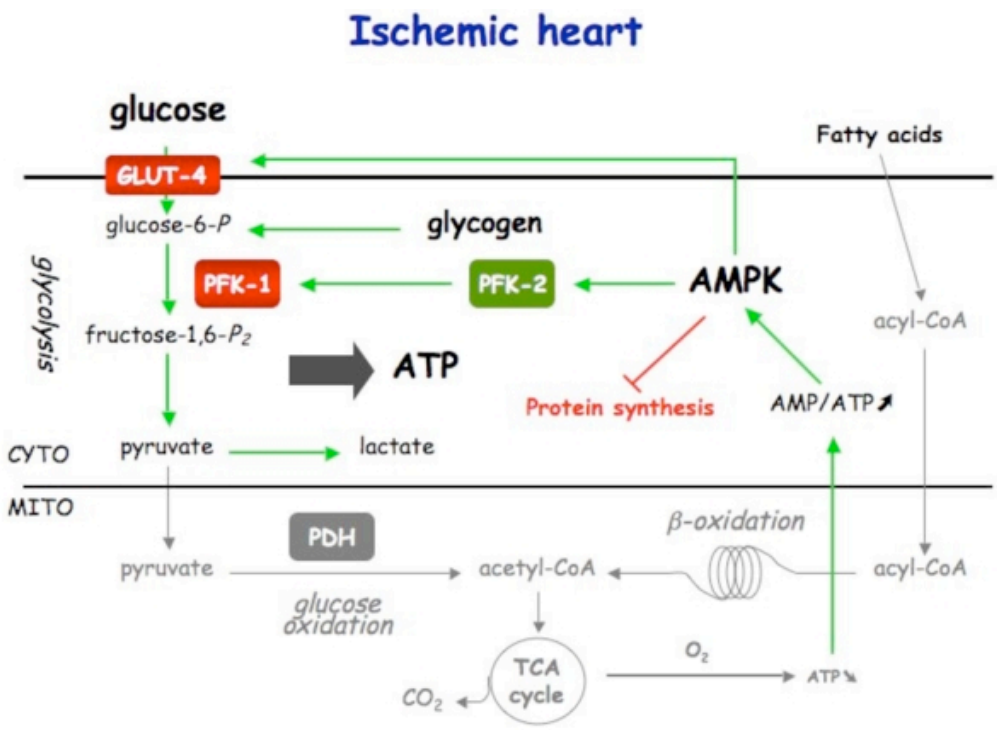

Figure 1.13 Cardiac metabolism during ischaemia: AMP:ATP ratio rises, activating AMPK and glycolysis becomes the sole ATP-provider. AMPK promotes GLUT-4 translocation and PFK-1 stimulation via PFK-2. (Viollet *et al.*[151])

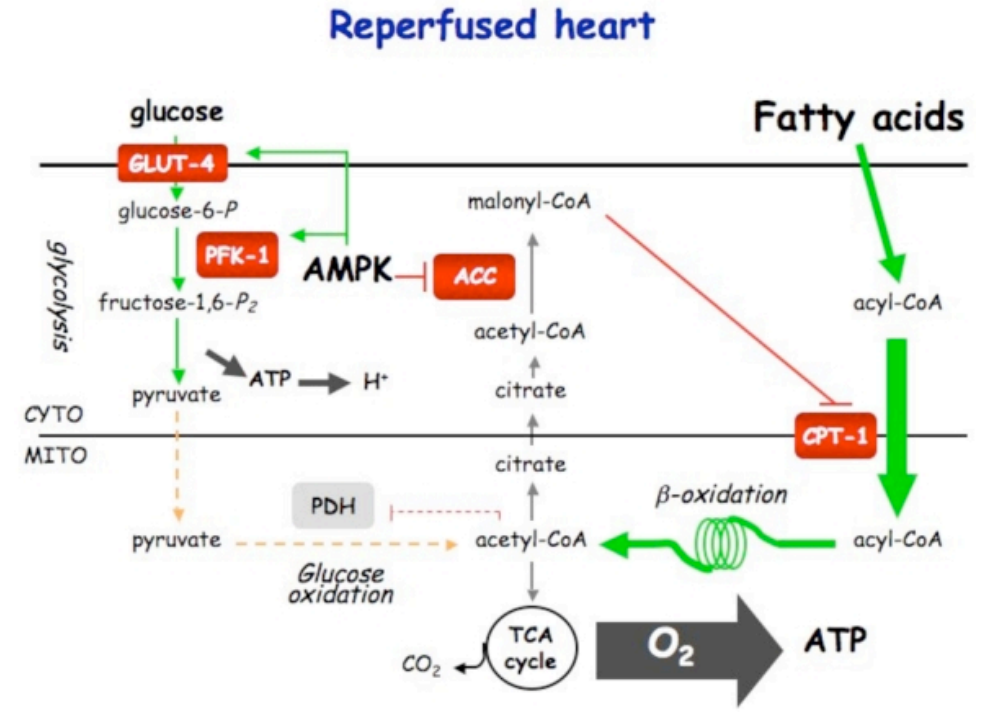

Figure 1.14 Cardiac metabolism during reperfusion. AMPK remains activated, phosphorylating and inactivating ACC, inducing fatty acid entry into the mitochondria to recommence fatty acid oxidation. The concomitant stimulation of glycolysis and fatty acid oxidation may induce uncoupling of glycolysis and glucose oxidation with the detrimental production of protons. (Viollet *et al.*[151])

Activation of AMPK with metformin in mouse model of ischaemia (left coronary artery ligation) demonstrates acute improvement in infarct size and troponin rise in both diabetic and non-diabetic mice[164]. In heart failure produced by a similar technique, metformin is able to improve animal survival by 47% and preserve LV dimensions and LV ejection fraction[165]. In both experiments this effect was lost in animals functionally lacking AMPK (dominant negative AMPK α2) or eNOS (eNOS-/-), indicating that these effects are mediated, at least in part, through eNOS/AMPK interaction[141].

Some of the cardioprotective effects of AMPK activation may arise from a reduction in apoptosis[139,162,166]. However, studies addressing the effect of AMPK activation on cardiomyocyte apoptosis have shown varying results. Studies in neonatal cardiomyocytes have indicated that AMPK activation may be both pro-apoptotic, via Bax translocation to the mitochondria, a step that occurs early in ischaemia[167] and anti-apoptotic, in palmitate-mediated apoptosis[168]. Palmitate is a major constituent of circulating fatty acids. Both AMPK α2 KD transgenic mice and adiponectin deficient mice have increased apoptosis following ischaemia and reperfusion[162,169]. In these adiponectin-deficient mice, adiponectin supplementation was shown to inhibit apoptosis secondary to AMPK activation. AMPK activation has been to be pro-apoptotic in many other cell types. In the liver, AMPK activation promotes apoptosis via c-Jun N-terminal kinase (JNK) and caspase-3 activation[170]. The explanation for these apparently contradictory effects remains unclear[139], although the duration of AMPK activation may play a role in mediating different effects[170].

Although AMPK activation clearly provides metabolic cardioprotection during ischaemia by increasing ATP availability, AMPK activation may also have detrimental effects on the heart, especially during myocardial reperfusion. At reperfusion, β-oxidation of fatty acids increases dramatically, due to increased levels of circulating fatty acids and to ACC phosphorylation by AMPK, with increased production of fatty acids derived-acetyl CoA. The presence of high levels of fatty acid-derived acetyl CoA reduces the production of glucose-derived acetyl CoA via the inhibition of the pyruvate dehydrogenase complex. This prevents pyruvate entering Randle's cycle and oxidative metabolism is reduced[171,172]. Furthermore, the uncoupling of glycolysis and glucose oxidation results in a build up of lactate and protons, which themselves require ATP for clearance. This reduces cardiac efficiency and may also decrease cardiac function[173] (Figure 4.15). For a review see Dyck and Lopaschuk[139].

1.14 AMPK and cardiac hypertrophy

Cardiac hypertrophy occurs for a range of reasons and through various mechanisms. It may arise as a result of physiological stimulus, such as exercise, or as a consequence of pathological mechanisms, such as pressure overload. Studies in several animal models of cardiac hypertrophy have demonstrated interactions with the AMPK system.

Left ventricular hypertrophy, achieved as a result of pressure overload (aortic ligation), is associated with a rise in AMPK activity that is thought to be a compensatory response to increased myocardial mechanical stress[174,175]. Mice unable to mount an AMPK response to pressure overload develop worse heart failure, including heart/body weight ratio, LV ejection fraction and mortality, than controls[175,176]. AMPK activation with metformin can attenuate pressure overload-induced cardiac hypertrophy[177,178]. Adiponectin appears to be an upstream mediator of this effect[175]. Hence, AMPK plays a key role in response to pressure overload and may also play a role in protection against the development of cardiac hypertrophy.

The finding that AMPK activation can reduce the development of cardiac hypertrophy might be predicted given that the development hypertrophy requires protein synthesis. Protein synthesis utilises energy and AMPK activation drives cell function away from ATP-consuming processes[179]. AMPK activation with AICAR has been shown to increase phosphorylation of eEF2 and reduce phosphorylation of p70S6 kinase and mTOR[178], all key regulators of protein synthesis. However, it is interesting to note that short-term AMPK activation (1 week) induces cardiac hypertrophy in angiotensin II-infused rats[180]. This suggests that either the effects of AMPK activation on the myocardium are time-dependent or that both hypertrophy induction and inhibition may be driven by AMPK activation, different effects having preponderance in different environments.

LKB1 appears to play an important role in the effects of AMPK activation on hypertrophy. Increased expression of LKB1 in neonatal rat cardiac myocytes inhibits the increase in protein synthesis observed in cells treated with phenylephrine[181] and inhibition of LKB1, in LKB1 KO mice or cells treated with siRNA to ablate LKB1 function, results in increased cardiac/body weight ratio and cardiomyocyte hypertrophy[182], with the predicted reduction in phosphorylation of AMPK and eEF2 and increased phosphorylation of mTOR and p70SK.

An interesting study from Kim *et al.* looked at exercise-induced cardiac hypertrophy in mice with a cardiac-specific KO of the IGF-IR[88]. Surprisingly they found that not only was the IGF-I receptor necessary for the development of exercise-induced cardiac hypertrophy but that exercised IGF-IR KO animals demonstrated a marked rise in cardiac AMPK activity. Exercised KO animals also had reduced cardiac glycogen stores and reduced peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) levels, potentially limiting exercise-induced increases in myocardial fatty acid oxidative capacity and mitochondrial function. The authors suggest these findings indicate increased energetic stress in the myocardium, probably at a mitochondrial level, accounting for AMPK activation. The same paper also reported that AMPK activation with AICAR was able to prevent IGF-I-induced hypertrophy in neonatal rat cardiomyocytes.

Short-term exercise has already been shown to increase cardiac AMPK activity in normal hearts[183], yet no difference was seen in this study between the WT exercised and sedentary groups, suggesting that exercise may induce transient changes in AMPK, which equilibrate with chronic training. The authors report that they repeated their experiments using transaortic constriction to induce pressure-overload hypertrophy and in this situation IGF-IR KO animals did not show any reduction in LV hypertrophy, suggesting that the IGF-I-induced hypertrophy pathway is specific to exercise-induced hypertrophy, although these data are not given in the paper.

In summary, AMPK activation can prevent IGF-I induced cardiomyocyte hypertrophy *in vitro* and can reduce the development of pressure overload-induced cardiac hypertrophy *in vivo*. The inability to mount an AMPK response to pressure overload results in worse cardiac outcomes. The inability to mount an IGF-I response to exercise results in a compensatory rise in AMPK activity.

1.15 AMPK in the clinical setting

Several drugs in clinical use have been shown to have effects on the AMPK system. Most notably, metformin is recognised as an activator of AMPK[184]. In addition the thiazolidinediones (TZDs)[146] and statins[185] have been shown to activate AMPK. Metformin and TZDs achieve normoglycaemia via different mechanisms; however, both have been shown to inhibit mitochondrial complex I activity and reduce cell respiration[186-188]. TZDs increase AMPK activity through changes in the cell AMP:ATP ratio[146]; metformin increases AMPK activity by altering the cytosolic AMP concentration[147]. Metformin has been shown to improve LV EDV and fractional shortening in dogs with heart failure produced by overdrive pacing, although it should be noted that the dogs received significantly higher daily doses of metformin than is currently used in patients[166]. Interestingly, these improvements were also seen when the dogs were treated with AICAR, indicating that the effect seen is due to metformin's ability to activate AMPK.

Metformin is known to activate AMPK in the clinical setting as well as in the laboratory. Ten weeks metformin treatment has been shown to increase skeletal muscle AMPKα2 activity and AMPKα2 phosphorylation at Thr172 in patients with type 2 diabetes mellitus (T2DM). These findings corresponded with increased glucose dispersal and muscle glycogen stores[189]. A similar study demonstrated increased AMPK activity in adipose tissue from patients taking metformin when compared to sulphonylureas[190]. TZDs and statins have only been shown to increase AMPK activity in animal and cell models.

There is evidence that metformin is able to exert a beneficial effect on cardiovascular outcomes in the clinical setting, although the prevalence of both T2DM and metformin use make head-to-head, long-term studies difficult. In the UK PDS trial for patients with newly diagnosed T2DM, patients receiving metformin showed a greater reduction in any diabetes-related end point ($p=0.0034$), all-cause mortality ($p=0.021$) and stroke ($p=0.032$) than patients treated with chlorpropramide, sulphonylureas or insulin, despite similar glycosylated haemoglobin levels in each group[191]. Metformin, used as a single agent in T2DM, is associated with lower rates of hospitalisation for myocardial infarction than sulphonylureas or combination therapy[192]. Administration of metformin to patients with T2DM reduces several markers of cardiovascular disease, including remnant lipoprotein cholesterol and soluble vascular cell adhesion molecule-

1[193]. At present, evidence in humans linking the AMPK-activating effects of metformin to its cardiovascular effects remains limited. However, Sasaki *et al*.'s study in dogs, demonstrating that the ability of metformin to improve markers of heart failure found similar cardiovascular improvements when dogs were treated with an alternative AMPK activator, AICAR, suggesting that AMPK activation is the mechanism by which metformin exerts its beneficial effect on the heart [166]. It is clear from ischaemia-reperfusion mouse models that AMPK activation plays a central role in cardiovascular protection.

1.16 AMPK gamma-2 subunit mutations

Despite a wealth of evidence that AMPK activation is cardioprotective, chronic constant AMPK activation may have deleterious consequences. Missense mutations in the gene encoding the AMPK-γ2 subunit (PRKAG2, located in human on chromosome 7q36[194]) cause ventricular hypertrophy, ventricular pre-excitation and progressive cardiac conduction system disease, resulting in Wolff-Parkinson-White syndrome[195]. At least ten mutations have been identified in the human PRKAG2 gene[196], which demonstrate varied clinical expressivity and typically present in late adolescence[197]. The role of AMPK activation in the development of this disease is still poorly understood. It is thought that constant, increased, unstimulated basal AMPK activity occurs, with excessive intracellular glycogen deposition (Figure 1.15)[196], yet a loss of ability to sense and respond to metabolic changes, with failure of AMPK activation and rising AMP concentrations[196].

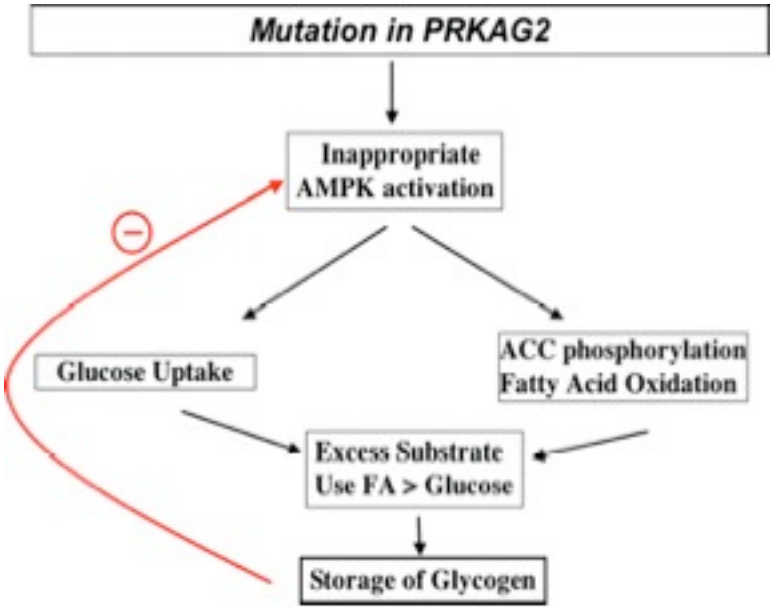

Figure 1.15 Model explaining glycogen accumulation in PRKAG2 cardiomyopathy. Mutations cause inappropriate AMPK activation under resting conditions. AMPK activation leads to mobilisation of fatty acids and increased entry of acyl-CoA to mitochondria. Concomitantly, there is an increase in glucose uptake. Increased substrate influx is not met by increased metabolic demand, allowing storage. Given the existing capacity of the heart to store glycogen, and given the preference to use fatty acids for mitochondrial oxidation, glycogen is stored. Assuming glycogen can inhibit AMPK a new steady state is reached under conditions of glycogen overload. FA indicates fatty acids (from Arad *et al.*[197])

There is some evidence that basal AMPK activity may fall later in the disease, perhaps influenced by the degree of glycogen accumulation, potentially contributing to hypertrophy through increased protein synthesis[198,199].

Transgenic mice expressing a human PRKAG2 mutation under the control of a tetracycline-repressible promoter demonstrate that cardiac hypertrophy can be reversed and cardiac dysfunction ameliorated by normalising glycogen content with mutant suppression, but ventricular pre-excitation can only be prevented with very early therapy prior to the development of accessory pathways[200]. Much work remains to understand fully how AMPK is able to produce the pathology seen with these mutations.

1.17 GH and AMPK

Thus both AMPK and GH have beneficial effects on the heart with short-term exposure but may cause cardiac dysfunction with chronic excess exposure. It is clear that both have similar cardiac effects in certain states: both AMPK activity[174] and IGF-I expression[46] are increased in pressure overload cardiac hypertrophy; both are involved in cardioprotection in ischaemia[151,169,201]; both increase eNOS activity[67,141].

Insulin has been shown to inhibit cardiac AMPK activity[202] and AMPK Thr172 phosphorylation[203]. In rat cardiac cells, this has been shown to occur through the phosphorylation of AMPK serine 485 (α1) or 491 (α2) through an AKT-dependent pathway[204]. More recently, in vascular smooth muscle cells, IGF-I has also been shown to phosphorylate AMPK serine 485, with a consequential reduction in AMPK activity and Thr172 phosphorylation, via AKT phosphorylation[205].

One study did not find any increase in AMPK Thr172 phosphorylation in human aortic endothelial cells (HAEC) treated with GH for 3 or 6 hours[66]. However, these cells had been serum starved for 16 hours prior to treatment with GH, a technique that has been shown to increase AMPK phosphorylation, even at 3 hours[206]. It may be that the AMPK contained within these cells was already maximally Thr172 phosphorylated or that it was phosphorylated to a level at which a relatively low dose of GH (30ng/ml) was unable to further increase phosphorylation. No other studies have been published looking at the effect of GH on AMPK.

The interactions between AMPK activity and the GH/IGF-I system are likely to be complicated and to alter with length of exposure. Furthermore, the multi-system impact of the GH/IGF-I axis *in vivo* means that multiple effects may occur synchronously. This area clearly needs further research to help delineate the processes that are taking place and provide a more detailed understanding of the chronic effects of GH on the heart and mechanisms by which these occur. The interaction of these two systems will be investigated in the following experiments along with work to address the role of CMR in states of GH excess and deficiency.

1.18 Hypotheses

- CMR will demonstrate increased LV mass and cardiac output in patients with acromegaly, which will improve with a reduction in GH levels after disease treatment
- CMR will demonstrate reduced LV mass in patients with ao-GHD which will improve with normalisation of GH levels after disease treatment

- Acute GH excess will increase cardiac AMPK activity in animal and cell models
- Chronic GH overexposure will result in chronic cardiac AMPK activation in animal models of acromegaly

The characterisation of growth hormone-related cardiac disease with magnetic resonance imaging

Objectives

- To use CMR to assess LV and RV volume and function and LV mass in patients with adult-onset GHD and acromegaly
- To use CMR to determine the impact of normalising IGF-I on heart morphology, mass and function
- To assess for fibrosis, as measured by late gadolinium enhancement, in the hearts of patients with acromegaly
- To examine for the presence of ischaemic heart disease using perfusion CMR in patients with acromegaly and GHD
- To assess ascending aorta area in patients with acromegaly and GHD

2 Materials & Methods

2.1 Study outline

Adult patients with GHD and acromegaly about to commence a therapy aimed at normalising GH levels (GH replacement, surgery, somatostation analogues or GH antagonists) were assessed using CMR. Cardiac function and morphology was assessed immediately before initiation of the chosen therapy and at six and twelve months after treatment. Echo was performed at each time-point and patients' clinical and biochemical response to their treatment was recorded (Figure 2.1). Power calculations performed on echo data suggested ten subjects were necessary for each arm[207] (see Section 2.14.1).

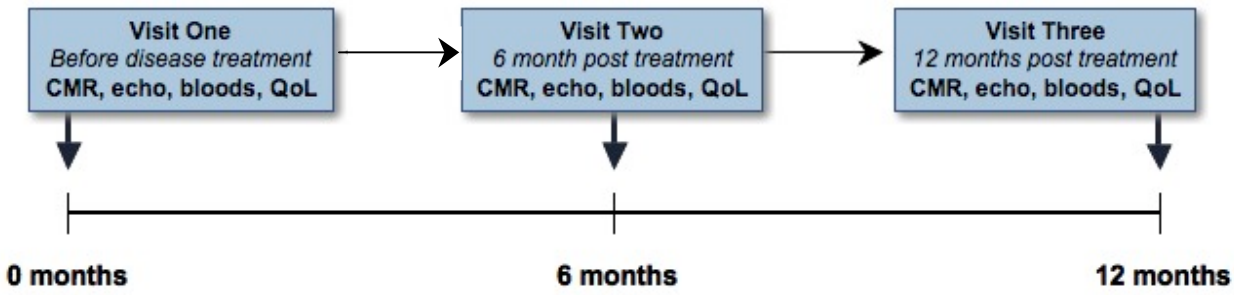

Figure 2.1 Study flow chart. Patients attended three visits in total, having a full set of investigations at each. QoL = quality of life assessment

2.2 Ethics

Multi-centre ethics approval (06/Q0401/53) was applied for and granted. The following centres directly recruited patients or referred patients to St Bartholomew's Hospital for consideration of inclusion in the study:

- St Bartholomew's Hospital, London (Prof A Grossman, Prof W Drake, Prof S Chew, Dr S Akker)
- King's Hospital, London (Dr S Aylwin)
- The Royal Free Hospital, London (Dr B Khoo)
- Addenbrooke's Hospital, Cambridge (Dr M Gurnell)
- The Lister Hospital, Stevenage (Dr F Kaplan)

2.3 Patient selection

In patients with pituitary disease, GHD was confirmed using provocation testing with insulin-induced hypoglycaemia (ITT) or glucagon stimulation. Both tests have been validated for the assessment of GH reserve[208,209]. Although the ITT is considered the gold standard in this assessment[210], the induction of hypoglycaemia with insulin is contraindicated in patients with a history of seizure, ischaemic heart disease and with unreplaced cortisol deficiency. Therefore, clinicians selected the choice of provocation test used based on patient suitability and local policies.

Age-matched IGF-I levels are not adequate in themselves for the diagnosis of GHD[208], although a markedly low IGF-I level is suggestive when taken in the clinical context of multiple pituitary hormone deficiencies, particularly in younger patients[211]. However, serum IGF-I level is used to monitor GH replacement in patients receiving recombinant (r)GH therapy and therefore levels were recorded at baseline[210].

NHS-funded GH replacement has been reviewed by the National Institute for Clinical Excellence (NICE)[27]. Only those patients exhibiting severe GH deficiency, as determined by a peak GH response to stimulation testing of <3.0 µg/L, in addition to impaired quality of life (QoL) because of GHD qualify for GH replacement. QoL is determined as a score of 11 or less on the disease-specific Quality of Life Assessment of Growth Hormone Deficiency in Adults (AGHDA) questionnaire[28]. Referring clinicians determined appropriateness for GH replacement, ensuring patients participating in this study fulfilled NICE criteria.

Acromegaly was confirmed clinically and biochemically. Patients' signs and symptoms of GH excess were documented along with their duration. The oral glucose tolerance test was used to demonstrate biochemical GH excess. This test looks for failure of GH suppression to <1.0 µg/L following an oral 75 g glucose load (GTT). Serum IGF-I levels were also measured as an indirect marker of GH excess. The referring clinician determined the patient's treatment, treating medically with somatostatin analogues or referring for transsphenoidal surgery as appropriate, using serum IGF-I as a marker of treatment success. Some patients were treated initially with somatostatin analogues and then went on to have transsphenoidal surgery later. Individual patients' treatments are given in the Results section (page 65).

For both groups of patients, other pituitary hormones were replaced with stable doses of medication for at least 3 months prior to entry to the study. This was an observational study only; no interventions were made. Inclusion and exclusion criteria are shown in Table 2.1.

Inclusion criteria	Exclusion criteria
Male or female	Pregnancy
Age 18-80 years	Significant renal impairment (eGFR <30)
Adult GH deficiency (GH <3 μg/L on ITT) or acromegaly (GH >1 μg/L on GTT).	Any preclusion to undergoing MRI (e.g. implanted metalwork, severe claustrophobia)
Fully replaced for any other pituitary hormone deficiencies (stable dosing for >4 weeks)	Documented moderate or severe reversible airway disease
Disease symptoms for at least 1 year	Second or third degree heartblock
Fulfils criteria for GH replacement therapy*	Known heart failure, severe valve disease or ischaemic heart disease
	Systolic blood pressure <90 or >180 mmHg; diastolic blood pressure >110 mmHg
	Childhood onset GHD
	GH therapy in the two years prior to study*

Table 2.1 Clinical study inclusion and exclusion criteria. ITT = insulin tolerance test; GTT = glucose tolerance test; *refers to GHD only

2.4 Patient recruitment

Recruitment took place between December 2008 and February 2010. Potentially suitable patients where identified by the Local Investigator at each site. The Local Investigator then had the choice of recruiting the patient into the study locally or referring to Professor Grossman at St Bartholomew's Hospital to be considered for involvement in the study. The majority of investigators chose the latter method of recruitment. Once referred, patients were contacted by telephone and screened for suitability for inclusion in the study by the researcher. Once eligibility for inclusion had been ascertained, and with their permission, patients were sent Patient Information Sheet v3 (Page 197). Once patients had been given time to read the Patient Information Sheet they were contacted again by telephone to determine if they wished

to take part in the study. Patients received the Patient Information Sheet at least 24 hours before participation in the study.

2.5 Study visits

Patients who wished to take part in the study were invited to attend for the First Visit in the Department of Endocrinology at St Bartholomew's Hospital. At this visit formal written consent was taken by the researcher, using the Patient Consent Form (Page 202). An interview was then conducted by the researcher, which included disease and treatment history, past medical history, medication details and current symptoms. Further details were supplied by the referring clinician, including previous imaging reports and biochemical results confirming diagnosis. Blood pressure (BP), height and weight were recorded. Patients gave a fasting blood test. In some cases, long travel times precluded fasting prior to the blood test. When this occurred it was noted. Patients also completed at disease-specific quality of life questionnaire: ACROQoL for patients with acromegaly[212] (Page 203) and QoL-AGDHA for patients with GHD[28] (Page 208).

Patients travelled to the London Chest Hospital, Bethnal Green, where they underwent echocardiography. They then atttended the Cardiac MRI department where a screening questionnaire for CMR safety was performed (Page 212). Two intravenous cannulae were sited, one for contrast agent and one for adenosine. Patients then underwent CMR as per the protocol below. Patients were asked to avoid caffeine from midnight to prevent interference with perfusion CMR results. An electrocardiogram (ECG) was performed on all patients who received adenosine, to demonstrate the absence of pro-arrhythmic heart rhythms. Patients with a history of significant bronchospasm were not given adenosine. Some patients declined perfusion CMR because of concern regarding side-effects from adenosine.

Six months and twelve months following the first visit, patients were invited to attend for Visits 2 and 3. Each visit involved a conversation with the researcher to update history, treatment and medication records, BP and weight measurements, QoL assessment and fasting blood tests, followed by echocardiography and CMR.

After each visit, patients submitted their receipts for travel costs to the researcher, who arranged for them to be reimbursed.

Echocardiograms were recorded and stored on CD for analysis at the end of the study. Each CMR was reviewed by a cardiologist as per the protocol for reporting routine clinical CMR scans. A report was issued and a copy of this was forwarded to the patient's GP and referring clinician. The report was also summarised and sent to the patient with written explanations regarding any mild abnormalities, along with the researcher's telephone number in case the individual had questions. In the case of patients with significantly abnormal scans, arrangement was made for follow up with a cardiology consultant for further assessment. CMR images were stored on the computer server to allow for future, detailed analysis.

2.6 Cardiac MRI controls

Age- and sex-match controls were recruited to match each of the study patients. Posters advertising for healthy controls (Page 213) were placed in the medical ward visiting areas and in outpatients. Advertisements were also e-mailed NHS Trust (Barts & the London) and Queen Mary University of London college staff. Applicants were asked to contact the researcher by telephone or e-mail. Each applicant was given brief information about the study and asked to complete a short screening questionnaire to assess suitability for participation. Completed questionnaires were used to match volunteers to patients. Patients were matched for sex and age, where at all possible matching within five years of age. Note was also taken of weight, height and any other medical problems and these factors were included in matching. Volunteers who were suitable matches were then provided with the Healthy Volunteer Information Sheet (Page 214). If, after reading this, the volunteer was still happy to go ahead an appointment time to attend the London Chest Hospital was offered. At least 24 hours took place between reading the Information Sheet and the appointment.

On arrival at the Cardiac MRI unit at the London Chest Hospital, informed consent was taken by the researcher. The volunteer completed an MRI safety questionnaire and then underwent a non-contrast enhanced CMR, using the same scanner protocols as the study patients. Height, weight and blood pressure were recorded. The patient gave a blood sample for IGF-I and serum for storage. CMR images were analysed as per study scans. Only one CMR was performed per volunteer, unlike the study patients who underwent three scans throughout the duration of the study. No intravenous contrast or adenosine was used in the volunteers.

2.7 Cardiac MRI

CMR was performed at the London Chest Hospital by experienced cardiologists using a Phillips Achieva CV 1.5 Tesla MRI scanner (Philips Medical Systems). Standard ECG-gated, weight-adjusted protocols were used, allowing image gathering to be timed to particular points of the cardiac cycle. To reduce breathing-artefact, patients were asked to hold their breath for up to 20 seconds at certain points. All patients received intravenous gadolinium as a contrast agent (Dotarem™ total dose 0.2 mmol/kg). Those patients who underwent perfusion CMR received intravenous adenosine 140 μg/kg/min given for 3 minutes, with continuous blood pressure and ECG monitoring throughout that time[118].

2.8 Image analysis

CMR images were analysed by a single observer, blinded to the underlying diagnosis. Images were analysed with "cmr[42]" computer software (Circular Cardiovascular Imaging Incorporated, Canada). Volume and mass parameters were measured using images derived from a balanced steady state free procession (b-SSFP) sequence. Perfusion analysis was performed on T_1-weighted images. Cardiac output and heart rate were both noted.

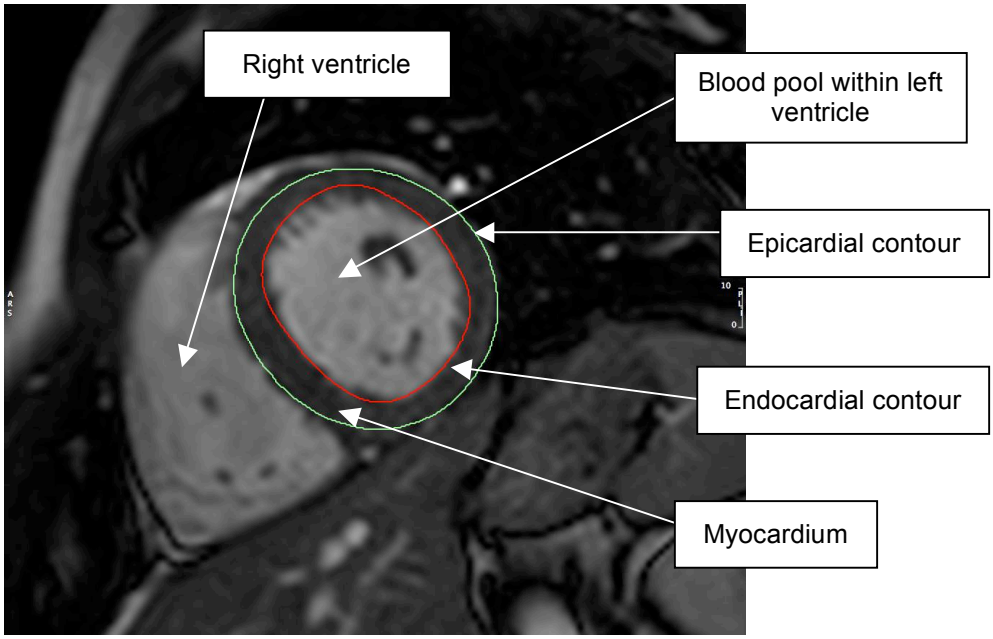

Figure 2.2 A short-axis slice in end-diastole with left ventricular endocardial and epicardial contours *(Courtesy of A. Dattani)*

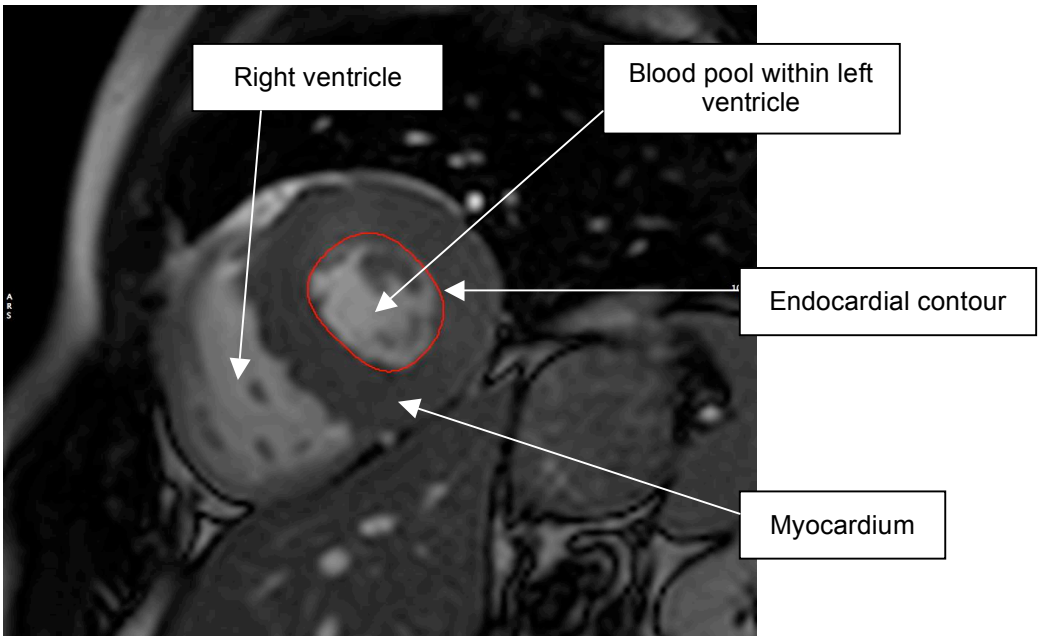

Figure 2.3 A short-axis slice in end-systole with left ventricular endocardial contour *(Courtesy of A. Dattani)*

To determine LV and RV volumes and LV mass, the scans were imported into cmr[42]. The series of short-axis cine images was opened. This is composed of 10-12 short-axis slices through the heart. Each slice represents 20-30 frames through one cardiac cycle[118]. The first frame was chosen as the end-diastolic frame. The frame with the smallest blood pool was selected as the end systolic frame. The point at which 50% of the blood pool was surrounded by the myocardium was presumed to be the basal slice. Manual endocardial contours were drawn for the end-diastolic and end-systolic slices (Figure 2.2). These were drawn outside the papillary muscles to provide consistent results. The epicardial contours were drawn for the end-diastolic slice only as the epicardium deforms in systole, rendering end-systolic epicardial contours less accurate in mass calculations[213]. From these data, the cmr[42] software calculated the following cardiac parameters: left ventricle – EDV, ESV, EF, SV, CO, LVM; right ventricle – EDV, ESV, EF, SV.

CMR image analysis and measurements were performed by Abhishek Dattani, medical student, and Dr Filip Zemrak, cardiology research fellow, under the supervison of Dr Ceri Davies and Dr Steffen Petersen.

2.9 Perfusion analysis and late gadolinium enhancement

All images were examined for evidence of late gadolinium enhancement (LGE) to look for evidence of fibrosis and myocardial scarring. Scans from patients who received adenosine were examined for areas of reduced myocardial perfusion to look for evidence of reversible ischaemia. Analysis was performed on T_1-weighted images acquired ten minutes after patients had received gadolinium-based contrast. Using cmr[42] software, endocardial and epicardial border contours were drawn on three short-axis slices through the left ventricle. The insertion point of the right ventricle was used as a marker to divide myocardium into segments[214]. These contours were used in each phase of contrast enhancement to calculate data for signal intensity in the different myocardial segments and for the ventricular cavity (Figure 2.4). Areas that demonstrated gadolinium enhancement more than two SD above the background myocardial level were recorded as showing LGE. Areas of reduced gadolinium enhancement during induction of hyperaemia with adenosine were recorded as showing reduced perfusion.

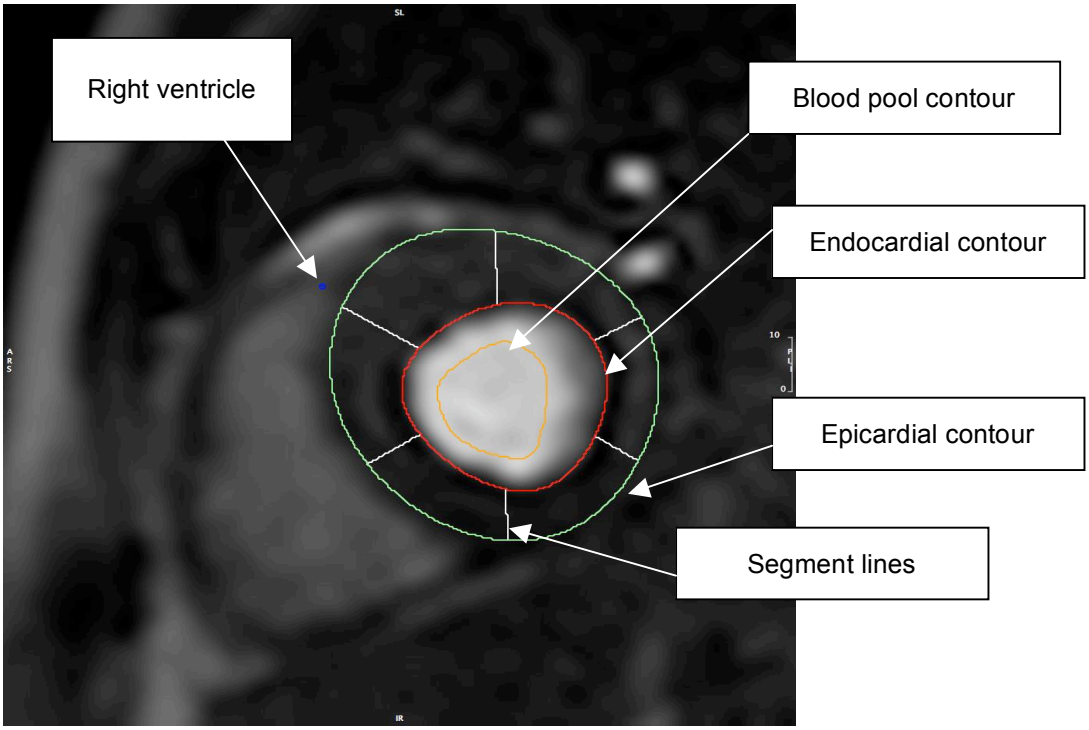

Figure 2.4 Endocardial, epicardial and blood pool contours drawn on a short-axis slice for perfusion analysis. The blue dot is the right ventricle indicator, which tells the software where the right ventricle inserts onto the left ventricle. This allows the software to produce segments separated by the segment lines as shown *(Courtesy of A. Dattani)*

Scans that demonstrated areas of LGE or reduced perfusion were reviewed by a senior cardiologist. Individuals who demonstrated evidence of possible reversible cardiac ischaemia were referred for cardiological assessment and consideration of additional imaging.

CMR images were analysed for LGE and perfusion by Dr Ceri Davies.

2.10 Aortic root analysis

Aortic dimensions were gathered from MRI scout images. Internal to internal distances in lateral and anterior-posterior planes were measured at the level of the pulmonary bifurcation (Figure 2.5). Area was calculated using the formula $\pi r1r2$ to allow for the elliptical nature of the aorta. Results were indexed to body surface area. Measurements were made by Dr Ceri Davies.

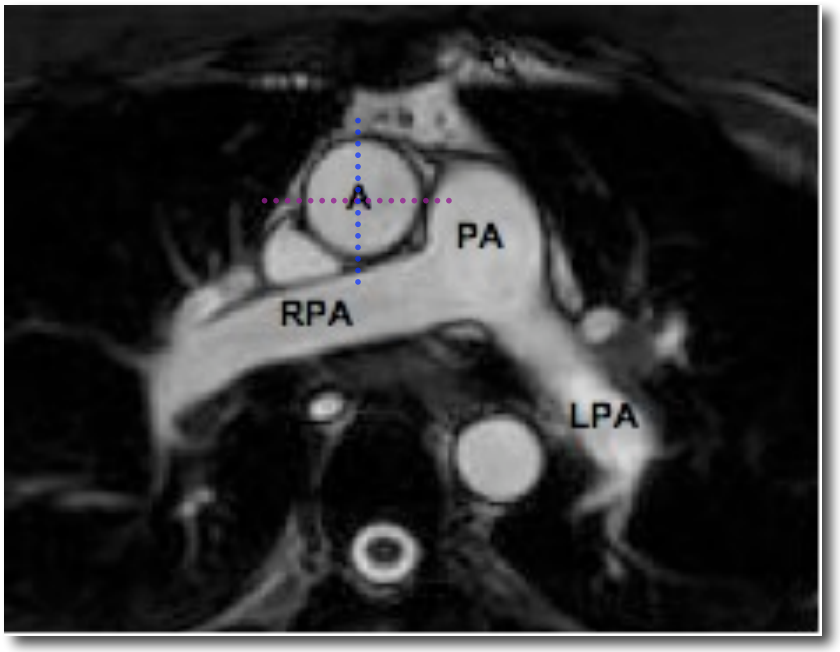

Figure 2.5 CMR at the level of the pulmonary bifurcation. Dashed line indicates measurement planes, blue = anterior-posterior plane, purple = lateral plane. A = aorta, PA = pulmonary artery, RPA = right pulmonary artery, LPA = left pulmonary artery

2.11 Quality of life assessment

Two well-validated disease-specific quality of life assessments (QoL) were used: the QoL-AGHDA[28] for patients with GHD or the AcroQOL[212] for patients with acromegaly. The QoL-AGHDA contains 25 yes/no questions regarding the symptoms of GHD. The presence of the symptom is awarded one mark; the absence of the symptom receives zero. The total score is the sum of all results to give a score out of 25, where 25 is the worst possible QoL. ACROQoL consists of 22 questions, each with five answer options. Answers range from "always" or "completely" which score one to "never" or "completely disagree" which score five. Results are summed and then converted to a score out of 100 using the following formula:

$$\text{ACROQoL} = \left(\frac{[\text{total score} - \text{min}]}{(\text{max} - \text{min})} \right) * 100$$

Where min = the minimum score possible (22) and max = the maximum score possible (110). The higher the result, the better the QoL.

Patients were given the questionnaire along with its accompanying instructions. Questionnaires have been designed so that patients can complete them independently without input from the healthcare provider. Patients were given adequate amounts of time to complete the questionnaire. Patients who spoke limited English were excluded. Copies of the questionnaires can be found in Appendix I.

2.12 Biochemical tests

IGF-I, fasting lipids, insulin and glucose, thyroid function tests (including T3), testosterone/oestrogen (as appropriate), urea, creatinine, blood count and serum for storage were taken from patients at each visit. IGF-I and serum for storage were taken from healthy volunteers on the day of their CMR. Samples were transferred on ice and then frozen at -20°C, or spun, separated and then frozen at -20°C, depending on the test required. All samples were processed in the Departments of Biochemistry and Haematology, Barts & the London NHS Trust.

2.12.1 IGF-I Assay

IGF-I levels were measured using a chemiluminescent immunoassay on the Siemens Immulite 2500 analyser (Surrey, UK). The assay reports IGF-I levels between 25-1600 ng/ml, with a reference range lying between 94-252 ng/ml. External quality assessment, performed by Guildford Peptide Hormones (Surrey, UK), reported the assay performance as "good". Internal quality assessment demonstrated the following results with two standard solutions: Solution 1, result = 48.6 ng/ml, mean 47.5, SD 4.2; Solution 2, result = 188 ng/ml, mean186, SD 15.9.

2.13 Biometric measurements

Blood pressure, weight and height were measured by trained healthcare professionals using standardised NHS equipment on Francis Fraser Ward at St Bartholomew's Hospital or in the Cardiac MRI Unit at the London Chest Hospital.

2.14 Statistics

2.14.1 Power calculations

Power calculations to look for a significant increase in LVMi were performed based on Valcavi *et al's* study of one year GH treatment on patients with adult-onset GHD[207]. As the literature suggests that changes in cardiac mass seen with GH replacement in GHD are subtler than those seen with normalisation of GH levels in acromegaly, it was felt appropriate to use a GHD study rather than an acromegaly study to perform power calculations[33,73]. Valcavi's study used echo to determine LVMi changes, however, CMR is reported as a more accurate measure of LV mass than echo and so it was felt that standard deviations (SD) seen at echo would be bettered by CMR[110]. Attempts were not made to determine clinical relevance of changes seen, as at present, there is inadequate data regarding this issue in the literature (discussed further in Section 4.4.2). SD produced by Valcavi's paper were used as a basis to predict changes in LVMi that would be seen in our study. A power of 80% and a significance of 0.05 (α) were used, power calculations were performed on StatsDirect. From these calculations, it was determined that ten subjects would be needed in each arm. Attempts were made to recruit 15 patients to each arm to allow for dropout. Two recently published studies also sought the same number of patients for the assessment of the effects of GH on cardiac mass, suggesting that these calculations were an accurate reflection of the available literature[128,215].

2.14.2 Data analysis

Images from study patients and healthy volunteers were compared to one another. For study patients, images before and after treatment were compared to one another. Additionally, published, standardised normal ranges for CMR were used[216]. These normal ranges were generated from the CMR data taken from 30 males and 30 females aged 20-65 years, who underwent CMR specifically to supply normative data.

Height and weight are major contributors to variations in LV between individuals[217]. To correct LV mass for these variations, results were corrected for body surface area (BSA) using the Mostellar formula:

$$\textbf{BSA in } m^2 = ([\textbf{height in cm x weight in kg}] / 3600)^{1/2}$$

CMR measurements of left ventricular mass, EDV, ESV, SV and CO were divided by BSA to create an indexed result and are notated as LVMi, EDVi, ESVi, SVi and CI. EF does not require indexing as it is reported as a percentage. This not only allows for comparison between individuals of the same sex but, with the exception of LVMi, allows for comparison of individuals of the opposite sex[218].

Serum IGF-I levels vary with age, the highest levels being seen in younger individuals. To allow for comparison, IGF-I standard deviation scores (SDS) were calculated using a standardised reference table provided by the assay manufacturer (Siemens, Surrey, UK). This provided mean and standard deviation (SD) results for men and women of different ages. The following formula was applied for each individual's IGF-I value:

$$\mathbf{SDS = (\mathit{x} - mean\ \mathit{x}) / SD}$$

Where *x* is the IGF-I value, mean x is the mean IGF-I value given for that individual's age and sex and SD is the SD value given for that individual's age and sex.

To assess for bias and error in the measurement of LV mass and to ensure reliability, interobserver variability was assessed. Inter-observer variability was investigated by the independent analysis of 34 scans by an experienced colleague, blinded to the original measurements. Bland-Altman plots were constructed by plotting the mean of the two values generated on the *x*-axis and the difference between the two values generated on the *y*-axis, subtracting the later value from the original one[219].

The Student's T test was used to compare patients with controls. Repeated measures ANOVA was used to compare patients before and after treatment. Paired T test was used to compared IGF-I SDS at the beginning and end of the study. A p value of 0.05 or less deemed as being statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Spearman's rank correlation test was used to compare IGF-I SDS and LVMi. Data are given as mean (standard deviation), except where specified. Error bars on graphs represent standard error of the mean (SEM). Statistical analysis was performed using StatsDirect, Excel and Prism.

3 Results

3.1 Patient characteristics

Ten patients with GHD (2 female, 8 male) and 14 patients with acromegaly (10 female, 4 male) were recruited and consented to take part in the study. A summary of the baseline characteristics of patients with GHD is given in Table 3.1 and of the patients with acromegaly in Table 3.2. One female with acromegaly dropped out before Visit One due to travel problems (Patient 5). The remainder of the patients all attended for the First Visit. All patients with GHD attended for Visit Two. Two patients with GHD did not attend for Visit Three, one (Patient 21, male) because he had discontinued GH as he failed to have ongoing clinical benefit, one (Patient 19, female) as she did not feel comfortable undergoing further CMR studies. Of the patients with acromegaly, three female patients underwent Visit One and no further visits, one because she did not feel comfortable undergoing CMR scans (Patient 8), one because of work pressure (Patient 2) and one because of a non-related chronic illness (Patient 12). One female patient could not attend Visit Two due to illness but did attend Visit Three (Patient 7). One female patient did not attend Visit Three as she did not feel comfortable undergoing further CMR scans (Patient 11).

3.2 Adverse outcomes

There were no significant adverse outcomes from this study. The only reports of side effects were of discomfort at venous cannulation (Patients 11 and 21) and of an uncomfortable sensation when receiving either the adenosine or the gadolinium contrast infusion. Effects from both were mild. Two patients requested that they did not have adenosine on future occasions and their wishes were complied with (Patient 19, 23).

Pt	*Age*	*Sex*	*Symp*	*Reason for GHD*	*Other medical conditions*	*Medications*	*Smoker*	*Visits attended*
1	73	M	3	NFPA TSS 2005 EBRT 2005	Mild OSA	Levothyroxine Testosterone Aspirin Hydrocortisone	Ex (30)	All
3	49	M	18	Prolactinoma, medical therapy	Type 2 DM Hypertension	Ramipril Candasartan Levothyroxine Aspirin Bromocripttine Metformin	No	All
14	56	M	10	NFPA TSS 1998 EBRT 1998		Levothyroxine Hydrocortisone Testosterone Aspirin Lansoprazole	Ex (30)	All
16	51	M	3	NFPA TSS 2006	Hypothyroidism Hypercholesterolaemia	Levothyroxine Testosterone Simvastatin	No	All
17	50	F	4	NFPA Craniotomy 1991 EBRT 1991 TSS 1992 TSS 2005 EBRT 2008	Renal stones	HRT	Yes	All
18	58	M	2	NFPA TSS 2003	Hypercholesterolaemia	Levothyroxine Hydrocortisone Testosterone Atorvastatin	No	All
19	34	F	6	Hypophysitis during pregnancy 2003	Depression	Levothyroxine Seroxat	Yes	1,2
20	76	M	3	NFPA Pituitary apoplexy 2007		Levothyroxine Hydrocortisone Testosterone	No	All
21	57	M	9	Meningioma EBRT 2000	Disordered sleep	Levothyroxine Carbemazepine Amitripyline	No	1,2
22	37	M	3	Meningioma Surgery Radiotherapy 2006	Acute lymphoblastic leukaemia – chemotherapy and cranial irradiation 1977	Levothyroxine Hydrocortisone Testosterone	No	All

Table 3.1 Characteristics of GHD patients in the study at the time of enrolment. Pt = patient, Symp = symptom duration in years, NFPA = non-functioning pituitary adenoma, TSS = transsphenoidal surgery, EBRT = external beam radiotherapy. Brackets represent number of years after stopping smoking

Pt	*Age*	*Sex*	*Symp*	*Proposed treatment*	*Other medical conditions*	*Medications*	*Smoker*	*Visits attended*
2	46	F	15	Sandostatin	Thyroidectomy	Levothyroxine Alfacalcidol	No	All
4	49	F	5	TSS	OSA Asthma	Lanreotide Amlodipine Diazepam Citalopram Tramadol Asthma inhalers	Yes	All
5	30	F	3	Pegvisomant (prev TSS)	Asthma	Sandostatin Cabergoline Octreotide sc HRT Pregabalin Salbutamol	No	None
6	50	F	10	TSS	Hypertension Breast cancer (treated)	Ramipril Venlafaxine	No	All
7	58	F	15	TSS	Hypertension OSA Back pain	Ramipril Bendrofluazide Amitriptyline Oxytosin	No	1,3
8	39	F	10	Lanreotide (prev TSS)	Hypertension OSA	Levothyroxine Bendrofluazide	Yes	1
9	30	M	5	TSS		Lanreotide	Yes	All
10	63	M	25	Lanreotide (prev TSS)	Hypertension	Cyclopenthiazide Amiloride Testosterone Hydrocortisone	Ex (15)	All
11	46	F	10	Lanreotide (prev TSS)			Ex (3)	1,2
12	58	F	10	TSS	Hypertension Depression	Atenolol	No	1
13	67	M	30	Cabergoline	Hypertension Mild OSA	Ramipril	Ex (30)	All
15	45	M	5	TSS	Encapsulated parotid carcinoma	Octreotide sc	No	All
23	58	F	30	Lanreotide & TSS	Hypertension Depression	Omeprazole	No	All
24	76	F	15	TSS	Hypertension OSA	Losartan Amlodipine Doxazosin Aspirin Simvastatin Diclofenac	No	All

Table 3.2 Characteristics of acromegaly patients in the study at the time of enrolment. Pt = patient, TSS = transsphenoidal surgery, OSA = obstructive sleep apnoea, Symp = symptom duration in years. Brackets represent number of years after stopping smoking.

3.3 Baseline comparison of patients and controls

There were no significant differences between the patients and the control group, with the exception that male patients with GHD were significantly heavier than their controls (87.8 versus 71.2 kg, p=0.007) (Table 3.3). This difference was not seen the female GHD patients or the acromegaly patients (Table 3.4). As might be predicted, acromegaly patients were more likely to have hypertension than controls (8 out of 13 *versus* 2 out of 13).

GHD	Controls (n=10)	Patients (n=10)	p value
Gender (M/F)	8/2	8/2	NA
Age (years)	54 (12.1)	55 (13.4)	0.890
Male weight (kg)	71.2 (9.54)	87.8 (11.6)	**0.007**
Female weight (kg)	58.8 (8.14)	75.0 (19.8)	0.395
Male height (cm)	175.9 (4.6)	173.8 (9.6)	0.580
Female height (cm)	166.5 (4.9)	156.5 (2.1)	0.120
Systolic BP (mmHg)	133 (14)	135 (19)	0.863
Diastolic BP (mmHg)	82 (10)	83 (12)	0.811
Hypertension (number)	0	1	NA
Diabetes (number)	0	1	NA
Smoker Y/N/Ex (number)	3/5/2	2/6/2	NA

Table 3.3 Baseline characteristics of GHD patients and their controls. Data are given as mean (SD). BP = blood pressure

Acromegaly	Controls (n=13)	Patients (n=13)	p value
Gender (M/F)	4/9	4/9	NA
Age (years)	52 (11.2)	53 (12.3)	0.980
Male weight (kg)	92.5 (18.7)	102.3 (10.6)	0.396
Female weight (kg)	69.1 (12.2)	79.2 (17.4)	0.176
Male height (cm)	178.3 (7.7)	181.5 (5.5)	0.517
Female height (cm)	165.6 (8.2)	163.8 (6.4)	0.614
Systolic BP (mmHg)	125 (17)	133 (19)	0.280
Diastolic BP (mmHg)	78 (10)	77 (12)	0.781
Hypertension (number)	2	8	NA
Diabetes (number)	1	0	NA
Smoker Y/N/Ex (number)	1/6/6	3/7/3	NA

Table 3.4 Baseline characteristics of acromegaly patients and their controls. Data are given as mean (SD). BP = blood pressure. Patient 5 is excluded as she withdrew from the study prior to Visit One.

3.4 Baseline LV characteristics

3.4.1 GHD baseline LV characteristics

Females with GHD had significantly reduced LVMi when compared to their controls (37.3 *v.* 55.4 g/m^2, p=0.027) and had an LVMi beneath the lower limit of published normal ranges (44.6 – 59.4 g/m^2)[216]. There was no significant difference in LVMi between males with GHD and their controls (58.0 v. 60.6 g/m^2, p=0.58). Both male patients and their controls had a mean LVMi within the published normal range (55.4 – 74.0 g/m^2)[216]. However, one male patient (Patient 3) had an LVMi two SD above the group mean and over upper limit of the normal range (76.0 g/m^2). This patient had diabetes and hypertension. When this patient's LVMi was removed from the analysis, the resultant mean LVMi lay right at the bottom of the normal range (55.4 g/m^2). Both control groups had mean LVMi within the normal range, as would be predicted (Figure 3.1).

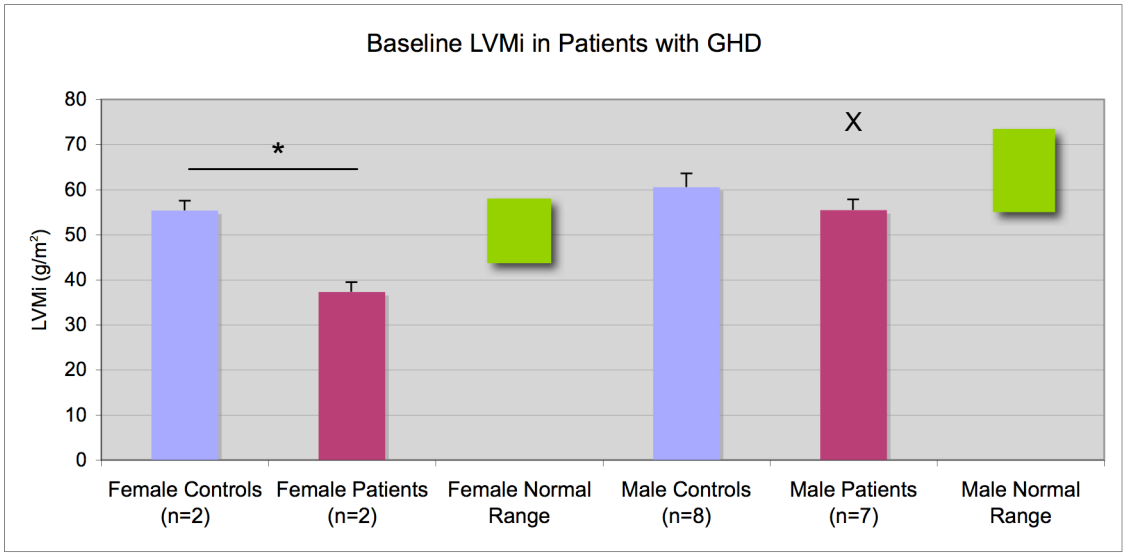

Figure 3.1 Baseline LVMi in patients with GHD and their controls. There is a significant reduction in LVMi in females with GHD (p=0.027). LVMi from Patient 20 has been excluded from analysis but is marked on the graph with "X". Data are presented as means, error bars represent SEM. Green bars indicated published normal ranges.

There were no differences in LV volumetric or functional indices between the GHD patients and their controls when all patients were analysed. However, two patients (Patients 3 and 16) had EDVi more than two SD above the group mean. When these results were excluded from the analysis, patients with GHD had significantly reduced EDVi when compared to controls (67.3 v.

76.0 ml/m^2, p=0.025). For the other indices, all other measurements were within two SD of the group mean. LV volume indices and EF results are given in Table 3.5.

GHD	Controls	GHD Patients	p value
EDVi ml/m^2	76.0 (8.84)	67.3 (5.1)	**0.0254**
ESVi ml/m^2	27.6 (7.0)	27.5 (5.2)	0.965
SVi ml/m^2	47.6 (5.8)	44.7 (3.5)	0.189
EF %	62.7 (2.7)	63.6 (6.9)	0.707
CI l/min/m^2	3.32 (0.59)	2.99 (0.55)	0.215
HR beats/min	69.8 (9.7)	66.7 (9.6)	0.482

Table 3.5 LV volumetric and functional indices for GHD patients and their controls. Values are means (SD). Two outliers are excluded from EDVi calculations.

3.4.2 *Acromegaly baseline LV characteristics*

Patients with acromegaly demonstrated increased LVMi when compared to controls, both in females (61.8 v. 40.8 g/m^2, p=0.0019) and in males (71.5 v. 56.7 g/m2, p=0.0055) (Figure 3.2).

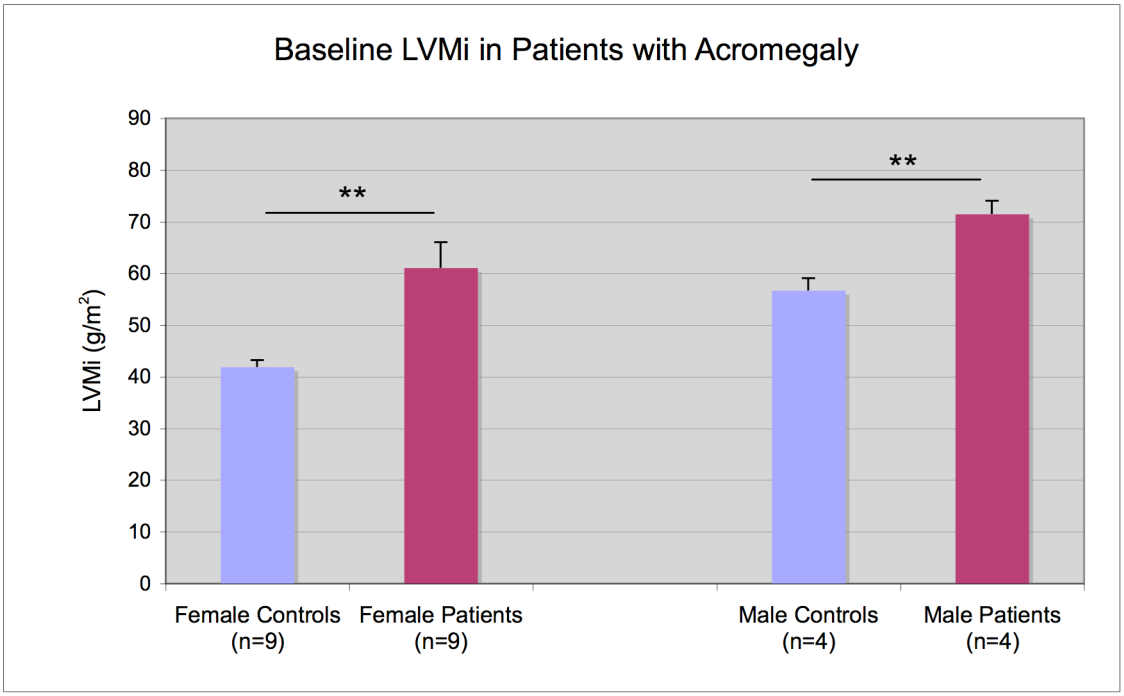

Figure 3.2 Baseline LVMi in patients with acromegaly, showing a significant increase in LVMI in both male (p=0.0055) and female (p=0.0019) patients compared to controls.

When all data were analysed, there was a borderline significant increase in LV EDVi in patients compared with controls (86.9 v. 72.9 ml/m^2, p=0.051). One female patient (Patient 4) had a particularly low EDVi, more than two SD from the mean. This patient's CMR demonstrated evidence of reversible ischaemia and she progressed to have a stent inserted into the left anterior descending coronary artery. When this patient's value was excluded from analysis there was a significant increase in LV EDVi when patients were compared to controls (90.7 v. 72.9 ml/m^2, p=0.0055).

There was no difference in ESVi between patients and controls (33.7 v. 29.8 ml/m^2, p=0.226). Once again, Patient 4 demonstrated an ESVi more than two SD from the mean. When this patients result was excluded from the analysis, there was a trend towards increased ESVi in patients when compared to controls (35.3 v. 29.8 ml/m^2, p=0.062).

When all results were included there was a significant increase in SVi between patients and controls (53.2 v. 43.2 ml/m^2, p=0.031). However, when Patient 4 was excluded, whose SVi lay more than two SD from the mean, this significance was amplified further (55.4 v. 43.2 ml/m^2, p=0.0048). Volume indices are summarised below in Figure 3.3.

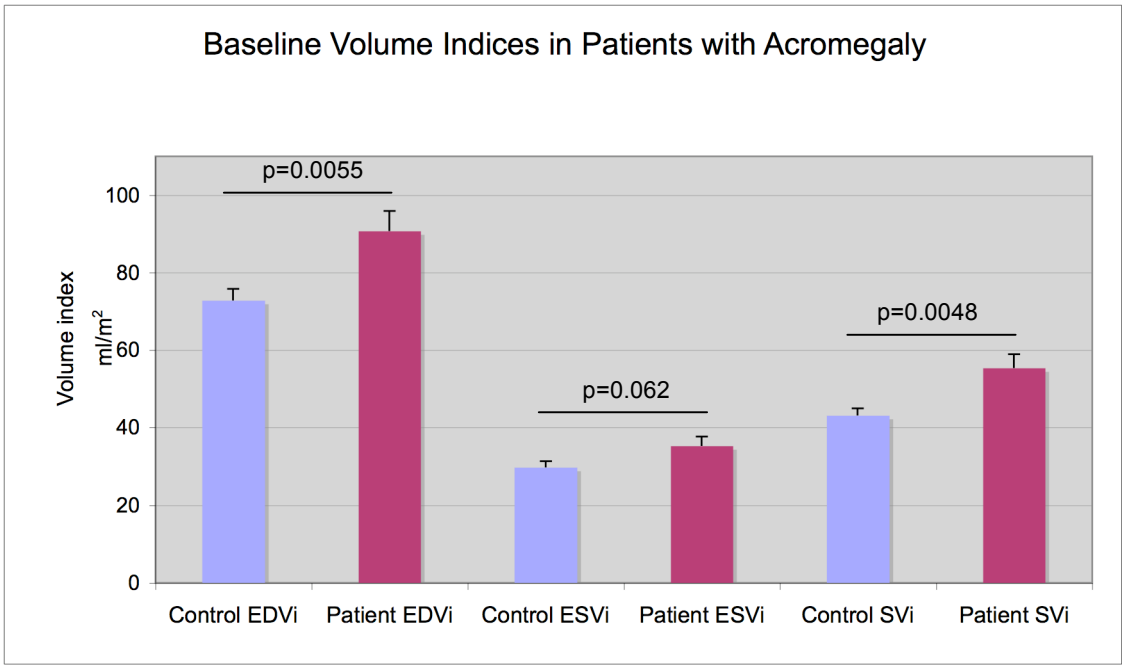

Figure 3.3 LV volume measurements in patients with acromegaly compared to their controls. There is a significant increase in EDVi and SVi and a borderline significant increase in ESVi in patients. Results from Patient 4 have been excluded as they lay two SD from the mean. Control group, n=13; Patient group, n=12.

Patients demonstrated a significantly higher CI when compared to controls (3.76 v. 2.91 l/min/m^2, p=0.020). When Patient 4 was excluded, whose CI was more than two SD from the mean, this difference became more marked (3.90 v. 2.91 l/min/m^2, p=0.0055).

There was no significant difference in EF between patients and controls (62 v. 59 %, p=0.2643) nor any significant difference in heart rate (72 v. 67 bpm, p=0.439).

3.4.3 LVMi and IGF-I SDS

In both patient groups, IGF-I levels were consistent with the underlying diagnosis: GHD mean IGF-I SDS -1.60, acromegaly mean IGF-I SDS +14.3. To ascertain whether cardiac mass was proportional to IGF-I, LVMi was plotted against IGF-I SDS (Figure 3.4 and Figure 3.5). There was no significant correlation between IGF-I SDS with LVMi in male patients with GHD (Pearson r -0.462, p=0.249). There were too few female patients with GHD to calculate a correlation. For patients with acromegaly, there was no significant correlation in females (Pearson r +0.389, p=0.301) or males (Pearson r +0.303, p=0.698).

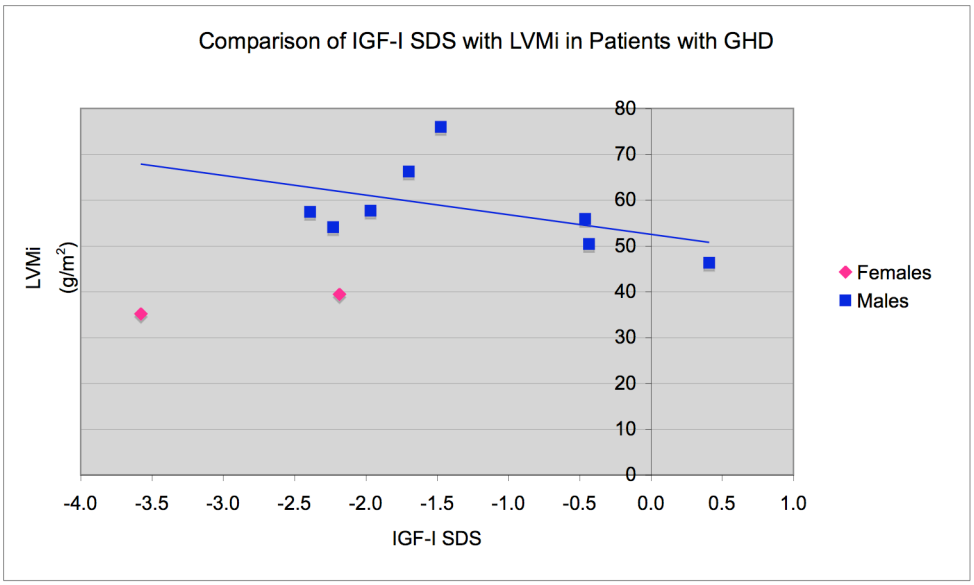

Figure 3.4 Comparison of IGF-I SDS and LVMi in patients with GHD. Line is best fit.

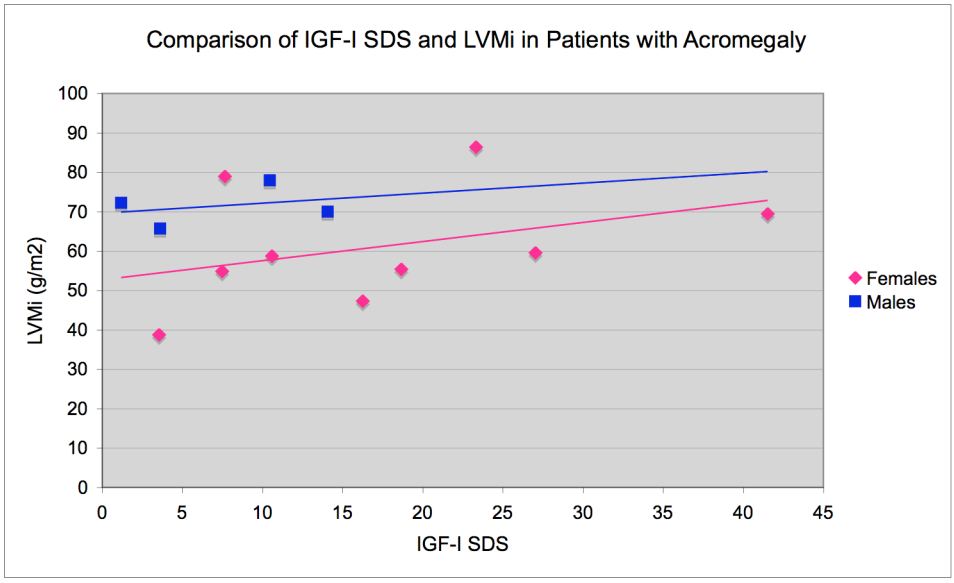

Figure 3.5 Comparison of IGF-I SDS and LVMi in patients with acromegaly. Lines are best fit.

3.5 Baseline RV characteristics

3.5.1 GHD baseline RV characteristics

There was no difference in RV EDVi, ESVi, SVi or EF between GHD patients and their controls when all data were analysed. Two male patients had EDVi more than two SD above the group mean (Patients 3 and 16). When these results were excluded from the analysis, patients with GHD had lower EDVi than controls (67.8 v. 78.9 ml/m^2, p=0.034). Although Patient 16 had a mean ESVi more than two SD from the group mean, excluding this result did not alter significance in this parameter. Patient 3 had a SVi more than two SD from the group mean. When this result was removed from the analysis, patients with GHD had SVi that was significantly lower than controls (44.1 v. 49.7 ml/m^2, p=0.045). RV mass was not assessed as this measurement has poor reproducibility on CMR. Results are summarised below in Figure 3.6.

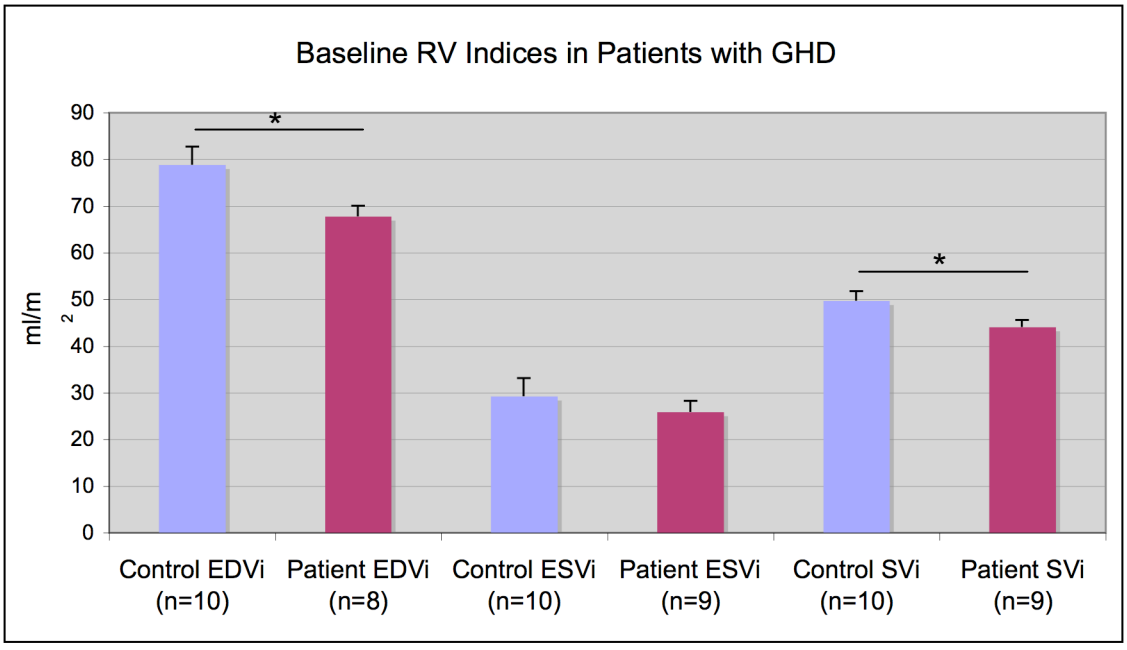

Figure 3.6 Baseline RV volume indices in patients with GHD. Patients with GHD had significantly lower EDVi (p=0.034) and SVi (p=0.045) than controls. Outliers >2 SD from the mean have been excluded.

3.5.2 *Acromegaly baseline RV characteristics*

Patients with acromegaly had no difference in RV EDVi, ESVi and EF when compared to controls when all results were analysed. One patient (Patient 4) had an EDVi more than two SD below the mean. When this result was excluded from the analysis, a trend towards increased EDVi was seen in patients with acromegaly (88.9 v. 76.5 ml/m^2, p=0.101). All patients had an ESVi and EF within two SD of the mean. Patients with acromegaly showed a significant increase in SVi when compared to controls (51.1 v. 43.5 ml/m^2, p=0.031). Patient 4 had an SVi more than two SD beneath the group mean. However, excluding this result did not alter significance (53.2 v. 43.5 ml/m^2, p=0.029). RV mass was not assessed as this measurement has poor reproducibility on CMR. Results are summarised below in Figure 3.7.

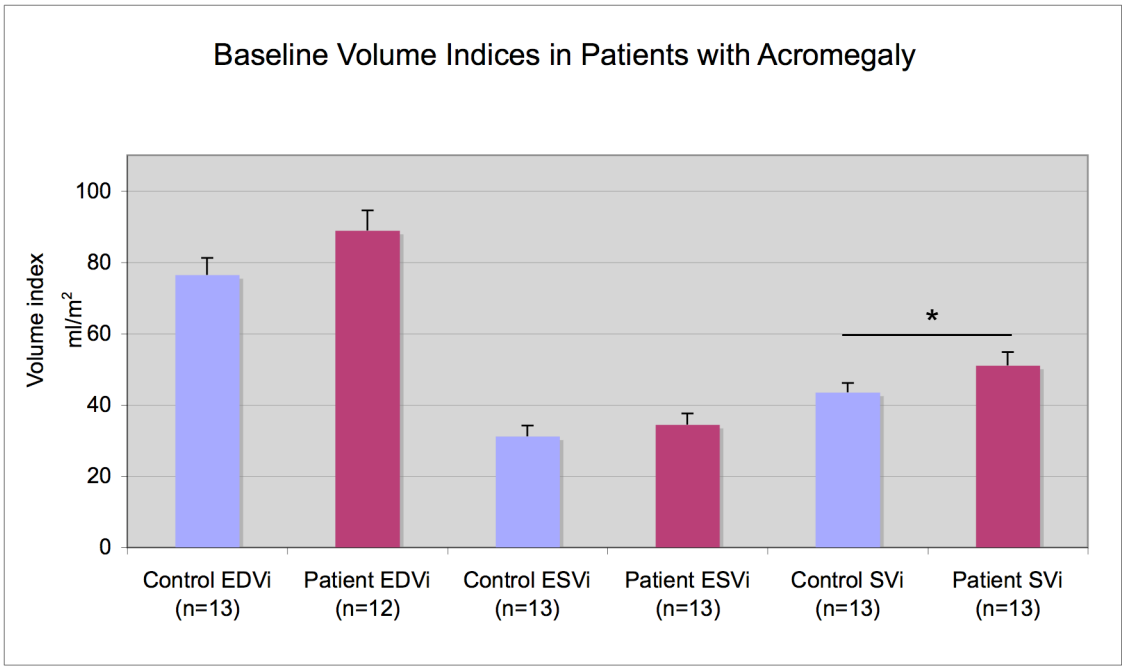

Figure 3.7 Baseline RV volume indices in patients with acromegaly. Patients with acromegaly had a trend towards increased EDVi (p=0.101) and had significantly increased SVi (p=0.031) compared to controls. Patient 4 has been excluded from the EDVi analysis as her result were more than two SD from the mean.

3.6 IGF-I changes with treatment

3.6.1 GHD IGF-I changes

By Visit Three, patients with GHD demonstrated a significant increase in IGF-I SDS with one year of GH treatment (paired T test, p=0.0032). At baseline mean IGF-I SDS was -1.60 and at one year +0.61. One patient (Patient 16) had an SDS less than -2.73 at Visit Three. This corresponded to a serum IGF-I of 47 ng/ml, a figure significantly lower than any other result, including the patient's own baseline IGF-I. This result was thought to arise from sample error and was excluded from calculations, though changes in IGF-I SDS remained significant even when it was included (p=0.037). Individual patient data are shown in Table 3.6 and Figure 3.7.

Pt	Age	Sex	Final GH dose (mg/day)	Initial IGF-I SDS	Final IGF-I SDS
1	73	M	0.30	- 1.70	0.40
3	49	M	0.30	- 1.47	0.06
14	56	M	0.50	- 2.23	3.02
16	51	M	0.35	- 0.46	- 0.01
17	50	F	0.60	- 3.58	- 1.83
18	58	M	0.20	- 0.43	0.86
19	34	F	0.40	- 2.19	- 1.46
20	76	M	0.50	- 2.39	- 0.27
21	57	M	0.20	0.41	1.44
22	37	M	0.30	- 1.97	1.88

Table 3.6 Treatment of patients with GHD. Where patients dropped out before the end of the study, IGF-I level at Visit 2 is given. IGF-I values have been converted into SDS to allow for comparison of different age and sex patients.

The diagnosis of GHD is made based on the failure of the patient to mount a GH response to an appropriate stimulus, such as hypoglycaemia. IGF-I cannot be used to make the diagnosis as there is marked crossover in the results between GH deficient and GH replete individuals. This is seen here, where only four individuals with GHD had baseline an IGF-I SDS of less than two. However, IGF-I levels are used to monitor response to treatment and an IGF-I in the upper third

of the age- and sex-matched reference range in usually aimed for. Patient 17 was taking oral oestrogen therapy, explaining her relatively low IGF-I SDS of -1.83 with a reasonable dose of GH. It should also be noted that her baseline IGF-I SDS was particularly low at -3.58. One patient was over treated with GH and had an SDS of 3.02. This result was reviewed by the referring clinician and the GH dose was reduced.

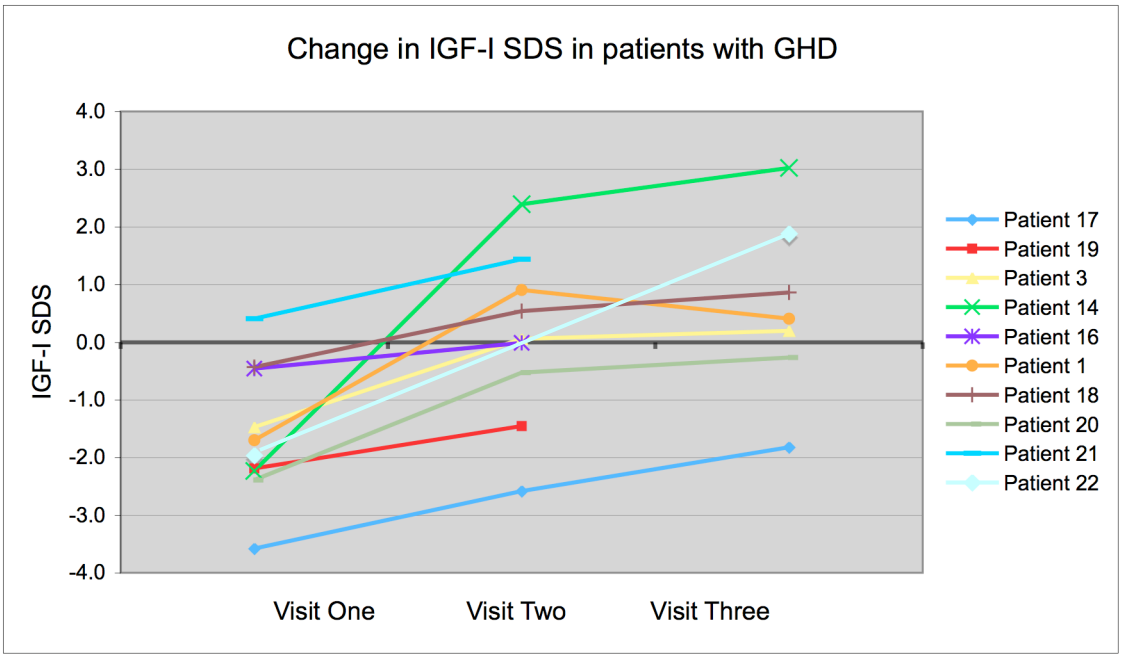

Figure 3.7 Change in IGF-I SDS in patients with GHD treated with GH over 1 year. Result from Patient 16 at Visit Three is excluded.

3.6.2 Acromegaly IGF-I changes

Patient with acromegaly had the converse response in IGF-I SDS, with a significant fall at one year (paired T test, p=0.036). At baseline mean IGF-I SDS was +14.3, SD 11.3, and at one year +3.02, SD 2.41. Individual patient data are shown in Table 3.7 and Figure 3.8. Only one patient achieved complete disease remission at one year (Patient 7, IGF-I SDS -1.93, treated with transsphenoidal surgery). Patient 11 achieved disease remission at 6 months but did not return for the one year scan (IGF-I SDS -2.04). It is possible that this patient may have had disease recurrence by one year. All patients achieved a marked improvement in SDS when compared to baseline with the exception of Patients 10 and 13, both of whom had mild disease at baseline.

Pt	Treatment prior to Visit 1	Treatment during Study (started after Visit 1)	Treatment after Visit 3	Initial IGF-I	Final IGF-I
2		Sandostatin – withdrew from study		16.2	
4	6/12 Lanreotide	Lanreotide –TSS delayed for medical reasons	TSS	7.46	4.46
5	TSS	Pegvisomant – withdrew from study			
6		TSS	Somatostatin analogue	27.0	6.53
7		TSS		18.7	- 1.93
8	TSS 6/12 earlier, Lanreotide for 6/12 prior to TSS	Lanreotide – withdrew from study		7.63	
9	6/12 Lanreotide	TSS		10.5	2.12
10	TSS 1986, intolerant of cabergoline	Lanreotide		3.60	3.36
11	TSS 1999	Lanreotide		3.55	- 2.04
12		TSS		23.3	
13		Cabergoline for 6/12 then stopped		1.19	2.05
15	Octreotide	TSS, followed by Lanreotide, Pegvisomant added several months later		14.1	3.59
23		Lanreotide for 6/12 followed by TSS, restarted lanreotide after TSS		41.5	2.61
24		TSS	Somatostatin analogue	10.6	3.53

Table 3.7 Treatment of acromegaly before, during and after study. 6/12 = 6 months, IGF-I levels are given as SDS. Patient 9 declined blood tests at Visit Three so result from Visit Two was used. Where patients dropped out the IGF-I SDS result is left blank.

Patients received a range of treatments, as shown above. These treatments were determined by referring clinicians and were based on clinical disease, clinician and patient choice and local guidelines. As this was an observational study, no attempt was made to influence the treatments given. Three patients were receiving somatostatin analogues when they attended for CMR1 (Patients 4, 9 and 15). Patient 8 had previously received somatostatin analogues but was not taking them at the time of CMR1. Seven patients took somatostatin analogues through the study, two of whom also had transsphendoidal surgery (TSS) during the study. Five patients had TSS alone during the study. One patient was treated with pegvisomant (Patient 15), in combination with other therapies. The other patient due to start pegvisomant dropped out before CMR1 (Patient 5).

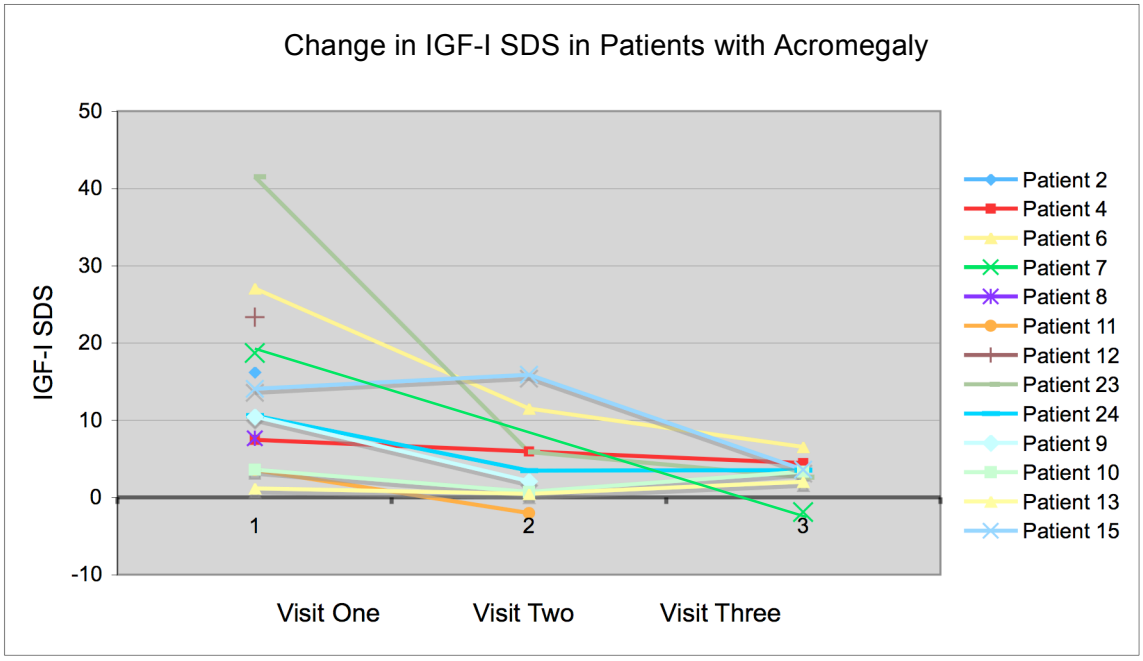

Figure 3.8 Changes in IGF-I SDS in patients with acromegaly before and at one year after treatment.

3.7 LV changes with treatment

3.7.1 GHD LV changes

Male patients with GHD showed a strong trend towards increased LVMi with one year of GH treatment (repeated measures ANOVA, p=0.052). There was no difference in LVMi between patients after one year of GH treatment and the control group (62.9 v. 60.6 g/m^2, p=0.603). Patients 3 and 16 continued to have LVMi two SD above the group mean. Removing these data from the analysis, did not improve significance.

There were only two females in the GHD group, one of whom withdrew after the second scan. This limited conclusions that could be drawn as paired analyses require at least three pairs. However, when unpaired T test was performed, no difference was seen between female patients at baseline and after six months of GH treatment (37.3 v. 37.9 g/m^2, p=0.8716). Furthermore, after six months of GH treatment, the significant difference in LVMi between patients and controls persisted (37.9 v. 55.4 g/m^2, p= 0.030). Individual LVMi changes over time are shown in Figure 3.9.

When numbers were increased by analysing males and females together, there was a significant increase in LVMi with one year of GH treatment (repeated measures ANOVA, p=0.023).

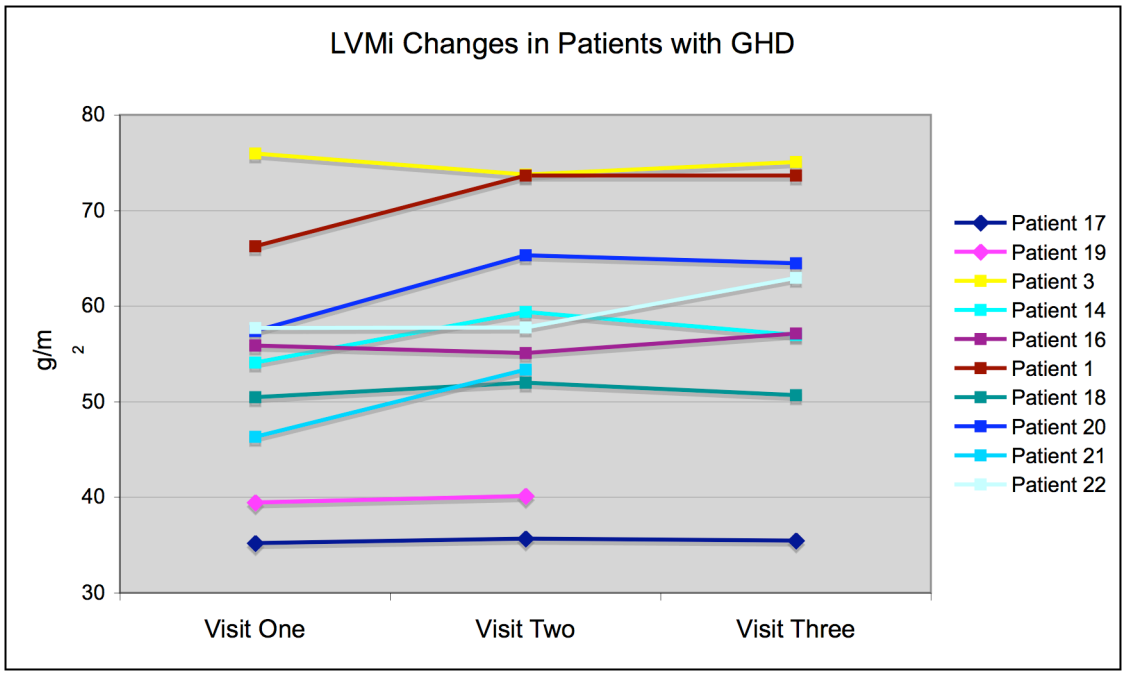

Figure 3.9 Individual changes in LVMi in patients with GHD treated with GH for one year. Females are shown with diamonds and males with squares.

Male patients with GHD moved from a mean LVMi right at the bottom of published reference ranges at Visit One (CMR1) to a mean LVMi well within the reference range by Visit Three (CMR3) (55.4 v. 59.5 v. 61.0 g/m^2, reference range 55.4 – 74.0 g/m^2). Patient 3 was excluded from this analysis as his LVMi was over two SD above the groups mean at CMR1. All LVMi values for females remained beneath the lower limit of the reference range.

There were no significant changes in LV EDVi, ESVi, SVi, CI, EF or heart rate in GHD patients treated with GH for one year. No difference was found in these parameters when CMR3 was compared to control values. No values were more than two SD outside the group mean at CMR2 and CMR3.

Only patient demonstrated evidence of reduced systolic function, as determined by LV EF. Patient 10 had an LV EF right at the bottom of the normal range on CMR1 (57%, normal range 54-68%)[220]. By CMR2, EF had fallen beneath the bottom of the normal range and was sitting at two SD from the mean (CMR2 44%, CMR3 51%). All other EF measurements were within two SD of the group mean. Patient 15 had an EF above the upper limit of the normal range on CMR1 (71%) but by CMR2 and 3 was within normal limits (both 67%).

3.7.2 *Acromegaly LV changes*

There were no significant changes in LVMi with one year of treatment of acromegaly in females (paired ANOVA p=0.1636) or males (paired ANOVA p=0.4375). Female patients continued to demonstrate a significantly increased LVMi when compared to controls (55.9 v. 41.9 g/m^2, p<0.001). For male patients, the significant increase in LVMi was lost and only a trend towards increased LVMi was seen (66.6 v. 56.7 g/m^2, p=0.1121). Mean data are shown in Figure 3.10. Individual data are shown in Figure 3.11.

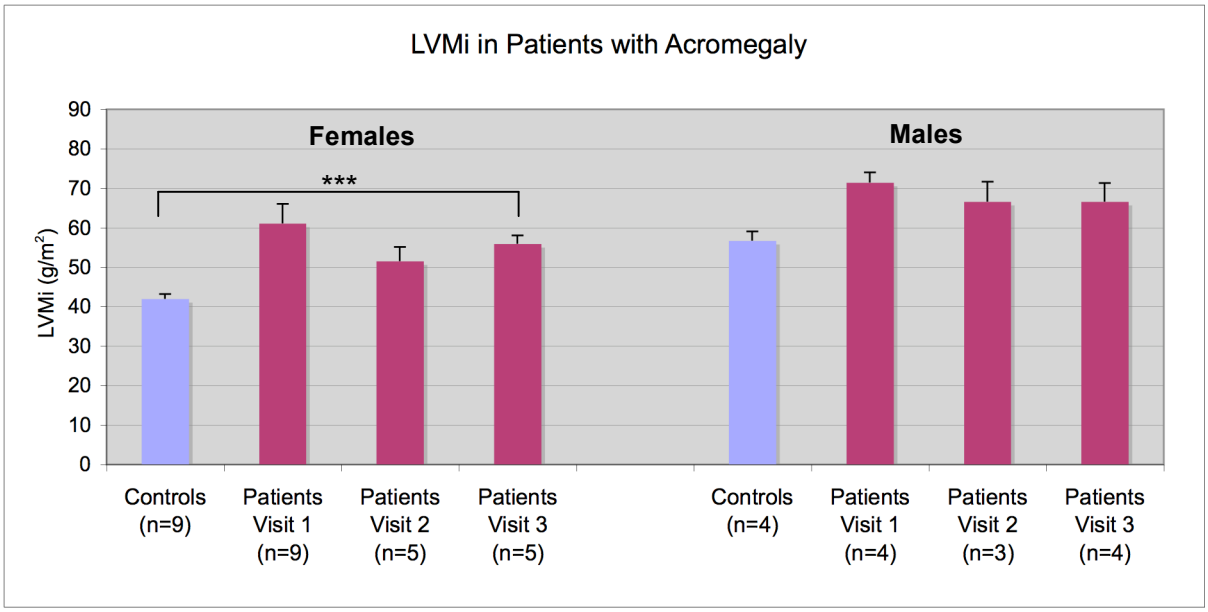

Figure 3.10 Changes in LVMi in patients with acromegaly over one year. There is no significant difference in LVMi over time. A significant difference in LVMi between patients and controls persists at one year in females (p<0.001) but only a trend towards increased LVMi persists in males (p=0.1121).

Patients with acromegaly did not demonstrate any difference in EDVi, ESVi, SVi, CI or EF when data from each visit were compared with paired ANOVA. All results lay within two SD of the group mean. The difference, at baseline, between patients and controls in EDVi was lost by CMR3 (78.3 v. 72.9, p=0.478) but the difference in CI persisted (3.51 v. 2.91 ml/min/m^2, p=0.027). Heart rate did not change significantly over the year (paired ANOVA p=0.1934).

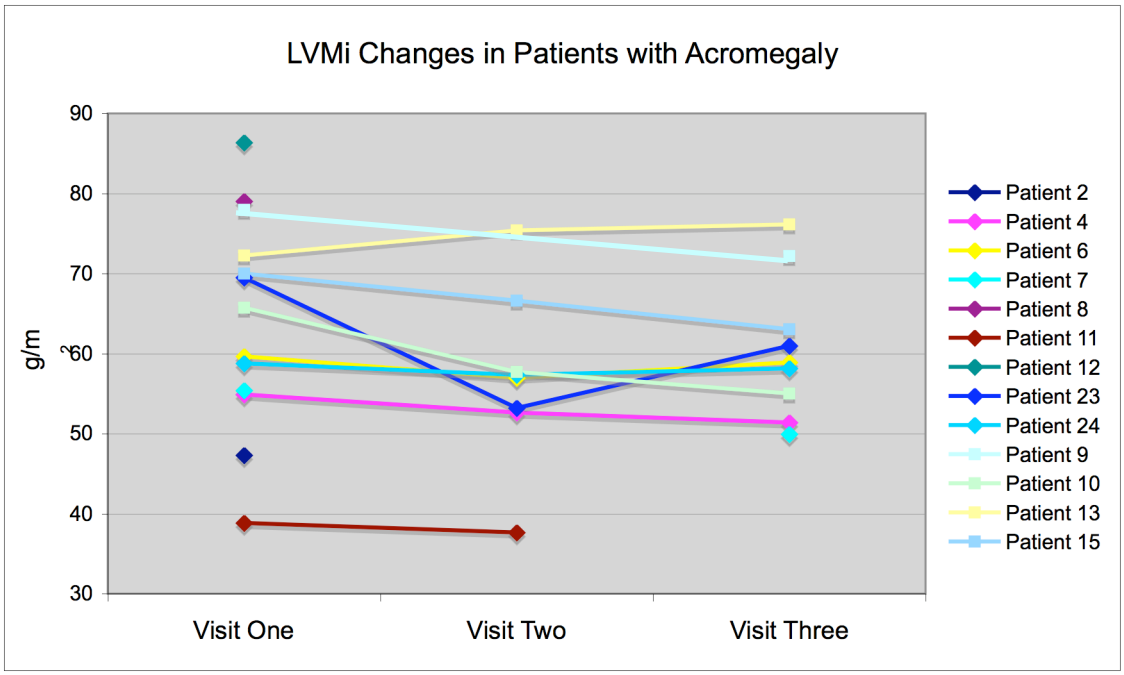

Figure 3.11 Individual changes in LVMi in patients with Acromegaly. Females are shown with diamonds and males with squares.

3.8 RV changes with treatment

3.8.1 GHD RV changes

There were no significant changes in RV volume indices in patients with GHD treated with one year of GH replacement. When patients previously excluded from baseline analysis (Patients 3 and 16) were excluded, changes remained insignificant, although a trend towards increased EDVi was seen (70.6 v. 77.6 v. 76.1 ml/m^2, p=0.068). There were no differences between results from CMR3 and the control group and the difference in EDVi seen between patients and controls at baseline was lost by one year (Table 3.8).

	CMR over three visits	CMR 3 v. control
EDVi ml/m^2	0.068	0.543
ESVi ml/m^2	0.146	0.833
SVi ml/m^2	0.774	0.665

Table 3.8 Change in CMR volume indices in patients with GHD over three visits (p value, repeated measures ANOVA) and comparison of volume indices between CMR 3 and controls (p value, T test). Patients 3 and 16 are excluded from EDVi analysis.

3.8.2 Acromegaly RV changes

When all results were analysed there were no significant changes in RV volume indices or EF after one year of treatment. When Patient 4 was excluded from EDVi and SVi analyses, as per the baseline analysis, there was a trend towards increased EDVi (92.5 v. 76.5 ml/m^2, p=0.060) and a significant increase in SVi (54.7 v. 43.5 ml/m^2, p=0.040) in patients at CMR3 when compared to controls. Results are given in Table 3.9.

	CMR over three visits	CMR 3 v. control
EDVi ml/m^2	0.919	0.060
ESVi ml/m^2	0.926	0.599
SVi ml/m^2	0.973	**0.040**

Table 3.9 Change in CMR volume indices in patients with acromegaly over three visits (p value, repeated measures ANOVA) and comparison of volume indices between CMR 3 and controls (p value, T test). Patient 4 is excluded from EDVi and SVi analyses.

3.9 Inter-observer variability

Thirty-four CMR scans were assessed independently by two observers blinded to diagnosis. LV mass, EDV, ESV, and EF were plotted on Bland-Altman plots (Figure 3.12, Figure 3.13, Figure 3.14 and Figure 3.15).

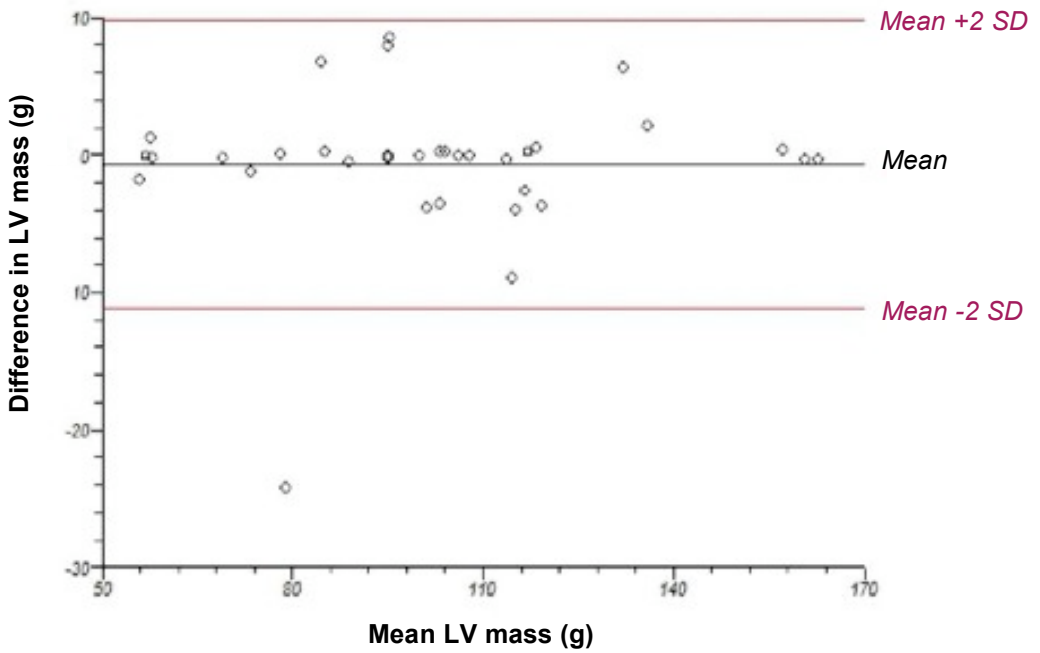

Figure 3.12 Inter-observer variability in measurement of LV mass. Mean difference is close to zero (-0.6 g). One result lay >4 SD from the mean difference. No trend is present. *Courtesy of A. Dattani.*

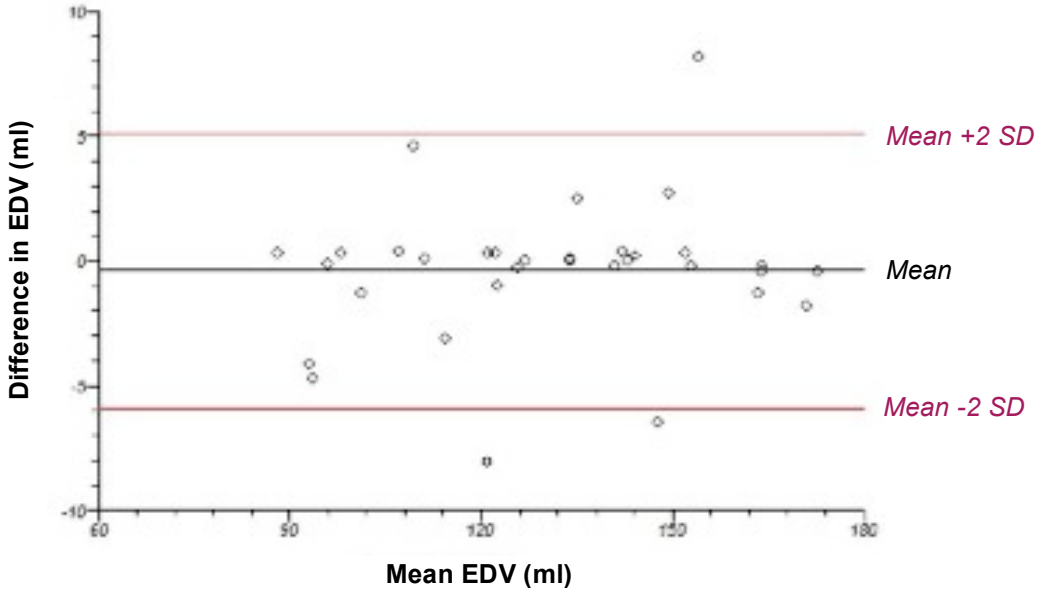

Figure 3.13 Inter-observer variability in measurement of LV EDV. Mean difference is close to zero (-0.4 ml). Three results lay slightly more than 2 SD from the mean difference. No trend is present. *Courtesy of A. Dattani.*

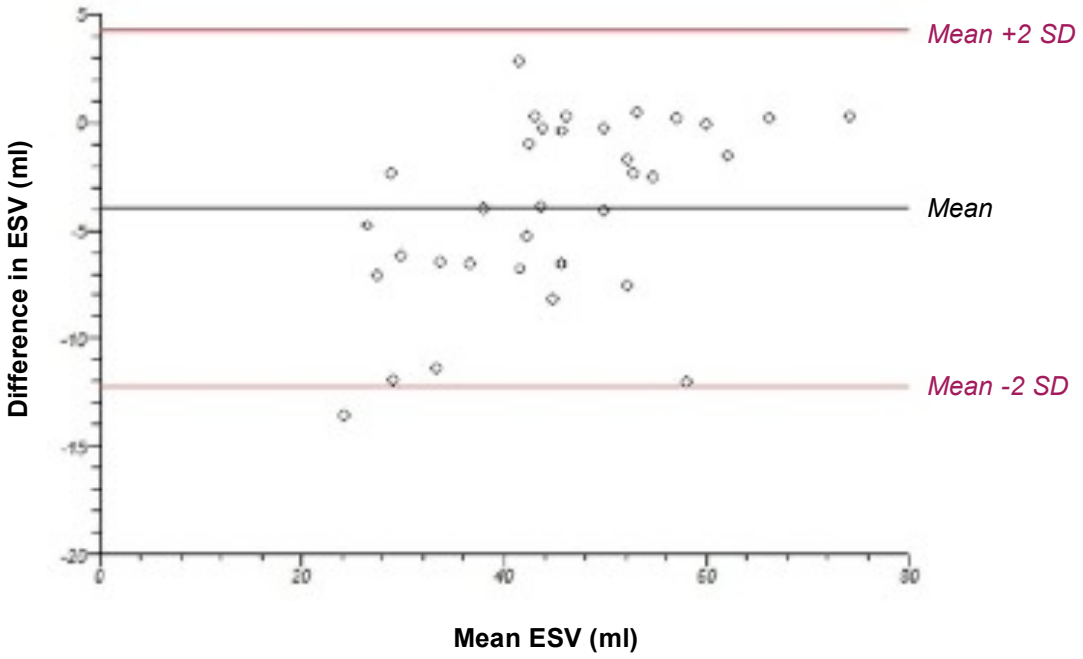

Figure 3.14 Inter-observer variability in ESV measurements. Mean difference is -4.0 ml. One result lay outside 2 SD. No trend is present. *Courtesy of A. Dattani*

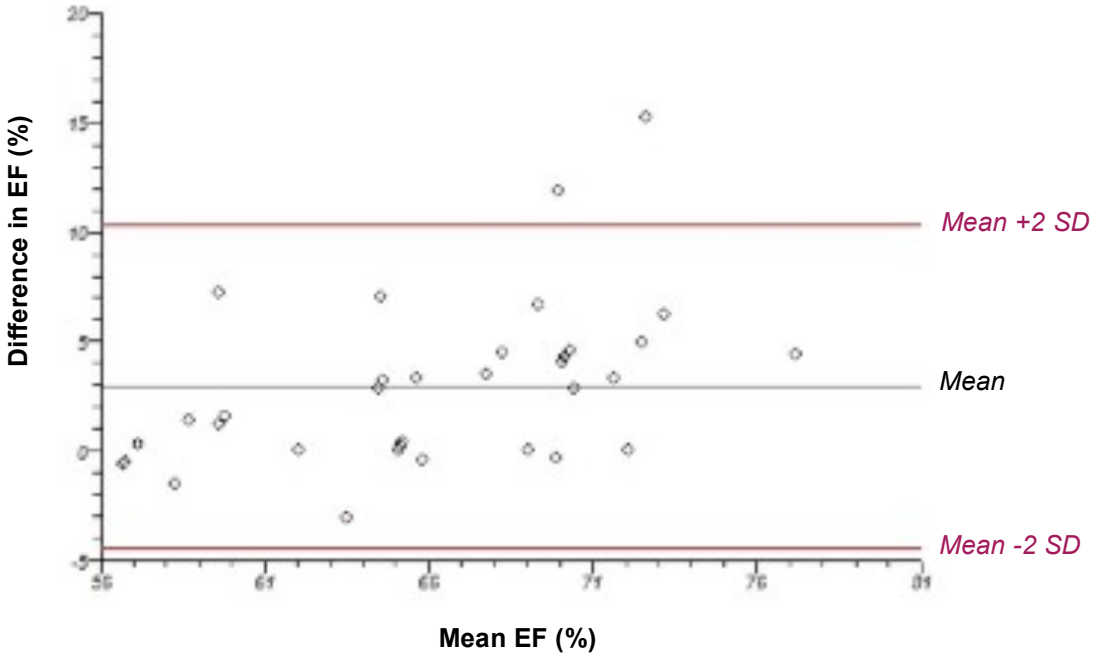

Figure 3.15 Inter-observer variability in EF measurements. Mean difference is 2.9%. Two scans are outside 2 SD. No trend is present. *Courtesy of A. Dattani*

3.10 Late gadolinium enhancement

All images were assessed for evidence of LGE that might be consistent with fibrosis. Two patients with GHD (Patients 1 and 14) and two patients with acromegaly (Patients 10 and 24) demonstrated evidence of LGE. Of the GHD patients, Patient 1 had the most extensive area of LGE, measuring 20.1 cm^3 at baseline. This area was transmural in nature, matched to an area of very mildly reduced perfusion but not associated with reduced wall motion abnormality. Patient 14 had very mild patchy basal and mid-inferolateral LGE on all three scans that was too little to quantify. Of the acromegaly patients, Patient 10 did not have any LGE seen at baseline but had patchy LGE in the inferolateral wall seen on both CMR2 and CMR3. Patient 24 had mild, patchy LGE in the mild inferior wall extending <25% transmurally. Results are summarised in Figure 3.16.

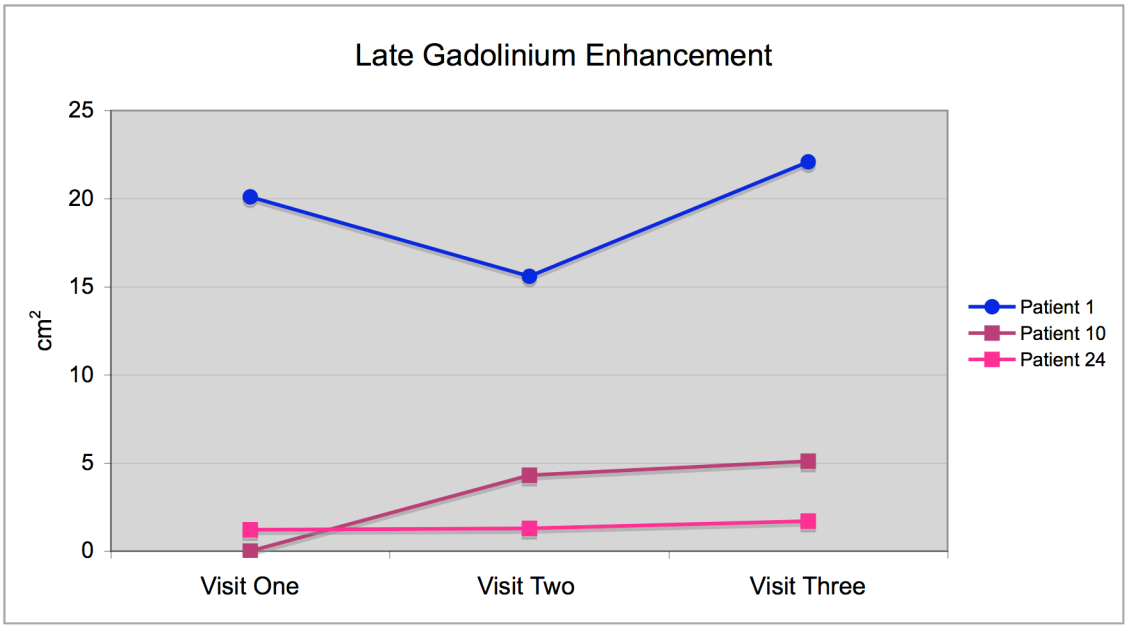

Figure 3.16 Late gadolinium enhancement demonstrated on study patient CMR scans. Squares represent patients with acromegaly, circle represents GHD. Patient 14 data are excluded as the cardiologist felt the LGE was too patchy to quantify

3.11 Perfusion CMR

Five patients had evidence of mycordial perfusion deficits when infused with adenosine. When scans were re-reviewed by a consultant cardiologist, three were thought to show mild, insignificant findings. On further questioning, all three patients were completely free from cardiac symptoms both at rest and on exertion, and thus no further investigations were performed. The patients were counselled to seek medical advice early in the event of the development of any cardiac-sounding symptoms. Results are summarised in Table 3.10.

Two patients (Patients 4 and 14) demonstrated more marked perfusion abnormalities. These patients each underwent a CT angiogram and clinical review by a consultant cardiologist. Patient 14 demonstrated no flow-limiting lesion on CT angiography. He was advised to follow a primary prevention strategy in the form of aspirin and a statin. Patient 4 demonstrated a 50% stenosis of the left anterior descending coronary artery (LAD). She underwent percutaneous stenting of this lesion.

Pt	*Group*	*Perfusion CMR finding*	*Outcome*
1	GHD	Mild perfusion defects matched to an area of late gadolinium enhancement	Changes mild and lay in an area of possible previous infarct. No further investigation needed.
4	Acro	Basal anteroseptal and inferoseptal perfusion defect	CT angiogram followed by percutaneous stenting of the LAD.
7	Acro	Perfusion defects in the basal to mid-inferoseptal wall and mid inferior wall.	Changes patchy, patient asymptomatic. No further investigation needed.
14	GHD	Persistence of a perfusion induced defect in the mid-inferoseptal segment	CT angiogram and cardiologist review. Primary prevention only.
20	GHD	Mild perfusion defect in the basal infero-septum	Changes patchy, patient asymptomatic. No further investigation needed.

Table 3.10 Patients (Pt) with abnormal perfusion following the induction of myocardial hyperaemia with adenosine. Acro = acromegaly.

Of the five patients who demonstrated perfusion abnormalities, only Patient 4, who required a coronary artery stent, was a current smoker. Two patients were ex-smokers but both of these stopped smoking approximately 30 years ago. Out of these five, only one patient had treated hypertension and no patient had diabetes. No patient was receiving an HMG-CoA reductase

inhibitor. The mean total cholesterol in these five patients was 5.24 mmol/l (Table 3.11). Numbers in this group were too small to draw statistically significant conclusions. However, rates of smoking, diabetes, hypertension and hypercholesterolaemia in these five patients did not differ noticeably from the rest of the patients.

Patient	*Group*	*Smoker*	*Diabetes*	*Hypertension*	*Statin*	*Cholesterol*
1	GHD	Ex (30 years)	N	N	N	4.5
4	Acro	Yes	N	N	N	5.7
7	Acro	No	N	Y	N	5.6
14	GHD	Ex (30 years)	N	N	N	6.1*
20	GHD	No	N	N	N	4.3

Table 3.11 Cardiac risk factors in patients with abnormal perfusion following the induction of myocardial hyperaemia with adenosine. Acro = acromegaly, Y = yes, N = No, Statin = HMG CoA-reductase inhibitor. Hypertension, diabetes and hypercholesterolaemia refer to known already diagnosed disease. Total cholesterol levels represent mean values over study in mmol/l, within the exception of * which indicates total cholesterol at Visit One, after which an HMG CoA-reductase inhibitor was started that lowered total cholesterol levels markedly.

All patients received adenosine on at least one of their scans except for Patient 8, who declined adenosine at Visit One and then dropped out of the study. Both patients with acromegaly and with GHD demonstrated adenosine-induced perfusion abnormalities.

3.12 Aortic area

Patients with GHD demonstrated reduced body surface area (BSA)-indexed ascending aorta area when compared to controls (14.4 v. 18.3 cm^2/m^2, p=0.039). One patient with GHD had a bicuspid aortic valve, with consequential aortic root enlargement (Patient 21). This finding was felt to be incidental and not related to GHD. When his result was removed from the analysis, significance increased further (p=0.002). There was no increase in BSA-indexed ascending aorta area with one year of GH therapy (ANOVA, p=0.357). Results are shown in Figure 3.17.

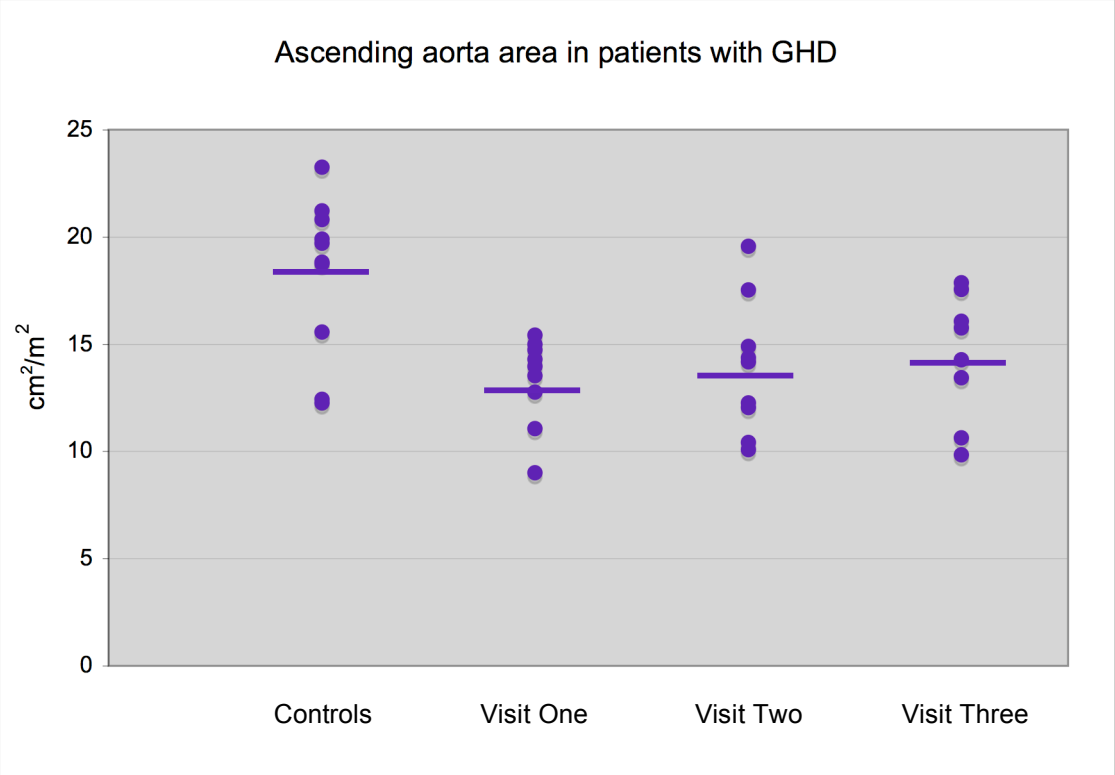

Figure 3.17 BSA-indexed ascending aorta area in patients with GHD. Patients have significantly lower aortic area than controls. There is no change in aortic area with one year of GH therapy. Results from Patient 21 are excluded.

There was no significant difference in BSA-indexed aortic area when acromegaly patients were compared to controls (p=0.982). There was no change in aortic area with one year of treatment for acromegaly.

3.13 Quality of life

3.13.1 Quality of life in GHD

Patients with GHD demonstrated a significant improvement in AGHDA score with one year of GH treatment (paired ANOVA p=0.0025). NICE guidelines advise that adults with GHD should score at least 11/25 on the AGHDA questionnaire prior to commencement of GH and that over the first nine months of treatment, patients' AGHDA score should reduce by at least seven points. All patients, except one (Patient 18, baseline AGHDA 10/25), started GH treatment with a baseline AGHDA of ≥ 11/25. Four patients achieved an improvement in AGHDA score of at least seven AGHDA points (Patients 16, 17, 20 and 22). Only one patient (Patient 14) had a rise in AGHDA score at twelve months, compared to six months. However, AGHDA score in this patient remained lower at 12 months that it was at baseline. See Figure 3.19.

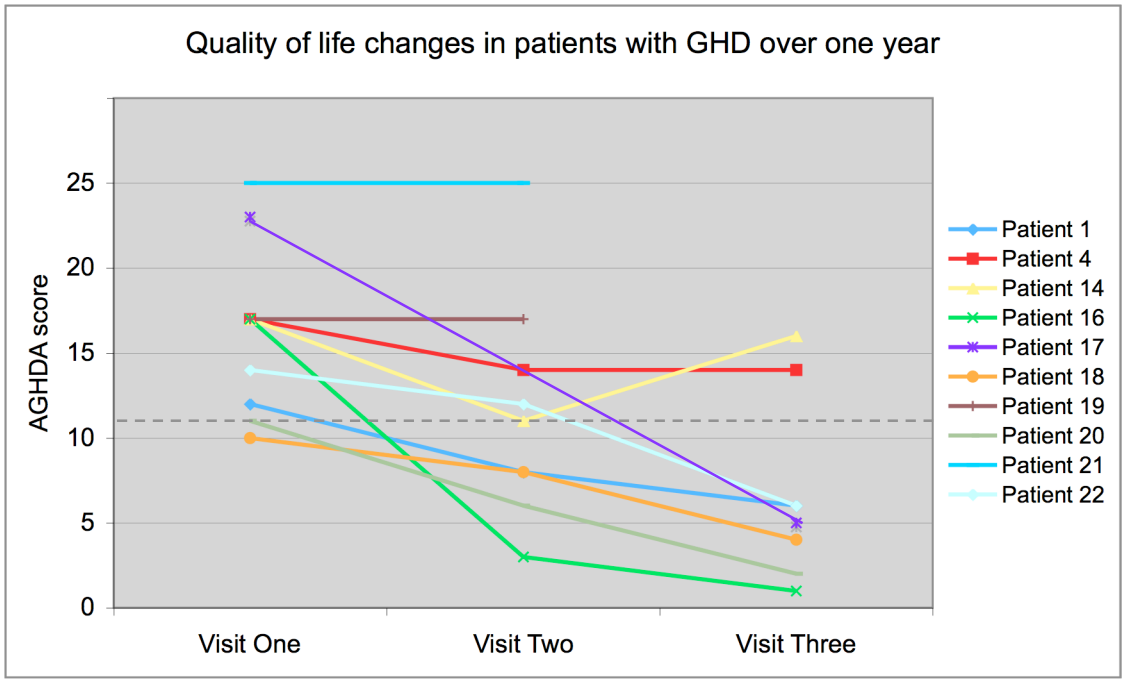

Figure 3.19 Quality of life changes over one year in patients with GHD treated with GH. Quality of life was measured using the disease-specific AGHDA questionnaire. The higher the score, the worse the quality of life, with 25 representing the highest score possible. An AGHDA score above 11 (dotted line) is judged by the NICE guidelines to represent impaired quality of life because of GHD.

3.13.2 Quality of Life in Acromegaly

Patients with acromegaly did not demonstrate a significant change in ACROQoL score by one year (paired ANOVA p=0.1064). The spread of ACROQoL scores was very broad, ranging from 7 to 81 out of 100 at Visit Three. Three patients had an improvement in ACROQoL score after 6 months treatment (Patients 10, 13 and 24), two patients had an improvement in ACROQoL score after one year (Patients 6 and 7). See figure Figure 3.20.

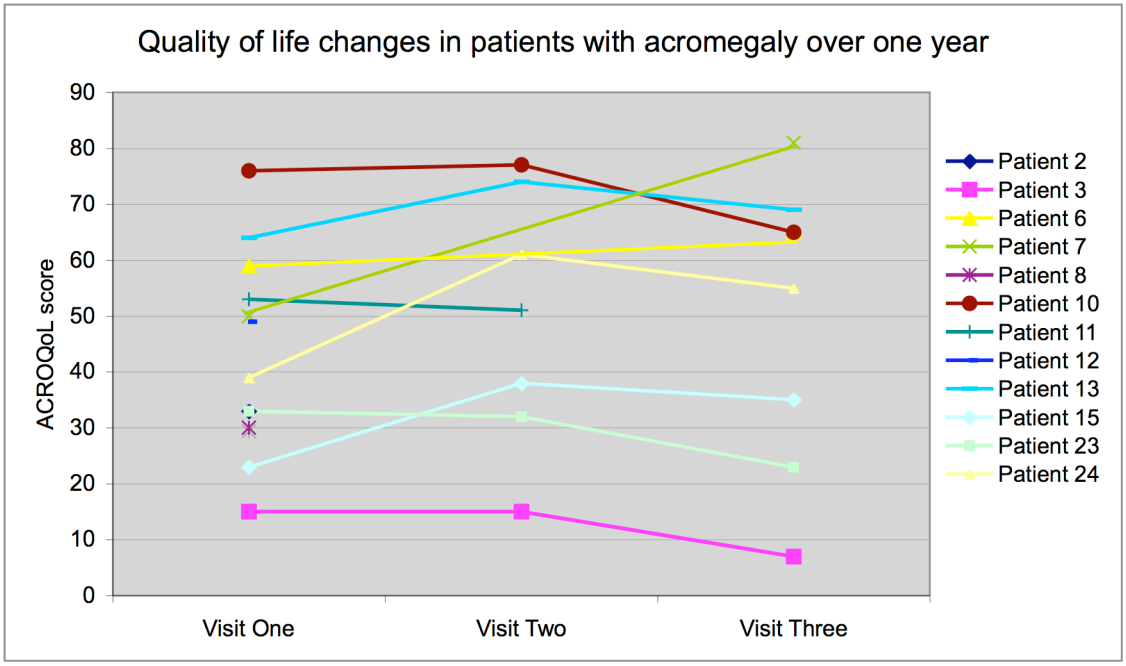

Figure 3.20 Quality of life changes over one year in patients with acromegaly. Quality of life was measured using the disease-specific ACROQoL questionnaire. The lower the score, the worse the quality of life, with 100 representing the highest score possible. Patient 9 was excluded as he spoke limited English and was unable to complete the questionnaire independently.

4 Discussion

4.1 Background

The cardiac complications of GHD and acromegaly remain poorly understood[23,73]. The multi-system impact of both these diseases complicates investigation; hypertension, diabetes, altered lipid profiles, changes in circulating volume and vascular changes may all contribute to cardiac changes; the relative rarity of acromegaly and ao-GHD makes large-scale studies difficult.

Due to these complexities, a form of cardiac assessment that provides highly detailed information about both heart structure and function is needed. CMR provides a more accurate assessment of cardiac mass and EF than echo or radionuclide ventography, with highly reproducible measurements, and also contributes information regarding reversible ischaemia and fibrosis[221,222]. It is, therefore, ideally suited to the assessment of GH-related heart disease. In order to gather all these data using other methods of investigation, a patient would need to attend a range of different cardiological tests, whereas CMR provides this information in a single set of scans. Additionally, CMR does not involve exposure to ionising radiation and is well tolerated.

CMR has its limitations: reproducibility of RV mass measurements and assessment of diastolic dysfunction are limited[223]. Caution is also needed in the assessment of fibrosis. CMR identifies fibrosis by comparing differences in gadolinium-based contrast uptake in healthy and fibrosed tissue and if diffuse fibrosis exists there is a possibility that it may be under reported[224].

To date, there are few CMR studies reviewing the impact of GH deficiency and excess on the heart. There is one study, published in 2011 just as our study was being completed, which used CMR to assess patients with ao-GHD (n=16)[128]. This study found patients had reduced LV EDVi (p=0.032) and LV ESVi (p=0.038) when compared to controls but no significant difference in LVMi or EF. After one year of GH replacement there were no significant changes in volume, mass or function indices, despite a good IGF-I response to GH replacement. CMR has also been used to track cardiac changes in patients with dilated cardiomyopathy treated with GH[129].

One study performed CMR on 14 patients with untreated acromegaly[130]. This found a greater prevalence of cardiac hypertrophy (LVMi) when measured by CMR (72%) when compared to

echo (36%) and noted the absence of cardiac fibrosis, previously considered to be a key feature of acromegalic cardiomyopathy[86]. A second study used T_2-weighted imaging to assess cardiac oedema before and 8-10 days after treatment for acromegaly. An improvement was demonstrated in this parameter but the study only addressed this very narrow time window and made no effort to correlate cardiac findings with true extent of acromegaly or its cure[131]. A third study compared eight patients with acromegaly to matched controls[132]. Patients were found to have increased LVMi and ESVi compared to controls at baseline. After three months acromegaly treatment, patients had a significant increase in EDVi but no change in any other cardiac parameters. All patients were treated with medical therapies, some in addition to surgery, and six out of eight received somatostatin analogues. The size of this study, short duration, failure to normalise IGF-I levels in patients make interpretation of these results difficult. Additionally, there is some evidence that somatostain analogues may also have an impact on cardiac function over and above their effect on GH and IGF-I levels[133].

4.2 Patient recruitment

Both acromegaly and ao-GHD are rare and recruiting affected individuals for studies has its challenges. Power calculations indicated ten individuals would be needed for each arm, hence fifteen individuals with each condition were sought for the study[128,207]. As previous studies did not separate males from females when assessing LVMi, this was not factored into the calculations[77,83,85,106,107,128,130,132,225]. However, a study of 60 healthy individuals which sought to determine normal ranges in CMR, has demonstrated a marked difference in LVMi between males and females (males 55.4 – 74.0 g/m^2, females 44.6 -59.4 g/m^2)[226]. Hence it was decided to present LVMi separately for males and females. As the groups did not contain equal numbers of males and females, some male/female subgroups were very small, particularly after dropout. Of note, only two female patients with GHD took part, one of whom dropped out after six months. This limited conclusions that could be drawn regarding these smaller subgroups. For completeness, combined analysis of males and females together has also been performed and is mentioned in the discussion where its findings differ from segregated data. As it has been demonstrated that there is no difference in BSA-indexed cardiac volume indices between males and females both males and female data were analysed together for these parameters[218]. The uneven distribtion of males and females recruited arose by chance, as both acromegaly and GHD have equal gender distributions. Of note, it has been reported that there is no gender-related difference in the cardiac response to GH replacement[227].

There was some patient drop out in both GHD and acromegaly groups. A range of reasons was given for this, including other medical problems, discontinuation of GH treatment and difficulties with taking time off work to attend for CMR. In a few cases, patients did not like undergoing CMR. It is interesting to note that the two patients who dropped out of the GH arm of the study had high baseline AGHDA scores that failed to improve with GH treatment and it is possible that low mood or lack of motivation influenced their dropout. The lack of improvement in AGHDA score with GH replacement in these individuals suggests that additional factors, other than GHD, may have been contributing towards reduced QoL. In patients with acromegaly there was no relationship between QoL and patient dropout.

The results in this study were analysed using paired T-tests and repeated measures ANOVA. The limitation of these tests is that they are dependent on a complete data set. As number of patients only attended for two out of three visits, their data sets are incomplete and so were excluded from ANOVA analysis. This reduced the power of the study. In each of the acromegaly and GHD groups the total number of male and females who attended for all scans and were included in paired analyses was eight (n=8). These numbers were less than had been planned for the study.

Controls were recruited to match patients for both age and sex. Controls for both the acromegaly and GHD groups were recruited on the same days and using the same recruitment method. CMR scans were performed simultaneously in both groups. There were no significant differences in height, systolic or diastolic blood pressure between patients and controls (Table 3.3 and Table 3.4). There was no difference in weight between patients with acromegaly and their controls (p=0.396). However, patients with GHD weighed significantly more than controls (88 v. 71 kg, p=0.007) and had a mean BMI in the overweight range (29.4 kg/m^2), whereas controls had a mean BMI in the healthy range (22.7 kg/m^2). This finding most likely represents the central adiposity seen in GHD[26]. However, individuals volunteering to provide control data have a tendency to be motivated individuals. This characteristic may also express itself as a motivation to maintain a healthy weight and the difference seen may reflect the bias seen in any group of volunteers. Alternatively, this is simply a matching failure. The indexing of ventricular mass and volume indices to BSA is designed to overcome this factor and so this difference between the two groups should not influence their comparison, although this does not factor in any differences in lean body mass that may exist between the groups.

4.3 Controls

Controls were recruited into the study to match the age (+/- 5 years where possible) and sex of the patients. With the exception of a higher body weight in male patients with GHD than controls, there were no other statistically significant differences between patients and their controls. However, it should be noted that the groups were very small, especially when female GHD patients were compared with their controls. In this case, the group number of two makes drawing statistical conclusions impossible. It should be noted that more patients with acromegaly had hypertension than their controls. However, it could be argued that this is the nature of acromegaly and, as this was treated in all cases so that there was no difference between systolic or diastolic blood pressure between groups, it has been corrected for as much as is reasonably possible.

The use of matched controls is only one way of comparing patients to normative data. Alternative methods include comparing patients to a “normal population” by using standard reference ranges and by comparing patients to themselves before and after treatment of disease. All three of these techniques were attempted in this study, to provide the most available information. The use of patients as their own controls was limited in the acromegaly group by the failure to achieve biological cure in the majority of the patients. Standard reference ranges are limited to the values that have been published in that field and comparisons can only be made when data are available. In this study, the majority of indices investigated, such as LV EDVi, had published normal range values. However some indices, such as RV indices and aortic area were less available. Matched controls are considered the best form of control groups in a study. However, matching controls to patients induces bias into the characteristics of the group that may cause them to deviate away from the “normal range”. Furthermore, small study numbers may amplify this, as demonstrated by the control populations in this study in whom mean group characteristics were, at times, outside of published normal ranges. Twenty-free controls were recruited, 10 for the GHD group and 13 for the acromegaly group. Although these controls were considered as “normal” controls, the CMR results between the two groups were significantly different from one another. Of particular note was the fact that

female acromegaly controls had a mean LVMi beneath the normal range (40.8 g/m^2, normal range 44.6-59.4), whereas female GHD controls had a mean LVMi in the middle of the normal range (55.4g/m^2). Although the female acromegaly patients had a mean LVMi above the normal range (61.8g/m^2), the significant difference seen between acromegaly patients and their controls (p=0.0055) may have been influenced by particularly low LVMi in the control group.

This finding is a consequence of the small numbers present in this study. Although it does not invalidate the comparison of patients with their matched controls, it does suggest a degree of caution is required. By using a range of methods to establish control data, patients themselves, normal ranges and matched controls, we have done our best to overcome this limitation.

4.4 Growth hormone deficiency

Recruitment of patients with ao-GHD was particularly difficult. Since the introduction of recombinant GH in 1985, the demonstration of the clinical benefits of GH replacement in adults with GHD and the introduction of the NICE guidelines in 2003[27], most patients fulfilling replacement criteria have been started on treatment. This has reduced the number of patients available for studies, a problem noted in another recent study[215]. It might be predicted that current patients would have shorter durations of GHD prior to replacement than those in historical studies, potentially reducing cardiac consequences of GHD. Our patients had a mean duration of GHD of 6.1 years. Duration of GHD in other studies ranges from similar durations to this study[103,207] to 13.2 years in Newman's study[215]. Previous studies in ao-GHD have shown significant changes in echo-measured LVMi when treated with GH with a sample size of ten, so the recruitment of ten patients was felt to be adequate[107].

4.4.1 Baseline findings

Despite there only being two female patients with GHD, these individuals demonstrated reduced LVMi when compared to controls, whereas in the larger group of males (n=8) there was no significant difference between patients and controls. When the group size was increased by analysing both male and female results together there was a trend towards reduced LVMi in patients (p=0.063) but significance was not achieved. When compared to published normal ranges, female patients had mean LVMi beneath the lower limit of normal and male patients had mean LVMi at the bottom of the normal range. These ranges were formulated using 30 males and 30 females aged 20-65 years, a much larger group than the control group in this study[216].

The mean age in this group was 55 years (range 34-76). It is interesting to note that in a similarly sized study in older patients with GHD (60-72 years), there was no difference in LVMi between patients and their controls, even in the absence of diabetes or long-term hypertension[228], whereas a study in younger patients showed differences between patients and controls[107]. This might suggest that differences in LVMi that have been noted in patients with GHD may be more marked in younger patients and lost with age when other contributors towards increased cardiac mass, such as hypertension, may come into play. It should be noted that the second study contained both ao- and co-GHD patients, which may confound

comparison. However, the lack of difference in LVMi between patients and controls in our study may, in part, represent the age of the patients enrolled.

4.4.2 One year of GH replacement

All patients in this study demonstrated an improvement in QoL score between presenting and final AGHDA-QoL measurement, itself a prerequisite of ongoing treatment[27], and an increase in IGF-I SDS (Table 3.6). GH dose was titrated locally by the referring clinician according to local protocols, so that the timings of dose increases varied from individual to individual. Those patients for whom GH dose was titrated up more rapidly may have had longer degrees of exposure to a "normal" GH level, which may have influenced the cardiac findings seen. However, it was felt that this variation would be minimal and unlikely to have any impact on cardiac findings at CMR.

With only two female patients, one of whom dropped out after Visit Two, it is impossible to describe the effects of GH replacement on females alone. Males alone demonstrated a strong trend towards a significant increase in LVMi (ANOVA p=0.052). When data from males and females were pooled together a statistically significant increase in LVMi was seen with 1 year of GH treatment (p=0.023). This change represented a 3.5 g/m^2 increase in LVMi. Whether or not this is clinically significant cannot be concluded at present, as there is no published work addressing this issue. Certainly, in males, mean LVMi rose from the bottom of the published normal range to the middle of the range (55.4 *v.* 61.0 g/m^2, normal range 55.4 – 74.0), which might suggest a clinical impact from this change. Of the volumetric and function parameters measured, only EDVi was reduced at baseline when compared to controls. Although this did not rise significantly with GH replacement, by one year the difference between patients and controls was lost, suggesting a subtle improvement in this parameter.

The mechanism behind the reduction in cardiac mass in GHD is likely to be multi-factorial. Withdrawal of GH replacement in eight teenagers with GHD resulted in a reduction in skeletal muscle fibre diameter to 86% of baseline values[229]. Three months GH replacement (12.5 μg/kg, to a maximum of 1.0 mg/day) in patients with ao-GHD resulted in increased area of both Type I and Type II skeletal muscle fibre area[230]. It must be noted that this is a high replacement dose of GH and treatment with GH increased the IGF-I levels above the upper limit of the normal range. Although there have not been any publications looking at the cardiac histology in GHD, in animal models of GHD cardiomyocyte area is reduced and increases with GH therapy[61]

suggesting that change in cardiac muscle fibre diameter is the likely mechanism for the increase in cardiac mass seen in this study.

Previous echo studies in patients with ao-GHD have had contradictory results regarding cardiac mass. Nass *et al.* found the even with 6 months of high dose GH treatment (12.5 μg/kg/day) there was no change in cardiac mass, volume or function, although a trend towards increased LVMi was seen (p=0.058) and maximal oxygen consumption and power output at exercise was increased[103]. The authors suggest that the improvements in exercise performance may represent increased cardiac output and increased respiratory muscle strength rather than changes in cardiac structure. Valcalvi *et al.,* using even higher doses of GH (16.7 μg/kg/day), reported no difference in cardiac mass in the first six months of treatment but by one year reported a significant increase in LVMi, that persisted even at three months after ceasing GH[207]. This study also reports that the reversal of diastolic dysfunction in patients with GHD did not persist after stopping GH, suggesting that the increased myocardial contractility seen with GH replacement is not simply due to increased cardiac mass. Colao *et al.* report reduced LVMi in patients with GHD compared to controls and an in increase in LVMi with one year of GH[107]. This paper reports GH titration to IGF-I levels but starts with a dose of 10 μg/kg/day, a dose higher than would be used in current UK practice. Additionally, it combines both co- and ao-GHD, which is likely to have skewed results.

Current UK practice is not to dose GH replacement on weight but to titrate against IGF-I and improvement in symptoms[210,231,232]. This reduces the unwanted side-effects of fluid retention, carpal tunnel syndrome and arthralgia and seeks to replace patients to physiological GH levels as best as can be achieved with a once daily injection. This usually results in patients receiving lower doses of GH than those used in the above studies. At the end our study, the average GH dose for men was 4 μg/kg/day (mean dose 0.35 mg/day) and for the single remaining woman was 10 μg/kg/day (0.6 mg/day). Of note, this female patient was taking oral oestrogen-containing hormone replacement therapy, which necessitates higher doses of GH to achieve equivalent IGF-I levels. In Maison and Chanson's review of the impact of GH on cardiac structure in 2003, none of the studies utilising lower GH doses, equivalent to those used in our study, contained ao-GHD patients, limiting conclusions that can be drawn about the cardiac effect of GHD in this population[33].

Since the above review, studies have taken place using GH doses more consistent with current practice. Some studies show increases in LVM in ao-GHD with one year of GH replacement[233,234] whereas others show no change[235]. However, problems with dose variations continue to confound comparison. Even a very recently published double-blind study seeking to examine cardiovascular function in GHD replaced patients with 12.5 μg/kg/day, which had to be reduced in a significant number of patients because of side effects, so that the mean dose given was 0.64 mg/day at 6 months[215]. Interestingly, when the study became open-label after the first six months and GH doses were titrated to IGF-I levels, the mean dose of GH given reduced to 0.5 mg/day. This study failed to show a difference in LVM in GHD patients treated with GH but did not correct the parameter to BSA, making comparison to other studies difficult.

Patients in Andreassen's CMR study had similar duration of GHD to those in our study (median 5 years) and received similar GH doses (mean dose 0.34 mg/day)[128]. Andreassen's study reported reduced in EDVi and ESVi in GHD patients compared to controls. The finding of reduced EDVi was confirmed in our study, although we failed to demonstrate any difference in ESVi. This may reflect the smaller numbers in our study, a situation which was amplified by two patients with volumetric parameters more than two SD from the group mean that were excluded from the analysis. Andreassen analysed LVMi in males and females together demonstrating a trend towards increased LVMi ($p=0.059$) with one year of GH treatment. When male and female data were pooled, we were able to show an increase in LVMi over this time (paired ANOVA, $p=0.023$). In neither study were any significant differences seen in other volumetric parameters or cardiac function at baseline or with one year of treatment. These two studies are relatively concordant in their results, the slight differences between the two may be explained by the size of the study population. A larger study would be necessary to confirm these findings.

Just as it is possible that age my mask cardiac changes in patients with GHD at baseline, it also remains possible that increasing age may mask differences before and after GH replacement. It is interesting to note that a study treating younger patients (median age 26.5 years)[107] saw improvements that were not seen in study treating only older patients (mean age 68 years)[235], although these studies were disparate and measured different parameters. In our patients, there was no correlation between ΔLVMi and age ($r=0.5102$, $p=0.1964$). However, the study was not powered to address this question. CMR studies separating younger and older patients would be needed to answer this question.

4.4.3 Right ventricular findings

Patients with GHD demonstrated reduced RV EDVi (p=0.034) and SVi (p=0.045) compared to controls. CMR is considered the most accurate a reproducible method for assessing RV volume parameters and has shown to be reproducible[223,226,236]. There are no published works looking RV parameters in patients with GHD, including the recent CMR study. Acromegaly is known to produce biventricular cardiac involvement[73,83]. Therefore, it does not seem unreasonable that lack of GH would also produce biventricular changes.

Early studies in hypophysectomised rats showed that GH treatment increased RV mass[237]. Studies in patients with GHD have not sought to examine the RV in depth. In this study, although there were no significant RV changes with one year of GH treatment, a trend toward increased RV EDVi was seen (p=0.068) and the difference in EDVi between patients and controls was lost (Visit One 70.6 v. 78.9 ml/m^2, p=0.034; Visit Three 76.1 v. 78.9 ml/m^2, p=0.543), suggesting a subtle increase in EDVi. Larger studies are needed to produce demonstrable changes in the RV than in the LV due to technical challenges in calculating parameters and consequential increased error[236]. As this study was powered to demonstrate changes in LV mass, it may have been inadequately powered to demonstrate significant RV changes.

4.4.4 Exclusions

Results more than two SD from the group mean were considered as outliers and excluded from the analysis. Grubbs defines an outlying observation, or outlier, as “one that appears to deviate markedly from other members of the sample in which it occurs”[238]. These may reflect measurement error, non-normally distributed data, a mixture of two result distributions in the population sampled or simple chance. In order to minimise measurement error, two observers reviewed the CMR scans, with good concordance of recorded measurements (Page 87). To confirm normality of data means and medians were compared with negligible difference found between the two. The most likely causes for outliers in this population were the presence of a mixed population or simple chance. GHD is a complex, multi-system disease and is associated with a range of other medical problems, such as dyslipidaemia, which may influence cardiac findings. Hence the population examined is likely to be heterogeous in nature. Additionally, chance remains a potential reason for outlying results.

Arguments for and against the exclusion of data points from analysis exist. The decision whether or not to exclude an outlier depends in part on the nature of the study. As our clinical study was both observational and long-term and the diseases examining were multi-system, it was felt appropriate to exclude outlying data that lay more than two SD from the group mean.

The results excluded from analysis were Patient 3 for LVMi and EDVi and Patient 16 for EDVi. Patient 3 was a 49-year-old man with hypertension and diabetes. His LVMi at CMR1 was not only well above the group mean but was above the upper limit of published references ranges (76.0 g/m^2, normal range 55.4 – 74.0). It is likely that hypertension contributed towards his LV hypertrophy. Given his risk factors it also is possible that ischaemic heart disease may have played a role, although perfusion CMR did not show any evidence of reversible myocardial ischaemia, nor was any evidence of previous infarction on perfusion CMR. Patient 16 had increased myocardial volume. His past medical history included well-replaced primary hypothyroidism and hypercholesterolaemia. He did not have documented hypertension and was not on any antihypertensives. He was not a smoker. No clear reason was identified for his raised EDVi that sat at two SD from the group mean (86.4 ml/m^2, group mean 71.0).

4.4.5 Other medical conditions and drugs

All patients included in the GHD arm of this study had multiple pituitary hormone deficiencies. All except one patient were receiving thyroid hormone replacement. Seven out of ten patients were receiving sex steroid replacement. Five out of ten patients were receiving hydrocortisone. No pharmacological hormone replacement exactly mirrors the tight physiological control that exists in nature and there will be, at times, supraphysioloigcal levels of that hormone within circulation. The replacement of GH may also impact on the availability of the active component of these substances to the cardiac tissues. GH increases conversion of cortisol to cortisone, reducing tissue exposure to the active form of the compound[239]. GH also increases conversion of levothyroxine to liothyronine, increasing tissue exposure to the active thyroid hormone[240]. It has not been determined if any of these additional hormonal factors contribute towards the cardiac findings in patients GHD.

GHD is associated with an atherogenic lipid and inflammatory marker profile. Two GHD patients in this study had LGE and three patients had evidence of perfusion defects with hyperaemia. Of these, one patient may have had a previously missed myocardial infarct (Patient 1). Control subjects did not receive gadolinium contrast or adenosine and so it is

impossible to determine if this level of findings was greater than would be expected in individuals without GHD. However, it should be noted that these findings were very similar to those seen in the acromegaly group. The only patient with hypertension and type 2 diabetes mellitus (Patient 3) did not demonstrate LGE or perfusion abnormalities with adenosine. However, this patient did have LV hypertrophy, presumed hypertensive in origin, and was excluded from the data analysis.

4.5 Acromegaly

4.5.1 Left ventricular findings

Cardiac enlargement is long recognised consequence of acromegaly[21]. Both male and female patients with acromegaly demonstrated increased LVMi when compared to controls. Patients also exhibited increased EDVi, SVi and CI and a trend towards increased ESVi compared to controls. There was no correlation between IGF-I SDS and LVMi.

Acromegaly is a disease associated with a long duration of symptoms prior to diagnosis[72]. Disease duration correlates with RV free wall thickness and may correlate with LVMi, so that early diagnosis is likely to influence the severity of hypertrophy seen[83]. Additionally, the presence of hypertension significantly increases the degree of LV hypertrophy patients develop and it remains unclear whether early treatment of hypertension may modify the development of acromegalic cardiomyopathy[83,85]. As national programmes, such as the Quality and Outcomes Framework (QOF), reward general practitioners for screening for hypertension and diabetes, these secondary consequences of acromegaly are being identified and treated earlier, potentially influencing the development of cardiac disease even prior to the diagnosis of acromegaly being made.

The biventricular, concentric nature of early acromegalic cardiomyopathy is one of the reasons that this condition is thought to arise from direct GH/IGF-I action, rather than purely as a compensatory response to the secondary features of acromegaly. If hypertension and diabetes mellitus were the only driving factors for the cardiomyopathy, a picture more akin to hypertensive cardiac disease might be expected. The heart disease of GH excess, at least in the early stages, differs in a range of ways from simple heart failure as shown below in Table 4.1 adapted from Sacca *et al.*[68].

	Chronic heart failure	*GH/IGF-I excess*
LVM	↑	↑
LV dimension	↑	↔
LV wall thickness	↔	↑
Relative wall thickness	↓	↑
Afterload	↑	↓
Contractility	↓	↑
Peripheral vascular resistance	↑	↓

Table 4.1 Changes in ventricular geometry, forces and haemodynamics in chronic heart failure and after short-term GH/IGF-I excess, from Sacca *et al.*[68]

4.5.2 Treatment of Acromegaly

Whereas in GHD patients recruited into studies may differ from historical cohorts due to shorter disease duration, in the case of acromegaly, patients may differ due to changes in treatment patterns. Ten years ago, transsphenoidal surgery, followed by depot somatostatin analogues and radiotherapy if GH excess continued, was standard care[17]. Now the practice of pre-treating tumours not confined to the pituitary fossa with six months somatostatin therapy prior to surgery has become more common[18]. Somatostatin analogues may influence cardiac disease over and above the effect on GH levels. A study by Colao *et al*. that compared the cardiac consequences of somatostatin analogues or transsphenoidal surgery as primary treatment for acromegaly showed that primary somatostatin analogue therapy was associated with an improvement in EF that was not seen with surgery[133]. Our own data, reported in this thesis, demonstrates a reduction in cardiac AMPK activity in GH3-implanted rats treated with somatostatin analogues. This could be a mechanism by which Colao's findings might be explained. Additionally, pegvisomant, a growth hormone receptor antagonist, has come into clinical practice for hard-to-control disease[241]. This means that current patients with acromegaly may be receiving far more heterogeneous treatment strategies than those involved in past studies, making outcomes harder to compare.

This variation in treatment strategies is clearly demonstrated in the treatments received by our patients in this observational study (Table 3.7). Patients were exposed to a range of methods to attempt to normalise GH levels: somatostatin analogues, TSS and pegvisomant. It is not clear if variation in treatment strategies influenced the findings at CMR. Furthermore, some of our patients were receiving somatostatin analogues on entry into the study, which may have influenced baseline CMR findings. This heterogeneity needs to be taken into account when interpreting findings, particularly when no change is seen in patients before and after treatment. To overcome this, it may be necessary for future studies to include much larger groups of patients, to allow for sub-group analysis.

In addition to heterogeneity in the treatments patients receive, study outcomes may also be influenced by variability in treatment success. The definition of "cure" in acromegaly has been debated. The consensus conference in Cortina d'Ampezzo in 1999 defined controlled acromegaly as a nadir GH <1 μg/L following oral glucose tolerance testing, an age-sex-normalised IGF-I and no clinical disease activity[242]. The terms "controlled" or "remission" are more appropriate than "cure" as even in the presence of these criteria, there is a 2-3% chance of recurrence[243]. It has been reported that treated acromegaly patients with basal GH levels between 2.5 and 5.0 μg/L have mortality rate twice that of matched controls[244]. Transsphenoidal surgery alone may provide a biochemical cure for half of patients[245], the main limitation to successful primary surgery being tumour size. Many patients with macroadenoma are now being pre-treated with somatostatin analogues with a view to reducing tumour size and increasing the chance of cure at surgery[246].

This study did not seek to define "cure" in patients but IGF-I was measured on each visit as a marker of disease activity. Out of 13 patients with acromegaly in this study, only four achieved an IGF-I SDS less than two, i.e. age and sex normalised. For two of these (Patients 10 and 13) this reduction had occurred at six months but by one year IGF-I SDS had risen again. Patient 11 dropped out the study at six months and so it remains unknown if her IGF-I SDS would have remained controlled. Only Patient 7 had an IGF-I SDS less than 2 at one year (SDS -1.93). Interestingly, this patient showed a marked improvement in QoL score, far greater than any other acromegaly patient. All acromegaly patients in this study had macroadenoma, a probable factor in the degree of ongoing disease. Failure to achieve biochemical remission is likely to have influenced cardiac mass and volume measurements, with patients failing to demonstrate the expected improvement in these parameters by one year. Inadequate numbers of patients

achieved biochemical remission in order to be able to analyse the data from these patients separately from those who did not. Hence, only the effect of reducing IGF-I, rather than the effect of normalising it, has been assessed by this study.

4.5.3 Findings after one year of treatment

After one year of treatment for acromegaly, there was no significant difference in LV mass in males or females, although when data from both sexes were pooled a trend towards a reduction in LVMi was seen (ANOVA, p=0.0817). Although females continued to demonstrate a raised LVMi at one year when compared controls (p<0.001), the difference between male patients and controls was lost (p=0.112), suggesting improvement of the cardiac hypertrophy, as has been reported elsewhere[133]. There were no differences in volume indices. Some published studies report a rapid improvement in LVMi with successful treatment of acromegaly, which persists at five years[92-95], whereas others failed to see an improvement in the short term[132].

The failure to demonstrate significant improvement in LVMi in this study is likely to reflect lack of control underlying disease, with persistence of GH and IGF-I excess. This may also be contributed to by loss of power as only 8 patients attended for all three scans. The paired nature of a repeated measures ANOVA test requires a complete data set and hence only data from 8 patients were compared for this part of the study, despite higher numbers than this taking part.

Patients continued to have a raised cardiac index at one year when compared to controls. Given the persistent ongoing raised IGF-I levels in the acromegaly group, this would be predicted. The increased circulatory volume of acromegaly is thought to arise directly from increased salt and water retention, mediated via the kidneys[90]. Hence, changes in cardiac index are likely to be one of the earliest alterations found in GH excess. Indeed, patients may report an improvement in oedema, a symptom mediated via the same mechanism, immediately on waking up after successful transsphenoidal surgery. The patients in this study had evidence of ongoing GH excess and therefore the raised cardiac index persisted.

Incomplete disease resolution appears to have caused an ongoing reduction in QoL. Figure 3.20 demonstrates that by one year of treatment, most improvements in QoL that were seen at six months have been lost (six patients) and that in the case of two patients (Patients 3 and 23)

QoL was worse at one year than at baseline. No statistical correlation was found between IGF-I SDS and QoL.

4.5.4 Medications

It is possible that medication, other than for direct control of GH levels, may also have had an impact on the cardiac findings seen. Of particular note are angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs). ACE inhibitors have been shown to reduce LV EDV and ESV and increase EF in patients with symptomatic heart failure[247] and reduce LV EDV in patients with asymptomatic heart failure[248]. Reduction in LVM using these drugs is associated with a reduction in myocardial infarction and stroke, independent of systolic blood pressure[249]. These findings may arise from both improvements in myocardial remodelling and from a reduction in preload and afterload.

Three patients with acromegaly were taking ACE inhibitors (Patients 6, 7 and 13) and one was taking an ARB (Patient 24). It is interesting to note that three of these patients (Patients 6, 7 and 24, all female) had baseline LV EDVi and ESVi at the lower end of the results range: mean EDVi 90.7 ml/m^2 (range 60.2 – 119.8) Patient 6 EDVi 60.2 ml/m^2, Patient 7 EDVi 67.7 ml/m^2 Patient 24 EDVi 75.6 ml/m^2; Mean ESVi 35.3 ml/m^2 (range 23.1 – 47.4) Patient 6 ESVi 24.1 ml/m^2, Patient 7 ESVi 23.1 ml/m^2, Patient 24 ESVi 24.2 ml/m^2 (Patient 4 results excluded, as per previous analyses). Patient 13 (male) had results close to the mean (EDVi 89.5 ml/m^2, ESVi 39.2 ml/m^2). Such small numbers make it impossible to draw any conclusions. The 2008 Italian meeting of the COMorbities evaluation and Treatment in Acromegaly study (COMETA) recommended that ACE inhibitors or ARBs should be the first-line antihypertensives used in acromegaly[250]. However, further work is needed to address the role of this family in improving cardiac indices in patients with acromegaly.

4.5.5 Other medical conditions

Obstructive sleep apnoea (OSA) is known to produce cardiac disease; in one study right ventricular hypertrophy was demonstrated in 71% of OSA patients[251]. Whether OSA causes frank heart failure or simply exacerbates pre-existing heart failure remains unclear[252]. However, untreated OSA is associated with increased cardiac morbidity and mortality[253]. Five out of 13 acromegaly patients in this study had OSA (Patients 4, 7, 8, 13 and 24). Of these, only Patient 4 had volume indices greater than two SD from the group mean. However, rather than these being increased, as would be predicted with OSA, this patient had reduced RV EDVi and

reduced LV EDVi, ESVi and SVi, representing the picture of cardiac constriction. The other three patients with OSA all had LVM and LV and RV volume indices well within the normal range. It must be noted that RV mass, which might be predicted to be increased in OSA, was not measured in this study as this measurement is less reproducibly measured on CMR[223].

OSA is a common problem in acromegaly. It is possible that identification of OSA and its appropriate treatment in the above patients is the reason that they do not exhibit any significant mass or volume changes (with the exception of Patient 4). It is possible that other patients within the study may have the cardiac consequences of undiagnosed OSA.

4.5.6 Exclusions

Patient 4 had a markedly reduced EDVi and ESVi, even when compared to controls rather than other patients with acromegaly, and was excluded from the analysis of these parameters. There was evidence of reduced perfusion on induction of hyperaemia and a basal anteroseptal and inferoseptal LGE. Further investigations demonstrated a 50% stenosis of the left anterior descending coronary artery, which required stenting. Additionally this patient had marked LV hypertrophy with chordal systolic anterior motion (SAM) of the anterior leaflet of the mitral valve and LV outflow tract turbulence. During systole there was apical and mid-ventricular cavity obliteration. This picture is similar to that found in hypertrophic obstructive cardiomyopathy.

With somatostatin analogue therapy there was a reduction in LVMi (59.6 to 56.9 g/m^2) and resolution of SAM. LV outflow tract turbulence persisted. This patients transsphenoidal surgery was delayed due to the discovery of reversible cardiac ischaemia that required intervention and then for a thyroidectomy, as a multinodular goitre was compressing the trachea. Therefore, at the end of one year, although there was a mild improvement in IGF-I, her acromegaly remained uncontrolled. Whether or not these cardiac findings will resolve with remission of the acromegaly remains to be determined.

4.5.7 Right ventricular findings

As has already been discussed, one of the distinctive features of acromegalic cardiomyopathy is biventricular involvement[83]. It was predicted that similar changes would be seen in the RV as were seen in the LV. In the LV, both EDVi and SVi were increased but in the RV only SVi was significantly increased at baseline (p=0.031), although there was a trend towards increased EDVi (p=0.10). After one year of treatment, there was no change in SVi or EDVi, and results remained comparable with baseline findings.

4.6 Late gadolinium enhancement

Whereas reduced wall motion on perfusion CMR strongly correlates with the presence of previous ischaemia, for it directly visualises function, the presence of LGE is far harder to interpret. Here, the relationship between perfusion and signal response is not linear, and indeed may be inverse [118]. Interpretation of LGE findings is dependent on scanning techniques used, timing of gadolinium injection, the pattern of LGE and the presence of other findings, such as regional wall motion abnormalities.

Gadolinium-based contrast agents are distributed throughout the extracellular space and are excreted renally in an unchanged form. These agents shorten T_1 relaxation times and consequentially appear bright on T_1 images[254]. The reasons for accumulation of gadolinium-based contrast in damaged tissue remain incompletely understood, but appears to arise from a combination of altered contrast wash-in/washout kinetics and expansion of the extracellular space[255]. The pattern of enhancement is important as LGE may occur in a range of settings, including infarction, fibrosis, cardiomyopathies and infiltration[256] and needs to be examined with care as artifactual high signal may arise from the chest wall, fat or cerebrospinal fluid[118]. Gadolinium-based contrast agents are well tolerated and are generally very safe. However, they are avoided in the presence of reduced glomerulofiltration rate because of an association with nephrogenic systemic fibrosis[257].

In this study we particularly wished to used LGE to examine the hearts of patients with acromegaly for the presence of fibrosis. Acromegaly has long been reported as being associated with increased myocardial fibrosis[21]. Fibrosis occurs when myofibroblasts produce increased quantities of extracellular matrix components, including collagen, resulting in hardening or scarring of tissue[258]. This process may occur in a very localised manner, such as following a regional myocardial infarction, or be a more diffuse process such as in hypertensive cardiomyopathy. Myocardial tissue collagen content is often used a surrogate for fibrosis and the terms are often used interchangeably in the literature. The point at which increased collagen deposition, especially when proportional to myocardial growth, becomes pathological has not been determined. Caution is needed in interpreting the literature in regard to this matter.

Original acromegaly autopsy studies report that 50-85% of hearts examined demonstrated interstitial fibrosis[86,259]. However, these studies demonstrated not only fibrosis but also a

lymphomononuclear infiltrate and coronary artery disease, suggesting that myocarditis and ischaemic heart disease may have contributed to the findings seen. Indeed, all these studies took place prior to the successful treatment of acromegaly and patients died with active disease. It is probable that multiple pathologies, including ischaemic heart disease, were at play in producing these findings. Frustaci *et al.* performed endomyocardial biopsies on 10 patients with acromegaly and compared them to samples from papillary muscles in patients without acromegaly undergoing mitral valve replacement[87]. This demonstrated a massive increase in cell apoptosis in both myocytes and non-myocytes and an eight-fold increase in interstitial collagen with small foci of replacement fibrosis. The mechanism behind these findings remains unclear as in other experiments IGF-I excess appears to be protective against apoptosis[260,261] and to cause increased myocardial growth with a proportional increase in collagen content[262,263] and GH excess reduces collagen I and III expression[264]. It must be noted that four out of ten patients in Frustaci's study had systolic dysfunction as determined by EF <50% and that collagen content and myocyte apoptosis correlated positively with duration of acromegaly and negatively with EF. It may be that collagen deposition is a feature of advanced acromegalic heart disease rather than the earlier stages. Furthermore, although the presence of hypertension did not correlate with collagen deposition in the study, whether the use of ACE inhibitors and ARBs may play a role in the modification of collagen deposition in acromegalic cardiomypathy remains to be determined. Certainly, both these families of drugs reduce the development of increased cardiac collagen content in heart failure[265,266].

Echocardiography can be used to assess myocardial fibrosis in patients with acromegaly. A study using interventricular septum echoreflectivity demonstrated evidence of increased fibrosis in patients with acromegaly compared to controls, which correlated with procollagen III, a serum marker of collagen synthesis. Control of acromegaly with surgery and somatostatin analogue if required normalised both echoreflectivity and procollagen III levels to that of the control group[267]. A study that used an alternative technique to assess collagen content and to measure subtle changes in wall contractility, both thought to reflect the presence of fibrosis, demonstrated increased evidence of fibrosis in patients with acromegaly that improved with somatostatin analogue therapy[268]. Interestingly, some patients demonstrated evidence of fibrosis in the absence of increased LVMi, suggesting that in the clinical setting the process of fibrosis may precede measurable hypertrophy, a finding that has not been demonstrated in animal studies.

In the one CMR study in acromegaly, although CMR was able to identify LV hypertrophy that was missed by echocardiography it was less successful in identifying fibrosis. CMR only identified one patient out of 14 with mild fibrosis as measured by LGE, whereas echo identified six patients with evidence of increased collagen[130]. In our study two out of 13 acromegaly patients demonstrated mild LGE. In both cases this was patchy and diffuse. LGE did not correspond with regional wall abnormalities and hence did not arise as a result of previous infarction. We did not assess for evidence of increased myocardial collagen content at echocardiography.

CMR utilises normal myocardium as the control against which increased signal from delayed gadolinium washout is compared. A weakness of this method is that it may fail to identify a diffuse fibrotic process, in which all the myocardium is affected[224]. The patchy fibrosis demonstrated in two scans in patients with acromegaly is likely to represented increased collagen content in the myocardium of these patients. However, it may be that biopsy of the myocardium of other patients in this study would also demonstrate increased diffuse collagen deposition, missed by this CMR. The lack of concordance seen in Bogazzi's study between the fibrosis demonstrated at echo and CMR suggests that LGE measurement by CMR is not the optimum method for identifying fibrosis in patients with acromegaly. It is possible that levels of myocardial fibrosis are less in our study population than in historical controls, due to control of risk factors for ischaemic heart disease and better treatments for patients with acromegaly. However, the finding of only two patients out of 13 in our study who demonstrated evidence of LGE seem to concord with Bozazzi's and CMR may not be the optimum method for determining the presence of fibrosis in the heats of patients with acromegaly.

Cardiac fibrosis is not a feature of GHD. Of the two patients with GHD who demonstrated LGE, one had a missed previous infarct and one had such mild, patchy fibrosis, it was impossible to quantify. Of note, this second patient, (Patient 14) also had a perfusion defect with induction of hyperaemia. Although computerised tomography angiography failed to demonstrate any distinct coronary artery lesions, he was felt to be at high risk from ischaemic heart disease and managed with a primary prevention strategy. Therefore, ischaemic heart disease was the probable cause of LGE in these two patients with GHD.

4.7 Perfusion CMR

Three CMR techniques are available to assess the myocardium for evidence of coronary artery disease: dobutamine infusion may be used to assess for inducible wall motion abnormalities, adenosine or dipyridamole may be used to induce hyperaemia to assess for abnormalities in myocardial perfusion, or the coronary arteries may be visualised using magnetic resonance coronary angiography (MRCA)[118]. In this study, the vasodilator adenosine was used.

CMR is a valuable, non-invasive method for investigation for coronary heart disease[269]. In a study of 513 patients, three-year cardiac event-free survival was 99.2% in those with a normal adenosine perfusion CMR, an abnormal scan was strongly associated with cardiac events (hazard ratio 12.5)[270]. However, just as stress echocardiography, nuclear medicine imaging and CT coronary angiography are all imperfect tests for the presence of flow-limiting coronary artery disease, so too perfusion CMR has its limitations. When compared to coronary angiography as a gold standard, adenosine perfusion is reported as showing 91% sensitivity but only 62% specificity in the detection of epicardial coronary stenoses[271]. Additional limitations of CMR perfusion studies are that only the most proximal flow-limiting lesion is identified with more distal lesions being missed and its qualitative rather than quantitative nature, that and cannot determine degree of stenosis[118].

In this study only two patients exhibited a reduction in perfusion with induction of hyperaemia, one patient with GHD and one with acromegaly. Further assessment was made with computerised tomography angiography and only Patient 4, an acromegaly patient, was felt to have evidence of coronary artery disease that required intervention. This patient was not experiencing exertional chest pain. It is impossible to know whether this lesion would have caused complications during the planned pituitary surgery, although its early identification and treatment was felt to be in the patient's best interest. In this case the use of CMR during the pre-operative work-up allowed for the minimisation of peri-operative cardiac risk. Only one patient out of fourteen enrolled in the study had evidence of reversible ischaemia as compared to a 30 year old study that showed evidence of reversible ischaemia using thallium scanning in eight out of 34 acromegaly patients[79]. Only one out of our 14 acromegaly patients was taking an HMG CoA reductase inhibitor and so this reduction in ischaemic heart disease is not explained by use of these medications. Using CMR for purely identifying reversible ischaemia is unlikely to be cost efficient.

Although acromegaly is associated with an increase in two of the main risk factors for ischaemic heart disease, hypertension and diabetes mellitus, it is not clear if there is increase in ischaemic heart disease in acromegaly, particularly in early disease. Autopsy studies have demonstrated evidence of myocardial infarction and atherosclerosis, although the level of disease seen was not thought to exceed background levels in similarly aged populations[86]; cardiac deaths continue to be increased in this patient group[96]. Studies looking at carotid intima media thickness (IMT), a marker of atherosclerosis, have conflicting results. One study found that patients with acromegaly have lower IMT than controls[97] and others that patient have higher IMT than controls[272,273]. Attempts to predict cardiac events using coronary artery calcium scoring and Framingham risk factors have not been successful in the medium term[274]. Both GH and IGF-I are known to increase eNOS activity in experimental settings and it has been suggested that this may have cardiovascular benefit[55,56]. However, studies on small arteries taken from patients with acromegaly indicate endothelial dysfunction, with reduced eNOS and endothelium-derived hyperpolarizing factor bioavailability[98]. This suggests that in acromegaly the small vasculature does receive the benefit that would be predicted from increased GH/IGF-I eNOS activation.

Cardiovascular mortality remains raised in patients with hypopituitarism who are receiving glucocorticoid, thyroxine and sex steroid replacement therapy; GHD is thought to be the main contributor to this discrepancy[99]. Indeed, GH replacement appears to improve this finding. Svensson *et al.*, in an open, prospective study, found that after three years of GH replacement mortality rates were no different to background population rates and were improved compared to mortality rates in an historical hypopituitary cohort not treated with GH[275]. In our study, only one patient with GHD had evidence of reduced perfusion with the induction of hyperaemia. Computerised tomography of the coronary arteries failed to show any flow-limiting lesions and the patient was managed with a primary prevention strategy. In addition, Patient 1, demonstrated evidence of a previous myocardial infarction on LGE. This patient had no known history of ischaemic heart disease. As he remained symptom-free, no intervention was performed.

The degree to which the mechanisms behind ischaemic heart disease contribute towards the development of acromegalic cardiomyopathy remains to be elucidated. Perfusion CMR indicates with a good sensitivity that, whatever is happening at the microvascular level, coronary artery flow-limiting lesions do not appear to be playing a role in the development of the abnormalities seen in this study. In acromegaly and GHD, the pharmacological control of any cardiovascular risk factors present seems prudent: in GHD a low threshold for statin therapy is indicated to counteract the raised LDL-cholesterol and in acromegaly tight diabetes control. In both conditions blood pressure should be closely monitored and kept with target ranges and smoking strongly discouraged[26,250].

4.8 Aortic area

It has been reported that acromegaly is associated with increased aortic diameter. Van der Klaauw *et al.* demonstrated increased aortic diameter at the level of the sino-tubular junction and ascending aorta in patients with acromegaly when compared to controls[81]. These findings did not improve with treatment for acromegaly. Savage *et al.* reported one case of increased aortic root diameter in a series of 25 patients with acromegaly[276]. Casini *et al.* showed increased aortic root diameter in patients with acromegaly compared to controls, noting that men had greater aortic root diameters than women and that aortic root diameter positively correlated with LVMi[277]. This study also demonstrated increased levels of aortic root ectasia in patients with acromegaly than in controls. All these studies used echocardiography to make these measurements. Levels at which measurements were taken are shown below in Figure 4.1, taken from Buchner *at al.*[278].

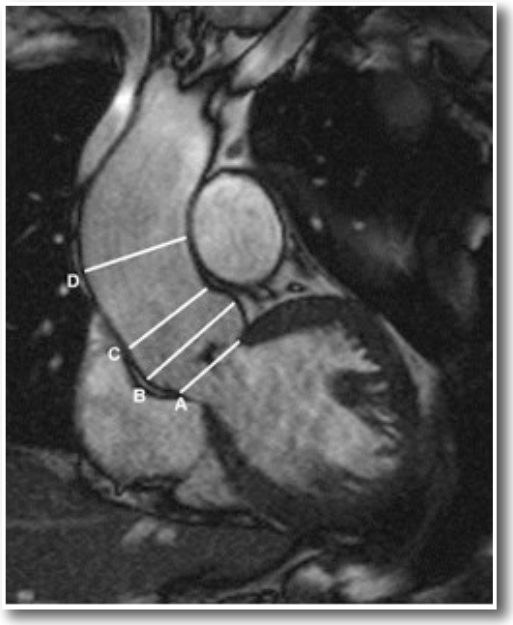

Figure 4.1 CMR oblique view showing the four aortic root diameter measurements at end diastole: A, aortic valve annulus (root); B, sinus of Valsalva; C, sinotubular junction; D, proximal ascending aorta. From Buchner *et al.*[279]

CMR is recognised as an accurate method for assessing aortic diameter; it has been used to study diseases such as Marfan's and Turners syndromes, both of which are associated with aortic abnormalities[279,280]. In this study, we measured the diameter of the ascending aorta in two planes and then calculated the area to allow for elliptical contours. Only one individual, a patient with GHD (Patient 21), had a significant aortic abnormity. This occurred due to he presence of a bicuspid aortic valve, a finding presumed to be incidental to his diagnosis of GHD.

No difference was seen in this study between ascending aorta areas in patients with acromegaly and their controls, even when findings were corrected for body surface area. In Savage's study only one patient out of 25 demonstrated increased aortic diameter, suggesting that this is not a frequent finding of acromegaly. We were not able to demonstrate the increase in ascending aorta diameter demonstrated in Van der Klaauw's sudy, in which 37 patients were compared to controls. Of these, 18 had active acromegaly and 19 were classified as having "inactive" acromegaly. No differences were found between patients with active acromegaly and those with inactive acromegaly, nor were any changes seen after one and a half years. At baseline, the combined acromegaly group had an ascending aorta that was on average 3 mm greater in diameter than controls. This very small difference may only have been significant due to the number of patients who took part in the study. The lack of significance seen in our study may reflect a difference in patient numbers.

Patients with GHD demonstrated significantly reduced ascending aorta area ($p=0.039$). When Patient 21, with a bicuspid aortic valve, was removed from the analysis, significance increased further ($p=0.0025$). Reduced aortic diameter has been demonstrated in adults with co-GHD, which is likely to reflect impaired development, particularly as it does not improve with GH replacement[281]. The only study that looked at aortic diameter in patients with mainly ao-GHD did not report any increase in aortic diameter with 60 months GH replacement but did not compare the aortic diameters of patients with GHD to those of healthy individuals[234]. To avoid variations in aortic diameter with the cardiac cycle measures are taken during diastole, so the reduced aortic distensibility seen in GHD should not be the cause of this finding[282]. GH replacement failed to increase aortic area and a significant difference persisted between patients and controls at one year. In published studies, even up to five years GH failed to increase aortic diameter[234]. The mechanisms behind the reduction seen in aortic area in patients are not clear. Given the novelty of this finding, further investigation would be valuable.

4.9 Inter-observer variability

Thirty-four patients' CMR scans were assessed by two independent observers blinded to diagnosis. The resulting Bland-Altman plots demonstrated very few data points more than two SD from the mean and for LVM and EDV the majority of data points lay close to zero, indicating very good correlation between the two sets of readings. A little more scatter was seen on the graphs for ESV and EF but still only three data points lay outside two SD.

Bland-Altman plots construct their points by subtracting two observers' measurements from one another, so that identical results give a score of zero. The further a point lies from zero, the more different the two measurements are. The degree of discrepancy that is acceptable is a clinical rather than a statistical decision, but it is recommended that results lie within two SD. Ninety-five percent of data points lay within two SD of the mean on these graphs.

No trend was seen in the data towards increased difference with increased mean measurement, nor did the scatter around the bias line get larger as the mean measurement increased, indicating consistent variability[283]. It was concluded that these results demonstrated a lack of bias and a lack of interobserver variability.

4.10 Conclusion & further work

This study importantly demonstrates that CMR can be used to identify and track the changes that occur in patients with GHD and acromegaly. There has been conflicting evidence regarding cardiac mass in patients with ao-GHD. The accurate assessment of LV mass by CMR allows for demonstration of low cardiac mass in females with ao-GHD and cardiac mass right at the bottom of the normal range in males with ao-GHD. Treatment with GH for one year caused a significant increase in LV mass. However, larger numbers are needed to demonstrate this finding in comparison to controls. Patients with acromegaly are known to demonstrate increased cardiac mass, a finding that was confirmed in this study. Patients underwent treatment for acromegaly; however, this was unsuccessful in controlling disease and LV mass remained increased at one year.

CMR provides a wealth of information regarding the cardiovascular system and here was able to demonstrate interesting findings, including reduced volume indices in patients with acromegaly receiving ACE inhibitors and ARBS and reduced aortic area in patients with ao-GHD. CMR was tolerated well and, in the case of one patient, was able to provide important information about reversible ischaemia, allowing for treatment prior to general anaesthetic.

CMR is useful for the identification of fibrosis following myocardial infarction. However, in acromegaly, where fibrosis is diffuse, CMR is not the optimum imaging modality for demonstrating this finding. Indeed, fibrosis may be missed if CMR alone is used for its identification in acromegaly.

Several areas have arisen as a result of this study that would benefit from further investigation and are listed below.

1. The comparison of CMR and echocardiographic findings

Patients who took part in this study also underwent echocardiography at each Visit. These results will be analysed and compared to CMR findings. This will allow for comparison of LV mass and volume between the two modalities and will assess for evidence of diastolic dysfunction, an early findings in acromegalic heart disease that is not well demonstrated using current CMR techniques.

2. Further CMR work addressing the effect of GHD on LV mass and volume

The results from this study suggest LVMi and EDV are reduced in ao-GHD. Andreassen's recent study demonstrated similar findings, although in that study the reduction in LVMi did not reach significance. A larger study, powered to allow for the separate analysis of men and women separately, would help address this finding further.

3. Impact of age on the cardiac consequences of GHD

Hypertension and ischaemic heart disease are common causes of cardiac disease in older patients. There are indications from several studies that patient age may impact on the findings seen, with changes more easily identifiable in younger patients than in older ones. This suggestion is confounded by the fact that studies in younger patients are more likely to include individuals with co-GHD. A study designed to measure CMR-determined cardiac changes in patients ao-GHD stratified into younger and older ages bands would be helpful in addressing this hypothesis further.

4. Aortic dimensions in patients with GHD

This study is the first to demonstrate reduced ascending aorta area in patients with ao-GHD. The mechanism of this finding remains unclear, particularly as aortic areas to do not increase with GH therapy in the intermediate timeframe. It would seen important to confirm this finding with further CMR studies in patients with ao-GHD. Animal studies using *dw/dw* rats may be helpful in determining the mechanism by which this finding occurs and would allow for histological examination of the aortic tissue.

5. The impact of ACE inhibitors on acromegalic cardiomyopathy

The finding that patients with acromegaly in this study taking ACE inhibitors or ARBs have lower EDV than those not taking this family of drugs suggests that these agents may have disease modifying actions. However, the numbers in this study were insufficient to draw statistically and clinically significant conclusions. Ideally, this should be studied using a randomised control trial. Given that current guidelines from the Italian COMETA study group already recommend these drugs as first-line antihypertensives, there may be ethical issues in producing such a study. However, there may be a role for treating non-hypertensive patients with acromegaly with ACE inhibitors or ARBs, with a view to determining if these should be given to all patients with acromegaly. It may also be possible to examine this hypothesis in mice, using bGH-transgenics and murine CMR.

The effects of growth hormone dysregulation on adenosine monophosphate-activated protein kinase in cardiac tissue

Objectives

- To determine the impact of acute GH and IGF-I excess on the cardiac AMPK activity in the GHD *dw/dw* rat and matching wild-type rats
- To determine the impact of chronic GH excess on the cardiac AMPK activity in GH3 cell-implanted rats and transgenic bGH-overexpressing mice
- To determine the impact of chronic GH deficiency on the cardiac AMPK activity in GHRKO transgenic mice
- To use primary neonatal rat cardiomyocytes to confirm the effects of GH and IGF-I on cardiac AMPK activity
- To compare cardiac AMPK activity, as measured by a functional assay, with Western blotting for phosphorylated AMPK levels in cardiac tissue and cells

5 Materials and Methods

- Full protocols are given in Appendix II
- Italics indicate buffers and mixes whose exact components are given in Appendix II
- All chemicals were supplied by Sigma unless specified otherwise

5.1 Experiment summary

To determine if any changes in cardiac AMPK activity occur with alteration in GH level, a pilot study was planned. This involved the treatment of C57BL mice with six weeks GH treatment. This pilot was performed as part of a larger study designed to investigate the effect of GH on obesity in high fat diet fed mice, in which the cardiac tissue was not required, and hence would be available for the measurement of AMPK activity. Mice fed with high fat diet demonstrated and increase in cardiac AMPK activity when treated with GH. However, in this study, no mice fed with normal chow received GH. The experiment was therefore repeated, this time containing a normal chow-fed arm treated with GH. In the repeat, neither the chow-fed nor the high fat diet animals demonstrated an increase in cardiac AMPK activity with GH treatment. It was not clear why this difference was found between the two experiments. As the author was already focussing on the effect of GH on cardiac tissue, and studies into the effect of high fat diet had never been planned, it was decided that only normal chow fed animals should be investigated in future experiments. Furthermore, it was not clear whether the six week GH treatment reflected acute or chronic effects of GH on the heart. To be better able to distinguish these effects, two separate groups of experiments were planned, to look at these timepoints separately. It was also decided at this time to focus on the effect of GH on cardiac AMPK activity. Other tissues collected from animals would not be studied for this thesis.

The second animal model to be studied was the GH3-implanted rat. These animals were raised, treated and sacrificed by Dr Herbert Schmid, Novartis. The experiment was initially designed to address the effects of various somatostatin analogues on tumour size and disease control in GH3 rats. The investigators provided cardiac tissue from these animals for AMPK measurement. The timing for this experiment was determined by Dr Schmid and was determined prior to the results from the CB57 black mice being available.

In order to address the acute effects of GH on cardiac AMPK activity, rats were injected with IP GH or IGF-I. To amplify any changes that might be seen, this was initially performed in GH deficient dwarf (*dw/dw*) mice. However, when the experiments were repeated in wild-type rats, results were disconcordant. Hence, the remainder of the acute studies were performed in WT animals.

To examine the chronic effects of GH excess and deficiency on cardiac AMPK, two and eight month-old bGH-overexpressing and GHRKO mice were studied. These timepoints were chosen to correlate with published studies describing the cardiac structure and function in bGH mice. The ages were felt to represent the early and late effects of GH excess and deficiency. The results from these models were disconcordant with a simultaneously published study looking at levels of pAMPK in the cardiac tissue of bGH mice. As only cardiac AMPK activity had been measured in our tissues, they were re-studied by Western blotting for pAMPK levels. This technique was then also applied to the cardiac protein solutions from the acute studies.

The opportunity to work with neonatal rat cardiomyocytes became available towards the end of the studies. This allowed for confirmation of the findings of the acute studies in an alternative model of GH excess, and allowed the focused examination of the effects of GH and IGF-I on the isolated cardiomyocyte. Both cardiac AMPK activity and pAMPK levels were measured in this experiment.

5.2 Animal models

5.2.1 C57BL/6J and transgenic mice

Male wild-type (WT) C57BL/6J mice and GH transgenic mice were supplied by Prof J Kopchick, Ohio University, Athens, Ohio, USA. Bovine-GH-overexpressing heterozygotic transgenics were produced by microinjection of a gene fusion of a mouse metallothinein transcriptional regulatory element and bGH cDNA containing the first intron into the pronucleus of C57BL/6J embryos. DNA analysis was used to confirm genotype. Age-matched littermates were used as controls. This model is fully described in Berryman *et al*.[284] The GH receptor KO mouse (GHRKO) was made by disruption of the fourth exon of the GHR/binding protein gene by homologous recombination. These mice were backcrossed for eight generations with C57BL/6J mice to give transgenics that were >99.61% congenic for the

C57BL/6J background. DNA analysis was used to confirm genotype. Age-matched littermates were used as controls. This model was originally published by Zhou *et al*.[285]

Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Animals were housed and treated at the Animal Facility of Ohio University (Athens, Ohio, USA). Sacrifice and dissection was performed by the staff of Ohio University. All procedures were approved by the Ohio University Institutional Care and Use Committee and fully complied with federal, state, and local policies and UK import laws.

5.2.2 Dwarf rats

Male AS and dwarf rats were supplied by Prof I Robinson and Dr D Carmignac, the National Institute for Medical Research, Mill Hill, UK. The dwarf (*dw/dw*) rat arose as a consequence of a spontaneous autosomal recessive mutation; the line has been continued for research purposes[60]. Normal age-matched rats of the same mixed background strain (AS) were used as control animals.

Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Animals were housed and treated at the Animal Facility of the National Institute for Medical Research, Mill Hill, UK in line with local and national animal care policies. Sacrifice was performed by Dr D Carmignac, dissection was performed by the author.

5.2.3 GH3-tumour implanted rats

GH3-tumour implanted rats were supplied by Dr H Schmid, Novartis, Basil, Switzerland. This is a long-established animal model of acromegaly in which cells from the GH and prolactin secreting GH3 cell line, a rat pituitary cell line, are injected subcutaneously in the flank of a rat[57]. Here they increase in size, exposing the animal to excess GH, simulating acromegaly. In this case, female Wistar-Furth (WF) rats were injected with 5 $x10^6$ GH3 cells in 100μl diluent (50% Hank's balanced salt solution / 50% Matrigel [BD Biosciences]) subcutaneously, in the right flank. Once tumour size was 100-200mm^3 (43-51 days) somatostatin analogue treatment was given. Animals were treated with vehicle only or the somatostatin analogues octreotide long-acting release (LAR) or pasireotide LAR. Animals were sacrificed day 35 post treatment with vehicle or somatostatin analogue.

Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Animals were housed and treated at the Animal Facility, Novartis, Basil, Switzerland, in line with local and national animal care policies. Sacrifice and dissection was performed by the staff at Novartis.

5.3 Neonatal rat primary cardiomyocytes

Three-day old Sprauge Dawley rat pups (Charles River) were sacrificed by cervical dislocation. The heart was rapidly removed and placed in *ADS buffer* on ice. Hearts were minced using fine scissors. *Enzyme solution*, containing collagenase (Worthington) and pancreatin was added and the mixture was incubated in a 37°C. The supernatant, containing blood and debris, was discarded. Fresh enzyme was added and the tube returned to the waterbath. After twenty minutes, the supernatant, this time containing released cells, was removed and re-suspended in a tube of foetal bovine serum (FBS), to inactivate the *enzyme solution.* The sample underwent four further cycles of fresh enyme solution, incubation and supernatant aspiration, each time adding the supernatant to the flask containing FBS. The contents of the flask were spun and the cell pellet re-suspended in warm FBS and incubated. Cells were plated in Primaria (BD Biosciences) petri dishes and cultured for one hour, prior to cells being replated and incubated. Experiments were performed once cardiomyocytes were seen to be spontaneously beating on microscopy[286,287].

This technique was performed under the supervision of, and with assistance from, Dr S Brouilette, Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Queen Mary University of London.

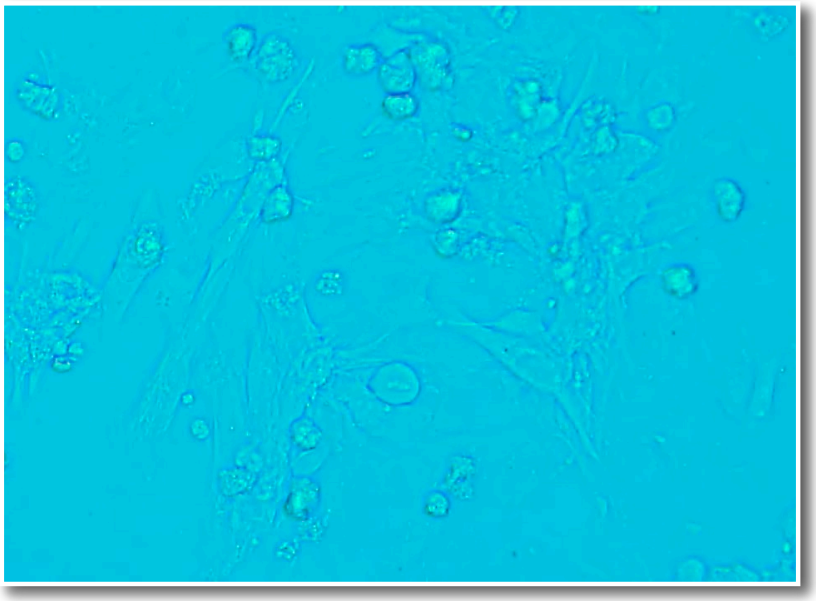

Figure 5.1 Neonatal cardiomyocytes. ***Courtesy of Dr Scott Brouilette***

5.4 Protein preparation

5.4.1 Protein solution preparation from tissues

Organs from sacrificed animals were flash frozen in liquid nitrogen and stored at minus 80°C. Tissue was homogenised in *tissue lysis buffer* (see Appendix for details) using the Precellys system (Stretton Scientific), which utilises ceramic beads and high-speed agitation. Protein content was determined using a bicinchoninic acid (BCA) kit (Pierce Perbio Science, #23227) and a Wallac plate reader.

5.4.2 Protein solution preparation from cells

Once cells had undergone treatment, the contents of each well was lysed with *cell lysis buffer* and wells were scraped. Well contents were aspirated and then spun at 10,000 rpm for five minutes. The fluid contents of the tube were aspirated and stored. Protein content was determined as above.

5.5 Functional AMPK Assay

AMPK protein was immunoprecipitated from cardiac tissue homogenate using G-protein beads (Amersham, #17-0618-01); 300 μg was mixed with AMPK α1 and α2 antibodies (Prof G Hardie, University of Dundee). This was divided into three aliquots. The first two were assayed for AMPK activity with *Positive Master Mix,* a γ^{32}P-ATP (Perkin-Elmer, #NEG002A250UC) containing reaction solution containing SAMS (Upstate, #12-355). SAMS is a synthetic substrate for AMPK which exactly corresponds to the AMPK binding site of cyclic-AMP-dependent protein kinase (Serine77) and is highly specific[288]. The assay measures the ability of the AMPK contained within the protein to phosphorylate the SAMS contained within the reaction solution, by measuring the amount of γ^{32}P transferred into the SAMS, hence determining AMPK activity. The third aliquot was assayed with *Negative Master Mix*, an identical reaction solution with the exception that it contains *Herpes-Brij buffer* in the place of SAMS. This determines the background level of AMPK activity, reflecting autophosphorylation, immunoglobulin phosphorylation and residual peptides not removed by immunoprecipitation, and acts as a negative control.

The reaction was performed on a shaker for 20 minutes at 30°C. Samples were then pipetted onto squares of P81 phosphocellulose papers (Millipore) and dropped into 1% orthophosphoric acid, to terminate the reaction. Phosphorylated proteins, including SAMS, bind to the paper, while the remainder of the reaction solution is rinsed away. Papers were dried and then counted by liquid scintillation (Wallac 1409 DSA). AMPK activity was calculated using the difference in counts between the SAMS positive and the SAMS negative samples, expressed as nmol of ATP incorporated per minute per mg of protein.

5.6 Western blotting

Western blotting was performed using 15-40 μg protein per lane, depending on the concentration of the protein and the well volume. When total and phospho-protein were being compared, two identical gels were loaded and run simultaneously, to reduce the need for stripping. SDS loading buffer was added to the maximum volume that the well would contain. Samples were briefly centrifuged and then heated to 95°C for 5 minutes to denature the protein. Samples were centrifuged briefly again and then placed immediately on ice. Proteins were separated by electrophoresis on bis-tris (Invitrogen) gels. With the exception of pACC blots, bis-tris 4-12% gradient gels were used. A pre-stained molecular weight marker was run in parallel. A commercial 2-(*N*-morpholino)ethanesulphonic acid (MES) running buffer was used (Invitrogen). Gels were run for 15 minutes at 95 volts and then for an hour at 125 volts. Larger proteins were run for longer in an ice box.

Separated proteins were transferred to a nitrocellulose gel using a semi-dry transfer, run for 40 minutes at 0.4 A for a single gel or 0.8 A for two gels. The membrane was placed in a 50ml falcon tube with 40ml of milk-containing *blocking buffer*. This was rolled at room temperature for 90 minutes to block non-specific binding sites. The buffer was removed and primary antibody, diluted in blocking buffer, was added to the membrane. Table 5.1 lists the primary and secondary antibodies used in these experiments. The membrane was incubated with primary antibody overnight at 4°C. The membrane was then washed with *washing buffer* before a secondary antibody was added, again diluted in *blocking buffer*. As the secondary antibody was light sensitive, the falcon tube was wrapped in aluminium foil. This rolled at room temperature for 90 minutes and then washed with *washing buffer*.

Primary Antibodies	Company	Dilution
Phospho-ACC (Ser79), Rabbit mAb	Cell Signalling, #3661	1:1000
GAPDH (FL-335), Rabbit pAb	Santa Cruz, #25778	1:2000
AMPKα (23A3), Rabbit mAb	Cell Signalling, #2603	1:1000
Phospho-AMPKα (Thr 172), Rabbit mAb	Cell Signalling #4188	1:1000
Secondary Antibodies		
Anti-rabbit IgG (800CW), Goat pAb	Li-Cor, #926-32211	1:10,000

Table 5.1 Primary and secondary antibodies

The Odyssey Infrared Imaging System (Li-COR Biosciences) was used to read the membrane. The densitometric readings of the resultant bands were analysed using ImageJ (Image Processing and Analysis in Java) or directly using the internal Li-COR software.

5.7 Statistics

Data were analysed using GraphPad Prism 5.0a. The Student's t-test for single comparisons and ANOVA was used for multiple comparisons. Figures are shown as mean ± SEM. A p value of < 0.05 was considered significant. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

5.8 Suppliers

Company	Address
Amersham	GE Healthcare Life Sciences, Amersham Place, Little Chalfont, Bucks. HP7 9NA
BD Biosciences	Edmund Halley Road - Oxford Science Park, Oxford. OX4 4DQ
Calbiochem	c/o CN Biosciences UK Ltd, Boulevard Industrial Park, Padge Road, Beeston, Nottingham. NG9 2JR
Cell Signalling	New England Biolabs (UK) Ltd. 75-77 Knowl Piece Wilbury Way, Hitchin, Hertfordshire. SG4 0TY
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley. PA4 9RF
Perkin-Elmer	Chalfont Road, Seer Green, Buckinghamshire. HP9 2FX
Pierce Perbio Science	Thermo Fisher Scientific, p/a Perbio Science UK Ltd, Unit 9 Atley Way, North Nelson Industrial Estate Cramlington, Northumberland NE23 1WA
Li-Cor	Li-Cor Biosciences Uk Ltd, St Johns Innovation Centre, Cowley Road, Cambridge, Cambridgeshire, CB4 0WS
Millipore	Suite 3 & 5, Building 6, Croxley Green Business Park, Watford. WD18 8YH
Pharmacia	c/o Pfizer Ltd, Ramsgate Road, Sandwich, Kent. CT13 9NJ
Santa Cruz	Santa Cruz Biotechnology, Inc., Bergheimer Str. 89-2, 69115 Heidelberg, Germany
Sigma	The Old Brickyard, New Road, Gillingham, Dorset. SP8 4XT
Stretton Scientific	Stretton House, Highstairs Lane, Stretton, Derbyshire. DE55 6FD
Upstate	c/o Millipore, Suite 3 & 5, Building 6, Croxley Green Business Park, Watford. WD18 8YH
Wallac	c/o Perkin-Elmer, Chalfont Road, Seer Green, Buckinghamshire. HP9 2FX

Table 5.2 Suppliers of chemicals, equipment and antibodies

6 Pilot project: the effect of 6 weeks bGH treatment on cardiac AMPK activity

6.1 Outline

To assess the effect of GH on cardiac AMPK activity, 25 week-old C57BL/6J black mice were treated with subcutaneous (sc) bGH, 5 μg/g bodyweight daily for 6 weeks. Animals were assigned to one of three treatment arms: normal diet + saline, high fat diet (HFD) + saline, HFD + GH. Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Mice were bred and raised as described previously. Mice were sacrificed by cervical dislocation and tissues flash frozen in liquid nitrogen. Tissues were homogenised and protein content determined and cardiac and liver AMPK activity was measured by functional assay run in triplicate, using 300 μg per sample, as per the protocols in *Materials & Methods* (page 126).

6.2 Results

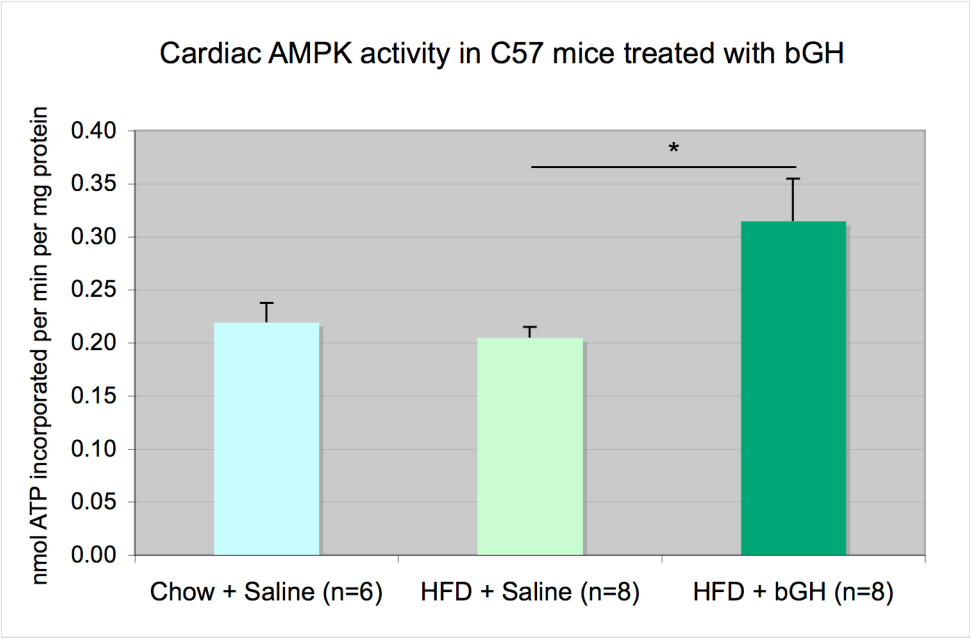

Figure 6.1 Mean cardiac AMPK activity in black mice treated normal chow or HFD and 6 weeks bGH treatment. ANOVA 0.0132. GH treatment caused a significant increase in AMPK activity. Error bars show SEM.

Results demonstrated a significant difference (ANOVA p=0.0132) in cardiac AMPK activity between the three groups (Figure 6.1) and a significant increase in AMPK activity in HFD + GH animals when compared to those treated with saline (0.316 ± 0.04 *v.* 0.205 ± 0.01 nmol ATP incorporated/min/mg protein). There was no difference seen in liver AMPK activity (Figure 6.2).

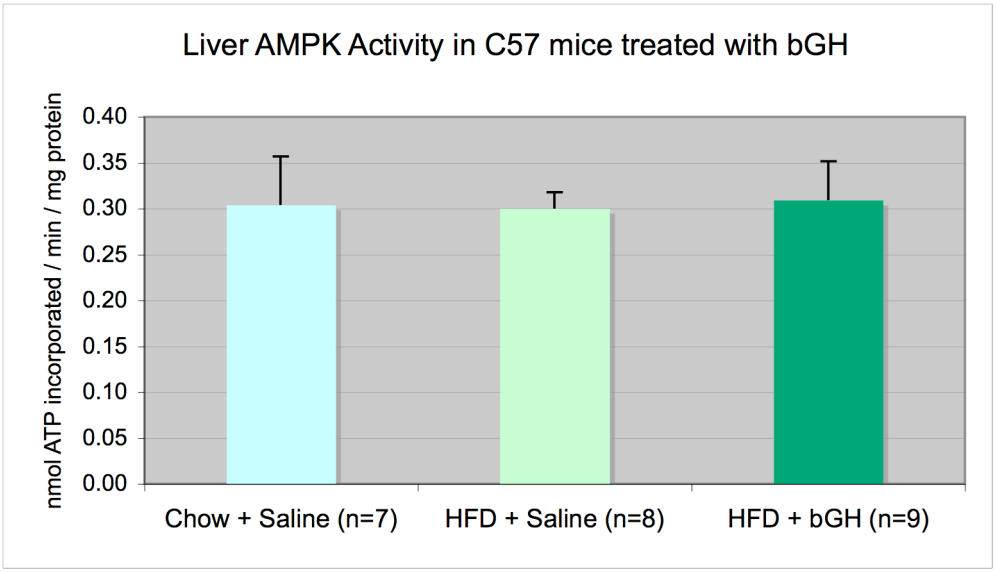

Figure 6.2 Liver AMPK activity in black mice treated with normal chow or HFD and 6 weeks bGH treatment. No difference was seen between groups.

6.3 Summary

The pilot study indicated that GH treatment is able to increase cardiac AMPK activity. However, the diets given to these animals confounded results that could be drawn and it was not clear whether bGH would have an effect on AMPK activity in mice fed a normal diet. Therefore, the experiment was repeated, this time including a normal diet + bGH group. As discussed in Section 5.1, this experiment formed part of a larger study, seeking to address the effects of GH on obesity in high-fat fed mice, in which the cardiac tissue was available. The focus of our study was the effect of GH, rather than the effect of high-fat diet.

The liver was analysed in this study as an example of metabolically active tissue. As GH treatment did not produce a significant effect on liver AMPK activity, and as the focus of our clinical study was the effect of GH on the heart, it was decided to focus on cardiac tissue and cells in future experiments.

7 The effect of bGH treatment on cardiac AMPK activity of C57BL/6J mice receiving normal or high fat diet

7.1 Outline

Three-week old C57BL/6J mice were divided into two groups and received either normal chow or high fat diet (HFD) for 16 weeks. The diet was then continued but half the mice in each diet group also received bGH 5 μg/g bodyweight sc daily for 6 weeks. Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Animals were sacrificed at 25 weeks by cervical dislocation. Tissues were homogenised and protein content determined. Cardiac AMPK activity was measured by functional assay run in triplicate, using 200-300 μg per sample, as per the protocols in *Materials & Methods*. Western blotting was performed on cardiac tissue lysates for pACC using 40 μg protein per lane.

7.2 Results

Neither normal chow-fed or HFD animals showed a significant difference in cardiac AMPK activity after 6 weeks bGH treatment when compared with saline-treated controls (ANOVA p=0.9425, Figure 7.1). However, pACC was significantly increased in the bGH treated animals (bGH 6908 ± 886 v. controls 1766 ± 268 arbitrary density units; p=0.0005) suggesting exposure to increased AMPK activity (Figure 7.2). These membranes did not respond well to stripping and re-blotting for GAPDH and so no housekeeping genes are recorded. The size difference between pACC (257kDa) and GAPDH (37kDa) limits demonstration of both proteins on the same membrane. The Western blot was repeated but this time demonstrated only multiple, non-specific bands, presumed to relate to protein degradation (Figure 7.3). Insufficient residual tissue was available to re-homogenise further protein solution.

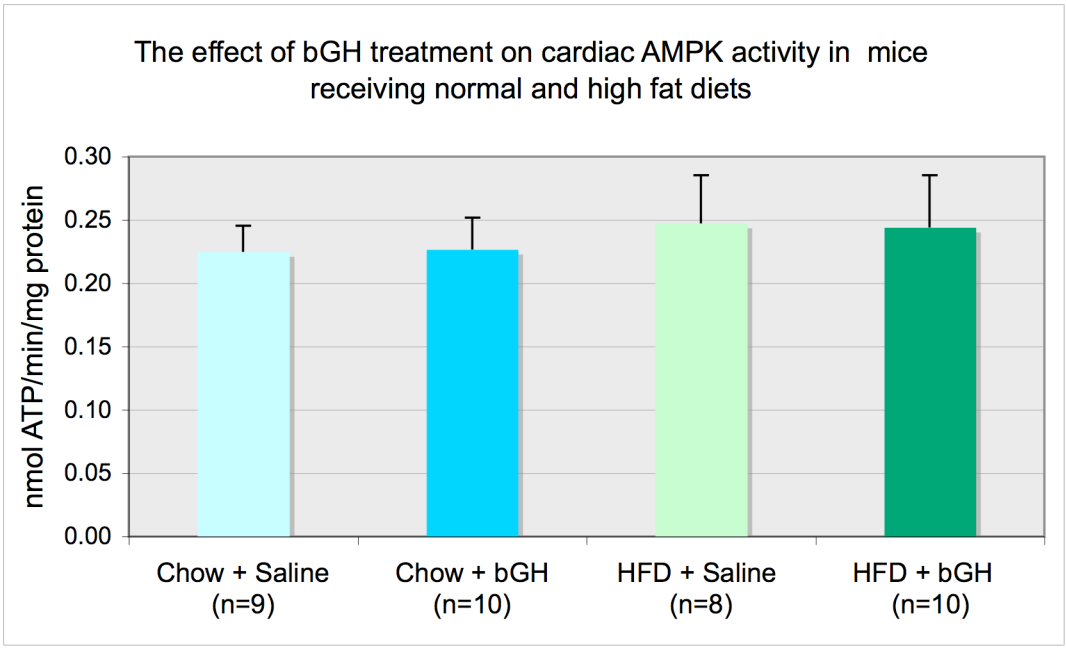

Figure 7.1 Cardiac AMPK activity in chow or HFD mice treated with bGH. No differences was seen between the groups (ANOVA, p=0.943)

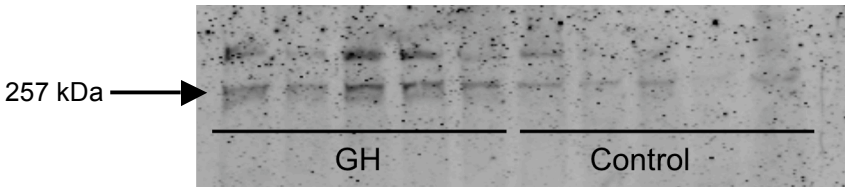

Figure 7.2 Normal chow-fed mice treated with 5 μg/g bodyweight/day bGH (left) or saline (right), demonstrating increased pACC (250 kDa) in bGH treated animals (p=0.0005). Gel loaded with 40 μg protein per well.

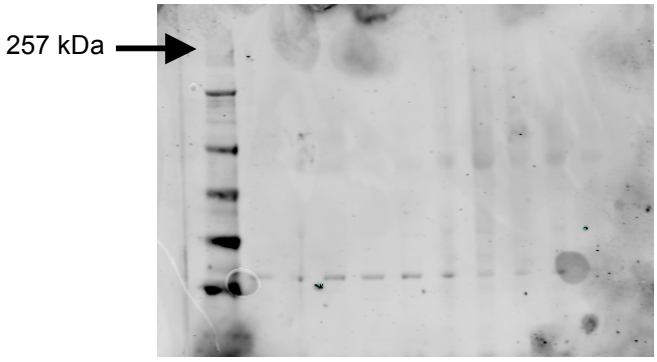

Figure 7.3 Normal chow-fed mice treated with 5 μg/g bodyweight/day bGH or saline. Non-specific bands are seen throughout the gel. There are no distinct bands at 257 kDa, which would correspond with pACC.

7.3 Summary

This experiment failed to reproduce the AMPK activity findings of the pilot study, despite being done under identical conditions. The reason for this discrepancy was not clear. It was unclear whether six weeks of treatment reflected the acute effects of GH on AMPK activity or the early chronic effects. Therefore, experiments were designed to examine the acute effect of GH/IGF-I on cardiac AMPK activity in dwarf and WT rats, and separate studies in GH3-tumour implanted rats and transgenic mice were designed to address the chronic effect of GH excess and deficiency on the heart.

As discussed in Section 5.1, this experiment formed part of a larger study, seeking to address the effects of GH on obesity in high-fat fed mice, in which the cardiac tissue was available. The focus of our study was the effect of GH, rather than the effect of high-fat diet.

8 Acute studies: The impact of acute growth hormone treatment on cardiac AMPK activity

8.1 Outline

Six week old *dw/dw* dwarf rats (weighing approximately 100 mg) were treated with 50 μg hGH intraperitoneally (ip) and then sacrificed at 1 hour, 6 hours or 24 hours. Control dwarf rats were treated with saline. Animals were sacrificed by cervical dislocation. Tissues were collected and flash frozen in liquid nitrogen. Hearts were quickly rinsed in saline prior to freezing to remove excess blood. Cardiac AMPK activity was measured by functional AMPK assay. As results were significant at 6 hours, the experiment was repeated at this timepoint but this time giving 25 μg recombinant human IGF-I. Dwarf rats were chosen for this study to amplify any changes that might be seen. To confirm the findings in animals with intact GH/IGF-I axes, the 6 hour experiments were repeated in six week old WT AS rats (weighing approximately 120 g). As results with IGF-I treatment were not significant in these animals at 6 hours, an IGF-I dose-curve was performed. The results are given as percentage of controls to allow comparison between experiments performed on different days.

8.2 Results

GH injection produced a *fall* in cardiac AMPK activity in dwarf rats that did not reach significance at 1 hour (p=0.10) but did achieve significance by 6 hours (71% of control value, p=0.0038) and remained highly significant at 24 hours (62% of control value, p = 0.0003, Figure 8.1).

Similar results were seen when the experiment was repeated at 6 hours using 25 μg human IGF-I instead on GH, with an 20% fall in cardiac AMPK activity compared to controls (p=0.0025, Figure 8.2). This dose was chosen as previous work by Prof Robinson's group indicated that a 50% decrease in dose is necessary for equivalent effect when using IGF-I instead of GH (personal communication from Prof ICAF Robinson).

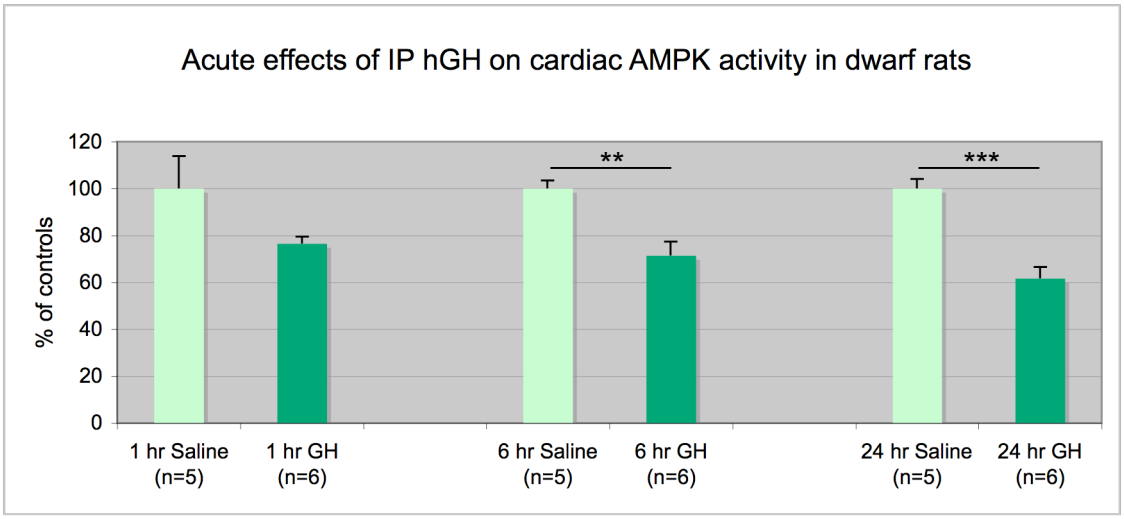

Figure 8.1 The acute effect of hGH on 6 week old dwarf rats when compared to saline-treated controls, demonstrating a trend towards a reduction at 1 hour and a significant fall in cardiac AMPK activity by 6 hours, which decreased further by 24 hours.

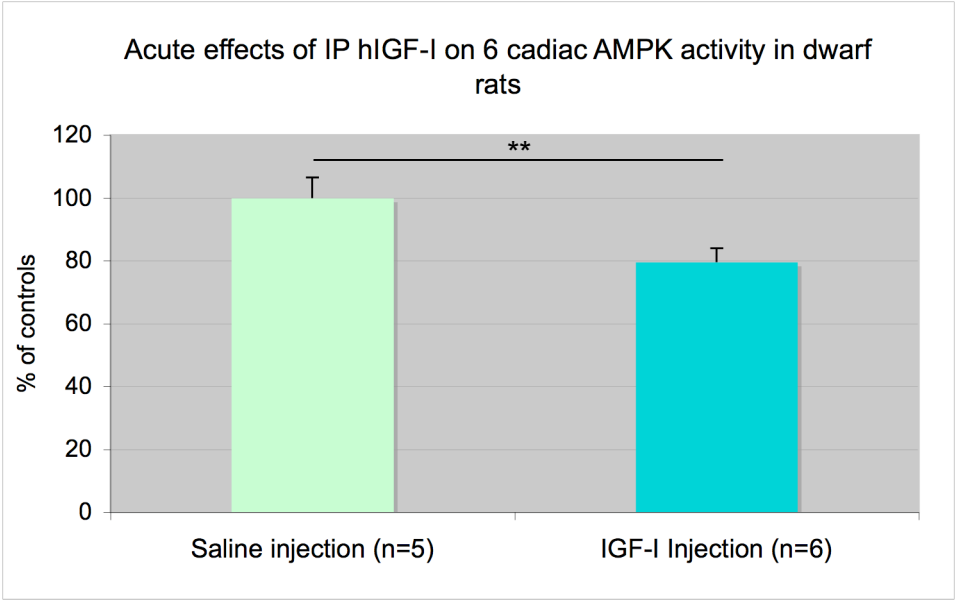

Figure 8.2 The acute effect of hIGF-I on 6 week old dwarf rats compared to saline-treated controls, demonstrating a significant fall in AMPK activity in animals treated with hIGF-I for 6 hours

Surprisingly, when the 6 hour experiments were repeated with WT AS rats, GH treatment had the reverse effect and resulted in a marked *rise* in cardiac AMPK activity (208% of controls, p=0.0027, Figure 8.3). In WT rats, 25 μg IGF-I IP failed to cause a significant change in AMPK activity (p=0.658, Figure 8.3), possibly due to a lower dose/gram bodyweight in these larger rats. Therefore, a dose curve was performed using 50 μg and 100 μg human IGF-I IP. Both of these doses resulted in a significant fall in the cardiac AMPK activity (84.64% and 84.04%, respectively), in line with findings in dwarf rats but of a lesser magnitude (Figure 8.4).

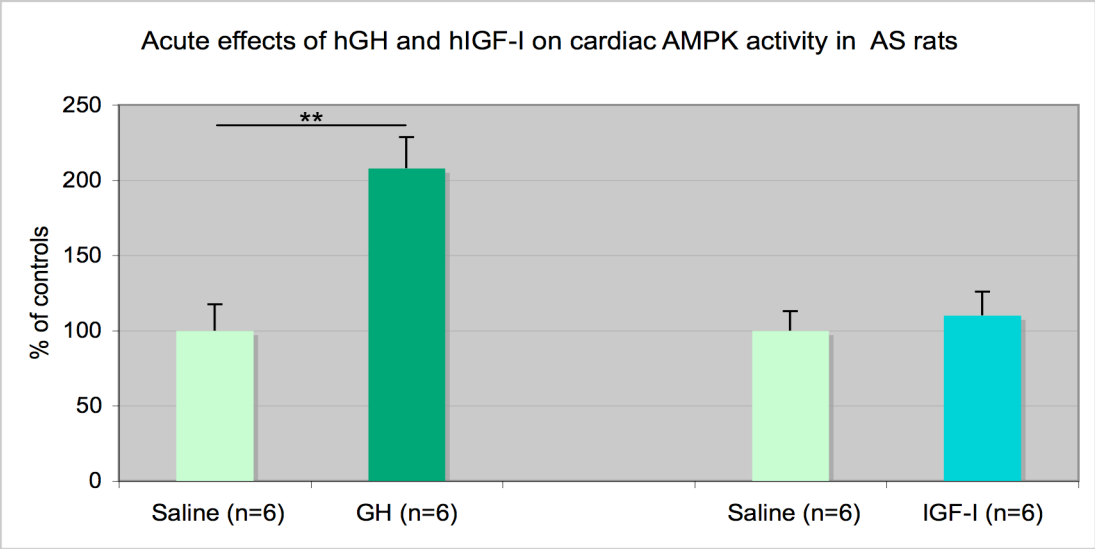

Figure 8.3 The acute effects of 50 μg hGH and 25 μg hIGF-I on 6 week old AS rats showing a marked rise in cardiac AMPK activity at 6 hours with GH but no change with this dose of IGF-I.

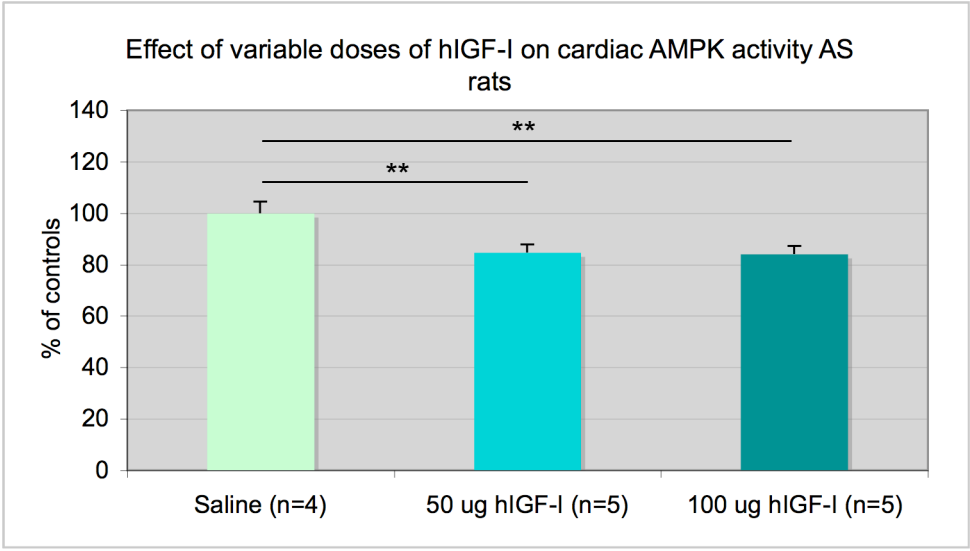

Figure 8.4 Treatment of 6 week old AS rats with variable doses of hIGF-I demonstrating a significant fall in cardiac AMPK activity at 6 hours with both 50 μg and 100 mg doses. ANOVA, p=0.011.

When WT and dwarf rats were compared directly with body-weight adjusted GH doses, there was no significant difference seen between WT rats treated with saline and WT rats treated with GH (p=0.690), and no significant difference between dwarf rats treated with saline and dwarf rats treated with GH (p=0.209, Figure 8.5). There was no difference in cardiac AMPK activity between WT rats treated with saline and dwarf rats treated with saline (p=0.930). The multiple groups in this experiment meant each group contained small numbers.

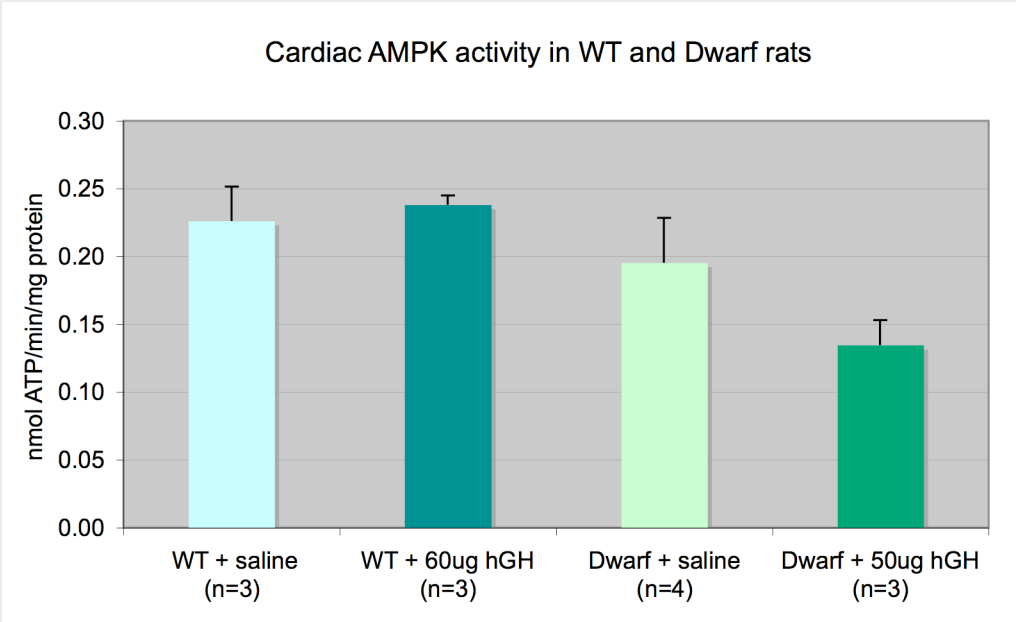

Figure 8.5 Cardiac AMPK activity in WT (AS) or dwarf rats treated with body-weight adjusted doses of saline or hGH IP for 6 hours, demonstrating no significant difference between WT rats treated with saline and those treated with GH (p=0.690) and no significant difference between dwarf rats treated with saline and those treated with GH (p=0.209). There was no difference between cardiac AMPK activity in WT and dwarf rats each treated with saline (p=0.930), suggesting that dwarf rats to do not have difference baseline cardiac AMPK activity than WT rats.

8.3 Summary

The initial hypothesis that acute GH exposure causes an acute increase in cardiac AMPK activity was confirmed in AS rats, when 50μg hGH IP was able to induce a marked rise in AMPK activity at 6 hours. However, surprisingly the converse finding was seen in dwarf rats. The dwarf rat was chosen as the initial model for this experiment as it was predicted that any results seen would be amplified by the GH-deficient state. When experiments were repeated with WT animal, converse results were found. This demonstrates the need for vigilance when drawing conclusion from models with altered physiology.

Experiments with both dwarf and WT rats demonstrated a subtle but significant fall in cardiac AMPK activity with an optimised dose of hIGF-I treatment. The finding in WT rats that GH and IGF-I induce opposite effects on cardiac AMPK activity suggests that GH and IGF-I have different direct actions on AMPK. Furthermore, whereas GH has an opposite effect on cardiac AMPK activity in dwarf rats when compared to WT animals, the ability of IGF-I to lower cardiac AMPK activity persists in both models.

9 Chronic GH dysregulation model I: GH3-implanted rats

9.1 Outline

Female WF rats were implanted with 5 x10^6 GH3 cells, sc into the right flank. When tumour size measured between 100-200 mm^3, animals were treated with vehicle, octreotide LAR 80 mg/kg sc once or pasireotide LAR 80 mg/kg sc once. Control animals received the same treatments with the exception that they were not implanted with a GH3 tumour. Body weight and tumour size were monitored and blood was taken for glucose levels. Animals were kept in cages on 12 hour light – 12 hour dark cycles and had free access to food and water. Sacrifice occurred 35 days after treatment under isoflurane 5% anaesthesia. Tissues were frozen in liquid nitrogen. Animals were bred, treated and sacrificed by staff at Novartis, Basel, Switzerland.

9.2 Results

GH3-implanted animals showed a marked increased in body weight when compared to animals without tumours (GH3 & vehicle 354 ± 10.5 g *v.* Vehicle Only 187 ± 9.75 g) demonstrating clinical evidence of GH excess (Figure 9.1). There was no difference in body weight between GH3-implanted rats treated with vehicle and those that received somatostatin analogues (incremental area under curve).

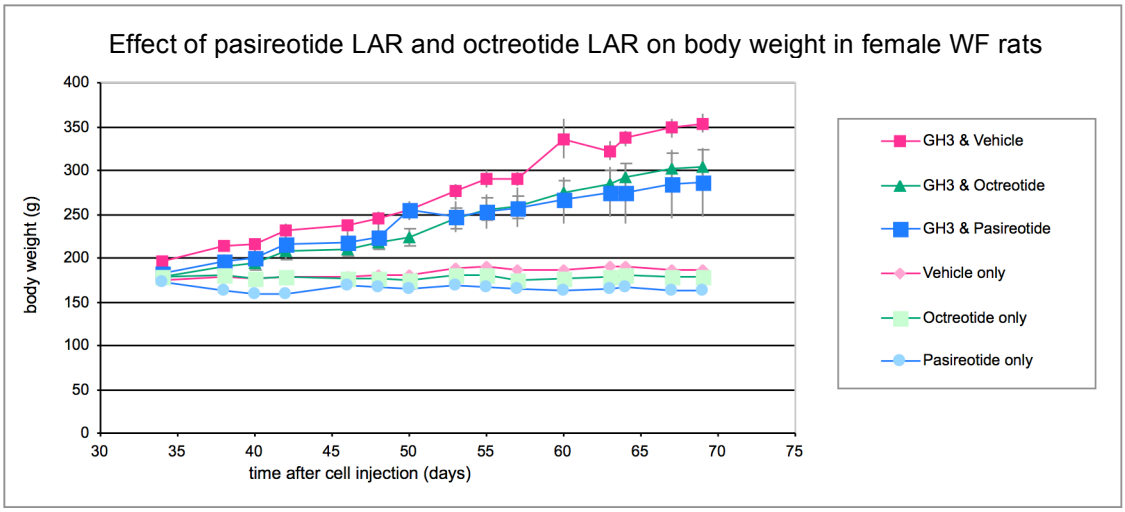

Figure 9.1 The effect of GH3 tumour implantation on WF rats demonstrating a clear increase in body weight with GH3 implantation, which was not ameliorated by either octreotide or pasireotide. ANOVA, p<0.0001

Cardiac AMPK activity was significantly different between different groups (ANOVA, p=0.0002). There was no difference in cardiac AMPK activity between Vehicle Only and GH3 & Vehicle groups but there was a marked reduction in AMPK activity in each group treated with somatostatin analogues, Octreotide LAR and Pasireotide LAR, when compared to their relevant control group (Figure 9.1). Neither of the somatostatin analogues was able to induce a significant reduction in GH3 tumour size (GH3 Only *v.* GH3 & Octreotide *v.* GH3 & Pasireotide, mean tumour volume 8.95 ± 1.78 *v.* 5.35 ± 1.01 *v.* 5.71 ± 2.82 cm^3 respectively, ANOVA p = 0.4129, Figure 9.2).

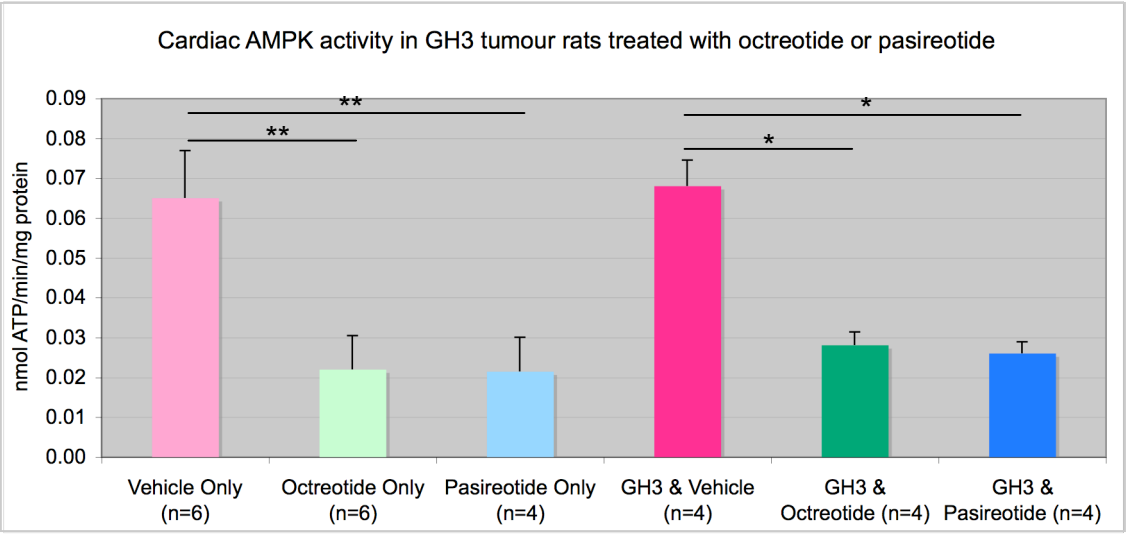

Figure 9.1 Cardiac AMPK activity in GH3-implanted WF rats demonstrating a fall in AMPK activity with both octreotide and pasireotide treatment. ANOVA, p=0.0002.

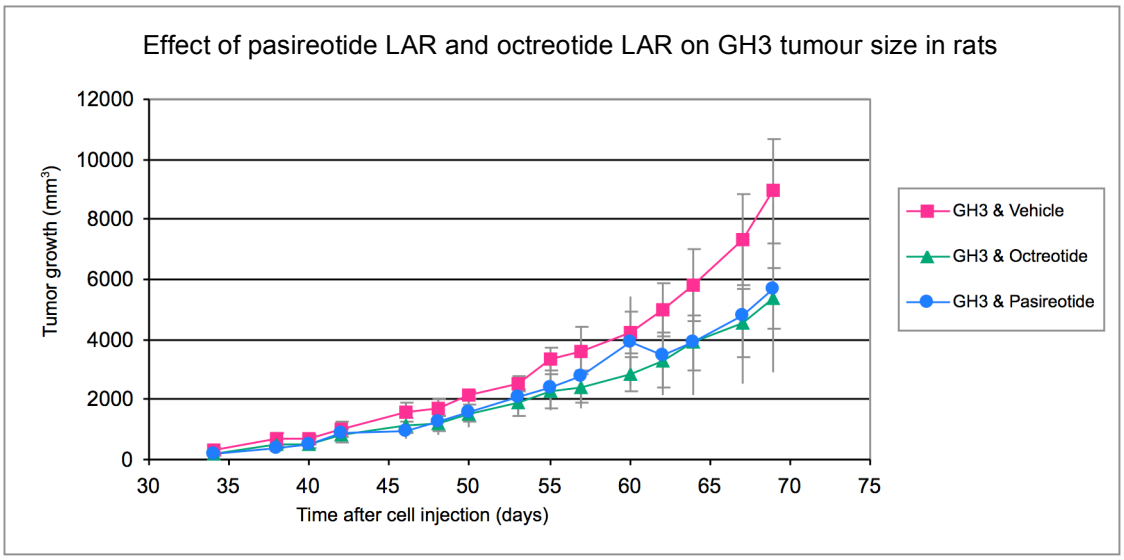

Figure 9.2 Tumour growth in GH3-implanted WF rats showing no alteration in tumour size with octreotide or pasireotide treatment when compared to controls. ANOVA, p=0.413)

9.3 Summary

Rats implanted with GH3 cells developed features of GH excess, as determined by increased body weight. Treatment with long-acting pasireotide or octreotide did not reverse this finding, indicating that these animals still had ongoing GH excess.

GH excess did not have any effect on cardiac AMPK activity when control and GH3-implanted animals were compared. However, animals treated with long-acting somatostatin analogues had markedly reduced cardiac AMPK activity, whether or not GH excess was present. This finding was unexpected and suggests an interaction between these drugs and the AMPK system. Suppression of AMPK activity may explain some of the influence of these drugs on the cardiovascular system in acromegaly. This is discussed further in section 12.6.

10 Chronic GH dysregulation model II: Transgenic bGH-overexpressing and GH receptor KO mice

10.1 Outline

The transgenic mouse model provides an alternative model of GH dysregulation. GH excess was produced by the introduction of bGH cDNA into the pronucleus of C57BL/6J embryos. GHD was produced by disruption of the fourth exon of the GHR/binding protein gene resulting in functional knock out of the GHR. Two groups of bGH-overexpressing mice and GHRKO mice were raised with age-matched controls. The first was sacrificed at two months of age, the second at eight months. These timepoints were chosen to reflect early and late acromegaly and to correspond with relevant studies in the literature. Animals were housed in cages kept on a 12 hour light, 12 hour dark cycle and received access to water and food *ad libitum*. Mice were bred and raised at the animal facility at Ohio University, Athens, Ohio, USA. Mice were sacrificed by cervical dislocation and tissues flash frozen in liquid nitrogen by staff at this facility. Hearts were rinsed in saline prior to freezing to remove excess blood. Tissues were homogenised, protein content determined and cardiac AMPK activity was measured by functional assay, using 200-300 μg per sample, as per the protocols in *Materials & Methods*. AMPK activity data are presented as percentage of controls to allow for comparison between groups of different genetic background and experiments performed on different days.

AMPK activity results in this study were disconcordant with recent publications. These had demonstrated increased cardiac AMPK phosphorylation at Threonine172 in three-month old bGH mice (n=5), usually considered to be a marker of increased AMPK activity. Our AMPK activity assay did not demonstrate any differences in bGH mouse cardiac tissue. We therefore undertook Western blotting to investigate this further. This was performed using antibodies for pAMPK and tAMPK, with 15-20 μg protein per sample. Each protein was corrected for GAPDH and results were expressed at pAMPK/tAMPK. Protocols are given in *Materials & Methods.*

10.2 Results

10.2.1 Model comparisons

GHRKO animals had reduced body weight compared to littermate controls at both two months (10.5 *v.* 23.7 g, p<0.0001) and eight months (18.0 *v.* 34.9 g, p<0.0001). Bovine-GH transgenics demonstrated increased bodyweight compared to littermate controls at both two months (35.9 *v.* 23.2 g, p<0.0001) and eight months (51.1 *v.* 35.1 g, p<0.0001). There was no difference in bodyweight at either timepoint between the two control groups (Figure 10.1).

Figure 10.1 Comparison of body weights of bGH and GHRKO mice and their littermate controls at 2 months and 8 months. bGH transgenics have significantly increased body weight at both timepoints; GHRKO transgenics have significantly reduced body weight at both timepoints. No difference is seen between the two control groups.

There was evidence of cardiac hypertrophy, as demonstrated by an increase in heart mass as a percentage of total body mass, in bGH transgenics compared to controls at both two months (0.52 *v.* 0.47 %, p=0.0008) and at eight months (0.62 *v.* 0.52%, p=0.0096). At two months there was no difference in cardiac weight as a proportion of body weight in GHRKO mice. However, by 8 months, transgenics had significantly smaller hearts as a proportion of their body weights compared to controls (0.39 *v.* 0.54%, p=0.0003). At neither timepoint was there any difference in cardiac weight as a percentage of body weight between the two control groups (Figure 10.2).

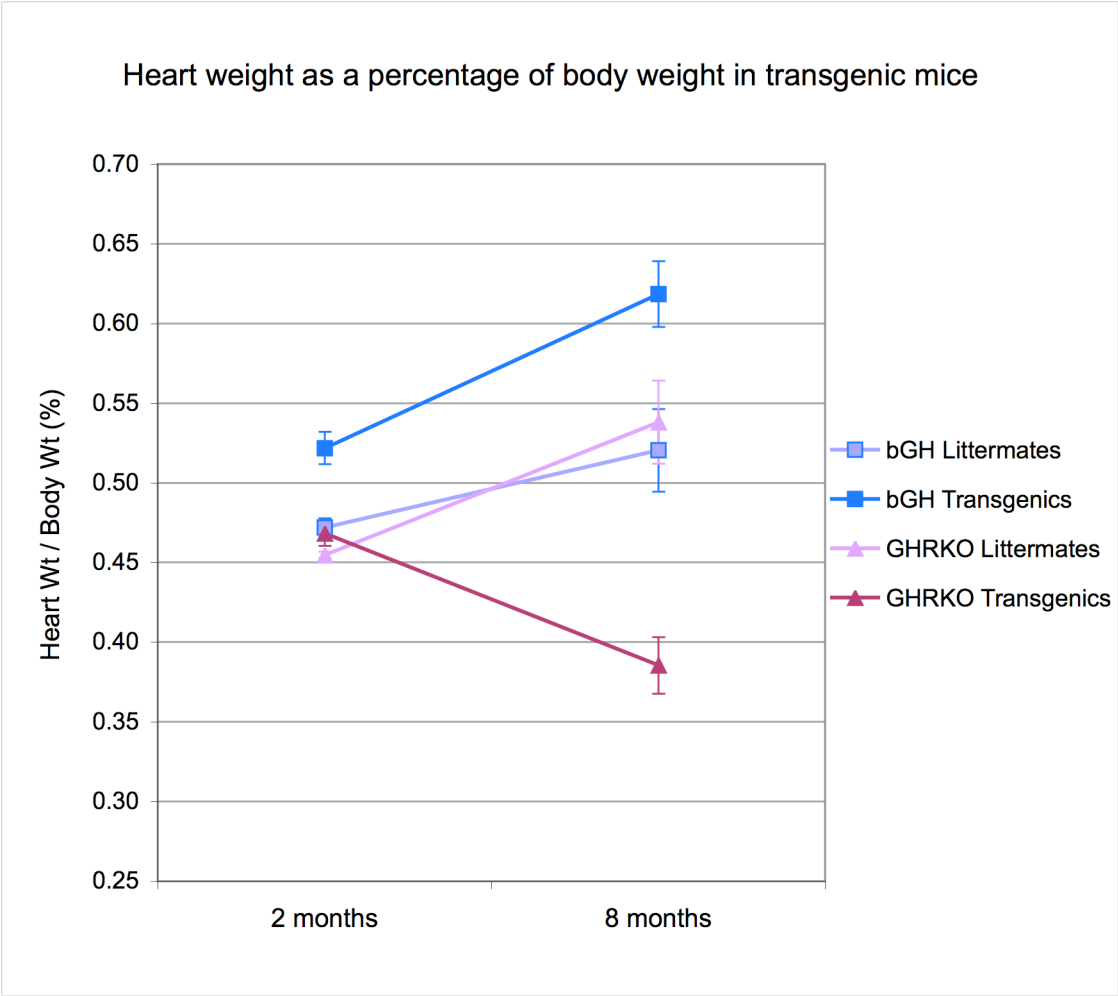

Figure 10.2 Heart weight as a percentage of body weight in transgenic mice. At 2 and 8 months bGH transgenics demonstrate cardiac hypertophy when compared to littermate controls. By 8 months GHRKO transgenics have reduced heart to body weight ratio that is not seen at 2 months

10.2.2 AMPK activity

At two months of age there was no difference in cardiac AMPK activity between bGH transgenics and their littermate controls (p=0.716) and no difference between GHRKO transgenics and their littermate controls (p=0.812) (Figure 10.3). At eight months there was still no difference bGH transgenics and their littermate controls (p=0.196) and no difference between GHRKO transgenics and their littermate controls (p=0.846) (Figure 10.4).

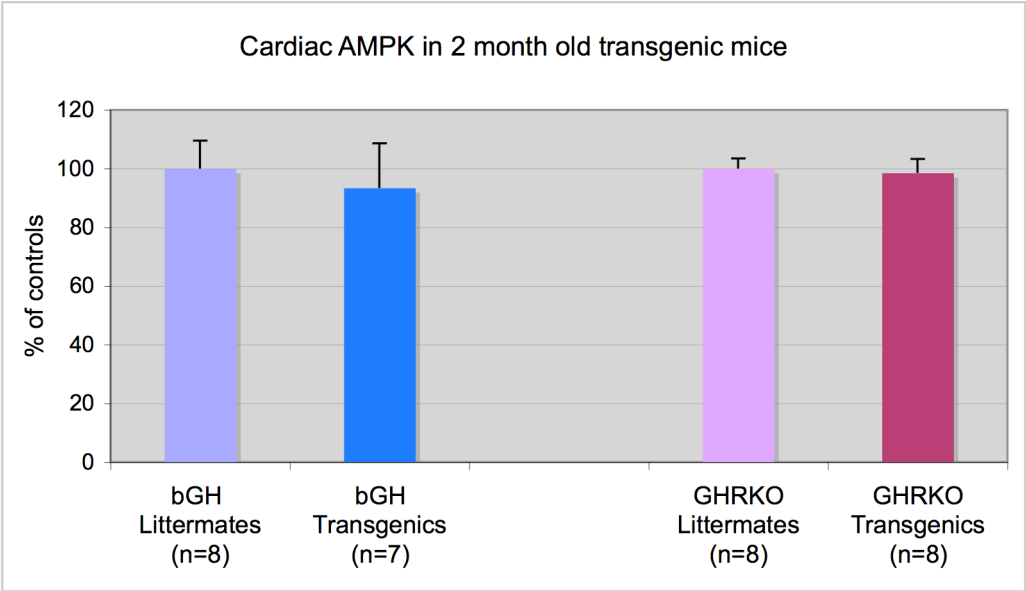

Figure 10.3 Cardiac AMPK in transgenic mice at 2 months showing no difference between bGH transgenics and their controls (p=0.716) and GHRKO transgenics and their controls (p=0.812).

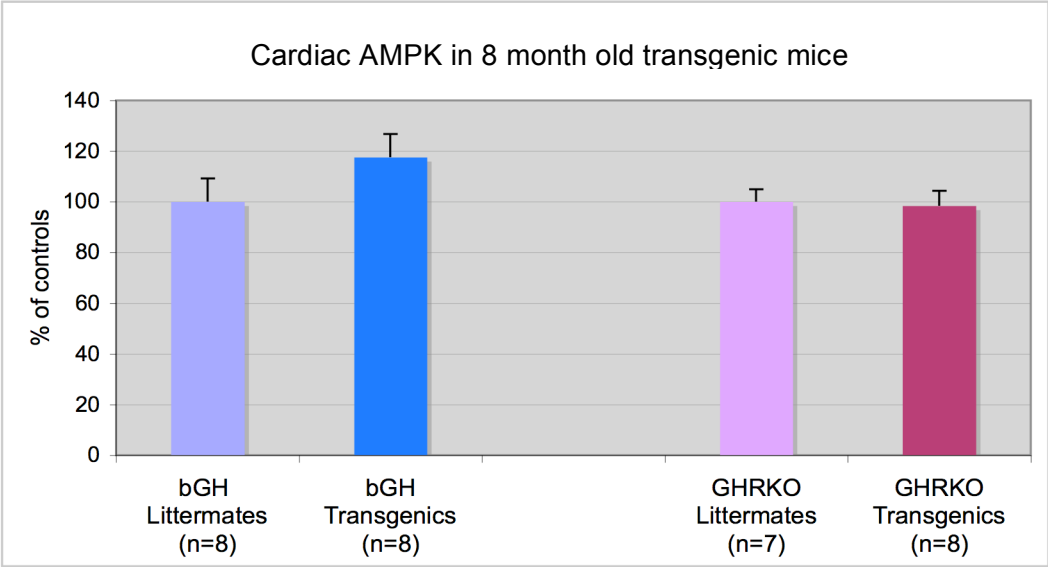

Figure 10.4 Cardiac AMPK in transgenic mice at 8 months showing no difference between bGH transgenics and their controls (p=0.196) and GHRKO transgenics and their controls (p=0.846).

10.2.3 Animal age

Two month old animals were compared directly with eight month old animals, on the same assay, to see if age impacted on cardiac AMPK activity. Healthy control animals (bGH littermates) showed a trend towards a reduction in cardiac AMPK with age, so that at eight months cardiac AMPK activity was 62.1% of what it had been at two months (p=0.056). Bovine-GH transgenics showed a trend towards the opposite finding, so that by eight months cardiac AMPK activity was 132 % of two month levels (p=0.083).

10.2.4 Western blotting

Two month old bGH transgenics demonstrated a significant increase in Thr172 pAMPK levels when compared to controls (p=0.014, Figure 10.5, Figure 10.6, Figure 10.7). However, by eight months this finding was reversed and bGH mice had significantly *reduced* pAMPK levels compared to controls (p=0.036, Figure 10.8, Figure 10.9, Figure 10.10). The findings are presented as pAMPK/tAMPK, each corrected for GAPDH.

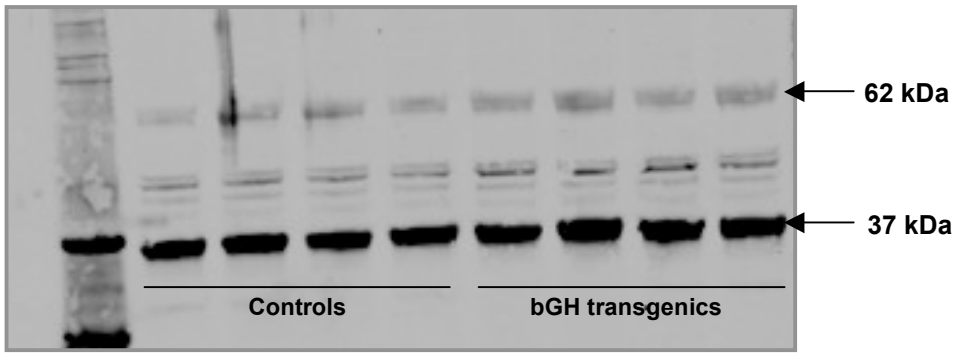

Figure 10.5 Western blot of cardiac protein from 2 month old bGH transgenics and their controls, demonstrating pAMPK at 62 kDA, corrected to GAPDH at 37 kDa.

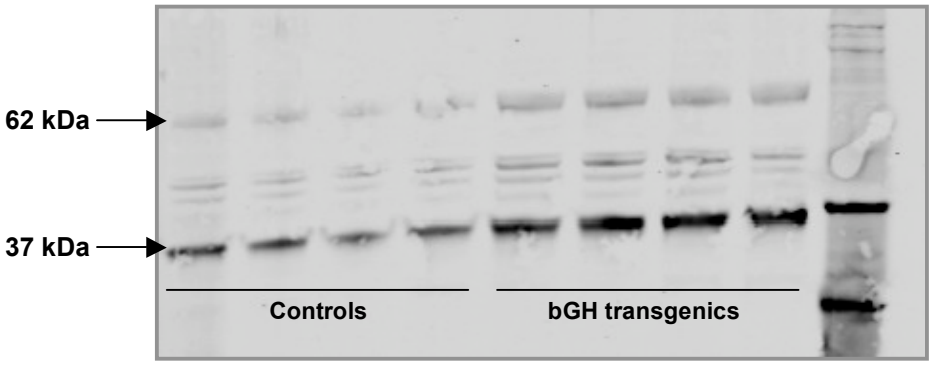

Figure 10.6 Western blot of cardiac protein from 2 month old bGH transgenics and their controls, demonstrating tAMPK at 62 kDA, corrected to GAPDH at 37 kDa.

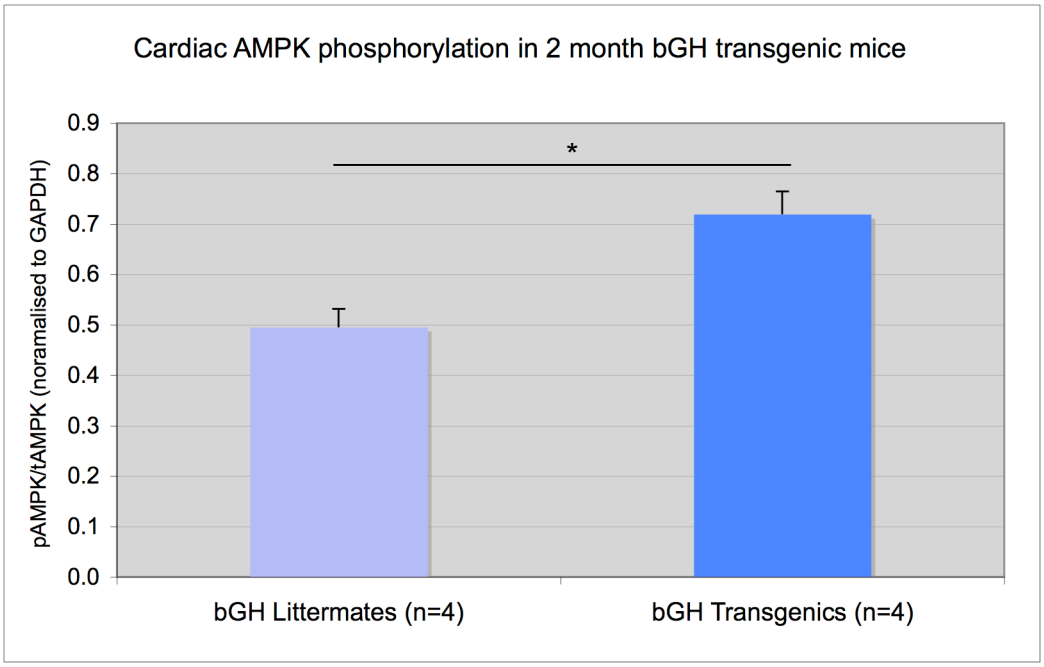

Figure 10.7 Results from Western blot for cardiac pAMPK levels in 2 month old bGH transgenic mice, showing a significant increase in transgenics compared to controls (p=0.014). Data are given as pAMPK/tAMPK, each having been corrected to GAPDH.

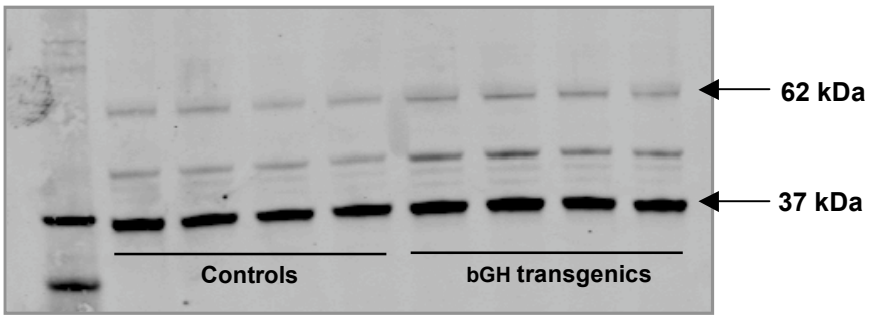

Figure 10.8 Western blot of cardiac protein from 8 month old bGH transgenics and their controls, demonstrating pAMPK at 62 kDA, corrected to GAPDH at 37 kDa.

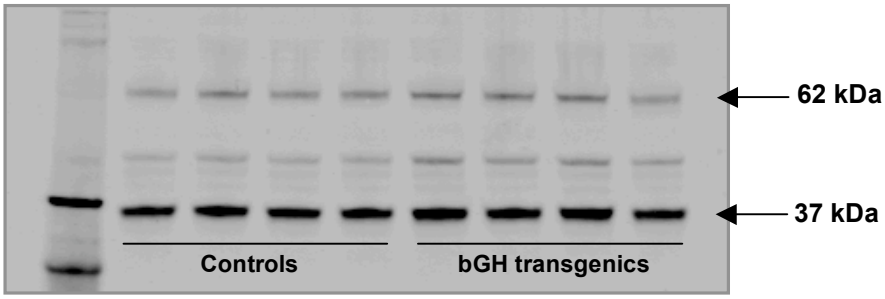

Figure 10.9 Western blot of cardiac protein from 8 month old bGH transgenics and their controls, demonstrating tAMPK at 62 kDA, corrected to GAPDH at 37 kDa.

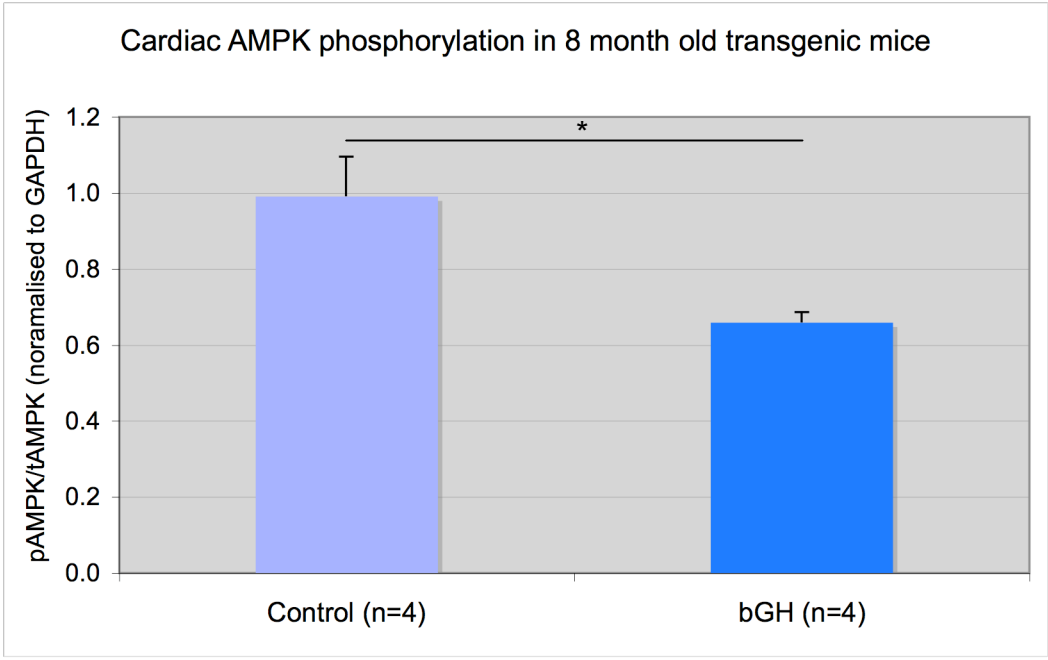

Figure 10.10 Results from Western blot for cardiac pAMPK levels in 8 month old bGH transgenic mice, showing a significant decrease in transgenics compared to controls (p=0.036). Data are given as pAMPK/tAMPK, each having been corrected to GAPDH.

Two month old GHKRO transgenic mice demonstrated a trend towards increased cardiac pAMPK levels but the levels did not achieve significance (p=0.093, Figure 10.11). When further studies with the four remaining samples in the group were performed and combined with these results, the analysis still remained non-significant (p=0.13). Eight month old GHRKO mice demonstrated a trend towards reduced cardiac pAMPK levels, which again failed to reach significance (p=0.14, Figure 10.12)

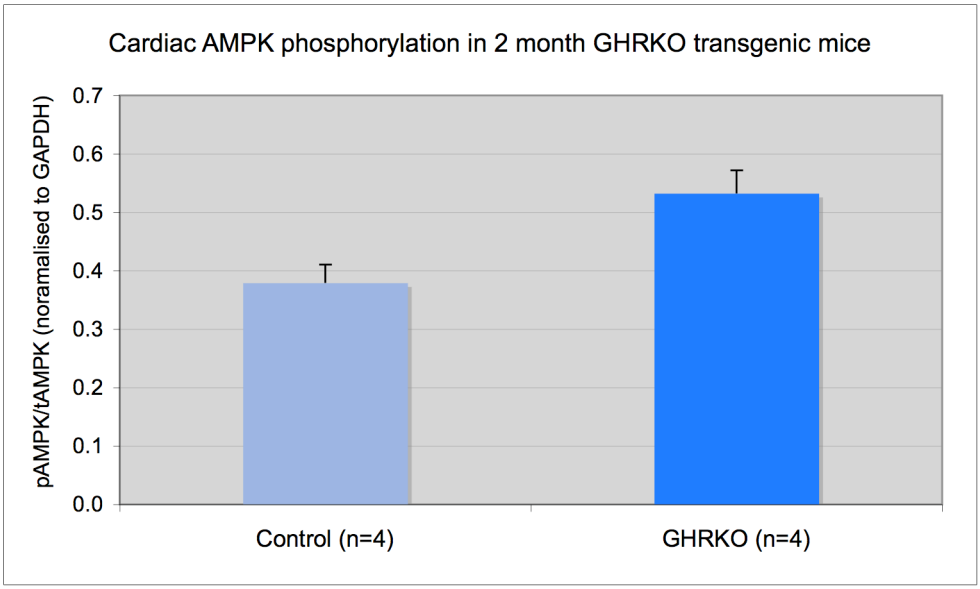

Figure 10.11 Western blot for cardiac pAMPK levels in 2 month old GHRKO transgenic mice, showing a trend towards increased pAMPK levels in transgenics (p=0.093). Data are given as pAMPK/tAMPK, each having been corrected to GAPDH.

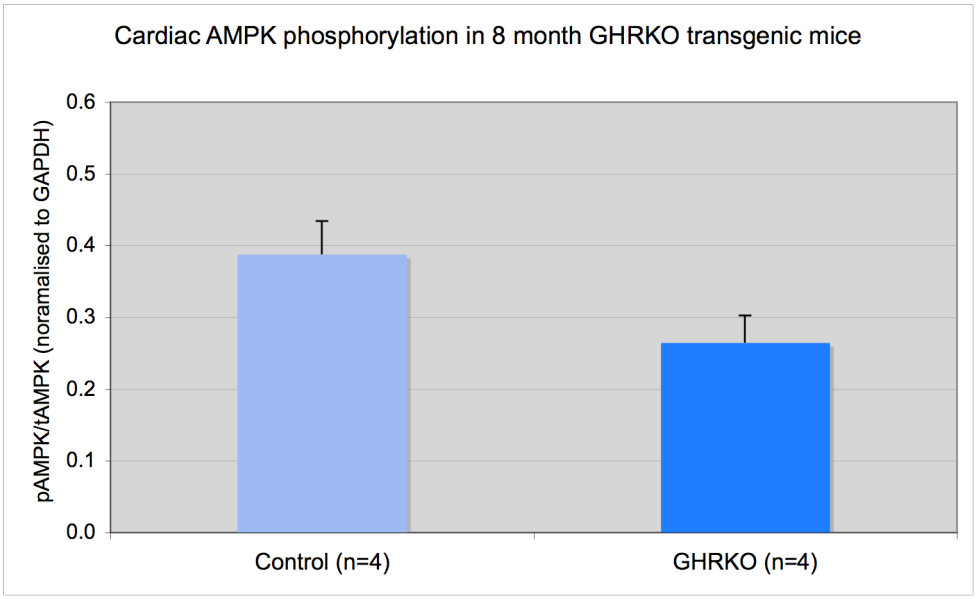

Figure 10.12 Western blot for cardiac pAMPK levels in 8 month old GHRKO transgenic mice, showing a trend towards reduced pAMPK levels in transgenics (p=0.14). Data are given as pAMPK/tAMPK, each having been corrected to GAPDH.

10.3 Summary

Functional AMPK assay failed to demonstrate any alteration in cardiac AMPK activity levels in bGH or GHRKO transgenic mice at either two or eight months of age. However, Western blotting was able to demonstrate increased pAMPK levels in two month old bGH transgenics and reduced pAMPK levels in eight month old animals. GHKRO transgenics demonstrated a similar pattern, increased pAMPK in two month animals and reduced pAMPK in eight month animals, although the results failed to achieve stastical significance.

The disconcordance between the functional AMPK assay and Western blotting for pAMPK was not expected as usually these methods are reported as being concordant. This is reviewed in the Discussion.

11 AMPK activity in neonatal rat primary cardiomyocytes treated with GH or IGF-I

11.1 Outline

Unstarved neonatal rat cardiomyocytes, prepared as given in *Methods and Materials*, were plated in 6 well plates, each containing 2 ml media (6 x 10^5 cells per well)[206]. Three days after harvesting, cells were treated with hGH 1 μg/ml (Gentotropin, Pharmacia #0022/0071)[289,290], hIGF-I 75 ng/ml (Sigma #I3769)[88,291,292], AICAR 1 mM (Calbiochem #123040)[293] or vehicle alone and incubated for six hours. Cells were lysed using *cell lysis buffer* and protein content quantified using BCA assay. Functional AMPK assay was performed in duplicate/triplicate using the maximum volume of protein solution available. The experiments were repeated on three occasions. AMPK activity data are presented as percentage of controls to allow for comparison of experiments performed on different days. Western blotting for pAMPK and tAMPK was also performed on the protein solution.

11.2 Results

This experiment was run three times. On the first two occasions six replicates were used. On the third occasion, nine replicates were used. Only the third experiment demonstrated significant findings (ANOVA, p=0.048), with hGH treatment increasing AMPK activity compared to controls (p=0.032, Figure 11.0). When the results of the three experiments were combined, hGH treatment resulted in a significant increase in cardiac AMPK activity when compared to both controls and to hIGF-I treated cells (Figure 11.1). There was no significant difference between the control group and the IGF-I treated group.

Western blotting for pAMPK levels was performed on protein solutions from the third experiment (Figure 11.2, Figure 11.3, Figure 11.4, Figure 11.5). This demonstrated differing results from the functional assay. Here there was a significance difference across all groups with treatment (ANOVA, p=0.0015). There was a significant increase in pAMPK in the IGF-I groups compared to the control group and the AICAR group compared to the control group. There was a trend towards increased pAMPK in the GH group compared to the control group but this difference failed to achieve significance (Figure 11.6). However, as can be seen from

Figure 11.2, there appears to a slight loss of bands which may have contributed towards this disconcordance.

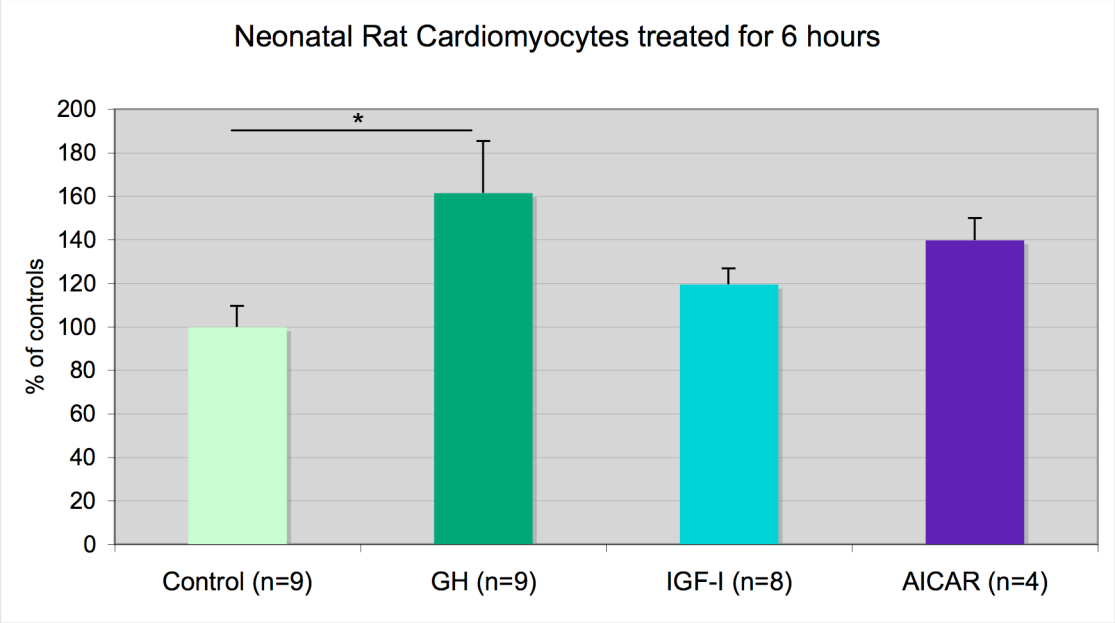

Figure 11.0 Neonatal cardiomyocytes treated with GH, IGF-I or AICAR from experiment three. There is a significant difference between groups, ANOVA, p=0.048. There is a significant difference between the control and the GH-treated group.

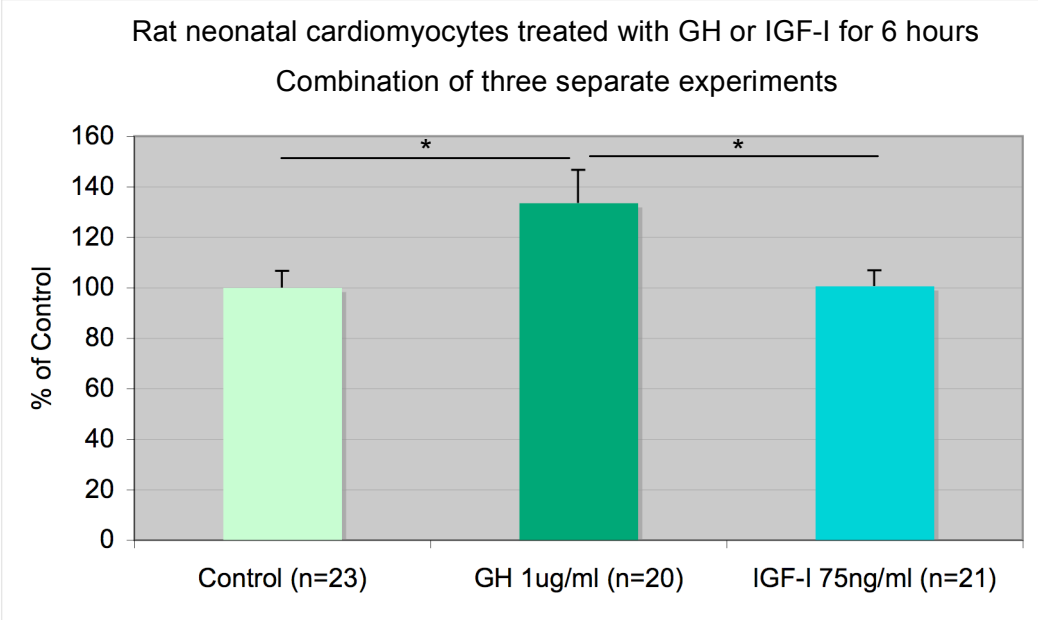

Figure 11.1 Pooled results from experiments with neonatal cardiomyocytes treated with GH, IGF-I or AICAR. There is a significant difference between groups, ANOVA, p=0.016. There is a significant difference between the control and the GH treated group and between the GH treated group and the IGF-I treated group. AICAR is not shown as this duration of this treatment varied between experiments and so the experiments were not comparable.

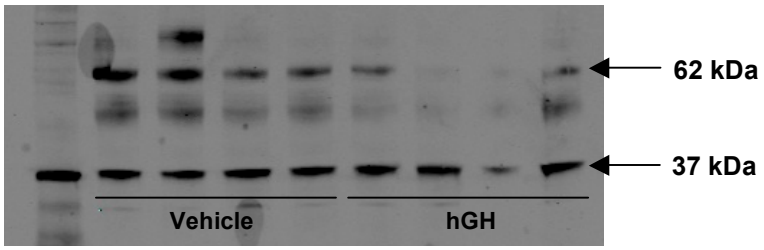

Figure 11.2 Western blot of Thr172 pAMPK levels in neonatal rat cardiomyocytes treated with vehicle or hGH, demonstrating pAMPK at 62 kDA, corrected to GAPDH at 37 kDa

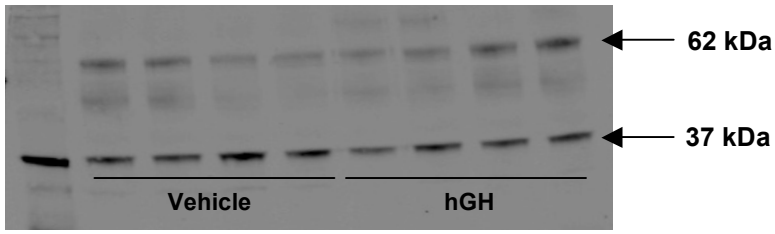

Figure 11.3 Western blot of Thr172 tAMPK levels in neonatal rat cardiomyocytes treated with vehicle or hGH, demonstrating tAMPK at 62 kDA, corrected to GAPDH at 37 kDa

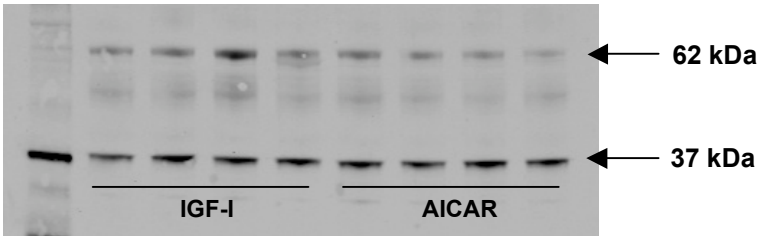

Figure 11.4 Western blot of Thr172 pAMPK levels in neonatal rat cardiomyocytes treated with IGF-I or AICAR, demonstrating pAMPK at 62 kDA, corrected to GAPDH at 37 kDa

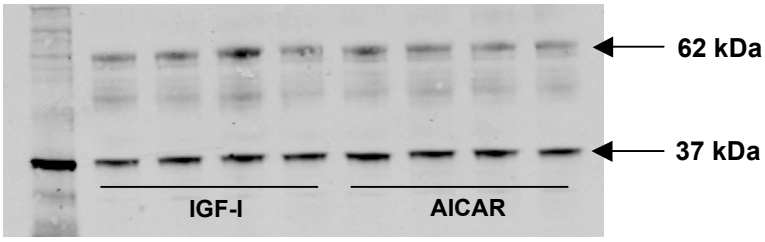

Figure 11.5 Western blot of Thr172 tAMPK levels in neonatal rat cardiomyocytes treated with IGF-I or AICAR, demonstrating tAMPK at 62 kDA, corrected to GAPDH at 37 kDa

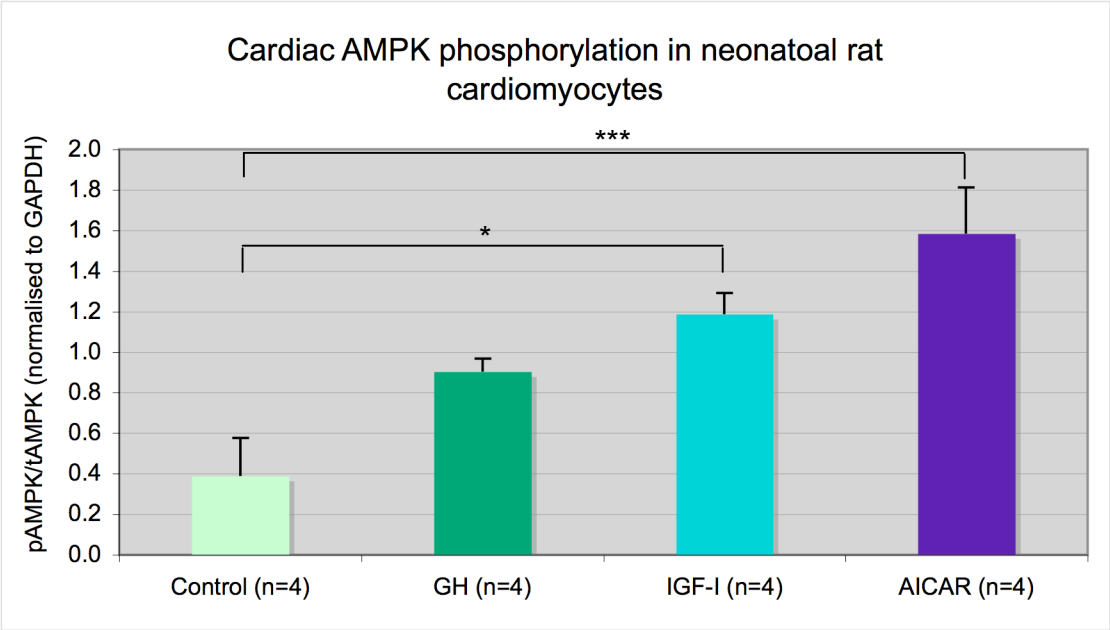

Figure 11.6 Cardiac AMPK phosphorylation levels as determined by Western blotting. There are significant differences between the groups, ANOVA p=0.0015. Both IGF-I and AICAR treatment induced a significant increase in pAMPK levels when compared to controls. GH treatment showed a trend towards increased levels. Results are given as pAMPK/tAMPK, each normalised to GAPDH.

11.3 Summary

Functional assay demonstrated an increase in AMPK activity with GH treatment. No significant difference was seen with IGF-I or AICAR treatment. When the same protein solutions underwent Western blotting, a significant increase in Thr172 pAMPK levels in cells treated with IGF-I or AICAR was seen. Increases in Thr172 pAMPK levels with GH treatment did not achieve significance, although a trend was present.

12 Discussion

12.1 Pilot studies

The pilot study demonstrated that six weeks of GH is able to activate cardiac AMPK in mice fed a high fat diet (Figure 6.1). However, repetition (Figure 7.1) failed to reproduce these findings, even though the same conditions were maintained throughout both experiments and homogenisation, protein quantification and the AMPK assay were done by the author on both occasions, following the same protocols. The reasons for this are not clear. Study mice were from the same genetic background in both experiments and were raised at the same facility. The same doses of bGH were used, although batch numbers differed.

The presence of increased pACC protein in the bGH-treated cardiac tissue in the repeated experiment suggests that these tissues have been exposed to increased AMPK activity. ACC is regarded as the main downstream target of AMPK[138] and there is no evidence that it can be directly phosphorylated by GH/IGF-I, although this is an area not addressed by the literature. It is known that amplification occurs along the AMPK pathway so that very subtle increases in AMPK activity may have much larger effects on ACC phosphorylation. This could, in part, explain this incongruous finding. Alternatively, this result could reflect that the tissue has previously been exposed to increased AMPK activity, with a persistence of a measurable pACC rise. Acute exposure to exercise has been shown to induce a temporary cardiac AMPK activity rise in mice[183] that is not longer seen with longer-term training[88]. It may be hypothesised that duration of exposure to GH affects AMPK activity, with acute exposure resulting in an increased activity that is lost as tolerance develops. By six week of GH treatment, AMPK tolerance has developed to acute GH excess but there is inadequate development of cardiomyopathy for it to influence AMPK activity. In order to separate out these two timepoints and address this question, experiments to look at both the acute and chronic effect of GH/IGF-I on cardiac AMPK activity were designed.

12.2 Acute GH excess

Acute treatment of both dwarf and WT AS rats with GH or IGF-I resulted in significant changes in AMPK activity, indicating that the GH/IGF-I axis interacts with the AMPK system in the acute setting. The ability of a single dose of GH to markedly increase cardiac AMPK activity in WT rats fits with our initial hypothesis that GH causes an acute rise in AMPK activity. Furthermore, the effect of GH on cardiac AMPK activity appears to be a direct action, as treatment with IGF-I failed to reproduce it. Findings that acute GH treatment increases AMPK activity were confirmed by performing similarly timed experiments in primary neonatal rat ventricular cardiomyocytes. Although the HL-1 cardiac cell line expresses the GH receptor and has been used with good effect in the study of cardiac functioning, signalling and metabolism[294], we have not been able to measure AMPK activity this line. Therefore, we chose to use primary neonatal rat cardiomyocytes for these experiments as it was felt they would be more suitable for a functional assay. One weakness of this model is that cells mainly express the neonatal V3 myosin isoform rather than the adult V1 form, limiting conclusions that can be drawn[295]. Interestingly, chronic GH excess in rats with GH3 tumours causes the same shift towards foetal myosin isoforms[296]. In addition, these cells carry all the usual challenges of working with primary cells rather than an immortalised line. However, they are a well-established model and provide reproducible results. It should be noted that Western blotting results in these cells did not appear congruous with functional assay results. This may in part reflect some loss of bands on the GH blot. Insufficient quantities of protein remained to perform further blots. The difference between findings on functional AMPK assay and Western blotting is discussed later.

The only published study looking at the direct effect of GH on AMPK failed to show any effect of GH on pAMPK levels in endothelial cells[66]. As reviewed in the Introduction, this may be due to serum starvation that the cells were subjected to prior to treatment[206]. Alternatively, this may represent tissue specific effects of GH, resulting in variable effects in different cells. The lack of published data in this field make the finding that GH increase cardiac AMPK activity in two models particularly interesting but clearly indicates a need for further studies in this area.

The discovery that GH had an opposite effect on cardiac AMPK activity in GHD dwarf rats than in WT rats was not predicted. These animals are known to have cardiac consequences

arising from GHD, specifically, reduced cardiomyocyte size, cardiac structure and myocardial contractility, which improve with GH treatment[61]. It remains unclear whether the effect seen on cardiac AMPK activity in the dwarf animal is a true effect of GH on cardiac AMPK activity in a GHD-state or if it represents a peculiarity of this model. Certainly, any conclusions drawn should be done so with caution. Studies that have been published with these animals tend to concentrate on the cardiac effects of GH on the dwarf itself, without any direct comparison to WT rats[61,297,298]. In order to determine if this is a model-related effect, studies in other animal models of GHD are needed.

If GH treatment in patients with GHD is confirmed to be associated with a fall in cardiac AMPK activity, it remains to be demonstrated how long this effect persists and why it occurs. One potential explanation is that the heart in the GHD individual exists in perpetual state of metabolic "stress"; replacing GH ameliorates this stress with a reactive fall in AMPK activity. Both defining and determining the presence of metabolic stress cause challenges. One method examining myocardial metabolic stress is to use cardiac MRS to look at PCr:ATP ratios, as a marker of energy availability. A reduction in PCr:ATP ratio occurs in diabetes prior to the development of demonstrable heart disease, indicating that this finding is very early marker of cardiac problems[299]. The only study that has used cardiac MRS to assess the GH/IGF-I axis treated isolated rat hearts with IGF-I[43]. This study failed to demonstrate any effect of IGF-I on myocardial energy supply, although it should be noted that exposure time was only 20 minutes. There are no studies addressing cardiac or skeletal muscle MRS in patients with GHD. This is an area that warrants further research (see page 123).

An alternative method of assessing myocardial stress is to look for early signs of physiological dysfunction, which may be done by measuring N-terminal pro-B-type natiuretic peptide (pro-BNP). This peptide is a marker of increased ventricular wall stretch, a prelude to systolic heart failure, increased volume expansion and diastolic dysfunction[300-302]. However, the only study to date in this area demonstrated that patients with GHD do not have alterations pro-BNP, either before or after GH treatment[132].

Our animal studies did not demonstrate any baseline difference between cardiac AMPK activity in WT and dwarf rats, although numbers were small (Figure 8.5). If chronic GHD produced cardiac metabolic stress it would be predicted that dwarf animals would have a higher baseline AMPK activity level. The reasons for the fall in cardiac AMPK activity seen in dwarf rats treated with GH remains unclear.

12.3 Acute IGF-I treatment

In both WT (AS) and dwarf rats, acute IGF-I treatment decreased cardiac AMPK activity. This was a consistent finding over several experiments, although higher IGF-I doses were necessary to achieve this finding in WT rats, which may relate to an increased volume of distribution, secondary to size. This finding was not seen in neonatal cardiomyocytes, where, despite a significant increase in cardiac AMPK activity with GH treatment, there was no difference between IGF-I treatment and the control group (Figure 11.1). Conversely, Western blotting demonstrated increased Thr172 pAMPK levels in neonatal cardiomyocytes treated with IGF-I

The reduction in AMPK activity with IGF-I treatment seen in the animal studies is likely to occur due to IGF-I induced phosphorylation of AMPK serine 485 (Ser485), with a consequential reduction in Thr172 phosphorylation reducing AMPK activity. This effect has already been demonstrated in vascular smooth muscle cells treated with IGF-I[205]. Insulin, which shares many effects with IGF-I due to partial homology and receptor cross-activation, has been shown to reduce cardiac AMPK activity through this mechanism[202-204]. However, at present there is only one published study demonstrating that IGF-I phosphorylats Ser485 and this was not performed in cardiomyocytes. Since human fibroblasts treated with IGF-I demonstrate both increased AMPK activity and Thr 172 pAMPK levels the possibility of tissue-specific effects of IGF-I on AMPK needs to be considered[303].

In this study WT rats required an IGF-I dose 50 μg to elicit a fall in AMPK activity, a dose equivalent to 417 μg/kg. Children with Laron syndrome received IGF-I replacement doses that range between 80 – 240 μg/kg a day[304,305]. Although, parallels that can be drawn between rat studies and human treatment doses are limited, this much higher IGF-I dose requirement to induce AMPK activation in rats may indicate that the effect was the result of insulin receptor activation rather than IGF-I receptor activation. In a study of patients with type 1 diabetes, IGF-I treatment with 100 μg/kg a day maintained stable glycaemia and yet reduced insulin requirements by 45%, indicating that the doses of IGF-I used in our study should be adequate to activate the insulin receptor[306]. Delineating the respective roles of the IGF-I and insulin receptors is complicated by the marked cross-talk between these systems. The insulin receptor and IGF-I receptor may exist as a hybrid dimer and in the heart up to 90% of receptors may exist in the hybrid form[307]. In vascular smooth muscle cells these

hybrid receptors are activated by physiological concentrations of IGF-I[308]. The selective cardiac insulin receptor knockout (CIRKO) mouse could be used to address this question further. CIRKO mice are unable to increase cardiac AMPK activity in response to exercise, whereas those with cardiac IGF-I receptor KO retain this ability[88], suggesting that the insulin receptor, rather than the IGF-I receptor, is the doorway to AMPK activation. CIRKO is a *cre/loxP* mouse that demonstrates a degree of cardiac abnormality, mainly reduced cardiac size due to reduced cardiomyocyte area and persistence of foetal β-myosin heavy chain isoform but also demonstrates impairment of cardiac output and power, ejection fraction and fractional shortening[309], hence results from studies need to be interpreted with caution.

An alternative model for studying this question would be the insulin receptor substrates (IRS)-1 KO mouse. IRS-1 is the major substrate of the insulin receptor. IRS-1 knockout mice are viable and, although growth-retarded, have histologically normal heart[310]. However, it must be noted the IRS-2 is still functional in these models and insulin can activate the PI3K pathway through phosphorylation of this substrate[311]. Furthermore, the reduction of insulin's actions may have a wealth of cardiovascular effects that impact on the AMPK system.

The supraphysiological doses of IGF-I given to these rats might explain the discordance between the results from our animal studies and cell studies, in which more doses of IGF-I more comparable with human circulating levels were used (75 ng/ml). However, it should be noted that Ning *et al.* demonstrated an increase in AMPK Ser485 phosphorylation and a reduction in Thr172 phosphorylation with only 10 ng/ml IGF-I in vascular smooth muscle cells[205]. Whether or not this effect also occurs in cardiomyocytes remains to be elucidated. Ning only measured AMPK phosphorylation levels and did not demonstrate AMPK activity with a functional assay. One limitation of using neonatal rat cardiomyocytes is the relatively low protein yield per pup requiring a large number or pups to be sacrificed to produce enough protein for a functional assay, which requires 200 – 300 μg protein per sample. An alternative method for clarifying that the changes in phosphorylation seen did indeed have biological effect would be Western blotting for pACC. Although this method requires much lower quantities of protein, pACC is a large protein, measuring 257 kDa, bringing technical challenges to producing good results. Western blotting for Ser485/491 would be useful in confirming the effect seen in WT and dwarf rats with IGF-I treatment occurs via the mechanism discussed above.

12.4 Chronic GH excess

Transgenic bGH-overexpressing and GHRKO mice demonstrated the phenotype of their GH status. Bovine-GH transgenics had increased body weight and cardiac hypertrophy at both two eight months (Figure 10.1 and Figure 10.2). GHRKO transgenics had reduced body weight and reduced cardiac mass as a percentage of body weight at eight months (Figure 10.1 and Figure 10.2). However, no alterations in cardiac AMPK activity were seen when transgenics were compared to littermate controls at two months or at eight months. These timepoints were chosen to reflect early and late GH-related cardiac disease. By eight months the bGH transgenic already demonstrates metabolic disruption in the heart, as shown by reduced PCr:ATP ratio, and evidence of reduced systolic function[312].

A recent study from Bogazzi *et al.* shows increased cardiac pAMPK in three month old bGH-overexpressing mice when compared to age-matched littermates but this difference was lost by nine months[174]. It should be noted that although pAMPK levels in Bogazzi's nine month old mice were no different from controls, pACC was reduced when compared to controls suggesting a fall in AMPK activity. With only five animals in each group it may be that the experiment was insufficiently powered to demonstrate a measurable decrease in cardiac pAMPK levels[313]. Reasons for the normalisation of AMPK activity with time in these studies remain unclear. It may be that although acute GH exposure is associated with increased AMPK activity, cardiac tissue acclimatises to chronic GH/IGF-I excess resulting in normalisation of AMPK activity. Alternatively, a mixed picture may be occurring with falling AMPK activity from a failing, cardiomyopathic heart cancelling out the GH/IGF-I drive to increased AMPK activity. However, as eight month old bGH-overexpressing mice have reduced cardiac energy status when measured by PCr:ATP ratio[312] and falls in PCr:ATP ratio have been associated with increased AMPK activity in pressure-overloaded rat hearts[174], it would be predicted that cardiac AMPK activity would be raised in the hearts of the eight month old bGH transgenic mice.

The mice used in Bogazzi's study were from a different source from ours but were made by similar protocol on the same C57B background. It was predicted that we would demonstrate similar findings in our mice when cardiac AMPK activity was measured by functional assay. However, no difference in cardiac AMPK activity was seen between bGH transgenics and their controls at either two or eight months. As these results neither seemed consistent with

our hypothesis nor the published literature, Western blotting was performed for Thr172 pAMPK levels. This showed a significant increase in cardiac pAMPK levels in two month old transgenics and a trend towards reduced cardiac pAMK in eight month old transgenics, results similar to those published in Bogazzi's paper. The incongruent results between functional AMPK assay and Western blotting for pAMPK have not previously been reported. The functional kinase assay, which utilises the ability of phosphocelluose paper to bind phosphorylated protein, is a well established method of quantifying enzyme activity[314]. In order to achieve specificity for AMPK activity, enzyme is immunoprecipitated using AMPK-α1 and AMPK-α2 antibodies and SAMS, a specific target peptide for AMPK, is used as a substrate for phosphorylation[288]. To exclude the consequences of phosphorylation arising from basal AMPK activity, samples are run that do not include this target peptide and this value is subtracted from the results gained. However, as with all enzymes measurements, the method of collecting the samples remains important. Stress to the animal prior to sacrifice or dissection without extremely rapid cooling to prevent enzyme activity is essential. Ideally, samples are processed immediately post-sacrifice, however, often this is not feasible. Furthermore, the steps of homogenisation and immunoprecipitation take some time, especially when a large experiment is being performed, making freezing between stages essential. In all the experiments performed here, samples were used as early as possible and care was taken to expose tissues and protein samples to minimal freeze-thaw cycles. Despite these limitations, the AMPK functional kinase assay is still regarded as the gold standard in the assessment of AMPK as it measures actual activity, rather than phosphorylation, a surrogate marker of activity *(Prof G Hardie, personal communication to M Korbonits).* For this reason, this method was used as our primary method for investigating AMPK in these experiments.

Bogazzi's paper used the other common method of measuring AMPK, Western blotting for Thr172 pAMPK levels. This method is also well established for the investigation of AMPK. It is based on the assumption that AMPK activity is determined by phosphorylation of Thr172[315]. Publications in the area of AMPK contain a mixture of results from the functional kinase assay and Western blotting for pAMPK. Few papers publish both results, although in those that do, results from the two methods are congruous, including those from our labarotory[316,317]. There have not been any reports of disconcordance between the two methods and the reasons for this occurrence here remain unclear. However, the neonatal cardiomyocyte experiments also demonstrated disoncordance. This was particularly marked with IGF-I treatment, which

appeared to trend towards reduced AMPK activity, in line with dwarf rat experiment results, but showed significantly increased levels of Thr172 pAMPK.

It has been noted that the method of preparing tissue may affect the levels of pAMPK. Mice killed by cervical dislocation have 8-fold higher levels of brain pAMPK levels than those killed by focused microwave irradiation to the brain[318]. However, these findings were on Western blotting and there is no reason to predict they would differ from a functional assay. Furthermore, as all our animals were sacrificed using a consistent method, ongoing enzyme activity post-sacrifice does not explain the discrepancy in our results. It should be noted, in all our experiments, functional kinase assay was performed prior to Western blotting for pAMPK levels, meaning that samples underwent an additional freeze-thaw cycle prior to Western blotting. If ongoing enzyme activity were occurring, this would reduce the significance of findings at Western blotting rather than increase them.

As discussed earlier, insulin and IGF-I are able to reduce AMPK activity through phosphorylation of Ser485[205]. One possible mechanism for the discordance seen between our functional assay and Thr172 pAMPK levels is that the increased levels of IGF-I in these chronic models of GH excess is phosphorylating Ser485 in addition to Thr172, resulting in increased levels at Western blotting but no increase in activity. However, experiments indicate the mechanism through which Ser485 phosphorylation exerts its inhibitory effect on AMPK activity is through reduction of Thr172 phosphorylation[205]. Hence even if Ser485 phosphorylation were affecting AMPK activity, results from Thr172 pAMPK levels and AMPK activity assays would be congruous.

Another possible explanations for this incongruity is that the kinase assay was not functioning correctly. However, the author performed all the AMPK assays, using the same protocols and chemicals on all occasions. Other experiments performed simultaneously to these demonstrated marked changes in AMPK activity levels indicating the assay was working well. There is no reason to believe assay fault contributed towards these findings, although this would be the most straight forward answer to the conundrum.

Previous work has demonstrated a clear association between phosphorylation of Thr172 and AMPK activity. It remains possible that further phosphorylation sites exist that do not affect Thr172 phosphorylation but impact AMPK activity. The animal models used in this

experiment are chronically exposed to GH, which a consequential increase in IGF-I levels. As in acromegaly, it is clear that these animals have multiple chronic metabolic and physiological consequences of these changes. This long duration of exposure may affect the AMPK complex in a way that acute exposure to GH and IGF-I did not. The most commonly measured target of AMPK is pACC. Western blotting for this protein could help determine whether or not Thr172 pAMPK levels were actually reflecting AMPK activity. It should be noted that, in common with other kinase systems, amplification occurs along this pathway, so even subtle changes should be measurable. Further work is needed to address this.

The hypothesis that chronic GH hormone excess would increase cardiac AMPK activity has not be borne out by our study. In fact, a more complex picture occurs, with evidence of increased cardiac Thr172 pAMPK levels in young "acromegalic" animals but reduced levels in older animals. The ages of these animals, two and eight months, were chosen to reflect early and late acromegaly[312,313]. In patients with acromegaly the cardiac findings vary with time. Early acromegalic heart disease has been likened to athletic cardiac hypertrophy, with hypertrophy, a hyperdynamic circulation and reversibility with removal of stimulus. With time, diastolic and then systolic dysfunction develops. The fibrosis of acromegalic cardiomyopathy has been reported as non-reversible, even with good control of GH[23]. The AMPK findings in our study appear to represent these two stages of disease: early increased AMPK phosphorylation but, as cardiac dysfunction develops, a fall in AMPK phosphorylation over time.

The mechanism behind the fall in AMPK phosphorylation in advanced acromegalic cardiomyopathy is unclear. As is discussed in the Introduction, ongoing AMPK activation in the presence of an ischaemic heart runs the risk of worsening the situation by uncoupling glycolysis and glucose oxidation[151]. It may be that protective mechanisms designed to switch off the AMPK pathway in presence of ongoing acute ischaemia become chronically activated in heart failure. Alternatively, the reduction in pAMPK may arise from downregulation of the AMPK pathway in response to chronic AMPK activation in early heart disease.

AMPK activation with metformin is able to prevent the progression heart failure in dogs[166]. There may be a role for the use of metformin for cardiac protection in patients with uncontrolled acromegaly. There have been concerns about using metformin in patients with heart failure because of the risk of lactic acidosis[319]. However, studies now indicate that not

only is metformin safe in chronic heart failure but that cardiac mortality is reduced in heart failure patients taking metformin when compared to patients on other diabetes therapies or combination therapies[320]. In patients with diabetes metformin is associated with less diastolic dysfunction than other diabetes medications[321]. There are no trials of metformin in patients with acromegaly and the rarity of the disease would make recruiting adequate numbers to such trials difficult. Furthermore, as seen in our clinical study, patients with acromegaly are often taking many drugs that may modify the development of cardiac disease, such as ACE inhibitors or HMG-CoA reductase inhibitors. However, there is a strong argument, particularly given the insulin resistance of this condition, to consider treating acromegaly patients with this cheap and relatively safe medication.

12.5 Chronic GH deficiency

There are no published data examining the effect of GHD on cardiac AMPK activity. Although we failed to demonstrate any changes in cardiac AMPK activity using the functional assay, we demonstrated a trend towards changes in cardiac AMPK Thr172 phosphorylation. Interestingly, the changes seen were similar to those seen in bGH mice, with increased cardiac pAMPK in younger animals and reduced cardiac pAMPK in older animals. This finding indicates a U-shaped relationship between GH AMPK: in the younger heart both excess and deficient GH levels cause a rise in AMPK activity, in the older heart both excess and deficient GH levels cause a reduction in AMPK activity.

The dramatic fall in cardiac AMPK activity seen in the dwarf rats treated with GH in the acute studies may indicate that the myocardium in the GHD state is under chronic metabolic stress. As GH normalisation improves the metabolic state and AMPK activity falls. This hypothesis fits with the raised cardiac AMPK activity seen in younger GHRKO mice. It does not explain the trend towards reduced AMPK activity in the older heart. One suggestion it that the reduced AMPK found in these samples indicates a failing myocardium. However, as discussed earlier heart failure is associated with falls in PCr:ATP ratios, which in animal models have been associated with increased AMPK activity (see Section 12.4). It is interesting to note that bGH animals demonstrate the same finding, with a reduction in AMPK activity by eight months.

The findings in GHRKO transgenics were not statistically significant, although a clear trend was present. It may be that these findings are incidental and do not represent any true change. Experiments with larger numbers would be necessary to investigate this further. The changes seen in the heart disease of GHD are far subtler than those seen in acromegaly and the factor by which GH and IGF-I levels vary from those of healthy individuals is far more marked, as discussed in our clinical study. If pAMPK levels are proportional to the degree of alteration in cardiac structure and function, it would be expected that pAMPK changes would be more marked in the hearts of animal models of acromegaly than in those of GHD.

The timepoints in this study were chosen to correlate with early and advanced acromegaly in humans and to allow for comparison with published data in mouse models of acromegaly[312]. A more recent paper chose similar timepoints for investigation of pAMPK levels and

demonstrated cardiac hypertrophy on histology in nine month old bGH mice[313]. To allow for comparison between animal models of acromegaly and GHD, matching time points were chosen with our bGH and GHRKO animals. Interestingly, GH status affects life span in mice. GHRKO mice demonstrate reduced aging and increased longevity, with the longest living mouse recorded living to almost 5 years old[322,333]. In contrast bGH mice have reduced lifespan[324]. Therefore, eight month old GHKRO mice and eight month old bGH-expressing mice may not be in comparable life stages. It is possible that ongoing examination of GHRKO may demonstrate further and more significant alterations in cardiac AMPK as time progresses. The trend towards reduced cardiac pAMPK may remain, resolve or become amplified.

12.6 Somatostatin analogue treatment

The GH3-implanted rat is a well-recognised model of acromegaly[325] and the somatostatin analogues, such as octreotide LAR and pasireotide LAR, have been shown to reverse, at least in part, the biochemical and clinical features of acromegaly[326,327]. Our animals demonstrated clear gigantism with GH3 implantation (Figure 9.1). Treatment with 80 mg/kg sc somatostatin analogue did not reduce body weight or tumour size. As rats with GH excess represent a model of gigantism, rather than acromegaly, it might be predicted that body weight would persist. Other published work with the same model, treating with a shorter acting somatostatin analogue, also do not show any reduction in tumour size but do show a reduction in the velocity with which the tumour grows[328].

In this experiment, the animals received 69 days of GH excess. This was not dissimilar to the length of GH exposure in the GH-injected mice (Section 7) and was chosen in advance of the results from the mouse experiment being available. As previously discussed, the failure to see the predicted increase in cardiac AMPK activity with this period of GH excess may reflect acclimatisation of the cardiac tissue to the hormone excess. Therefore, if the impact of GH on cardiac AMPK activity it U-shaped, it may well be that this model's timings are unsuitable to provide further data regarding the question of the influence of GH/IGF-I on AMPK activity.

Despite this, there was a clear effect of pasireotide and lanreotide on cardiac AMPK activity. The ongoing effect of a single dose results from encapsulation of the drug in microspheres of polyactide-polyglycolide. When placed as a depot, the drug is released in a biphasic pattern. Human studies demonstrate that after a single dose of octreotide LAR, octreotide levels are still measurable in the serum for up to 60 days[329].

It has been suggested that somatostatin analogues may be superior to surgery in improving cardiovascular outcomes in patients with acromegaly, particularly systolic function and lipid profile[133]. The exact mechanism for this finding is unknown but it has been shown that human cardiomyocytes express somatostation receptor subtypes (SSTR) 1 and 2 and that cardiac fibroblasts express SSTR 1, 2, 4 and 5 and it has been suggested that somatostatin analogues may directly affect the heart[330]. To date, there is no published work examining the interaction between the somatostatin and AMPK systems[331].

The finding that somatostatin analogues reduce cardiac AMPK activity may supply a potential mechanism for this. Our experiments with young adult bGH mice demonstrate an increase in cardiac pAMPK levels. Somatostatin analogues may exert some of their beneficial cardiac effects in acromegaly by acting directly to counteract the effects of increased AMPK activity. However, our data raise questions about the long-term safety of such drugs. A heart unable to mount an AMPK response to acute ischaemic stress is likely to have a worse outcome in the event of an ischaemic event. Furthermore, the use of metformin, an AMPK-activator, is associated with a reduction in cardiovascular morbidity and mortality[191,192]. However, the serious deleterious cardiac consequences of acromegaly or carcinoid syndrome, another endocrine disease treated with somatostatin analogues, may well outweigh any risks arising from drug-induced cardiac AMPK activity alterations. Whether or not AMPK activity normalises with acclimatisation to these drugs remains to be determined.

12.7 Conclusion & further work

This work demonstrates that the GH/IGF-I and AMPK systems interact with one another. In the acute setting, GH increases cardiac AMPK activity, whereas IGF-I appears to act in a converse manner to reduce it, possibly acting via the insulin receptor. With both chronic GH excess and deficiency, the picture is less clear. In early disease cardiac pAMPK levels rise but in late disease they fall. Functional AMPK assay has not been able to demonstrate alterations in cardiac AMPK activity in these situations. This may represent a failure on behalf of the assay or arise from additional AMPK modification in a yet unknown manner, most likely additional phosphorylation. The multi-system impact of acromegaly and GHD creates challenges in fully investigating and understanding these diseases.

Several areas have arisen as a result of this study that would benefit from further investigation and are listed below.

1. AMPK Ser485/491 phosphorylation

The phosphorylation of Ser485 has been demonstrated as a mechanism by which insulin reduces AMPK activity. I suggest performing Western blotting for Ser485/491 pAMPK on the cardiac protein from the animals in the studies described in this thesis. I hypothesise that IGF-I treatment will result in increased Ser485/491 pAMPK levels, whereas levels will be reduced with GH treatment.

2. Further work with cells to examine the effect of GH/IGF-I on AMPK

We demonstrated that acute GH treatment increased AMPK activity in primary neonatal rat cardiomyocytes and WT rats. The only published study looking at the direct effect of GH on AMPK didn't show any effect of GH on pAMPK levels in endothelial cells[66]. However, this study was confounded by serum starvation of the cells were subjected to prior to treatment[206]. The lack of published data in this field make the finding that GH increase cardiac AMPK activity in two models particularly interesting but clearly indicates a need for further studies in this area. I suggest repeating this experiment in HL-1 cells, an immortalised cardiac cell line that is known to express the GH receptor[332]. Previous work in our group has not been able to measure AMPK activity in these cells. Through using the techniques described in this thesis I hope to be able to measure both AMPK activity and pAMPK in these cells. Confirming our neonatal rat cardiomyocyte findings in a second cardiac cell type will add validity to my

findings. Furthermore, the immortalised nature of HL-1 cells will allow for more extensive experiments to take place.

3. The effect of GH on AMPK activity in the GHD state

Dwarf rats demonstrated a fall in cardiac AMPK activity with GH treatment. It remains unclear whether the effect seen on cardiac AMPK activity in the dwarf animal is a true effect of GH on cardiac AMPK activity in a GHD-state or if the finding represents a peculiarity of this model. I plan to repeat this experiment, treating the GHRKO mouse with injections of GH, using the protocol followed with the dwarf rats. By using an alternative model of GHD I hope to determine if the results reported here were due to a peculiarity of the model used or if they represent a true finding.

It remains unclear whether the GHD heart is under a form of constant metabolic stress, arising from low basal energy levels. To investigate this further I plan to perform cardiac MRS on patients with GHD before and after GH replacement. We already have ethical approval for this study. With this I hope to determine the available energy for the myocardium. Performing MRS before and after GH replacement will indicate whether GH reverses the impact of GHD on the heart but will not address whether the findings corresponded to AMPK changes.

4. Further investigation of the activity of the pAMPK in bGH and GHRKO mice

These transgenic animals demonstrated changes in pAMPK that did not result in measurable changes in AMPK function. Firstly, I plan to repeat this experiment to ascertain assay failure did not cause the incongruous results seen. Additionally, I plan to measure downstream makers of AMPK to determine the effect of increased Thr172 phosphorylation. The most commonly measured target of AMPK is pACC. I suggest performing Western blotting for this protein to help determine whether or not the functional AMPK assay was accurately reflecting AMPK activity. Amplification occurs along this pathway, so even subtle changes should be measurable